Declaration

I declare

a) that this thesis is composed by myself and
b) that the work is my own, except where otherwise stated.

Ralph Henry Holme
June 1998
Abstract

Members of the vertebrate Msx gene family encode homeodomain-containing transcription factors that are expressed in a variety of tissues during mouse development, including the eye. Msx 1 and Msx 2 are reported to be essential for eye development, but their precise function during the formation of this organ is not clear.

Msx function in the eye was investigated by performing a detailed analysis of Msx 1 and Msx 2 expression in E9.5-E13.5 mouse embryos. The optic vesicle gives rise to the pigmented retina epithelium (PRE) and neural retina. In situ hybridisation showed that Msx 2 was expressed in cells of the optic vesicle that are presumed to give rise to the neural retina. Expression was not detected in the PRE. These observations suggest that Msx 2 may play a role in specifying neural retina cell fate or in suppressing PRE fate.

This function was investigated by ectopically expressing mouse Msx 2 in primary cultures of dissociated 5 to 9 day-old chick PRE cells. This resulted in a small proportion of expressing cells acquiring a neural phenotype, but had no effect on non-expressing neighbouring cells. Dedifferentiated PRE, cultured for up to 14 days, remained responsive to Msx 2. Ectopic expression of mouse Msx 1 in chick PRE cultures also resulted in a small proportion of cells acquiring a neural morphology.

At least two classes of neural cell were produced by Msx 2; bipolar cells expressed the neuronal marker TuJ1, and cells with several processes were either positive or negative for TuJ1. Both groups were negative for the ganglion marker Islet 1, while preliminary observations suggested that Msx 2-induced neural cells were also negative for two further ganglion markers, NF68 and Map 2. These ganglion markers labelled only a subset of cultured neural retina. These results suggest that Msx 2-induced neural cells may be equivalent to this subset of neural retina cells.
The possibility that Msx 2 function in the developing retina is conserved between chick and mouse was explored by analysing the expression of Msx 2 in the chick retina. cmMsx 2 expression was not detected in the presumptive neural retina of the optic vesicle or optic cup, suggesting that an alternative gene, perhaps cmMsx 1, functions in chick.

To confirm that the function of mouse Msx 2 in chick PRE cultures reflects the function of this gene in vivo, a mouse transgenic line has been generated that carries a transgene, composed of Msx 2 upstream of an IRES/βGeo cassette, expressed in the PRE under the control of the Trp2 promoter. Preliminary results show that these mice exhibit no obvious eye defects and that the transgene is expressed in patches of the PRE at the stages analysed (E9.5-E13.5). In contrast, Trp2-LacZ mice express LacZ uniformly throughout the PRE. Whether or not these differences are a consequence of Msx 2 function remains to be investigated.

The results presented in this thesis suggest that mouse Msx 2 may function in chick PRE cells in one of two ways; either to generate multipotent neural retina precursors or to directly drive the differentiation of individual PRE cells into different neural cell types. Msx 2 expression in retinal progenitors of the optic vesicle, but not progenitors within the differentiating neural retina is consistent with Msx 2 functioning in the optic vesicle to recruit neural retina precursors rather than to drive their final differentiation. The Msx genes may play a similar role at other sites of expression.
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<td>---------------</td>
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<td></td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
<td></td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>βGal</td>
<td>β-Galactosidase</td>
<td></td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
<td></td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl-terminal</td>
<td></td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
<td></td>
</tr>
<tr>
<td>DAB</td>
<td>3-diaminobenzidine tetrahydrochloride</td>
<td></td>
</tr>
<tr>
<td>Dapi</td>
<td>4', 6-diamidino-2-phenylindole</td>
<td></td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxyctydine triphosphate</td>
<td></td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
<td></td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfide</td>
<td></td>
</tr>
<tr>
<td>DNA</td>
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<td>dNTPs</td>
<td>deoxynucleotide triphosphate</td>
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<tr>
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<td>dithiothreitol</td>
<td></td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
<td></td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
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</tr>
<tr>
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<td>ethanol</td>
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</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>G</td>
<td>guanine</td>
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<td>hrs</td>
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<tr>
<td>kb</td>
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<tr>
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<td>minutes</td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
<td></td>
</tr>
<tr>
<td>NaAc</td>
<td>sodium acetate</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
<td></td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
<td></td>
</tr>
<tr>
<td>PRE</td>
<td>pigmented retina epithelium</td>
<td></td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
<td></td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
<td></td>
</tr>
<tr>
<td>sdH₂O</td>
<td>sterile distilled water</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
<td></td>
</tr>
<tr>
<td>secs</td>
<td>seconds</td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>thymine</td>
<td></td>
</tr>
<tr>
<td>TESPA</td>
<td>3-aminopropyl-triethoxy silane</td>
<td></td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet light</td>
<td></td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
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All units are Standard International (SI) units.
Chapter 1

Introduction

1.1 Transcription factors

During embryonic development, cells exhibit complex changes in gene expression affecting diverse biological processes, from proliferation to differentiation. Transcription factors interact with the transcription machinery in a sequence-specific manner, either activating or repressing transcription. Their importance for normal cellular function is highlighted by developmental malformations, disruption of physiological pathways and tumorogenesis that can arise from gain or loss-of-function mutations in transcription factors (Engelkamp and van Heyningen, 1996).

A typical transcription factor contains at least two functional domains; a sequence-specific DNA-binding domain and an effector domain. The former targets the protein to gene regulatory elements and the latter either activates or represses transcription. An alternative mode of action for many transcription factors is to alter the overall topology of the DNA, bringing components of the transcription machinery together, thus either activating or repressing transcription. The high mobility group (HMG)-containing protein lymphoid enhancer-binding factor (Lef 1) acts in such a way, binding to the minor groove of DNA, causing it to bend and allowing transcription factors, bound to flanking sequences, to interact (Tjian and Maniatis, 1994).

Regulation of complex gene expression patterns by a relatively small number of transcription factors can be considered at two levels. At the level of the individual transcription factor, specificity can be generated by a specific DNA-binding domain. The first DNA-binding domain to be described was the homeodomain. Proteins containing this domain can be grouped into subclasses according to sequence similarities. Different subclasses recognise different DNA sequences (Treisman et al., 1992). Other unrelated DNA-binding domains have also been identified, zinc finger
domain (contained in WT1), basic helix-loop-helix leucine zipper domain (contained in MITF), paired domain (contained in PAX 6) and pou domain (contained in POU3F4; Ton et al., 1991; De Kok et al., 1995; Little et al., 1995; Moore, 1995). Specificity can be increased further by combining more than one DNA-binding domain in any one protein. The second DNA-binding domain can either be linked to the first, as in POU3F4 which has a pou-specific domain linked to a pou-subclass homeodomain, or be separated as in PAX 6 which contains a paired domain 5' of a paired-subclass homeodomain. At the level of DNA binding, specificity can be generated by combining distinct binding sites, thus bringing together specific combinations of transcription factors.

1.2 Msx transcription factors

The murine Msx gene family consists of three members, termed Msx 1, Msx 2 and Msx 3 (Davidson, 1995; Shimeld et al., 1996). These transcription factors contain a 60 amino acid homeodomain that differs by only two amino acids between the three proteins. The Msx gene family has probably evolved via gene duplication and divergence in the lineage leading to vertebrates. The Msx gene family is related to the Drosophila gene Muscle segment homeobox (Msh). Over the 60 amino acid homeodomain, Msx 1, Msx 2 and Msx 3 are 92%, 92% and 90% homologous to Msh respectively (Holland, 1991). In the N-terminal region of Msx 1 and Msx 2, a domain of 19 amino acids is also highly conserved (fig. 1.1). This conservation is observed across higher vertebrates. In addition, Msx 1 and Msx 2 have regions conserved among higher vertebrates, but unique to each protein (Davidson, 1995). These domains may indicate regions of shared and distinct function respectively.
1.2.1 Msx homeodomain structure

The Msx homeodomain can be classified as a class II or Engrailed-like homeodomain based on sequence similarities with other homeodomain-containing transcription factors (Treisman et al., 1992). Determination of the Engrailed homeodomain 3D structure revealed that the protein folds to produce a helix-turn-helix structure composed of an extended N-terminal arm and three alpha helices. Helix 1 and helix 2, closest to the N-terminal, are too far away from the DNA to make many contacts. Helix 3, which is perpendicular to the first two helices, fits directly into the major groove making extensive contacts with the DNA (Kissinger et al., 1990). The glutamine residue, at position 9 in helix 3, appears to be critical for specific-binding to the Engrailed consensus binding site (TAATTG). Conversion of this residue to a lysine in Engrailed switches the binding specificity to the Biciod consensus binding site (TAATCC), normally only recognised by the Biciod class of homeodomain-containing proteins (Treisman et al., 1992).

In vitro gel retardation assays have suggested that the consensus binding site for both Msx 1 and Msx 2 is CTTAATTG. Msx 2 binds to this site at a higher affinity than Msx 1 (Catron et al., 1993, 1996). Residues in both the N-terminal arm and the three helices of the homeodomain, ensure that the overall conformation of the homeodomain is stable and functional (Isaac et al., 1995). In these studies the Msx-binding sites were composed of small oligonucleotides. Consequently, the
contribution that the sequence flanking the consensus binding site \textit{in vivo} has on Msx function was not addressed.

\subsection*{1.2.2 Msx biochemical function}

The biochemical function of Msx I has been explored by co-transfecting NIH 3T3 cells with an \textit{Msx I} expression construct and one of two reporter-gene constructs. The reporter constructs expressed \textit{Luciferase} from a basal promoter and contained either a consensus homeodomain-binding site or a genomic homeodomain-binding site, from the \textit{Wnt 1} enhancer. \textit{Luciferase} expression was repressed in cells expressing \textit{Msx I} (Catron \textit{et al.}, 1995, 1996). Msx 2 was shown to have the same effect as Msx 1 in this cell culture assay (Catron \textit{et al.}, 1996). Therefore, Msx 1 and Msx 2 can both function as transcription repressors. Their shared biochemical function suggests that they could be functionally redundant at sites of the embryo in which they are co-expressed. Repression of transcription may not require the homeodomain to bind DNA. This was shown in the cell culture system previously described by mutating either the consensus or the genomic homeodomain-binding sites, thus abolishing DNA-binding. These mutations did not effect the repression of the reporter gene by Msx (Catron \textit{et al.}, 1995, 1996). In a second assay using NIH 3T3 cells, the transcription activator VP16 was fused to Gal 4, thus directing it to Gal 4-binding sites positioned upstream of a reporter gene. In this assay, Msx 1 repressed VP16-activated transcription demonstrating that an Msx-binding site is not required for repression by Msx (Catron \textit{et al.}, 1995).

In transient transfection assays, truncated forms of Msx 1 and Msx 2 which lack the homeobox and C-terminal portion, maintain the ability to function as transcription repressors (Catron \textit{et al.}, 1996), suggesting that the repression domain is located in the N-terminal. Engrailed also functions as a transcription repressor, with multiple structural domains contributing to this activity (Smith and Jaynes, 1996). One of these domains, eh 1, is located in the N-terminal of the protein. This domain is conserved in numerous homeoproteins from both fly and mouse, including Msx 1 and Msx 2.
Therefore, the eh 1 domain of Msx may also contribute to the repression of transcription.

Although the homeodomain is not required for repression, it does have the ability to repress transcription in transient transfection assays (Catron et al., 1996). This was demonstrated in a cell culture assay comprising a reporter gene driven by a basal promoter, downstream of Gal 4-binding sites. In this assay, transcription was shown to be repressed by Gal 4 when fused to the Msx homeodomain (Catron et al., 1996). However, though a peptide composed of only a homeodomain can be transported to the nucleus and can bind to an Msx consensus-binding site in vitro, it cannot repress transcription when targeted to the promoter of the reporter gene by an Msx binding site. This is in contrast to either full-length or the N-terminal portion of Msx (Catron et al., 1993, 1995).

Given that repression of transcription by Msx is not necessarily mediated by direct DNA-binding, repression may function through protein-protein interactions. Co-immunoprecipitation and glutathione s-transferase (GST) interaction assays reveal that the homeodomain can bind to TATA binding protein (TBP), a general transcription factor (Zhang et al., 1996). This interaction is specific to the homeodomain and does not require the presence of DNA (Zhang et al., 1996). In vivo, TBP is a component of a multi-protein complex termed TFIID. Msx 1 can interact with TBP when incorporated in this complex (Zhang et al., 1996). Mutations in the homeodomain which abolish TBP-binding also abolish transcription repression suggesting that repression of transcription by Msx is mediated via an interaction with TBP (Zhang et al., 1996). It will be important to establish if a similar interaction exists in vivo.

The mechanisms employed by a cell to ensure that the correct genes are repressed may be far more complex than merely targeting transcription factors to gene regulatory elements through specific DNA-binding. In vivo, a combination of tissue-specific and promoter-specific proteins could interact with Msx, either directing
it to a specific target gene or sequestering it away from transcription machinery. Recent evidence illustrates that both Msx 1 and Msx 2 can directly interact with the Distal-less (Dlx) homeoproteins (Zhang et al., 1997). The genes encoding these proteins are expressed in a spatial/temporal specific manner during mouse development (Zhang et al., 1997). GST interaction and yeast two-hybrid assays reveal that homodimeric complexes between individual Msx and Dlx proteins, heterodimeric complexes between members of the same family and heterodimeric complexes between members of the Dlx and Msx families can form (Zhang et al., 1997). The interaction between Msx 1 and Dlx 5 is mediated specifically via the homeodomains of the two proteins. (Zhang et al., 1997). The presence of an Msx 1-binding site in the GST assay abolished the interaction between Msx 1 and Dlx 5 (Zhang et al., 1997). This suggests that DNA- and Dlx 5-binding by Msx 1 is mutually exclusive.

Msx 1 can bind directly to the enhancer of MyoD (Woloshin et al., 1995). In Myoblast cells, this enhancer can drive the expression of Luciferase. However, over-expression of Msx 1 in cells containing this reporter construct represses Luciferase expression (Zhang et al., 1997). Repression of transcription by Msx 1 can be alleviated by increasing amounts of Dlx 2 or Dlx 5. Conversely, increasing amounts of Msx 1 represses the expression of Dlx 2-activated reporter constructs (Zhang et al., 1997). Thus the formation of Msx-Dlx dimers cancels their action. The loss of function maybe due to the Msx-Dlx dimers being unable to interact with the transcription machinery, or alternatively, having an altered DNA-binding specificity. Msx and Dlx genes are co-expressed in parts of the developing chick limb (chapter 1.3.7), and could therefore interact in vivo. Closer examination of the phenotype of mice null for Dlx 2 may reveal possible interactions between these two genes in vivo (Qiu et al., 1995).

1.3 The function and regulation of Msx

Expression of both Msx 1 and Msx 2 begins during gastrulation along the entire anterior-posterior axis of the embryo in the lateral plate mesoderm and in the lateral
parts of the neural plate (Davidson and Hill, 1991). This is maintained, after neural tube closure, in the dorsal neural tube and roof plate. Neural crest-derived cells that migrate from this region also express these genes. *Msx 1* is expressed in the dorsal and ventral tips of the somites (MacKenzie et al., 1997a). *Msx 3* is also expressed in the lateral neural plate, becoming restricted to the dorsal neural tube after closure (Shimeld et al., 1996). In comparison to *Msx 1* and *Msx 2*, *Msx 3* expression is much more restricted; with no expression in the roof plate, lateral mesoderm or migrating neural crest-derived cells. The anterior limit of *Msx 3* expression lies at the rhombencephalon and mesencephalon boundary. In contrast to *Msx 2*, *Msx 3* is down-regulated in rhombomeres 3 and 5 at E10.5. *Msx 3* expression is subsequently switched-off along the entire length of the neural tube at E12.5, while both *Msx 1* and *Msx 2* continue to be expressed in the dorsal neural tube.

During organogenesis, *Msx 1* and *Msx 2* are expressed in a specific manner in a number of developing organs. The function and regulation of *Msx 1* and *Msx 2* at these sites is reviewed in this section.

1.3.1 Limb development

During mouse embryogenesis, forelimbs start to develop at E9.5, and hindlimbs at E10.5, on the flank of the embryo from lateral plate mesoderm. The ectoderm around the distal tip of the developing limb bud forms a pseudostratified columnar epithelium termed the apical ectodermal ridge (AER). Cells of the progress zone, immediately beneath the AER, remain undifferentiated and proliferative. As the bud elongates, cells leave the progress zone and differentiate, thus a proximal-distal gradient of differentiation is observed in the developing limb (Hinchliffe and Johnson, 1980). Reciprocal interactions between the AER and underlying mesenchyme are required for normal limb development. The AER promotes the underlying mesenchyme to remain proliferative and undifferentiated, while the progress zone maintains the AER state of differentiation (Summerbell et al., 1973). The spatial/temporal distribution of
Msx mRNA in the developing limb coincides with these reciprocal interactions suggesting a possible involvement.

Msx 1 is strongly expressed in the progress zone but only weakly expressed in the AER, conversely Msx 2 is strongly expressed in the AER (Davidson and Hill, 1991). Msx 1 expression in the progress zone is dependant on the AER. If chick AER, or the whole apical ectoderm in mouse, is removed, Msx 1 expression is lost from the progress zone (Ros et al., 1993; Wang and Sassoon, 1995). Furthermore, if proximal limb mesoderm, which no longer expresses Msx 1, is grafted under the AER then activation of Msx 1 in the limb mesoderm is observed (Davidson and Hill, 1991). Both these experiments suggest that there is a diffusible factor(s), produced in the AER, which acts on the progress zone to induce Msx 1. Bone morphogenetic protein 4 (Bmp 4) and Fibroblast growth factor 4 (FGF 4) are both expressed in the AER and could fulfil this role. Indeed, recombinant Bmp 4 activates Msx 1 when added to cultures of proximal limb mesenchyme (Wang and Sassoon, 1995). FGF 4-soaked beads can substitute for the absence of AER, promoting limb outgrowth and patterning in chick (Niswander et al., 1993). Msx 1 expression in cultured mouse limbs, after the removal of the AER, can also be maintained in the presence of FGF 4 (Wang and Sassoon, 1995).

The limbs of mice null for either Msx 1 or Msx 2 develop normally and are indistinguishable from wild type limbs (Satokata and Maas, 1994; Chen et al., 1997). Msx 1 and Msx 2 are co-expressed in the developing limb bud and share similar biochemical properties (chapter 1.2). It is therefore conceivable that in the absence of either one of the genes, the other can function in its place. In support of this idea, mice null for both Msx 1 and Msx 2 display a range of limb defects including the loss of anterior-posterior polarity. This defect is characterised by the absence of Sonic hedgehog (Shh) expression in the zone of polarising activity combined with a shift in FGF 4 expression from posterior to anterior apical ectoderm (Chen et al., 1997). The Msx genes are therefore essential for the initial stages of limb development and could
be involved in reciprocal signalling processes between the AER and the underlying mesenchyme.

### 1.3.2 Tooth development

Initiation of mouse molar tooth development occurs at E11 and is characterised by a thickening of the oral epithelium. By E13, a bud of epithelium has formed, around which the surrounding neural crest-derived mesenchyme from the first branchial arch condenses. In turn the epithelial bud undergoes a progressive morphological change, first into a cap and then into a bell-like structure. The mesenchyme enclosed within the bell differentiates into dental papilla cells which eventually give rise to odontoblasts and dental pulp. The remaining condensed mesenchyme forms the dental sac which surrounds the dental epithelium. The dental sac eventually differentiates into the periodontal tissue that anchors the tooth to the alveolar bone. Cells of the inner layer of the epithelial bell-like structure differentiate into ameloblasts which ultimately secrete the organic matrix of the enamel (Thesleff and Sahlberg, 1996; Stock et al., 1997).

Tissue recombination experiments, in which epithelium and mesenchyme of different source and stage are recombined, highlight the need for successive and reciprocal epithelial-mesenchymal interactions at each stage of tooth development. The primary capability to initiate tooth development resides in the oral epithelium, since teeth can form when mouse presumptive dental epithelium is recombined with mesenchyme from the second arch that does not normally give rise to teeth (Lumsden, 1988). As development continues, there is a secondary requirement for the newly specified dental mesenchyme to signal back to the epithelium to maintain tooth development. Remarkably, this reciprocal signal can actually instruct non-dental epithelium to undergo tooth differentiation (Kollar and Baird, 1970).

The expression of the Msx genes coincides with these sites of reciprocal signalling, suggesting a possible involvement. Msx 1 is expressed in the dental mesenchyme
during the bud, cap and bell stage, but not in the dental epithelium. *Msx 2* is also expressed in the dental mesenchyme, becoming restricted to the dental papilla and activated in the epithelial enamel knot (MacKenzie *et al.*, 1991a, b, 1992). The enamel knot is thought to be a signalling centre regulating patterning of the tooth cusps (Thesleff and Sahlberg, 1996).

Tooth development in mice null for *Msx 1* is arrested at the bud stage (Satokata and Maas, 1994). The formation of a tooth bud illustrates that *Msx 1* is not required during the initial stages of tooth development but is required during the later stages, either for receiving or replying to the epithelial signal.

The activation of *Msx 1* and *Msx 2* in the dental mesenchyme correlates with the proposed first inductive-signal from the dental epithelium to the underlying mesenchyme. This implies that the expression of the *Msx* genes in the mesenchyme is controlled by the epithelium. Tissue recombination experiments do indeed show that the dental epithelium can induce the expression of *Msx 1, Msx 2, Bmp 4, Syndecan 1* and *Tenascin C* in the mesenchyme. Furthermore, these experiments indicate that the epithelial signals are diffusible molecules (Thesleff and Sahlberg, 1996). The gene encoding the diffusible protein *Bmp 4* is expressed in the dental epithelium at E11.5, expression then shifts to the underlying dental mesenchyme at E12.5, making this protein an attractive candidate signalling molecule. To test this, beads releasing *Bmp 4* were implanted into dental mesenchyme and were shown to be able, in part, to replace the function of dental epithelium, causing the induction of *Msx 1, Msx 2* and *Bmp 4*, but not *Syndecan 1* and *Tenascin C* (Vainio *et al.*, 1993). It would therefore appear that *Bmp 4* is not the only molecule signalling to the mesenchyme. *FGF 4* has also been shown to induce *Msx 1* and *Msx 2*, but not *Bmp 4* in dental mesenchyme. However, unlike *Bmp 4*, *FGF 4* is capable of inducing *Syndecan 1* and *Tenascin C* (Chen *et al.*, 1996; Thesleff and Sahlberg, 1996). Although *FGF 4* can induce many of the markers associated with induced-dental mesenchyme, the gene is not expressed in the dental epithelium. However, *FGF 8* is expressed in the dental epithelium and may therefore be the in vivo signalling molecule (Thesleff and Sahlberg, 1996).
Tooth development is arrested at the bud stage in mice null for the transcription factor Lef 1. These mice exhibit a similar phenotype to Msx 1-knockout mice (Kratochwil et al., 1996). Lef 1 is also expressed in a very similar manner to Msx 1 suggesting that these genes may lie in the same genetic pathway. Lef 1 is first expressed in the dental epithelium at E11 with expression moving to the dental mesenchyme, similar to Bmp 4. As development progresses, expression is detected in the enamel knot, the mesenchymal papilla and pre-odontoblasts (Kratochwil et al., 1996). Normal dental epithelium combined with Lef 1-mutant dental mesenchyme results in tooth formation, irrespective of the developmental stage used. In contrast, the capability to form teeth when Lef 1-mutant dental epithelium is combined with normal dental mesenchyme is dependent on developmental stage. Teeth do not develop if E10-12 tissue is used, but are produced when E14-17 tissue is used, presumably because the older mesenchyme has already received the Lef 1-dependant signal from the epithelium (Kratochwil et al., 1996). These results demonstrate that Lef 1 is required only in the dental epithelium before or during the initial inductive signal from the epithelium to the underlying mesenchyme.

Recent investigation into the expression of these various genes, together with ectopic application of Bmp 4 in both the Msx 1 and Lef 1 mutants has indicated the order of these molecules in a genetic hierarchy affecting tooth development. Analysis of Bmp 4 and Lef 1 expression in Msx 1-knockout mice revealed that when the tooth bud arrests, both Bmp 4 and Lef 1 are down-regulated in the dental mesenchyme, Bmp 4 is unaffected in the epithelium (Chen et al., 1996). In Lef 1-mutant mice, Bmp 4 and Msx 1 expression are unaffected (Kratochwil et al., 1996). In the dental mesenchyme, Msx 1 is therefore upstream of both Bmp 4 and Lef 1, with Bmp 4 being upstream of Lef 1. Further evidence supporting this hierarchy comes from experiments in which beads soaked in Bmp 4 are implanted into the presumptive dental mesenchyme of either wild type or Lef 1-mutant mice. This results in the expression of Lef 1 and Msx 1 in wild type tissue around the implanted bead, but only Msx 1 in Lef 1-mutant tissue.
(Kratochwil et al., 1996). Activation of *Lef 1* by Bmp 4 may therefore function through *Msx 1*.

The down-regulation of mesenchymal *Bmp 4* in *Msx 1*-knockout mice, suggests that epithelium-derived Bmp 4 may act through *Msx 1* to activate mesenchymal *Bmp 4*. To test this possibility, Chen *et al.* (1996) implanted beads soaked in Bmp 4, or wild type dental epithelium, into E11.5 dental mesenchyme from both wild type and *Msx 1*-knockout mice. Expression of *Bmp 4* around the grafted bead or epithelium was only seen in wild type mesenchyme (Chen *et al.*, 1996). This confirms that induction of *Bmp 4* in the presumptive dental mesenchyme, perhaps by dental epithelium-derived Bmp 4, requires *Msx 1*. In contrast, *Lef 1* expression can be induced in presumptive dental mesenchyme not only from wild type mice but also *Msx 1*-knockout mice by implanting a Bmp 4-soaked bead (Chen *et al.*, 1996). This demonstrates that activation of *Lef 1* by Bmp 4 does not require *Msx 1*, although *Msx 1* is required upstream of *Bmp 4*.

In *Msx 1*-mutant mice, Syndecan 1 immunoreactivity in the mesenchyme is reduced. *Syndecan 1* expression in mutant mesenchyme can not be induced by implanting FGF-soaked beads, as in wild type mesenchyme (Chen *et al.*, 1996). This indicates that, like Bmp 4, the FGF (FGF 8) inductive signal is mediated by *Msx 1*.

In summary, *Msx 1* function during tooth development can be divided into two parts. During the initiation stage, E11.5, the dental epithelium signals to the underlying mesenchyme triggering its differentiation into dental mesenchyme. This epithelial signal may comprise of a cocktail of diffusible proteins including Bmp 4 and FGF (FGF 8). The presumptive tooth mesenchyme responds to these proteins by expressing numerous genes including *Msx 1*. Msx 1 then activates *Bmp 4* expression in the mesenchyme, which in turn induces *Lef 1*. During the bud stage (E12.5) Bmp 4 produced in the mesenchyme could act on the dental epithelium in a reciprocal fashion supporting further epithelial development. Indeed, *Msx 1*-mutant tooth buds are able to progress to the cap stage when cultured in medium containing Bmp 4 (Chen *et al.*, 1996).
A second possible role for $Msx\;I$ could be as an amplifier of the Bmp 4 signal. Since Msx 1 activates $Bmp\;4$ and in turn Bmp 4 activates $Msx\;I$ a positive-feedback loop is created. This would allow a stronger and more rapid spread of the Bmp 4 inductive-signal through the presumptive mesenchyme than diffusion of Bmp 4 from the dental epithelium.

1.3.3 Facial development

The face develops from a series of primordia which share many similarities with developing limb buds. These primordia consist of buds of undifferentiated mesenchyme surrounded by epithelium. As in the developing limb buds, outgrowth of the facial primordia is dependent on epithelial-mesenchymal interactions associated with a progressive differentiation of cartilage and bone (Wedden, 1987).

During facial primordia outgrowth, the expression patterns of both $Msx$ genes and $Bmp\;4$ suggest that they may be involved in epithelial-mesenchymal signalling. Stage 20 chick embryos express $Bmp\;4$ in the epithelium of the medial region of the mandibular primordia, distal tips of the maxillary primordia and frontonasal mass. $Msx\;I$, $Msx\;2$ and $Bmp\;2$ are expressed in the underlying mesenchyme. After stage 24, there is gradual transition of $Bmp\;4$ expression from the epithelia of the facial primordia to the underlying mesenchyme (Barlow and Francis-West, 1997).

Mice null for $Msx\;I$ exhibit a reduction in both mandibular and maxillary outgrowth leading to a range of craniofacial abnormalities. $Msx\;I$ is therefore essential for proper facial primordia development (Satokata and Maas, 1994). Experiments in which beads soaked in Bmp 4 are implanted into various parts of the facial primordia, suggest that a similar genetic pathway to that in the developing limb and tooth operates in this system too. Barlow and Francis-West (1997) illustrated, using stage 20 chick embryos, that implantation of beads soaked in either Bmp 2 or Bmp 4 into regions of mesenchyme of the maxillary or mandibular primordia, which do not normally express $Bmp\;2$, $Bmp\;4$, $Msx\;I$ or $Msx\;2$, results in the activation of both $Msx$
genes in the mesenchyme around the beads. Bmp 4 was also expressed in mandibular mesenchyme, whilst extended expression of Sonic hedgehog and FGF 4 was observed in maxillary epithelium (Barlow and Francis-West, 1997).

The ectopic application of Bmps in the facial primordia results not only in altered gene expression but also changes in skeletal development. These changes may be a true consequence of altered patterning in the primordia or a secondary consequence due to physiologically abnormal effects of Bmps. Cell proliferation and cell death was observed around grafted Bmp-soaked beads, but not around control beads (Barlow and Francis-West, 1997). These processes could account for the altered skeletal development and may not represent the normal action of Bmps when present in the correct cells at the correct concentration.

Similar molecules and mechanism appear to operate during facial primordium, tooth and limb development. It is not possible to order these genes with any certainty into a genetic hierarchy controlling facial primordium outgrowth, but it is possible to make a speculative model based on the similarities with tooth bud development. Epithelium-derived Bmp 4 may signal to the underlying mesenchyme, activating numerous genes including the Msx genes. As in tooth development, the Msx genes may be required to turn on mesenchymal Bmp 4, creating a positive-feedback loop that may amplify the epithelium-derived Bmp 4 signal through the mesenchyme. Mesenchymal Bmp 4 may then act in a reciprocal fashion to affect the expression of genes such as Shh and FGF 4 in the epithelium.

1.3.4 Mammary gland development

Development of murine mammary glands starts at E10.5 with a thickening of the epithelium between the limb buds. During the next 48 hrs of development, the epithelium begins to form a bud and invaginates into the mesenchyme in a manner analogous to the tooth bud. Mesenchyme around the epithelium eventually forms the
stromal fat pad. Post-natal growth and morphogenesis of the mammary gland is controlled by a combination of hormones (Phippard et al., 1996).

The initial stages of mammary gland development require epithelial-mesenchymal interactions (Cunha et al., 1992). The mammary bud at stage E13.5 expresses Msx 1, Msx 2 and Bmp 2 in the epithelium only, while Bmp 4 is expressed in the underlying mesenchyme (Phippard et al., 1996). Given that these genes have been shown to be involved in epithelial-mesenchymal interactions in the structurally similar tooth bud, they may also perform a comparable role during the initial stages of mammary gland development, though this remains to be confirmed. In the tooth bud Msx 1 is expressed in the mesenchyme and not in the epithelium, demonstrating that both differences and similarities exist between these two systems. A more detailed investigation of the temporal expression patterns of these genes in the developing mammary gland is required before conclusions can be drawn from comparisons between these two systems. Lef 1-null mice exhibit arrested mammary gland development at the bud stage (van Genderen et al., 1994). During tooth development, Lef 1 was shown to be downstream of Msx 1, raising the possibility that these two genes may also function in the same genetic pathway during mammary gland development.

Mammary glands develop normally in mice null for Msx 1 implying that this gene is not required for the development of this organ (Satokata and Maas, 1994). Alternatively, functional redundancy between the Msx genes could account for the absence of a mutant phenotype. This is supported by the observation that these genes are co-expressed at E13.5 in the mammary gland and that Msx 1/Msx 2 double-knockout mice are reported to exhibit defects of the mammary glands (Phippard et al., 1996; Maas, personal communication).
1.3.5 Acquisition of neural tube dorso-ventral polarity

Floor plate and motor neurons develop from the ventral neural tube, while neural crest and roof plate cells develop from dorsal regions of the neural tube. Floor plate and motor neurons do not develop in chicks which lack a notochord implying that the notochord is a ventral signalling centre (van Straaten and Hekking, 1991). Shh is initially expressed in the notochord, with expression later extending to the overlying presumptive floor plate. This makes Shh an attractive candidate for a ventralising molecule (Echelard et al., 1993). Neural plate explants grown in the absence of the notochord fail to produce motor neurons or floor plate cells. The differentiation of these cell types can be rescued if grown with COS cells producing Shh or transfected with a Shh expression construct (Roelink et al., 1994; Tanabe et al., 1995). These experiments confirm that the ventralising signal, originating from the notochord, is mediated by Shh.

Transgenic mice have been created in which Shh is ectopically expressed in the dorsal neural tube under the control of the Wnt I promoter, a dorsally restricted gene. Within the developing brain of these mice at E10.5, Hepatocyte nuclear factor 3β (Hnf3β), a floor plate marker, is ectopically expressed dorsally. This is consistent with Shh functioning as a ventralising factor. However, although the dorsal part of the spinal cord appears abnormal at E9.5-E11.5, there is no ectopic expression of Hnf3β (Echelard et al., 1993). This may reflect a difference in the competence of these cells to respond to Shh or activate a ventralising pathway. These cells may have already received a dorsalising signal.

Msx 1 and Msx 2 are expressed in the lateral margin of the neural plate and consequently restricted to the dorsal neural tube after closure. Expression is also detected in the dorsal surface ectoderm overlying the neural tube (Davidson and Hill, 1991). Ventral neural plate explants can be forced to express Msx and Hnk 1, the latter a neural crest marker, if co-cultured with dorsal surface ectoderm (Liem et al., 1995). These experiments suggest that the dorsal surface ectoderm, initially adjacent
to the neural plate and subsequently overlying the neural tube, can produce a
dorsalising signal. *Bmp 4* is expressed in the ectoderm flanking the neural plate and
the lateral margin of the neural plate. After neural tube closure *Bmp 4* is expressed in
both the dorsal neural tube and the overlying surface ectoderm (Liem *et al.*, 1995).
The distribution of this diffusible molecule implies that it could mediate the dorsalising
signal. Ventral neural plate explants grown in the presence of Bmp 4 contain *Msx*- and
*Hnk 1*-expressing cells, showing that Bmp 4 can mimic the effects of dorsal surface
ectoderm (Liem *et al.*, 1995).

The overlapping expression domains of *Bmp 4* and *Msx* along the dorsal axis,
together with the observation that Bmp 4 can induce *Msx* expression in ventral
explants, suggests that these proteins may be functioning in a manner analogous to
their role during epithelial-mesenchymal interactions. In contrast to
epithelial-mesenchymal interactions, the Bmp 4 inductive signal might be transmitted
through the plane of the ectoderm to induce *Msx* in the lateral neural plate. It has been
shown that after neural tube closure a reciprocal interaction between the dorsal neural
tube and the overlying surface ectoderm may occur. If beads soaked in Bmp 4, or
pieces of dorsal neural tube, are implanted between the somite and the neural tube
then ectopic expression of *Msx 2* in the overlying surface ectoderm is observed
(Takahashi *et al.*, 1996). The maintenance of *Bmp 4* and *Msx* expression in the
overlying dorsal ectoderm may therefore involve reciprocal interactions with the
dorsal neural tube.

The co-expression of *Bmp 4* and *Msx* in the dorsal neural tube may result in the
formation of a positive-feedback loop, also suggested to occur during tooth
development (chapter 1.3.2). If unchecked, this regulatory mechanism could result in
the rapid spread of the dorsalising signal ventrally through the neural tube. This is not
observed, perhaps suggesting that the ventralising signal can inhibit the spread of the
Bmp 4-mediated signal. In support of this idea, dorsal neural tube explants grown
adjacent to the notochord or in the presence of Ssh, exhibit a decrease in the number
of *Msx*-expressing cells (Liem *et al.*, 1995). Equally, the progressive spread of *Shh*
expression from the notochord into the floor plate is suggestive of a positive-feedback loop, that if unregulated would ventralise the entire neural tube. Ventral neural plate explants grown in contact with the notochord but in the presence of Bmp 4, do contain Msx- and Hnk 1-expressing cells (Liem et al., 1995). It would therefore appear that Bmp 4 limits the range of the Shh mediated signal.

1.3.6 Myogenesis

In mouse, segmented blocks of paraxial mesoderm form on either side of the neural tube. Within these uniform blocks of mesoderm, the somites, cells differentiate dorsally into dermomyotome and ventrally into sclerotome tissue, subsequently giving rise to muscle and cartilage respectively. Cells of the dermomyotome adjacent to the dorsal neural tube migrate under the dermomyotome and form the myotome. Muscle precursor cells of the limb and hypaxia, migrate from the lateral side of the dermomyotome to their respective target sites (Cossu et al., 1996a).

Rotation of the somite in the embryo does not affect the subsequent formation of muscle and cartilage. This implies that all parts of the early somite can give rise to both dermomyotome and sclerotome, with the surrounding environment dictating where in the somite these cell types form (Christ and Ordahl, 1995). The neural tube, which is close to the somite, is itself patterned dorsal-ventrally and may be involved in the patterning the somite. Indeed, the notochord and floor plate induce Pax 1, a marker of sclerotome differentiation in the somite. Shh can mimic the effect that the notochord and floor plate has on activating Pax 1 in sclerotome, suggesting that it may mediate the signal from the notochord and floor plate (Cossu et al., 1996a). In an analogous manner, a dorsalising signal emanating from the dorsal neural tube may induce the differentiation of the dermomyotome. This signal could be mediated by members of the Wnt gene family, which are expressed in the dorsal neural tube (Munsterberg et al., 1995). Alternatively, the Msx/Bmp 4 signalling mechanism could mediate this dorsalising signal. Both genes are expressed in the dorsal neural tube (Davidson and Hill, 1991; Pourquie et al., 1996), though only Msx 1 is expressed in
the dorsal tips of the somites (MacKenzie et al., 1997a). It will be interesting to learn if beads soaked in Bmp 4 can mimic the effect that the dorsal neural tube may have on dermomyotome differentiation and if this induces Msx 1 expression in the dermomyotome.

Although an axial-derived signal may be required for differentiation of dorsal muscle, the removal of the neural tube before limb and hypaxial muscle precursors migrate from the ventro-lateral dermomyotome does not affect the formation of hypaxial and limb muscle (Rong et al., 1992). However, these muscle types are not produced if the somite is cultured free of all surrounding tissue types (Cossu et al., 1996b). This argues that myogenesis in the ventro-lateral dermomyotome is influenced by some of the surrounding tissue types, but not by the neural tube. Investigation of the surrounding tissue demonstrated that the overlying surface ectoderm and adjacent lateral mesoderm influence ventro-lateral dermomyotome differentiation, but act in an opposing manner. Paraxial mesoderm co-cultured with dorsal surface ectoderm undergoes myogenesis, but the addition of lateral mesoderm delays myogenesis (Cossu et al., 1996b). In vivo, this correlates with the requirement to restrain muscle differentiation in the ventro-lateral dermomyotome and during the migration of these cells to the limb. The onset of myogenesis is associated with the activation of a battery of transcription factors termed myogenic determination factors (Edmondson and Olson, 1993). These genes are not expressed in the ventro-lateral dermomyotome which is consistent with suppression of myogenesis at this site (Cossu et al., 1996a).

The inhibition of myogenesis in ventro-lateral dermomyotome by lateral mesoderm can be mimicked by implanting Bmp 4-expressing cells (Pourquie et al., 1996). This suggests that the signal emanating from lateral mesoderm is mediated by Bmp 4. In keeping with this hypothesis, Bmp 4 is expressed in lateral mesoderm of stage 12 chick embryos (Pourquie et al., 1996). The co-expression of Msx in the lateral mesoderm raises the possibility that these genes may be functioning in the same pathway. It is not yet known if Bmp 4 can activate Msx 1 in the lateral mesoderm and what the function of Msx 1 is at this site. Bmp 4 is not expressed in the ventro-lateral
dermomyotome, therefore there must be a mechanism to prevent Msx 1 activating Bmp 4 at this site. In 10T ½ cells, Msx 1 can bind directly to an enhancer element in MyoD, inhibiting its expression (Woloshin et al., 1995). Dissociation of chick limb mesenchyme results in the down-regulation of Msx 1 which is associated with the up-regulation of MyoD (Wang and Sassoon, 1995). This suggests that in the developing limb bud, Msx 1 expression and muscle differentiation are mutually exclusive.

A speculative model of how myogenesis in the ventro-lateral dermomyotome may be suppressed is presented here. Within the lateral mesoderm, a Bmp 4-mediated myogenic-inhibitory signal is produced. The co-expression of Bmp 4 and Msx 1 at this site suggests that these genes could be acting in a positive-feedback loop. The ventro-lateral dermomyotome may respond to lateral mesoderm-derived Bmp 4 by activating Msx 1. This in turn may inhibit muscle differentiation by repressing myogenic determinant genes such as MyoD. During the migration of ventro-lateral dermomyotome muscle precursor cells to their target sites in the limb, the undifferentiated state could either be maintained by further Bmp 4 signals or the initial Bmp 4 signal may be "remembered". No defects in limb muscle formation have been reported in mice null for Msx 1, implying that Msx 1 is not necessary for myogenesis in the limb (Satokata and Maas, 1994). This observation may be explained by functional redundancy between Msx 1 and Msx 2. Indeed, Msx 2 can also inhibit MyoD expression in 10T ½ cells (Woloshin et al., 1995), however it will be important to establish if Msx 2 is expressed in the somites of both wild type and Msx 1-null mice. Alternatively, functional redundancy of complete mechanisms may account for the absence of limb muscle defects in Msx 1-null mice.

1.3.7 Chondrogenesis

Each vertebra of the vertebral column, separated by intervertebral discs, comprises a vertebral body, neural arch and spinous process. The vertebral bodies surround the notochord, the neural arches surround the ventral part of the neural tube and the
spinous process close the neural arches dorsally. Development of the ventral vertebral body and neural arch occurs independently, and via a different mechanism from the spinous process. The vertebral body and neural arch arise from sclerotome, requiring signals from the notochord that may be mediated by Shh (chapter 1.3.5). Mesenchymal cells between the dorsal neural tube and the overlying surface ectoderm differentiate into the spinous process, but the developmental origin of these cells is not known. They either migrate from the somite or arise from neural crest derived from the dorsal neural tube (Monsoro-Burq et al., 1994, 1996).

The developmental processes that control the differentiation of the sclerotome are very different from those controlling the differentiation of the dorsal cartilage. The notochord, which is essential for the development of cartilage from the ventral somite, inhibits the formation of the spinous process if grafted above the roof plate (Monsoro-Burq et al., 1994). A different set of signalling molecules from those controlling ventral chondrogenesis may therefore exist dorsally. Grafting of the dorsal neural tube to a position lateral to the host neural tube results in the ectopic expression of Msx 2 and the formation of extra cartilage around the site of the graft (Takahashi et al., 1992). Bmp 4 is present in the dorsal neural tube and is therefore an obvious candidate molecule to mediate the dorsal neural tube cartilage-inducing signal (chapter 1.3.5). Cells producing Bmp 4 cause the activation of Msx 1 and Msx 2 in surrounding cells when grafted dorso-laterally or directly dorsal to the neural tube in E2 or E3 chick embryos. In these experiments Bmp 4 mimics the dorsal neural tube graft in terms of Msx activation and extra cartilage formation (Monsoro-Burq et al., 1996; Watanabe and Le Douarin, 1996).

The expression patterns of Msx and Bmp 4 during the development of axial structures have been discussed previously (chapter 1.3.5). With respect to the development of the spinous processes, both Msx 1 and Msx 2, but not Bmp 4, are expressed in the mesenchymal cells that lie between the roof plate and surface ectoderm (Monsoro-Burq et al., 1995). An Msx 1/Bmp 4 positive-feedback loop may operate in the dorsal neural tube to maintain the chondrogenic Bmp 4 signal in an analogous
manner to the function played by these genes during tooth development. However this remains to be investigated. As cartilage differentiation begins, Msx 1 expression is down-regulated (Monsoro-Burq et al., 1996).

Ectopic activation of the dorsal chondrogenic inductive pathway ventrally, has profound consequences for the derivatives of the ventral somite. Implantation of cells producing Bmp 4 or Bmp 2 between the somite and neural tube resulted in the absence of vertebral bodies and neural arches (Monsoro-Burq et al., 1996). These effects are probably a consequence of interfering with the maintenance of pattern within the somite, since implantation of Bmp 2-producing cells between the neural tube and the paraxial mesoderm in a yet unsegmented region prevented the formation of both dermomyotome and sclerotome (Monosoro-Burq et al., 1996). Nonetheless it does demonstrate that chondrogenesis in dorsal and ventral regions is regulated by different mechanisms.

Although Bmp 2 was shown to be functionally equivalent to Bmp 4 in its ability to induce chondrogenesis in the vertebral axis, the gene encoding this protein is not expressed at any stage at this site (Monsoro-Burq et al., 1996). However, in the developing limb Bmp 2 is expressed at sites of mesenchyme condensations (Kingsley, 1994). Addition of Bmp 2 to cultured chick wing mesenchyme from stage 21/22 embryos results in an increase in chondrogenesis and a decrease in myogenesis (Duprez et al., 1996). This assay was repeated using mesenchyme from chimeric chicks in which the somites had been replaced at stage 14 with quail somites. Cartilage cells were never of quail origin and therefore not of myogenic lineage (Duprez et al., 1996). Therefore, unless there were remnants of chick cells left after the removal of the somite or if cells had already migrated from the somite prior to removal, this demonstrates that the increase in cartilage differentiation does not result from the recruitment of myoblasts and that the Bmp chondrogenic inductive signal acts only on cells of non-somitic origin.
In the chick limb, Dlx 5 (ortholog of mouse Dlx 5) expression overlaps with Msx 2 expression in many but not all regions. Both genes are expressed in the AER, mesoderm at the anterior margin of the limb bud and a discrete group of mesodermal cells at the mid-proximal posterior margin. At stage 23, Dlx 5 is also expressed in the proximal central core of the limb bud at the onset of chondrogenesis. Msx 2 does not share this domain of expression (Ferrari et al., 1995). This implies that the onset of cartilage differentiation may be incompatible with Msx 2 expression. The same is true for the onset of spinous process differentiation. Msx may function to hold back the differentiation of possibly already specified cartilage precursors until they are at the correct location. This proposed function shares striking similarities to that of Msx 1 function during myogenesis. It is interesting to note at this point that Msx 2 and Dlx 5 interact, abolishing one another's ability to regulate transcription (chapter 1.2.2). It is therefore possible that Dlx 5 function in the central core of the limb bud, in which Msx 2 is not expressed, differs from regions in which it is co-expressed with Msx 2.

In support of the proposed role Msx 2 may have in restraining chondrogenesis, mandibular arches from stage 23 chick embryos cultured in the presence of anti-sense Msx 2 oligonucleotides show enhanced chondrogenesis when compared to explants cultured alone or in the presence of sense oligonucleotides (Mina et al., 1996). Consistent with this observation, quail Msx 1 expression in early bone precursors of the mandibular mesenchyme is down-regulated at the onset of terminal differentiation (Takahashi et al., 1991). As reviewed in chapter 1.3.3, the expression of Msx 1 and Msx 2 in the mesenchyme of the facial primordia can be controlled by epithelial-derived Bmp 4. Furthermore, ectopic activation of this pathway in the facial primordia by implanting Bmp 4-soaked beads affected chondrogenesis (Barlow and Francis-West, 1997). The role of Msx during chondrogenesis may be far more complex than simply acting as repressors of differentiation. This is highlighted by the observation that rat calvarial-derived osteoblasts, which can undergo chondrogenic differentiation in culture, do not show the predicted increase in chondrogenesis when cultured in the presence of anti-sense Msx 1 or Msx 2 oligonucleotides but rather a significant decrease in comparison to cultures treated with random oligonucleotides.
This unpredicted observation could be explained if these cells are not equivalent to mandibular mesenchyme, so undergo an Msx independent chondrogenic pathway. This is perhaps unlikely since both are derived from cranial neural crest. Alternatively, Msx could have distinct developmentally early and late functions, initially to specify neural crest-derived mesenchyme to the chondrogenic lineage in response to a Bmp 4-mediated signal, therefore priming the cells for differentiation. Msx could then function to restrain any further differentiation, allowing precursor cells to proliferate and migrate into position before receiving appropriate cues to terminally differentiate. The effects seen when Msx function was blocked in these two different systems may therefore be a consequence of affecting the two different functions.

Analysis of promoter regions controlling the expression of bone-specific genes supports the idea that Msx suppresses terminal differentiation of osteoblasts. The promoter of the Osteocalcin gene, which is expressed in post-proliferative osteoblasts at the onset of terminal differentiation, contains three regulatory elements that are required for tissue-specific expression. One of these elements, the OC box, contains a consensus Msx-binding site (chapter 1.2.1; Hoffmann et al., 1996). Hoffmann et al. (1996) has demonstrated that a second unknown protein, OCBP, binds independently to the OC box. A mutation in the OC box that alters the nucleotides flanking the Msx consensus binding site, results in an increase in OCBP binding and a decrease in Msx binding. This results in increased promoter activity when assayed in ROS 17/2.8 cells. Conversely, mutations in the OC box that decrease OCBP binding results in significant loss of promoter activity (Hoffmann et al., 1996). It would therefore appear that OCBP is a positive regulator of this gene, whilst Msx functions to suppress transcription.

It will be interesting to learn at what level in the pathway leading to bone formation Msx acts as a repressor. Msx-binding sites have been identified in the promoters of several bone-specific genes, including the avian Bone sialoprotein (Bsp) encoding a major non-collagenous protein located in the extracellular matrix of skeletal tissue.
(Yang and Gerstenfeld, 1997). However, it has not been shown that Msx binds to these sites in vivo.

A missense mutation in human MSX 2 (a histidine substitutes for a proline at position 7 within the N-terminal arm of the homeodomain) causes Boston-type craniosynostosis. This highly penetrant, dominantly inherited syndrome, is characterised by the premature closure of the cranial sutures (Jabs et al., 1993). In mouse, Msx 2 is expressed in the cranial sutures in a region termed the osteogenic front (Jabs et al., 1993). This is a zone in which precursor, neural crest-derived mesenchyme undergoes chondrogenesis in a developmentally controlled manner, allowing the calvarial bones to grow without fusing around the expanding brain. The dominant nature of Boston-type craniosynotosis suggests that either the amount of Msx 2 is critical for normal maintenance of the osteogenic front or that mutant Msx 2 has a dominant-negative effect. Transgenic mice ectopically expressing either wild type or mutant Msx 2, from the CMV promoter, also show premature fusion of the cranial sutures (Liu et al., 1995). This observation perhaps argues against haploinsufficiency.

Miz 1, a zinc finger transcription factor, can interact with Msx 2 in the yeast two-hybrid system and in GST affinity purification experiments (Wu et al., 1997). The expression of this protein overlaps with Msx 2 at many sites; facial primordia, neural tube and hindbrain derived neural crest, but its expression during chondrogenesis has not been reported. In common with Msx 2, Miz 1 can bind to sequences in the OC box of the Osteocalcin promoter. The interaction between these proteins greatly increases the ability of Msx 2 to bind the OC box (Wu et al., 1997). Mutant Msx 2, carrying the Boston-type craniosynotosis mutation, interacts 30 % more avidly with Miz 1 than wild type Msx 2. This enhanced interaction results in a two-fold increase in binding affinity to the OC box of Osteocalcin, compared to wild type Msx 2 interacting with Miz 1. In isolation, mutant Msx 2 binds the OC box with similar affinity to wild type Msx 2 (Wu et al., 1997). The Boston-type craniosynotosis phenotype may therefore be due to an increase in the DNA-binding affinity of Msx 2.
to OC box-like motifs, either as a consequence of increased interaction with Miz 1, in the case of mutant Msx 2, or increased amounts of Msx 2, in the case of over-expression.

Although the Msx and Bmp genes have been implicated in regulating chondrogenesis at numerous sites; dorsal components of the vertebrae, limbs, facial primordia and calvaria, they are by no means universal players in chondrogenesis. This suggests that other cell types utilise an alternative set of genes.

1.3.8 Apoptosis

In the hindbrain region, the neural tube is subdivided into segments termed rhombomeres. Neural crest cells originate and migrate from the dorsal side of the rhombomeres to the branchial arches. Fate mapping experiments have demonstrated that this movement of cells is precisely patterned (Lumsden et al., 1991). Crest originating from rhombomeres 1, 2 and the posterior midbrain occupy the first branchial arch, cells from rhombomere 4 the second branchial arch and cells from rhombomere 6 the third branchial arch. No migratory cells are produced by rhombomeres 3 and 5 (Lumsden et al., 1991). The lack of cells emerging from these rhombomeres may enable neural crest from even rhombomeres to migrate in discrete streams.

Apoptosis (a process of programmed death) of cells in the dorsal side of rhombomeres 3 and 5 is responsible for the depletion of neural crest at these locations (Graham et al., 1993). Rhombomeres 3 and 5 can produce neural crest that does not undergo apoptosis when isolated from neighbouring rhombomeres and grown in culture (Graham et al., 1993). This might suggest that signals from neighbouring rhombomeres induce cell death in 3 and 5. This idea was supported by the observation that addition of the neighbouring rhombomere to the culture assay restored apoptosis. In vivo, replacement of rhombomere 4 with rhombomere 3, from a stage-matched donor, allowed the production of neural crest by the donor.
rhombomere 3 and also the host's rhombomere 3, indicating that such a mechanism also operates in the embryo (Graham et al., 1993).

Msx and Bmp 4 are expressed in the rhombencephalon during neural crest production. In stage 11 chicks, Msx 1 is expressed in the dorsal hindbrain up to the level of rhombomere 5, whilst Msx 2 is exclusively expressed in rhombomeres 3 and 5 (Graham et al., 1993). Increased Bmp 4 expression is also observed at this stage in rhombomeres 3 and 5, overlapping with Msx 2 expression (Graham et al., 1994). These expression patterns suggest a positive correlation between Msx 2/Bmp 4 and apoptosis. This is further supported by the observation that Msx 2 and Bmp 4 are down-regulated in rhombomere 3 when either cultured in isolation, or when this rhombomere is grafted into the rhombomere 4 position (Graham et al., 1993, 1994). In common with other sites of Msx and Bmp 4 function, Bmp 4 appears to activate Msx 2 in rhombomeres 3 and 5. Addition of Bmp 4 to cultured rhombomeres 3 or 5 restores normal Msx 2 expression and the onset of apoptosis (Graham et al., 1994). Interestingly, addition of Bmp 4 to rhombomere 4 does not induce Msx 2 or apoptosis implying that the neighbouring rhombomeres, that induce apoptosis, are themselves unable to respond to Bmp 4 (Graham et al., 1994). This may be due to the absence of Bmp 4 receptors.

In summary, Bmp 4 is induced in rhombomeres 3 and 5 in response to a signal emanating from neighbouring rhombomeres. The nature of this signal is unknown but if an analogous situation exists to that which is found in tooth bud development, the signal could be Bmp 4, derived from even rhombomeres. Bmp 4 within rhombomeres 3 and 5 then activates Msx 2, the single or combined action of these genes resulting in apoptosis. Alternatively, the expression of these genes predisposes cells in rhombomere 3 and 5 to additional cues that initiate apoptosis. There is no direct evidence to suggest that Msx 2 is involved in apoptosis and this process may be an independent event triggered by Bmp 4. It would be interesting to learn if forced expression of Msx 2 in an isolated rhombomere 3 would restore apoptosis.
An association between apoptosis, *Msx 2* and *Bmp 4* expression has also been shown in the developing limb. During limb development the separation of individual digits is achieved through apoptosis of the undifferentiated interdigital mesenchyme. The expression of *Bmp 4*, *Msx 1* and *Msx 2* prior to apoptosis in this region suggests that these genes may be involved in this cellular process. The involvement of Bmp 4 has been tested by grafting Bmp 4-soaked beads at the tip of the growing digit. This resulted in the expansion of both the *Msx 1* and *Msx 2* expression domains around the bead and was associated with ectopic apoptosis (Ganan et al., 1996). Cells respond to Bmp signals through the action of two types of Bmp receptor. Upon ligand binding, these two receptor types interact, activating the signal transduction pathway via phosphorylation of target proteins. Infection of developing chick limbs with a virus which produces a dominant-negative form of one of these receptors, containing a mutated kinase domain, leads to a reduction of interdigital apoptosis and the down-regulation of *Msx 2*, but not *Msx 1* or *Bmp 4* (Zou and Niswander, 1996). These two experiments indicate that interdigital apoptosis requires Bmp signalling that may act through the activation of *Msx 2*, though there is no evidence to suggest that *Msx 2* is directly involved in apoptosis.

A further positive correlation of *Bmp 4* and *Msx* expression with apoptosis was observed when beads soaked in Transforming growth factor (TGFβ1 or TGFβ2) were implanted into the interdigital mesenchyme of stage 29 chick limbs. This changed the cell fate of the surrounding cells from one of cell death to chondrogenesis. This was associated with the down-regulation of *Bmp 4* and both *Msx* genes in the cells surrounding the bead (Ganan et al., 1996). The addition of a second bead containing FGF 2 in close proximity to the TGFβ bead inhibited the chondrogenic-inducing properties of TGFβ (Ganan et al., 1996). FGF 2 and FGF 4 can also inhibit interdigit apoptosis when ectopically applied to this region, but in contrast to the effect of the TGFβs the expression of *Msx 1* and *Msx 2* is not affected (Macias et al., 1996). It would be interesting to know if *Bmp 4* is also down-regulated. If *Bmp 4* is not down-regulated, it would suggest that although Bmp 4 is required for apoptosis it is not sufficient. FGF 4 is expressed in the AER and beads soaked in this protein can
substitute for the absence of the AER in terms of maintaining Msx 1 expression in the progress zone (chapter 1.3.1). The normal function of FGF 4 could therefore be to maintain an undifferentiated population of cells through the action of Msx. Thus, the function of Bmp 4 and Msx 2 in the interdigital mesenchyme may not be to directly initiate apoptosis. These proteins may merely suppress differentiation, in keeping with their proposed function during myogenesis and chondrogenesis (chapter 1.3.6 and 1.3.7). In contrast to these latter processes, cells in the interdigital region fail to receive a cue to differentiate, or stop receiving a survival cue, perhaps FGF, and as a consequence of which enter either a default or Bmp 4-primed apoptotic pathway. Whether or not the Msx genes function in this apoptotic pathway remains to be tested.

P19 cells, an embryonal carcinoma cell line, do not express Msx 1 or Msx 2 when grown as monolayers, but do express these genes when grown as aggregates (Marazzi et al., 1997). Addition of Bmp 4 to P19 cells grown as aggregates increases Msx 2 expression, whilst addition of Bmp 4 to cells grown as monolayers did not alter Msx 2 expression (Marazzi et al., 1997). Aggregated P19 cells display a low level of apoptosis, which is increased by approximately two-fold in the presence of Bmp 4 or if stably transfected with Msx 2. Bmp 4 or constitutive Msx 2 expression has no apoptotic effect on P19 monolayers (Marazzi et al., 1997). These observations are consistent with the idea that Bmp 4 and Msx 2 are not sufficient to directly initiate apoptosis but merely confer apoptotic potential. The presence or lack of cues from the surrounding cellular environment governs the fate of such cells. In this culture system, adherence to the substratum is sufficient to silence the cell death pathway.

1.3.9 Other sites of Msx expression

The vertebrate sensory organs, ear, nose and eye, all develop from epithelial placodes. An additional shared feature is that they all express Msx 1 and Msx 2 during their development (Monaghan et al., 1991; Davidson, 1995). The possible functions performed by these genes in the developing eye will be explored later (chapter 1.5).
During chick ear development, \textit{Msx 1} is expressed in the dorsal aspect of the invaginating otic vesicle, subsequently becoming restricted to the endolymphatic apparatus, cristae, lagena, macula neglecta and parts of the semicircular canal (Suzuki \textit{et al.}, 1991; Wu and Oh, 1996). Although shown to be expressed in the ear, a detailed analysis of \textit{Msx 2} expression has not been reported. In keeping with many other sites of \textit{Msx} expression, an association with both \textit{Lef 1} and \textit{Bmp 4} expression is observed in the developing ear. \textit{Bmp 4} in chick, and \textit{Lef 1} in mouse, are expressed in the otic placode at the onset of invagination. \textit{Bmp 4} is expressed preferentially in the dorsal otic vesicle, correlating with the expression pattern of \textit{Msx 1} (van Genderen \textit{et al.}, 1994; Wu and Oh, 1996).

It is not yet clear if these genes function within the same genetic pathway during the development of the ear or what type of cellular process they may control. Mice null for \textit{Msx 1} do exhibit abnormalities in one of the middle ear ossicles implying that \textit{Msx 1} is required for normal ear development (Satokata and Maas, 1994).

Additional sites of \textit{Msx} expression during mouse embryogenesis, but where the function remains largely unknown, include the uterus, cervix, vagina and vibrissae which are all sites of epithelial-mesenchymal interactions. The \textit{Msx} genes are also expressed in the uterine wall, ectoplacental cone, amnion, allantosis, umbilical vein, Rathke’s pouch, heart, pericardium and genital tubicle (Davidson, 1995).

1.3.10 \textit{Msx} regulatory elements

To produce tissue-specific expression of \textit{Msx 1}, multiple elements within the promoter of the gene are required. These elements respond to different cell-type specific factors, repressing or activating transcription individually or in combination. Two such regulatory elements have recently been identified in the mouse \textit{Msx 1} promoter (MacKenzie \textit{et al.}, 1997a). The distal-most element (DE), -4006 to -4252 (bp from translation start site), is solely responsible for driving expression in the lateral nasal epithelium and the buccal mesenchyme of the first arch. A more proximal
element (PE), -2198 to -2276 (bp from translation start site), drives expression in the neural tube roof plate, epiphysis, eye, ventral tips of the myotome, proximal limb mesenchyme, AER and second pharyngeal arch. A combination of either DE and/or additional uncharacterised elements with PE accounts for expression in the otic vesicle and dorsal myotome. Msx 1 expression in the nasal mesenchyme, external ear precursor mesenchyme, dentition, Rathke’s pouch, skull bones and heart requires additional, as yet unknown, regulatory elements located more than five kb 5’ or four kb 3’ from the coding region (MacKenzie et al., 1997a). These elements are not contained within the intron (MacKenzie et al., 1997a).

It is interesting to note that Msx 1 expression within the dorsal and ventral myotome is controlled by a different combination of elements. In isolation the PE element can drive expression in both dorsal and ventral myotome like the native gene, but in combination with sequences 5’ to the PE element it only drives expression in the ventral myotome (MacKenzie et al., 1997a). This suggests that a negative element, on responding to a factor present only in the dorsal myotome, can suppresses PE activity. The fact that expression in the dorsal myotome is achieved when the gene is in its normal genomic position implies that there is yet another element involved, perhaps suppressing the negative regulatory element of PE.

The involvement of different sets of elements controlling dorsal and ventral myotome expression may be expected, since the signals required for Msx 1 maintenance at these two sites are thought to originate from different tissues. It has been proposed that myogenesis is inhibited in the ventral myotome by Bmp 4 originating from the lateral mesoderm (chapter 1.3.6). The dorsal myotome comes under the influence of the dorsal neural tube. The nature of this signal is not known but Bmp 4, expressed in the dorsal neural tube, is a possible candidate. The function of Msx 1 in the dorsal myotome is also unclear, but might be involved in repressing chondrogenesis in precursor spinous process cells that may be recruited from this site (chapter 1.3.7). Thus there are both similarities and differences in the regulation and function of Msx 1 at these sites. The possible shared requirement for Bmp 4 and function as a repressor
of differentiation may account for the shared requirement for the PE element, whilst
the differences in Bmp 4 origin and terminal fate of the Msx 1-expressing cells
account for the requirement of other different regulatory elements.

The normal regulation of Msx 1 in the limb and eye would appear to be controlled by
at least two elements, operating sequentially during development. The PE initiates the
erly expression of the gene in the limb bud mesenchyme and dorsal margin of the
optic cup, but fails to drive later expression. In the limb, this results in ectopic
expression in the proximal mesenchyme instead of the normal AER-dependant
eexpression in the distal mesenchyme. In the eye, expression at the dorsal margin fails
to expand around the optic cup rim (MacKenzie et al., 1997a). These observations
imply that there is a requirement for additional regulatory elements on which a
different set of upstream molecules act in the distal limb mesenchyme and ventral
margin of the retina.

Analysis of the regulatory regions of Msx 2 have revealed that the majority of
elements required to drive the normal expression of the gene are contained within 5.2
Kb of 5’ flanking sequence (Liu et al., 1994a). This region of DNA is unable to drive
expression in the developing tooth and results in ectopic expression in the spinal
ganglia indicating that other elements must be located outside this region. An
AER-specific element has been shown to be located within the first 439 bp of 5’
flanking sequence (Liu et al., 1994a). A corresponding element has also been found
within the chick Msx 2 promoter which can direct expression specifically to the AER
in transgenic mice (Sumoy et al., 1995).

The acquisition of a new regulatory element during evolution could produce new
domains of Msx expression. The protein would only have to have a desirable
function at one of these new sites for the expression to be maintained at all other sites
under the control of this element. It is therefore possible that at sites of gene
expression controlled by the same element, the encoded protein at some of these sites
may have no function. Great caution should therefore be given to functional assumptions based solely on expression evidence.

1.4 Anatomy of the developing eye

In the mouse embryo, the first visible sign of eye development occurs at stage E8 when the optic sulcus forms in the neural ectoderm of the forebrain. This evagination becomes more pronounced, forming a pit at E8.5 and the optic vesicle at E9 (Coulombre, 1965; Kaufman, 1992; fig. 1.2). The optic vesicle is connected to the neural tube by the optic stalk. As the optic vesicle develops it comes into close contact with the overlying surface ectoderm coinciding with the formation of the lens placode in the surface ectoderm. The induction processes occurring at this point are reviewed in chapter 1.5.3. The lens placode, which forms at E9.5, is essentially a thickening of the surface ectoderm and invaginates to form the lens (Coulombre, 1965; Kaufman, 1992).

By E10, the optic vesicle has formed a cup-shaped structure with an inner layer and an outer layer that eventually give rise to the neural and pigmented layers of the retina respectively (Coulombre, 1965; Kaufman, 1992; fig. 1.2). The first morphological distinction that can be made between the two layers of the optic cup is a marked increase in the thickness of the presumptive neural retina compared to the presumptive pigmented retina epithelium. Differentiation of the neural retina occurs in an asynchronous manner, the innermost layers differentiating first, the outer layers last and with mitotic activity ceasing first towards the back of the cup (Young, 1985). The neural retina eventually differentiates into seven distinct cell types, the cell bodies of which are arranged in one of three layers. Ganglion cell bodies are located in the ganglion cell layer which is closest to the lens. A plexiform layer of fibres and synapses separates this layer from the inner nuclear layer in which the cell bodies of bipolar, horizontal, amacrine and Muller cells are located. The external nuclear layer is adjacent to the pigmented epithelium and contains the cell bodies of rod and cone photoreceptor cells. The external and inner nuclear layers are also separated by a
plexiform layer (Burkitt, Young and Heath, 1993; fig. 1.3). The most distal region of the cup gives rise to the ciliary body, clearly recognisable by E14.5, and the iris (Coulombre, 1965; Kaufman, 1992). Epithelial cells, in the most ventral region of the cup, invaginate producing a groove (termed the choriod fissure) that runs from the optic margins down the optic stalk. This groove allows mesenchyme to invade the optic cup, where it can from the hyaloid vascular plexus (Coulombre, 1965).

The surface ectoderm, overlying the optic vesicle, starts to invaginate at E10, first forming a pit which by E11 has internalised forming the lens vesicle (Coulombre, 1965; Kaufman, 1992). Posterior lens vesicle epithelium quickly starts to elongate and differentiate into lens fibre cells which eventually fill the entire vesicle. Secondary fibres are produced throughout the life-time of the lens. Secondary fibres are derived from anterior lens epithelium which retains the ability to undergo mitosis. This germinal population of cells is thought to be located slightly anterior to the lens equator. Daughter cells produced in this region move posteriorly, differentiating into lens fibres at the equator. It is interesting to note that the retina tips are in close proximity with the lens equator, suggesting that they may influence the proliferation and differentiation events occurring at this site.

The surface ectoderm overlying the lens can be considered as presumptive corneal epithelium. This epithelium, together with neural crest-derived mesenchyme, gives rise to the transparent cornea (Coulombre, 1965). By E16, the anterior chamber develops in the perioptic mesenchyme between the lens and cornea. On the anterior side of the cornea, the conjunctival sac is formed by the fusion of the eyelids (Coulombre, 1965; Kaufman, 1992).

The perioptic mesenchyme differentiates into two layers. The inner-most forms a highly vascularised pigmented layer known as the choroid and the outer-most forms a layer known as the sclera which is continuous with the mesenchyme component of the cornea (Coulombre, 1965).
Fig. 1.2 Schematic diagram of early eye development in mouse. At E8 the optic sulcus forms in the neural ectoderm, giving rise to the optic pit by E8.5. The optic pit evaginates forming an optic vesicle by E9. At E10, the optic vesicle invaginates to form a cup-shaped structure. The outer layer differentiates into the pigmented retina epithelium (PRE), whilst the inner layer gives rise to the neural retina. At E9.5, the surface ectoderm starts to thicken over the optic vesicle to form the lens placode. The placode then invaginates forming a pit by E10.5 and a vesicle by E11.5.
Fig. 1.3 Schematic diagram of the laminar structure of the retina. Ganglion cell bodies are located in the inner most layer of the retina (ganglion cell layer). The cell bodies of bipolar, amacrine and horizontal cells are located in the inner nuclear layer. The external nuclear layer is adjacent to the pigmented retina epithelium (PRE) and contains rod and cone cell bodies. Ganglion cells make connections with cells of the inner nuclear layer within the inner plexiform layer. Cells of the inner nuclear layer make connections with photoreceptors (rod and cone cells) within the outer plexiform layer. Muller cells stretch between the inner limiting membrane and external nuclear layer. Diagram adapted from Burkitt, Young and Heath, 1993.
The morphological processes taking place during the formation of the avian eye are essentially the same as those occurring during mouse eye formation. However, the major difference is the speed at which these occur. At the start of the second day of incubation the optic vesicle of a chick embryo is morphologically equivalent to an E9 mouse optic vesicle (Hamburger and Hamilton, 1951; Kaufman, 1992). After 48 hours of incubation, the chick lens has been induced and the optic vesicle is becoming cup-shaped (Hamburger and Hamilton, 1951). This is equivalent to the optic cup of an E10 mouse embryo (Kaufman, 1992). By 72 hours, the chick eye is equivalent to an E12-E12.5 mouse eye in that the lens vesicle has separated from the ectoderm and lies within the optic cup, the two layers of which are clearly distinguishable (Hamburger and Hamilton, 1951; Kaufman, 1992).

1.5 The molecular basis of eye development

1.5.1 Possible master control genes

Components of the vertebrate eye develop from tissues of different developmental origin; surface ectoderm, neural ectoderm and neural crest-derived mesenchyme. A unifying feature is that they all share the expression of specific genes during their development. This raises the possibility that the same genes are required for the formation of different components of the eye, possible representing master control genes. For a gene to function as a master control gene it should not only be necessary for eye development but also capable of initiating eye formation. Whilst numerous genes have been shown to be essential for eye development, few have been shown to be able to induce ectopic eye formation. Furthermore, this capability is limited to specific sites of the embryo suggesting that essential co-factors are required.

The transcription factor, Pax6, may be a master control gene for eye development. This paired-box and homeodomain-containing gene is expressed in the optic pit at E8, expression being maintained in the optic vesicle and cup (Grindley et al., 1995). Pax6 transcripts are evenly distributed in both layers of the optic cup but progressively
become restricted to the distal margins of the developing retina. Transcripts within the neural retina also become progressively restricted to the inner-most layer (Grindley et al., 1995). The broad domain of Pax 6 expression, observed in head surface ectoderm at E8, progressively becomes restricted to the lens and nasal placodes. Pax 6 expression continues in the developing lens; detected in the placode, pit, vesicle and differentiating lens. The developing cornea also expresses Pax 6 (Grindley et al., 1995).

Small eye (Sey) in mice and aniridia (AN) in humans are semi-dominant conditions caused by mutations in Pax 6 (Hill et al., 1991; Ton et al., 1991). Homozygous Sey mice fail to develop a lens placode and to progress from an optic vesicle to an optic cup, consequently these mice lack eyes (Grindley et al., 1995). Mice and humans heterozygous for Pax 6 do form eyes, but these are associated with a variety of anterior eye abnormalities. In humans, this includes the absence or reduction in the size of the iris, characters diagnostic for aniridia, or the fusion of the cornea to the lens, diagnostic for Peter’s anomaly (Ton et al., 1991; Hanson et al., 1994). In addition to the above defects, Sey heterozygous mice also show a general reduction in the size of the eye (Hill et al., 1991). Whilst one functional copy of the Pax 6 gene is sufficient for lens placode and optic cup formation, gene dosage does appear to be critical during the later stages of eye development. Indeed, both haploinsufficiency and over-expression of Pax 6 lead to abnormalities of the ciliary body, iris and cornea in mice (Schedl et al., 1996).

Eyeless (Ey) is the Drosophila homologue of Pax 6, possessing 94% amino acid sequence identity to human and mouse Pax 6 (Quiring et al., 1994). In common with mouse and human Pax 6, Ey is essential for eye development. Targeted expression of Ey or mouse Pax 6 to wing, leg and antenna imaginal discs resulted in the formation of complete and morphologically normal eyes at these sites (Halder et al., 1995). Pax 6 may therefore be a master control gene for eye development since in mouse it is expressed in both surface and neural ectoderm derived structures of the eye, is essential for eye formation and, in Drosophila, can single-handedly trigger a
developmental pathway leading to eye formation. It is unlikely that \textit{Pax 6} is the sole master gene for eye development since it may not be able to induce eye formation at all sites of the embryo, suggesting that essential co-factors are expressed in imaginal discs.

\textit{Drosophila Sino oculis (So)} may also be a master control gene for eye development. This gene encodes a homoedomain-containing protein which when mutated leads to the failure of eye development (Cheyette \textit{et al.}, 1994). Three mouse homologues of this gene have been identified (\textit{Six} family), one of which is widely expressed during eye development (Oliver \textit{et al.}, 1995). At E9.5, \textit{Six 3} is expressed in the optic vesicle and optic stalk, with expression becoming confined to the neural retina and subsequently the inner nuclear layer (Oliver \textit{et al.}, 1995). \textit{Six 3} is also expressed in the developing lens, with expression becoming stronger in the anterior epithelium of the lens at later stages of development (Oliver \textit{et al.}, 1995). Thus, \textit{Six 3} is co-expressed with \textit{Pax 6} in derivatives of both the surface and neural ectoderm. Although the consequence of \textit{Six 3} loss-of-function on mouse eye development is not known, the fact that in \textit{Drosophila} both \textit{So} and \textit{Ey} mutants lack eyes indicates that these two genes may function in the same genetic pathway.

Ectopic expression of mouse \textit{Six 3} in killifish medaka has been achieved by injecting CMV-driven \textit{Six 3} cDNA into 2–4 cell stage medaka. This resulted in the formation of ectopic lenses in a region of the head which would normally give rise to the otic vesicle (Oliver \textit{et al.}, 1996). The ectopic lenses had a characteristic lens morphology and expressed \textit{Crystallin} and \textit{Pax 6}. Ectopic \textit{Six 3}-expressing cells were uniformly distributed throughout the embryo, yet ectopic lens were only observed at the site of the otic placode. This implies that only the otic placode region is competent to respond to \textit{Six 3}. Thus, for \textit{Six 3} to be sufficient for lens formation, additional co-factors are required. No ectopic \textit{Pax 6} expression was observed in \textit{Six 3}-expressing cells outwith the otic placode region. Therefore, the observed \textit{Pax 6} expression in ectopic lenses may reflect a late requirement for Pax 6 rather than direct activation by \textit{Six 3}. 

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A third *Drosophila* gene, which when mutated leads to an eyeless phenotype and consequently may be a master gene for eye development, is *Eyes absent* (*Eya*, Bonini *et al.*, 1993). Three mouse homologues of this gene have been identified (*Eya* family) and are all widely expressed in the developing eye. *Eya 1* is expressed in the lens placode at E9.5 (Xu *et al.*, 1997). Expression of *Eya 1* continues in the lens vesicle, becoming stronger in anterior epithelial cells and weaker in lens fibres (Xu *et al.*, 1997). *Eya 3* is not expressed in the lens placode, but expression can be detected in the lens vesicle after E10.5.

In derivatives of the neuroepithelium, *Eya 1* is expressed in the distal margins of the optic cup, presumptive pigmented retina epithelium and the optic stalk. *Eya 2* is expressed in more proximal parts of the eye including the presumptive sclera and the neural retina. *Eya 2* expression in the retina later becomes confined to the inner nuclear layer. *Eya 3* is expressed in the optic vesicle and perioptic mesenchyme (Xu *et al.*, 1997). The consequence of loss-of *Eya* function for eye development in mouse is not known.

The combined expression of *Msx 1* and *Msx 2* encompasses all the early structures of the mouse eye. At E9.5, *Msx 2* is expressed in the neuroepithelium-derived optic vesicle, in cells that are presumed to form the inner layer of the retina (Monaghan *et al.*, 1991). *Msx 1* is also expressed in the optic neuroepithelium, but is localised to the distal tips of the neural retina (presumptive ciliary body). *Msx 1* is not detected in this region until E12.5 (Monaghan *et al.*, 1991). Monaghan *et al.* (1991) report that *Msx 2* is not expressed in the distal tips of the neural retina and that detectable expression in the remaining neural retina ceases at E12.5 (Monaghan *et al.*, 1991). However, a more detailed analysis of *Msx 2* expression reveals that this gene is expressed in the dorsal distal tips of the neural retina (chapter 3).

Components of the eye derived from surface ectoderm also express *Msx 2*; lens placode, lens vesicle, lens and corneal epithelium (Monaghan *et al.*, 1991). *Msx 1* is
expressed in mesenchymal cells surrounding the optic vesicle and later, at E11.5, in mesenchymal cells between the surface epithelium and the lens (Monaghan et al., 1991).

Mice null for Msx 1 or Msx 2 exhibit no detectable eye abnormalities, but mice null for both these genes display a range of eye defects (Satokata and Maas, 1994; Rauchman et al., 1997). These defects range from arrested eye development at the optic vesicle stage, phenotypically similar to Sey/Sey, to general microphthalmia. Msx genes are therefore essential for mouse eye development and in the absence of one the other can substitute for its function. With the exception of a small region of the distal neural retina, these genes are not co-expressed during eye development. The apparent functional redundancy between these two genes could be explained if both genes controlled the production of the same secreted molecule. In such a scenario, provided Msx 1- and Msx 2-expressing tissues were located close to one another, and indeed this is the case, the lack of secreted molecule production in one tissue might be compensated for by secretion from the neighbouring tissue.

The Msx genes are therefore possible master control genes for eye formation since they are expressed by neuroepithelium-, surface ectoderm- and neural crest-derived components of the eye, and are essential for the formation of the eye. However, whether these genes are sufficient for eye development remains to be investigated.

The fact that Msx 2 and Pax 6 are co-expressed in regions of the developing eye and that there is a requirement for both Msx and Pax 6 in order to proceed to the optic cup stage, implies that these genes may function in a common pathway. In support of this Msx 2−/−/Pax 6−/− mice exhibit an arrested optic vesicle phenotype similar to both Sey/Sey and Msx double-knockout mice (Rauchman et al., 1997). In Sey/Sey E9.5 mice, Msx 2 and Eya 1 expression is reduced, but not absent, in the lens placode (Rauchman et al., 1997, Xu et al., 1997). Therefore, it is possible that Pax 6 is not required for the initiation of Msx 2 and Eya 1 in the lens placode, but is required to maintain expression at maximum levels. Six 3 expression is unaffected in the brain of
E12.5 Sey/Sey mice, but whether expression is affected during the initial stages of eye development is not reported (Oliver et al., 1995).

1.5.2 Retina development

1.5.2.1 Recruitment of retinal cells

The first process to occur during the development of the retina is the specification of the optic sulci from the forebrain and their evagination. Genes expressed in the early optic neuroepithelium and required for the formation of both the neural and pigmented retina epithelium may function to control the recruitment of retinal cells. However, the mechanisms that these genes control are not known.

The murine gene Rx encodes a paired-like homeodomain-containing protein that is transcriptionally active during this initial stage of retina development (Mathers et al., 1997). Expression is first detected at E7.5 in the anterior neural plate and progressively becomes restricted to the optic sulci and a small region of the ventral forebrain. Rx expression is maintained in the optic vesicle and across all layers of the neural retina. The literature does not state whether the pigmented retina epithelium expresses this gene. Down-regulation of Rx coincides with the terminal differentiation of each cell type in the neural retina, so that by postnatal day 6.5 Rx is only expressed in the photoreceptor and inner nuclear layers (Mathers et al., 1997).

Mice null for Rx fail to develop eyes as a consequence of the failure to form optic sulci (Mathers et al., 1997). This phenotype suggests that Rx function is vital during the initial formation of the optic sulci, either for the recruitment of neural plate cells to a retinal fate or for their subsequent proliferation. This proposed function is supported by the observations that ectopic over-expression of Xenopus Rx, by injecting Xrx 1 RNA into 4-8 cell stage Xenopus embryos, resulted in the formation of ectopic retinal tissue and hyperproliferation of the endogenous retina (Mathers et al., 1997).
Sey/Sey mice do develop optic sulci which form optic vesicles, but fail to form optic cups (chapter 1.5.1). This implies that during retina development, Rx function is required before that of Pax 6. Rx may therefore be upstream of Pax 6 or, alternatively, these two proteins may function in parallel pathways.

Mouse Lhx 2, a LIM homeobox gene, is also expressed during the early stages of optic vesicle formation. At E8.5, this gene is expressed in the developing optic vesicle, with expression later becoming confined to the neural retina. Postnatally, Lhx 2 is confined to the inner nuclear layer of the retina (Porter et al., 1997). In contrast to Rx, Lhx 2 function is not required for the initial specification of the presumptive optic vesicle or their subsequent evagination, but is required for the transition from vesicle to cup. The failure of optic cup formation in Lhx 2−/− mice is also associated with the absence of lens and retina structures (Porter et al., 1997).

The Lhx 2−/− phenotype is very similar to that observed in both Sey/Sey and Msx double-knockout mice, raising the possibility that these three genes may be operating in a common pathway (chapter 1.5.1). However, Pax 6 expression is maintained in the optic vesicles of Lhx 2−/− mice and conversely Lhx 2 expression is maintained in the optic vesicles of Sey/Sey mice (Porter et al., 1997). Pax 6 and Lhx 2 are therefore both independently essential for the transition of the optic vesicle to optic cup. The relationship between Lhx 2 and Msx genes has not yet been investigated, but it will be interesting to learn whether or not these genes function in a common pathway.

Lhx 2−/− mice do not express Pax 6 in the surface ectoderm overlying the optic vesicle. This probably accounts for the failure of lens placode formation in these mice (Porter et al., 1997). Lhx 2 expression has not been reported in the surface ectoderm of the eye, this implies that Lhx 2 function in the optic vesicle is essential for Pax 6 expression in the lens placode. Whether Lhx 2 is directly involved in the regulation of a signalling molecule, or the failure to express Pax 6 in the surface ectoderm merely reflects the general consequence of arrested optic vesicle development, is not known. It is interesting to note that Msx double-knockout mice, which also exhibit arrested
optic vesicle development and consequently anophthalmia (chapter 1.5.1), do still express \( Pax \) 6 in the surface ectoderm, albeit at a reduced level. This supports the argument that \( Lhx \) 2 in the optic vesicle could be directly involved in regulating \( Pax \) 6 expression in the presumptive lens placode. This observation also suggests that if \( Lhx \) 2 and \( Msx \) 2 operate in a common pathway during optic vesicle development, \( Lhx \) 2 is more likely to be upstream of \( Msx \) 2 than downstream.

Other genes that may play an important role in the initial specification and outgrowth of the optic vesicle is the murine homologue of \textit{Drosophila tailless (Mll)} and members of the \textit{Zic} gene family. At E9, \textit{Mll} is expressed throughout the evaginating optic neuroepithelium, expression being maintained in both layers of the optic cup and the optic stalk at E10.5. Expression is absent from the ciliary body region at this stage (Monaghan \textit{et al.}, 1995). The \textit{Zic} gene family comprises three members which are homologues of the \textit{Drosophila Odd-paired} gene. At E9.5, all three \textit{Zic} genes are expressed in the optic vesicle and optic stalk (Nagai \textit{et al.}, 1997). The expression of the \textit{Zic} genes is dynamic with different members becoming expressed in different derivatives of the optic vesicle. This will be discussed in more detail later. Until loss-of-function mutants for \textit{Zic} and \textit{Mll} are reported, any involvement of these genes in the process of optic vesicle evagination remains speculative.

1.5.2.2 Recruitment of neural retina and pigmented retina epithelium from the optic vesicle

Cells of the optic vesicle are recruited into two distinct cell types; the presumptive neural retina and the presumptive pigmented epithelium. Numerous genes are progressively restricted to, or expressed \textit{de novo}, in one or other of these cell types, and thus may function in recruiting neural retina and pigmented retina epithelium cells.

\textit{Chox 10} encodes a paired-type homeodomain protein which is transcriptionally active at E9.5 in the developing optic vesicle. In contrast to \textit{Rx}, \textit{Lhx 2}, \textit{Pax 6}, \textit{Zic} and \textit{Mll}, expression of \textit{Chox 10} is not detected uniformly through the optic vesicle but is
confined to the region that will give rise to the presumptive neural retina (Liu et al., 1994b). *Chox 10* is specifically expressed in the neural retina until the onset of terminal differentiation, at which point expression is down-regulated in the differentiating cell types with exception of bipolar cells which continue to express *Chox 10* (Liu et al., 1994b). *Msx 2* expression also becomes restricted to the neural retina before being down-regulated prior to the onset of differentiation (chapter 1.5.1). These two genes may therefore play a role in recruiting optic vesicle cells to a neural retina cell fate, with *Chox 10* having an additional role in cells of the inner nuclear layer. A null mutation of *Chox 10* causes *Ocular retardation (Or)*, a recessive mouse mutation (Burmeister et al., 1996). Mice homozygous for this mutation exhibit microphthalmia as a consequence of reduced cell proliferation within the neural retina and are associated with the complete absence of bipolar cells (Burmeister et al., 1996). These mice do have a neural retina, demonstrating that *Chox 10* is not essential for the specification of neural retina cells, as might have been suggested by its expression pattern, but does control the proliferation of neural retina precursor cells and the specification or differentiation of bipolar cells.

At E10.5, as the optic vesicle forms the optic cup, *Zic 1* expression is maintained in both layers of the cup. In contrast, both *Zic 2* and *Zic 3* are restricted to the inner layer of the optic cup (Nagai et al., 1997). By E13, *Zic 1* is strongly expressed in the inner nuclear layer, while *Zic 2* is strongly expressed in the external nuclear layer of the retina. Expression of *Zic 1* and *Zic 2* progressively moves towards the distal margins of the neural retina. By E16.5, both genes are expressed in the ciliary body region (Nagai et al., 1997). The expression of these genes overlaps with the *Msx* genes, in particular *Zic 2* expression overlaps that of *Msx 2*, suggesting that they may be functioning in a common pathway, though this remains to be tested. *Zic 1*, *Zic 2* and *Zic 3* are also expressed in the dorsal neural tube (Nagai et al., 1997), a region of the embryo that also expresses the *Msx* genes (chapter 1.3.5). This is consistent with the possibility that the *Msx* and *Zic* genes may function in a common pathway. Whether *Zic 2* and *Zic 3* are involved in specifying neural retina cell fate or if their
expression patterns are merely a secondary consequence of neural retina specification remains to be investigated.

The mouse mutant, *Microphthalmia*, is caused by mutations in the basic helix-loop-helix-leucine zipper transcription factor *Mitf* which is expressed in the pigmented retina epithelium. These mice have small unpigmented eyes associated with abnormal thickening of the presumptive pigmented retina epithelium (Hodgkinson *et al.*, 1993).

The chick homologue of *Mitf* is expressed within cells of the optic vesicle that are presumed to give rise to the pigmented retina epithelium. As the retina develops, expression remains restricted to the pigmented retina epithelium, though expression was not detected after hatching (Mochii *et al.*, 1998). Thus the *Mitf*-mutant phenotype and expression pattern suggest this gene may play a role in establishing a pigmented retina epithelium fate in cells of the optic vesicle.

A chick pigmented retina epithelium culture system has been used to further explore the function of *Mitf*. Pigmented retina epithelium grown in the presence of FGF 2 lose the expression of pigment cell-specific genes, *Mmp115* and *Tyrosinase*. This correlated with the loose of *Mitf* expression. Conversely, when these cells were redifferentiated, by removing FGF 2 from the medium, *Mitf*, *Tyrosinase* and *Mmp115* expression was restored (Mochii *et al.*, 1998). These experiments demonstrate that *Mitf* expression correlates with the differentiation of pigmented retina epithelium cells. Cultured pigmented retina epithelium cells, constitutively expressing *Mitf*, fail to exhibit a down-regulation in *Tyrosinase* and *Mmp115* expression when treated with FGF 2 (Mochii *et al.*, 1998). Therefore, *Mitf* regulates at least part of the pigmented retina epithelium differentiation pathway.

Within the eye, *Mitf* may therefore regulate the expression of genes encoding enzymes that play a role in the synthesis of pigment. These genes could include *Tyrosinase*, *Tyrosinase-related protein 1* (*Trp 1*) and *Tryosinase-related protein 2*.

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They are all specifically expressed in the presumptive pigmented epithelium layer of the optic cup and their expression is subsequently maintained in the differentiated pigmented retina epithelium (Beermann et al., 1992; Steel et al., 1992). *Trp 2* expression starts at E9.5 in part of the optic vesicle destined to form the pigmented epithelium. *Trp 1* expression commences at E11.5, in the outer layer of the retina, and *Trypsinase* expression between E10.5 and E11.5, in the outer layer of the optic cup (Beermann et al., 1992; Steel et al., 1992).

1.5.2.3 Neurogenesis in the retina

A central to peripheral gradient of neurogenesis occurs within the retina (Young, 1985). Thus, the expression of genes involved in the maintenance of undifferentiated neural retina precursors may progressively become restricted to the distal margins of the optic cup. The expression of *Msx 2* in the developing mouse retina has this pattern suggesting a possible involvement in maintaining undifferentiated precursors.

Members of the *Notch* family encode transmembrane receptors. *rNotch 1* in rat, *cNotch 1* in chick and *Xotch* in *Xenopus* are all expressed in undifferentiated cells of the neural retina and become progressively confined to the distal margins of the retina as they differentiate (Ahmad et al., 1995; Austin et al., 1995; Dorsky et al., 1995). *cNotch 1* has two known membrane bound ligands, *cDelta* and *cSerrate*. *cSerrate* is co-expressed with *cNotch 1* in the lens, but not the retina, whilst *cDelta* is co-expressed with *cNotch 1* in the developing retina (Myat et al., 1996; Ahmad et al., 1997). The Notch/Delta signalling pathway controls the progression of precursor cells to a more differentiated state via lateral inhibition (Fortini and Tsakonas, 1993). Differentiating retinal cells may express *Delta* at an increased level, which results in the stimulation of Notch receptors on neighbouring cells which inhibits their differentiation. Indeed, cell-cell contact is important for restraining differentiation. Cultures of dissociated chick neural retina grown at low density have an increased percentage of differentiated ganglion cells compared to cultures grown at a high density (Austin et al., 1995). This could be due to physically preventing Notch/Delta
interactions. In support of this, addition of anti-sense Notch 1 or Delta 1 to cultured chick retina explants also results in an increase in ganglion cells (Austin et al., 1995; Ahmad et al., 1997). Conversely, over-expression of an activated form of Notch in chick retinal cells, resulted in a decrease in the number of ganglion cells (Austin et al., 1995).

Neurogenic genes, such as Notch and Delta, prevent neural differentiation by inhibiting the expression of proneural genes. Proneural genes promote neural differentiation. Mash 1 is the mammalian homologue of the Drosophila Achaete-scute proneural gene complex and is expressed in the developing mammalian retina (Guillemot and Joyner, 1993a). This basic helix-loop-helix transcription factor is an excellent candidate target for the Notch signalling pathway. Indeed, Notch 1-mutant mice exhibit increased Mash 1 expression in the midbrain and hindbrain and an expansion of the Mash 1 expression domains in the dorsal and ventral neural tube (Pompa et al., 1997). These observations are consistent with the Notch pathway suppressing Mash 1.

In the mouse retina, Mash 1 expression is detected at only low levels within the optic vesicle, significant levels of expression become evident only at E14.5 in proliferating neural retina progenitor cells (Guillemot and Joyner, 1993a). The ganglion layer does not express Mash 1. Terminal differentiation of the majority of rod, bipolar and Muller cells occurs at postnatal day 7; at this time Mash 1 expression ceases (Guillemot and Joyner, 1993a; Tomita et al., 1996a). The expression of Mash 1 at this late stage of retinal development, suggests that this proneural gene may play a role in promoting the differentiation of cells born late in development.

Mice null for Mash 1 die soon after birth but have not been reported to have retinal defects (Guillemot et al., 1993b). To explore later defects, retinal explants from Mash 1-null mice were cultured while observing the differentiation of rod, horizontal, bipolar and Muller glial cells (Tomita et al., 1996a). Differentiation of rod and horizontal cells was found to be delayed in the absence of Mash 1 but the final
quantity of each cell type was unaffected. In contrast, fewer bipolar and more glial
cells were observed in these cultures. Thus, *Mash I* is not required for the
specification of retinal cell types, but is involved in promoting the differentiation of
committed precursor cells at the correct time. *Mash I* may also function to ensure
that the correct proportions of Muller glial and bipolar cells are produced.

The *Drosophila* Notch/Delta signalling pathway acts through *Suppressor of hairless
(Su(H))* and *Enhancer of split (E(spl))* to regulate *Achaete-scute* (Fortini and
Tsakonas, 1994; Lecourtois and Schweisguth, 1995). The vertebrate homologue of
*Su(H)* is *RBP-Jk*, whilst *E(spl)* has five homologues termed *Hes 1-5* (Furukawa *et al.*, 1992; Sasai *et al.*, 1992; Takebayashi *et al.*, 1995). The expression of *RBP-Jk* in the
developing retina has not been reported, but the encoded protein is located in the
nucleus of cells in the neural tube and is the putative downstream effector of Notch
(Pompa *et al.*, 1997). Notch-activated *RBP-Jk* may result in the expression of *Hes*
genes (Jarriault *et al.*, 1995; Pompa *et al.*, 1997).

*Hes 1* is a basic helix-loop-helix transcription factor and is expressed in the dividing
precursor cells of the developing mouse retina (Tomita *et al.*, 1996b). Expression
ceases around postnatal day 7, at the end of retinal differentiation. In contrast to *Mash
I*, *Hes 1* suppresses neurogenesis and is therefore classed as a neurogenic gene. Mice
null for *Hes 1* display premature neuronal differentiation in the retina, associated with
This implies that *Hes 1* prevents neural differentiation by repressing the expression of
*Mash 1*. Retinal explant cultures from *Hes 1*-null mice produce ganglion cells in a
comparable manner to wild type explants. However, rod and horizontal cells
differentiate prematurely, consistent with *Hes 1* functioning in a pathway which
controls the differentiation of retinal precursor cells at later stages of development
(Tomita *et al.*, 1996b). Persistent expression of *Hes 1* in retinal precursor cells, in
cultured retinal explants infected with a constitutively *Hes 1* expressing vector,
prevented the differentiation of rod, bipolar or indeed any other neural retina cell type,
further demonstrating the role of Hes 1 as an inhibitor of neurogenesis (Tomita et al., 1996b).

Proliferation of the pool of precursor cells, produced by the action of the Notch signalling pathway, may be stimulated by Shh. Shh is expressed by postmitotic cells in the ganglion layer of E14.5 mouse retinas (Jensen and Wallace, 1997). It is not reported when expression starts in the retina, but expression continues into adulthood. Cells respond to Shh through the membrane bound protein Patched (Ptc). Precursor cells expressing Ptc, and therefore able to respond to Shh, are located adjacent to the Shh-producing cells (Jensen and Wallace, 1997). Addition of recombinant Shh to pellet neural retina cultures resulted in cell proliferation. The proportion of different cell types remained constant with or without Shh suggesting that Shh stimulates the proliferation of neural retina precursor cells but not their differentiation (Jensen and Wallace, 1997).

Retinal progenitor cells are multipotent implying that local environmental cues control the fate of these cells (Turner and Cepko, 1987). The antibodies VC1.1 and HPC 1 recognise N-Cam and Syntaxin respectively. These antigens are present on the surface of differentiated amacrine and horizontal cells of the rat retina (Alexiades and Cepko, 1997). The antibodies also label a population of proliferating retinal precursor cells, the majority of which differentiate into amacrine and horizontal cells (Alexiades and Cepko, 1997). However, a small proportion do differentiate into rod photoreceptors and bipolar cells. Thus, retinal precursors may be intrinsically biased toward a particular fate, but are not committed to a particular fate.

Ciliary neurotrophic factor (CNTF), or a related cytokine, may be one environmental cue regulating the fate of retinal precursor cells. Addition of recombinant CNTF to retina explants from postnatal day 0 rats resulted in a decrease in the number of rod photoreceptor cells and an increase in bipolar cells (Ezzeddine et al., 1997). Ezzeddine et al. (1997) demonstrate that CNTF acts on the rod precursor cells, switching their fate to one of bipolar cells. This implies that these precursor cells are
not irreversibly committed to the rod lineage. Indeed, even postmitotic cells that would normally become rod cells are blocked from doing so, and become bipolar in the presence of CNTF (Ezzeddine et al., 1997). CNTF has no effect on cells expressing opsin, indicating that by this stage of differentiation rod cells are committed to their lineage (Ezzeddine et al., 1997).

It is not clear if CNTF performs a similar role in vivo, since no retinal defect has been detected in mice null for Cntf (Masu et al., 1993). Functional redundancy between CNTF and related proteins could account for the lack of retinal phenotype. In support of this, CNTF and related molecules share receptors, whilst mice null for two of these receptors, Cntfα and Lifrβ, do exhibit retinal defects (Ware et al., 1995). Cntfα and Lifrβ null mice die soon after birth, though continued retina development can be achieved using explant cultures. It was observed, in such cultures, that there was an increase in the number of rod photoreceptors and that the addition of recombinant CNTF to these cultures had no effect on rod differentiation. This confirms that CNTF acts through Cntfα and Lifrβ (Ezzeddine et al., 1997).

1.5.2.4 Dorso-ventral polarity of the retina

Retinal cells need positional identity in order to make specific connections with cells in the optic tectum. It has been suggested that a cartesian co-ordinate system would allow cells to be positionally specified with respect to their position along the dorso-ventral and anterior-posterior axis (Sperry, 1963). Numerous genes have been identified that are asymmetrically expressed along the dorso-ventral axis. The encoded proteins have a variety of functions, from cell surface molecules to transcription factors. These proteins may either play a role in establishing the axis or be regulated as a consequence of the axis.

The transcription factors Brain factor (Bf), Sensory organ homeobox 1 (SOHo 1) and Xbr 1 in mouse, chick and Xenopus respectively, are all asymmetrically expressed along the dorso-ventral axis. Two Bf genes have been identified and encode
winged-helix proteins that can interact with DNA (Hatini et al., 1994). At E9.5, Bf 2 expression is restricted to the temporal half of the optic vesicle and stalk, expression being maintained in these regions as the retina develops (Hatini et al., 1994). Bf 1 is expressed in a very similar manner except that its domain of expression is adjacent to Bf 2, and therefore is expressed in the nasal half of the developing retina and optic stalk (Hatini et al., 1994). The level of expression is graded, so that strongest expression is detected towards the periphery of the retina (Hatini et al., 1994).

SOHo 1 encodes a novel homeodomain-containing protein which is expressed early in retina development (Deitcher et al., 1994). Expression is first detected, by in situ hybridisation, in the optic vesicle and later in both layers of the optic cup. It has not been shown whether SOHo 1 is asymmetrically expressed at these early stages but by stage 23, when the outer layer of the optic cup is becoming pigmented and the lens is well developed, SOHo 1 expression is restricted to the nasal half of the neural retina (Deitcher et al., 1994). Postnatally, SOHo 1 is expressed in all three layers of the retina (Deitcher et al., 1994).

Xbr 1 contains a homeobox similar to that found in the Drosophila Bar genes. However the homology is low, suggesting that Xbr 1 is not a Bar homologue but rather a member of the same class of gene (Papalopulu and Kintner, 1996). Expression of this gene is restricted to the dorsal half of the optic vesicle but unlike SOHo 1 and the Bf genes, becomes restricted to the dorsal ciliary margin (Papalopulu and Kintner, 1996). Bmp 4, encoding a secreted growth factor, is also expressed in the ciliary margin of Xenopus and, like Xbr 1, is restricted to the dorsal side of the retina. This raises the possibility that these two genes may be operating in a common pathway (Papalopulu and Kintner, 1996).

The expression of Xbr 1 correlates with at least two possible processes. In lower vertebrates, the ciliary margin contains a population of precursor cells that are able to generate all cell types found in the retina (Wetts et al., 1989). Xbr 1 may therefore function in maintaining this population of cells. Alternatively, Xbr 1, Bf 1, Bf 2 and
SOHo 1 could all be involved in establishing a dorso-ventral axis. This could involve the activation of cell signalling molecules, that diffuse producing a gradient of information. In the case of Xbr 1, this molecule could be Bmp 4. It will be interesting to learn if higher vertebrates have an Xbr 1 gene and to examine the functional relationship between Bmp 4 and Msx 1 in the ciliary margin.

It remains possible that Bf 1, Bf 2 and SOHo 1, which are not restricted to the periphery of the retina, are expressed in response to a dorso-ventral signal, rather than being the primary cause of the signal.

In addition to *Xenopus* Bmp 4, Radar (a TGF superfamily member) is a candidate dorso-ventral signalling molecule. *Radar* expression cannot be detected in the optic vesicle of the developing zebrafish, but the gene is activated later in the dorsal half of the retina, directly opposite the choroid fissure (Rissi *et al.*, 1995). Sequence comparisons strongly suggest that *Radar* is the zebrafish homologue of mouse *GDF 6*. However, it is not known if *GDF 6* is expressed in the mouse eye.

There is good evidence that retinoic acid could also establish dorso-ventral polarity. The synthesis of retinoic acids are mediated by aldehyde dehydrogenase enzymes. The expression of *Basic aldehyde dehydrogenase class 1* isoform (*AHD2*) is restricted to the dorsal half of the neural retina, whilst an *Acidic dehydrogenase* (*V2*) is expressed in the ventral half of the retina (McCaffery *et al.*, 1991, 1993). *V2* expression can be detected before *AHD2*, but by E9.5 both genes are expressed in their respective domains of the optic vesicle. The asymmetric expression of *AHD2* is maintained postnatally in Muller cells (McCaffery *et al.*, 1991, 1993). Higher levels of retinoic acid are detected in the ventral half of the retina compared to the dorsal as a consequence of the increased efficiency of *V2* over *AHD2* (McCaffery *et al.*, 1993). At least two forms of retinoic acid exist, trans-retinoic acid and 9-cis-retinoic acid. It is not yet known if these two types are differentially produced by the different dehydrogenases (McCaffery *et al.*, 1993).
There are two families of nuclear retinoic acid receptors; RARs and RXRs. Each family contains three members, encoded by different genes which in turn can be alternatively spliced and transcribed using different promoters to give several isoforms of each receptor type (Chambon, 1994). *In vitro*, RAR and RXR can bind DNA elements as homodimers and heterodimers adding to the complexity of the receptor unit (Chambon, 1994). Thus, depending on the combination of retinoic acid receptor types, different cellular responses to retinoic acid could be achieved.

The absence of abnormal phenotypes associated with knocking-out many of the RAR genes, suggests that either a high degree of functional redundancy exists between these receptors or that they do not have an essential function (Kastner *et al.*, 1995). Functional redundancy between receptors is supported by the fact that mice with null mutations in more than one receptor gene display developmental defects, including eye abnormalities. These include the absence of lens, cornea, anterior chamber, retinal degeneration, retina dysplasia, pigmented retina epithelium defects, failure of the eyelids to fuse and poor differentiation of the lens fibres and corneal stroma (Lohnes *et al.*, 1994; Grondona *et al.*, 1996). The majority of these defects could be secondary, arising from the failure of an interacting tissue to differentiate. To identify the primary source of defects in the RAR mutants, the distribution of these receptors in the developing eye was analysed. Throughout eye development, RARα is expressed ubiquitously, RARβ and γ are only expressed in the pigmented retina epithelium and the pericellular mesenchyme. This suggests that the primary defect may either occur in the pericellular mesenchyme or the pigmented retina epithelium (Lohnes *et al.*, 1994; Grondona *et al.*, 1996). A careful examination of the retina pigmented epithelium defects in RARβ 2−/RARγ 2− mice, revealed that sites of disorganised or abnormal pigment epithelium were always adjacent to abnormal neural retina (Grondona *et al.*, 1996). Pigmented retina epithelium is essential for the correct development of the neural retina (Raymond and Jackson 1995). Therefore the failure of pigmented retina epithelium to develop
correctly in RAR double-mutants could account for the secondary eye defects observed.

RXRa is widely expressed (possibly ubiquitously) and mice null for this gene display a range of eye defects (Dolle et al., 1994; Kastner et al., 1994). In E12.5-E16.5 embryos these defects included a lack of pigment in the ventral side of the retina, reduced size of the ventral neural retina, incomplete closure of the optic fissure and several anterior segment defects (Kastner et al., 1994). Again it is difficult to distinguish between primary and secondary defects. RXRa−/RARγ−/− and RXRa−/RARγ− mice both exhibited an increase in the severity of the RXRa−− phenotype, including a shortening of the ventral retina. This implies that these two genes function together (Kastner et al., 1994). RARγ is not expressed in the retina, therefore this co-operation would have to be in an adjacent tissue, perhaps the pigmented retina epithelium.

In common with mice, zebrafish also have two dorsal/ventral restricted retinaldehyde dehydrogenases, the ventral enzyme being more efficient. Exposure of zebrafish embryos, at a critical stage of development (towards the end of gastrulation) to retinoic acid results in a duplication of the retina (Hyatt et al., 1996). Paxb is normally expressed in the optic stalk and ventral regions of the retina surrounding the choroidal fissure. In retinoic acid-treated embryos, this ventral marker is expressed throughout the original and duplicated retina (Hyatt et al., 1996). Conversely, the expression of two dorsal retina markers, Mshc and the dorsal restricted Retinaldehyde dehydrogenase, could not be detected in retinoic acid-treated embryos (Hyatt et al., 1996). Beads soaked in retinoic acid and grafted next to the retina cause choroidal fissure-like structures to develop adjacent to the grafted bead (Hyatt et al., 1996). This morphological feature would normally develop on the ventral side of the retina. These results suggest that the retinoic acid signalling system plays a critical role in specifying ventral identity within the retina. The amount of retinoic acid to which cells are exposed might underlie dorso-ventral identity, since retinoic acid can be detected in both sides of the retina and dorsal identity is only lost by increasing the amount of
retinoic acid. Alternatively, the individual dorsal and ventral restricted retinaldehyde dehydrogenases may produce specific forms of retinoic acid.

The retinoic acid system and the transcription factors mentioned are likely to play a role in establishing positional identity with respect to the dorso-ventral axis. The genes that these systems regulate or the mechanism by which they actually confer positional information are not known. However, numerous cell surface proteins are asymmetrically expressed in the retina and may well be regulated by the retinoic acid system or the asymmetrically expressed transcription factors. Interestingly, these cell surface proteins are also asymmetrically expressed in the optic tectum. It is therefore possible to envisage a situation in which the amount or type of protein could allow the cells' axon to make spatially-specific connections within the optic tectum.

*Mek 4* and *Elf 1*, members of the Eph family, are receptor tyrosine kinases. *Elf 1* is the ligand for *Mek 4*. In chick, *Mek 4* expression is observed in a gradient within the retinal ganglion cell layer, low expression in the nasal aspect increasing temporally, whilst *Elf 1* is expressed in a complimentary gradient within the optic tectum, low expression in the anterior increasing towards the posterior (Cheng *et al.*, 1995). Temporal retinal axons make connections with the anterior tectum, whilst nasal retinal axons make connections with the posterior tectum. Ectopic expression of *Elf 1*, in patches of the anterior tectum, caused temporal axons to actively avoid *Elf 1*-expressing patches, but had no effect on nasal axons (Nakamoto *et al.*, 1996). This implies that *Elf 1* functions as a repellent axon guidance factor. Thus, these cell surface proteins may control topographic mapping of retinal cells to their target sites in the optic tectum in the manner outlined earlier.

*Qek 5* is also a member of the Eph family of receptor tyrosine kinases that may be involved in topographic mapping of the visual system. In quail, this gene is expressed in a ventral to dorsal gradient in the neural retina and in a posterior to anterior gradient in the optic tectum (Kenny *et al.*, 1995). The function of this protein has not been investigated.
1.5.2.5 Gene function in the presumptive ciliary body

During neural retina differentiation, the expression of numerous genes, like Msx 2, become progressively restricted to the distal tips of the retina (presumptive ciliary body), whilst other genes, such as Msx 1, are expressed de novo in this region (Monaghan et al., 1991). As previously suggested (chapter 1.5.2.3) the expression of genes involved in the maintenance of undifferentiated retinal precursors may progressively become restricted to the ciliary body as a consequence of the central to peripheral gradient of neurogenesis within the retina. The function of Msx 1, Msx 2 and Notch 2, also expressed in the mouse ciliary margin (Williams et al., 1995), may therefore be to maintain a population of retinal precursors. These precursors may give rise to components of the iris. Incidentally, the expression domains of other members of the mouse Notch gene family overlap with those of Msx in parts of the eye. At E10.5, the expression of Notch 1 and Msx 1 overlaps in the mesenchyme between the lens epithelium and surface ectoderm, whilst Notch 3 and Msx 2 expression overlaps in the surface ectoderm surrounding the eye (Monaghan et al., 1991; Williams et al., 1995). This suggests that members of these two gene families may function in a common pathway.

TGFβ2, a member of the transforming growth factor family, and α1(IX)Collagen are both expressed in the inner layer of the optic cup, progressively becoming restricted to the ciliary body (Gatherer et al., 1990; Liu et al., 1993). TGFβ2 has been implicated in extracellular signalling, inhibiting cell growth, modulating cell differentiation and inducing extracellular matrix synthesis during various developmental processes (Roberts and Sporn, 1992). In support of TGFβ2 functioning in the developing retina, Type II and Type III TGFβ receptors are expressed in the chick retina, whilst mice null for TGFβ2 have a reduced corneal stroma layer, an enlarged neuroblastic layer and hypercellular infusion (Barnett et al., 1994; Sanford et al., 1997). The precise function of TGFβ2 and α1(IX)Collagen in the ciliary body remains unknown.
1.5.3 Lens development

1.5.3.1 Lens induction

"Induction is the process initiated when one group of cells signals to a neighbouring group of cells to change its developmental fate" (Bard and Lehtonen, 1996). This process is essential for lens formation and has been the focus of much research for more than a century. Early experiments, performed by Spemann (1901) and Lewis (1904), led to the conclusion that the optic vesicle was the only structure required for lens induction. This conclusion was drawn from the observations that destruction of the retina prevented lens development, and transplantation of the optic vesicle to under the belly ectoderm resulted in ectopic lens development (Spemann, 1901; Lewis, 1904). Recent work suggests that lens induction is not as simple as first thought and the validity of these early experiments must be questioned. Rudimentary lenses can form even in the absence of the retina in some species, whilst it would have been difficult to prove that the isolated optic vesicle was free from surface ectoderm in Lewis's transplant experiment (Saha et al., 1989).

According to the current model, lens induction can be divided into four stages; competence, bias, specification and differentiation (Grainger, 1992, 1996). Competence defines the ability of the surface ectoderm to respond to an inducing signal. Towards late gastrulation, the entire animal cap ectoderm becomes transiently lens competent (Grainger, 1992, 1996).

Early lens-inducing signals, acting on competent ectoderm, do not originate from the optic vesicle, as this structure is not formed until the neural tube stage. By the neural tube stage, the surface ectoderm has lost its ability to respond or receive the lens-inducing signals. There is good evidence to suggest that the signal is planar, transmitted through the ectoderm from the presumptive neural plate or perhaps even from the presumptive retinal region. Tissue recombination experiments performed by
Henry and Grainger (1990) demonstrated that when Xenopus anterior neural plate tissue was cultured with surrounding ectoderm, lens formation was observed. Furthermore, when this tissue was isolated and cultured as a congruent sheet, there was a higher lens-formation success rate. The lens-inducing signal may be enhanced by mesoderm underlying the presumptive lens ectoderm. This is suggested by Henry and Grainger (1990) who observed an increase in lens formation when anterior neural plate tissue, surrounding surface ectoderm and mesoderm were cultured together. Ectoderm responding to the lens-inducing signal becomes biased towards forming lens tissue, but not all of this ectoderm will necessarily form lens tissue.

During specification, the area of induced-surface ectoderm that will actually form the lens is specified. At this stage the optic vesicle has formed and therefore could play a role in this process and subsequently in the differentiation of presumptive lens cells into functional lens fibre cells. The effect the optic vesicle might have on lens differentiation was investigated by Henry and Grainger (1990) by culturing Xenopus presumptive lens ectoderm, which had already been exposed to the earlier lens-inducing signals, with or without optic vesicle tissue of the same stage. Lenses derived from the presumptive lens ectoderm cultured without optic vesicle tissue contained no fibrous cells, an indicator of an advanced state of differentiation, whilst 66% of lenses derived from the presumptive lens ectoderm cultured with optic vesicle tissue contained fibrous lens cells.

The molecular mechanism controlling lens competence may be linked to other embryonic patterning events, either directly or indirectly. The expression of certain homeobox-containing genes within particular anterior-posterior domains of the neural plate is important for patterning axial neural tissues, but it is not clear whether these genes also have a direct role in patterning adjacent non-neural ectoderm (Couly and LeDouarin, 1990). Many of these genes are restricted to the neural plate, thus a secondary planar-induction from the patterned neural plate to the surrounding ectoderm would be required (Grainger, 1992). The nature of this signal is not known.
Mice null for *Pax 6* fail to develop lens placodes, demonstrating that this gene is essential for lens formation (Grindley *et al.*, 1995). The expression pattern of *Pax 6* correlates well with the described model of lens formation. In E8.5 mice, a broad domain of surface ectoderm expresses *Pax 6*. As development proceeds, expression progressively becomes restricted to the developing lens and nasal placodes (Grindley *et al.*, 1995). This is consistent with the view that a large area of surface ectoderm is initially biased towards a lens fate, with this area progressively becoming restricted to the presumptive lens placode. *Pax 6* function may therefore bias cells towards a lens fate.

In the model described, the developing optic vesicle was suggested to play a role in restricting lens-biased cells to the presumptive lens placode. However, a normal restriction of *Pax 6* expression is observed in chicks lacking an optic vesicle (Li *et al.*, 1994), suggesting that the optic vesicle may not be required for the specification of lens cells.

Although the mechanism by which *Pax 6* is restricted to the lens placode is not understood, it would appear that functional *Pax 6* is required to maintain expression within the surface ectoderm. In *Sey/Sey* mice, *Pax 6* is expressed normally in a broad domain of the surface ectoderm up to E9. However, instead of the domain being restricted to the lens and nasal placodes, expression is down-regulated throughout the surface ectoderm. This suggests that the factor that initially establishes the broad domain of expression is later replaced by a mechanism that requires functional *Pax 6* (Grindley *et al.*, 1995). *Pax 6* is also expressed in the developing optic vesicle of wild type mice and though the optic vesicle develops in *Pax 6*-null mice, the absence of *Pax 6* function in this tissue may effect *Pax 6* expression in the overlaying surface ectoderm (Grindley *et al.*, 1995). This is an unlikely explanation as normal restriction of *Pax 6* expression is observed in the surface ectoderm of chicks lacking an optic vesicle (Li *et al.*, 1994). It has been shown that quail *Pax 6* is capable of auto-regulation (Plaza *et al.*, 1993). Therefore, an alternative explanation is that a *Pax 6* auto-regulatory loop maintains expression.
The transcription factors, Msx 2 and Sox 2 are also expressed in the lens placode and its derivatives (Monaghan et al., 1991; Kamachi et al., 1995). These genes may therefore play a role in the specification of lens cells. Sox 2 and Pax 6 expression continues during the differentiation of lens fibre cells, suggesting that they may have an additional or continued function at later stages of lens development. In contrast, Msx 2 expression cannot be detected after E11.5-E12.5 (Monaghan et al., 1991). In common with other tissues of the eye and indeed other organs, Msx 2 expression is restricted to undifferentiated cells of the lens. This is consistent with Msx 2 playing a universal role in maintaining a state of dedifferentiation. Alternatively, the expression of Msx 2 in the lens placode is consistent with Msx 2 functioning in the inductive processes that occur at this site.

1.5.3.2 Lens differentiation

Lens fibre cells differentiate from the posterior half of the lens vesicle, while cells of the anterior half remain as an epithelial monolayer. Polarity of the lens is maintained throughout life, with epithelial cells proliferating just above the lens equator and differentiating into fibre cells just below. The differentiation of lens fibre cells is associated with expression of crystallin genes followed by denucleation.

Pax 6 and Sox 2 appear to have a role in the differentiation of the lens. Sox 2 binds to an enhancer element in the promoter of δl crystallin. In the presence of δEF3, an uncharacterized protein, Sox 2 activates the transcription of δl crystallin (Kamachi et al., 1995). In support of the involvement of Pax 6 in the differentiation of lens fibres, binding sites have been identified in crystallin promoters (Cvekl and Piatigorsky, 1996). Prox 1, the mouse homologue of Drosophila Prospero, is also expressed in the developing lens (Oliver et al., 1993). At E12.5, this homeobox-containing gene is expressed in a region of the lens associated with elongation of lens fibre cells. This might imply that Prox 1 is involved in the activation of genes required for terminal
differentiation of these cells (Oliver et al., 1993). These genes could include crystallins.

Coulombre and Coulombre (1963) demonstrated that the polarity of differentiation is controlled by the surrounding environment. By rotating the lens through 180 degrees, so that the anterior epithelium faces the vitreous, it was shown that the anterior epithelium can differentiate into lens fibre cells (Coulombre and Coulombre, 1963). The vitreous contains significantly more FGF than the aqueous, suggesting that FGF may be a good candidate for stimulating lens fibre differentiation (Schulz et al., 1993).

In rats, immunohistochemistry has demonstrated the localisation of FGF 1 (acidic FGF) to a zone of cells around the lens equator, the inner epithelium of the ciliary body and iridial regions of the retina directly apposing the equatorial region of the lens (McAvoy et al., 1991). Thus, extracellular signalling from the ciliary body, mediated by FGF 1, may control the differentiation of lens fibre cells at the lens equator.

FGFs bind to surface receptors which possess an internal tyrosine kinase motif that phosphorylates proteins within the cell, initiating a cascade of protein interactions ultimately leading to altered gene expression. FGFR1 (Flg), FGFR2 (Bek) and FGFR3 are all expressed in the lens, further suggesting a role for FGF in the developing lens (Orr-Urtreger et al., 1991; Peters et al., 1993). FGFR1 is also expressed in both the cornea epithelium and sclera, whilst FGFR2 is expressed in the cornea epithelium. This suggests that FGF may also effect other tissues of the eye (Orr-Urtreger et al., 1991).

Different receptors may have different affinities for FGF, and therefore become activated at different FGF concentrations (Orr-Urtreger et al., 1991; Peters et al., 1993). Thus a cell could evoke a specific response depending on the receptor type or combination expressed, therefore triggering different cellular responses. Alternatively,
different FGF receptors might phosphorylate different intracellular molecules. However, this possibility has not been tested.

Proliferation, migration and differentiation of cells in cultured rat lens epithelial explants can all be stimulated by FGF 2 (basic FGF), in a concentration-dependant manner. This lends support to FGFs functioning in lens development (McAvoy and Chamberlain, 1989). The function of FGF has also been tested in vivo, by generating transgenic mice that express a dominant-negative FGFR1 under the control of the αA crystallin promoter. In these mice, lens fibre cells fail to elongate properly or denucleate and subsequently undergo apoptosis (Chow et al., 1995; Robinson et al., 1995a). The observation that lens fibre differentiation is not completely blocked may indicate that other factors are involved. Alternatively, the amount of dominant-negative receptor may not be sufficient to completely disrupt the FGF signalling mechanism. However, these experiments demonstrate that lens cell survival, and to a degree differentiation, is dependant on FGF. Furthermore, over-expression of a secreted form of FGF 1 in transgenic mice using the αA crystallin promoter, resulted in the anterior lens epithelium losing its normal cuboidal morphology and becoming elongated (Robinson et al., 1995b).

1.6 Stability of the differentiated state and transdifferentiation

In vivo, cells maintain their differentiated state through appropriate interactions with their surrounding environment. These may be mediated through cytokine signalling, direct cell-cell contact or contact between cells and the extracellular matrix. Disruption of these interactions may result in the loss of a stable state of differentiation. This phenomenon underlies the regenerative potential exhibited in numerous organisms following tissue damage and tumorogenesis following altered gene expression.

Differentiated chick pigmented retina epithelium (PRE), from nine-day-old chick embryos, will maintain its differentiated state, even when dissociated, if grown at high
density (Itoh and Eguchi, 1986). This differentiated state is characterised morphologically by tightly packed, hexagonal cells that produce pigment and at the molecular level by the expression of *Mmp115*, encoding a Melanosomal matrix protein, and *pP344*, encoding a secreted serine protease inhibitor (Itoh and Eguchi, 1986; Mochii *et al.*, 1988; Agata *et al.*, 1993). Subcultivation of these cells at low density and in the presence of phenylthiourea (a potent inhibitor of melanogenesis) and testicular hyaluronidase, resulted in the cells becoming highly proliferative, large, loosely packed together and de-pigmented. *Mmp115* and *pP344* expression was also down-regulated (Itoh and Eguchi, 1986; Mochii *et al.*, 1988; Agata *et al.*, 1993). Similarly, repeated subcultivation of dissociated porcine PRE cells also resulted in a progressive loss of pigment and change in cell morphology. This change was associated with the redistribution of filamentous Actin from compact cortical rings in differentiated PRE cells, to linear fibres in dedifferentiated cells (Grisanti and Guidry, 1995). However, in this instance loss of the differentiated state was achieved solely by subcultivation without any requirement for phenylthiourea or testicular hyaluronidase.

Phenythiourea and testicular hyaluronidase affect cell surface properties (Itoh and Eguchi, 1986). Therefore, they could reduce cell-cell communication or interactions with the extracellular matrix. The observation that mechanically interfering with cell-cell communication, by passaging cells at low density, has a comparable effect on PRE cells, might suggest that phenylthiourea and testicular hyaluronidase merely enhance the dedifferentiation process. These experiments suggest that close cell-cell contact is required to maintain the differentiated state of PRE cells.

FGF 2 was found to be present in the crude preparation of testicular hyaluronidase and shown to have the same effect on PRE cells as testicular hyaluronidase (Hyuga *et al.*, 1993). FGF 2 might therefore be the active component of the testicular hyaluronidase preparation. Bost *et al.* (1994) have demonstrated that FGF 2 is expressed by cultured human PRE cells and that expression levels vary depending upon cell density (Bost *et al.*, 1994). Cells grown at high density express low levels of FGF 2, whilst cells grown at low density express high levels of FGF 2. When added
to cultured cells, EGTA depletes Ca\(^{2+}\), consequently cell-cell contacts are disrupted. Addition of EGTA to high density human PRE cultures resulted in a rapid three-fold increase in \(FGF\,2\) expression (Bost et al., 1994). This implies that \(FGF\,2\) expression is suppressed by cell-cell interactions, raising the possibility that increased levels of \(FGF\,2\), as a result of interfering with cell-cell contact, could underlie the loss of PRE identity. Indeed, the addition of \(FGF\,2\) to dissociated cultures of chick PRE cells results in enhanced de-pigmentation and a gain of large, loosely packed cell morphology without any requirement for subcultivation (Pittack et al., 1991; Opas and Dziak, 1994). Therefore in summary, breakdown in cell-cell communication is sufficient for dedifferentiation but it is also associated with up-regulation of \(FGF\,2\) which itself is sufficient to dedifferentiated PRE cells. It is therefore difficult to define the primary cause of PRE dedifferentiation. It will be interesting to learn how \(FGF\,2\) exerts its action and whether this involves a modification of cell surface properties.

Differentiated PRE cells possess well organised gap junctions that allow direct cell-cell communication, whilst dedifferentiated PRE cells have almost no gap junctions (Kodama and Eguchi, 1994). It has also been demonstrated that adherence of PRE cells to components of the extracellular matrix can be blocked by the addition of anti-\(\beta1\) Integrin antibody, resulting in the cells dedifferentiating (Mazaki et al., 1996). Therefore, Gap junctions and interactions with the extracellular matrix, mediated via \(\beta1\) Integrin, may be critical for maintaining a stable state of PRE differentiation. These proteins may well be affected by the action of \(FGF\,2\).

*In vivo*, the stability of chick Muller glial cells is maintained by direct cell contact with neurons. Disruption of these contacts, by culturing dissociated neural retina, results in the dedifferentiation of Muller glial cells in a manner analogous to the dedifferentiation of PRE cells (Moscona, 1986). Neural retina and PRE are both derived from the optic vesicle. It is therefore tempting to speculate that dedifferentiated cells derived from both Muller glial and PRE have an equivalent state of differentiation to cells of the early optic vesicle.
Dedifferentiated PRE cells can differentiate into lens-like cells or re-differentiate into PRE cells depending upon culture conditions. By removing phenylthiourea and testicular hyaluronidase/FGF 2 and growing at high density, dedifferentiated cells can restore cell-cell contacts and re-differentiate into PRE cells that produce pigment, have a tightly packed hexagonal morphology and express Mmp115 and pP344 (Itoh and Eguchi, 1986; Agata et al., 1993). Alternatively, dedifferentiated PRE cells grown at extremely high density in the presence of phenylthiourea, testicular hyaluronidase/FGF 2 and ascorbic acid, give rise to lentoid bodies, immunoreactive for the lens-specific genes αA crystallin, β crystallin and δ crystallin (Itoh and Eguchi, 1986; Agata et al., 1993). These lens-specific genes were not detected at significant levels in either differentiated or dedifferentiated PRE cells. This switch from one differentiated state to another is termed transdifferentiation (Eguchi and Kodama, 1993).

Gliocytes, dedifferentiated Muller glial cells, when cultured at high density also transdifferentiate into lentoids which express the lens marker δ crystallin (Moscona, 1986). In this system it is not known if gliocytes can transdifferentiate into PRE. However, there is a report of neural retina cells transdifferentiating into PRE cells in other systems (Okada, 1976). After several passages, quail neural retina cells start to express QNR 71 which encodes a Melanosomal matrix protein expressed in the PRE, but not the neural retina (Turque et al., 1996). The observation that gliocytes (destabilised neural retina) and dedifferentiated PRE can both give rise to lens-like cells and PRE further supports the idea that these destabilised cells have an equivalent potential for differentiation.

Viral infection of quail neural retina cells with an Myc-expressing retrovirus enhanced the transdifferentiation of these cells into PRE, as marked by QNR 71 expression and pigmentation (Turque et al., 1996). Conversely, PRE cells infected with the same Myc-expressing retrovirus, developed long cellular processes and expressed the neural marker Neuron specific enolase (Plaza et al., 1995). This paradoxical situation could be explained if Myc expression in both PRE and neural retina causes the destabilisation of these cell types, producing a common multipotent dedifferentiated
cell type with the potential to re-differentiate as either neural retina or PRE. It is interesting to note that dedifferentiated PRE cells, generated by low density subcultivation in the presence of phenylthiourea and testicular hyaluronidase/FGF 2, show a marked increase in Myc expression, compared to differentiated PRE (Agata et al., 1993).

It is surprising that PRE and Muller glial cells, both derived from neuroepithelium, can transdifferentiate into lens-like cells which are normally derived from surface ectoderm. However, during the development of the lens and derivatives of the optic neuroepithelium, numerous genes are expressed in both tissues (chapter 1.5.1). It is conceivable that such genes establish a lens differentiation pathway in both tissues, but that this pathway is normally suppressed during retina development. In support of this idea, low levels of crystallin mRNA can be detected in both chick PRE and the glial precursor layer of the neural retina (Clayton et al., 1986). Destabilisation of the PRE or Muller glial state of differentiation may result in these cells adopting a lens-like differentiation pathway under the appropriate conditions.

PRE cells can also transdifferentiate into neural retina-like cells. Between 40% and 80% of cells in stage 24 (E4.5) chick PRE explants treated with FGF 2 or FGF 1 lose their pigmentation and gain a neural morphology. This phenotypic change is accompanied with the loss of PRE markers, such as Tyrosinase and Retinaldehyde binding protein, and the gain of neural markers, such as N-Cam, Neuron specific enolase and Neurofilaments (Pittack et al., 1991; Guillemot and Cepko, 1992). Several different neural retina cell types are produced during this transdifferentiation process. Rat PRE explants treated with FGF give rise to amacrine, photoreceptor and ganglion cells, identified by the presence of Syntaxin, Opsin and Thy 1 respectively (Zhao et al., 1995).

PRE explants from stage 27 or older chicks fail to respond to FGF (Pittack et al., 1991). This demonstrates that in contrast to PRE transdifferentiation into lens-like cells, transdifferentiation into neural retina is dependant on the age of the parental
tissue or the degree of commitment to the PRE lineage. Dissociated chick PRE cells also fail to transdifferentiate into neural cells following FGF treatment, implying that cell-cell or cell-extracellular matrix interactions are necessary (Pittack et al., 1991). Certainly, there is no requirement for cells to pass through a proliferative phase since the presence of mitomycin C, which prevents cell division, does not effect FGF-driven PRE to neural retina transdifferentiation (Zhao et al., 1995). Thus, it appears that FGF can either destabilise the differentiated state of PRE cells or trigger a neural retina differentiation pathway. The presence or absence of cell interactions, that are only maintained when PRE cells are cultured as intact explants, seem to govern whether FGF acts to destabilise PRE or induce neural differentiation. It is possible that FGF-induced transdifferentiation of PRE to neural retina is a two step process, requiring both FGF and appropriate cues from the surrounding environment. FGF may function to destabilise the differentiated PRE state, whilst cues from the surrounding environment may act on these destabilised cells triggering a neural differentiation pathway.

The in vitro experiments described in this section suggest that during normal development FGF may play a role in establishing a neural retina differentiation pathway in cells of the optic vesicle. This was investigated by culturing chick optic vesicles in the presence or absence of FGF 2 neutralising antibody (Pittack et al., 1997). This resulted in a significant reduction in the number of Neurofilamant-positive cells in optic vesicles grown in the presence of neutralising antibody compared to those not grown in the presence of neutralising antibody. This suggests that the neural retina fails to differentiate appropriately when FGF function is disrupted. In contrast, differentiation of PRE cells was unaffected by FGF 2 neutralising antibody (Pittack et al., 1997).

1.7 Summary and aims

The Msx gene family encodes homeodomain-containing transcription factors that are specifically expressed during mouse development. Two emerging themes of Msx
function are the co-expression of Msx with Bmp 4, and a role in suppressing or delaying differentiation.

During limb, tooth and facial development, Msx is thought to be involved in reciprocal signalling processes between epithelial and mesenchymal tissues. At all these sites, epithelium-derived Bmp 4, or a Bmp 4-like molecule, activates Msx expression in the underlying mesenchyme. In the case of tooth development, Msx 1 then activates Bmp 4 expression in the mesenchyme, establishing a positive-feedback loop. Whether or not a similar mechanism operates in limb and facial mesenchyme remains to be established.

Msx prevents the differentiation of both myoblasts and chondrocytes, in addition to being associated with apoptosis of hindbrain neural crest. This may reflect a common role for Msx in delaying or inhibiting differentiation. In the case of hindbrain neural crest, the absence of a cue to differentiate, or the absence of a survival cue, may result in these cells entering a default apoptotic pathway.

In the developing eye, Msx 1 is expressed within the perioptic mesenchyme and the presumptive ciliary body while Msx 2 is expressed in the early optic vesicle and derivatives of the surface ectoderm. Unpublished data suggests that these genes are essential for eye development but the processes they control or the genetic pathways in which they function remain unknown.

The vertebrate eye develops from surface ectoderm, neural ectoderm and neural crest derivatives which undergo several morphological changes and differentiate in a highly orchestrated manner. Clues as to the molecular control of these processes have come from studying the expression patterns of functionally diverse genes active in the developing eye and the phenotype of mice null for, or ectopically expressing, some of these genes.
Transcription factors, by their very ability to regulate gene expression, are likely to lie at the centre of many molecular mechanisms controlling eye development. *Pax 6, Six 3* and *Eya* are expressed in all the major cell types of the developing mouse eye. *Pax 6*, at least, is essential for the formation of this organ. These genes have a conserved function in the structurally different *Drosophila* eye, highlighting the fundamental importance of such genes during eye development. Other transcription factors are expressed more selectively in one particular component of the eye such as *Lhx 2* in the optic vesicle, but are of no less importance as demonstrated by the lack of eyes in *Lhx 2*<sup>−/−</sup> mutants.

Growth factors and cell surface proteins allow cells to communicate with one another and are involved in controlling many aspects of eye development. FGF affects the differentiation of lens-fibre cells, while the cell surface proteins, Notch and Delta, are likely to be involved in controlling the onset of neurogenesis within the retina. The Notch and Delta pathway suppresses neural differentiation by inhibiting the expression of proneural genes such as *Mash 1*. In general, this type of hierarchical integration of genes into pathways controlling specific processes is poorly understood. Therefore, there is a need to increase our understanding of how the action of genes, expressed in the eye, are integrated together to control eye development.

Transcription factors, by their definition, alter gene expression. Therefore transcription factors, such as *Msx*, make excellent points from which to explore genetic pathways. The aim of this thesis is therefore to investigate the function of *Msx 1* and *Msx 2* during vertebrate eye development. The current understanding of where and when the *Msx* genes are expressed during eye development is very limited. Therefore, the existing description of *Msx 1* and *Msx 2* expression in the developing eye will be extended. This will enable *Msx* expression to be correlated with specific processes, so highlighting possible functions for these genes during eye development.

An appropriate cell culture system will then be developed to further explore these candidate functions. To address whether *Msx* function in cell culture reflects the true
in vivo function, the in vitro functional assay will be complemented with an equivalent in vivo system using transgenic techniques.

In the future, an in vitro Msx functional assay could be used to identify genes functioning in the same pathway as Msx. Many of these genes may be novel, so more of the genes functioning in eye development would be identified. In addition, the association of Msx with known genes would increase our understanding of the cascades of gene action controlling eye development. An understanding of Msx function in the developing eye will also help elucidate Msx function at other sites of the embryo.
Chapter 2

Materials and Methods

All chemicals were supplied by BDH unless otherwise stated.

2.1 Bacterial cell culture and plasmid DNA preparation

2.1.1 Media and solutions

Media and solutions were prepared as described by Sambrook et al. (1989) and sterilised by autoclaving.

L-broth: 10g tryptone, 5g yeast extract, 10g NaCl and 2.46g MgSO$_4$ dissolved in 1 litre of water.

L-agar: 10g tryptone, 5g yeast extract, 10g NaCl, 2.46g MgSO$_4$ and 15g agar (Oxoid Ltd) dissolved in 1 litre of water.

Ampicillin: (Boehringer Mannheim) Stock solution made at 50mg/ml in dH$_2$O, filter sterilised and stored at -20°C. Added to autoclaved media to give a final concentration of 50μg/ml.

2.1.2 Growing bacterial cells on agar plates

A small volume (200μl) of suspended bacterial cells was placed onto the surface of L-agar plates and spread evenly with a sterile bent glass rod. For bacterial cells obtained from a growing colony or glycerol stock, cells were streaked out onto L-agar plates using a sterile loop. For selection of ampicillin resistant colonies, ampicillin was added to the L-agar prior to pouring the plates. The plates were then inverted and incubated for 12-16 hrs at 37°C. Plates could then be stored at 4°C for several weeks.
2.1.3 Glycerol stocks

For long term storage of bacterial strains, 10ml of L-broth (plus ampicillin if desired) was inoculated by a single colony and grown at 37°C for 4 hrs. The cells were then pelleted by centrifugation at 3500rpm for 10 mins, resuspended in 15% glycerol (Fisher Scientific; FS) in L-broth and aliquoted into freezing vials and stored at -70°C.

2.1.4 Transformation of bacterial cells

For all procedures the E.coli. strain DH5α were used. The DH5α genotype is as follows:-

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F-endA1 hsdR17(rKm) supE44 thi-1 recA1 gyrA96 relA1
δ(argF-lacZYA)U169 (φ80d lacZ ΔM15)
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2.1.4.1 Preparation of competent cells

Using a sterile loop, bacterial cells from a frozen stock were streaked out onto a L-agar plate and grown at 37°C for 12-16 hrs. A single colony was then used to inoculate 10mls of L-broth which was grown at 37°C in a shaking incubator for 12-16 hrs. The entire culture was then used to inoculate 500mls of L-broth and grown at 37°C in a shaking incubator until the culture had an absorbance of 0.7 at 590nm. The cells were then harvested by centrifugation for 5mins at 5000rpm at 4°C. The pelleted cells were washed in 250mls of ice cold 0.1M MgCl₂ and re-centrifuged as before. The cells were then resuspended in 250mls of ice cold 0.1M CaCl₂ and incubated on ice for 20 mins. The cells were then pelleted as before, resuspended in 42.5mls of 0.1M CaCl₂ and 7.5mls of glycerol (FS), aliquoted into freezing vials, snap frozen on dry ice plus ethanol and stored at -70°C.

2.1.4.2 Heat-shock transformation of bacterial cells

An aliquot (80μl) of competent cells was thawed on ice, then mixed with the DNA to be transformed (usually the entire ligation reaction). This mixture was left on ice for
15 mins, then heat-shocked for 2 mins at 42°C and incubated for a further 15 mins on ice. The cells were then spread onto ampicillin containing L-agar plates and incubated at 37°C for 12-16 hrs.

2.1.5 Preparation of plasmid DNA

Mini preparation - A single colony was used to inoculate 6 ml of L-broth containing ampicillin and grown in a shaking incubator at 37°C for 12-16 hrs. The cells were then harvested by centrifugation and plasmid DNA isolated using the QIAprep Spin Plasmid Kit (Qiagen) as per manufacturers instructions.

Maxi preparation - A single colony was used to inoculate 20 ml of L-broth containing ampicillin and grown for 7 hrs at 37°C in a shaking incubator. The entire culture was then used to inoculate a further 200 ml of L-broth containing ampicillin and grown for 12-16 hrs at 37°C in a shaking incubator. The cells were then harvested by centrifugation at 4000 rpm for 20 mins at 4°C and plasmid DNA isolated using a Qiagen Plasmid Maxi Kit as per manufacturers instructions.

2.2 Enzymatic manipulation of DNA

2.2.1 Solutions and plasmid vectors

TE: 10 mM Tris, 1 mM EDTA (pH 7.5).

pSK: pBluescript II SK (Stratagene).

pCl: Mammalian expression vector (Promega).

2.2.2 Purification of DNA

One of two methods were used to concentrate and remove salts from DNA solutions between enzymatic reactions.
2.2.2.1 Ethanol precipitation

A 1/10 volume of 2M NaAc, pH5.5 was added to the DNA solution followed by 2 volumes of 100% ethanol. The contents was mixed and incubated at -20°C for at least 30 mins. The DNA was then pelleted by centrifugation at 11000rpm for 15mins at 4°C, washed in 70% ethanol, briefly dried under vacuum and resuspended in the desired volume of dH₂O or TE.

2.2.2.2 Microcon purification

The DNA solution was passed through a microcon 30 filter (Amincon) as per manufacturers instructions. To remove salts, a further 400µl of TE or dH₂O was passed through the column. To elute the DNA, the desired volume of TE or dH₂O was added to the column which was then placed on ice for 5 mins. The column was then inverted into a fresh tube and centrifuged as per manufacturers instructions.

2.2.3 Quantification of DNA

The concentration of double-stranded DNA was determined by measuring the absorbance at 260nm in a spectrophotometer. An absorbance reading of 1 corresponds to 50µg of DNA per ml.

2.2.4 Restriction-enzyme digestion of DNA

Digestions were performed in the buffer and at the temperature recommended by the supplier. Restriction enzymes were supplied by Boehringer Mannheim and New England Bio Labs. Up to 1µg of DNA was generally digested in a total volume of 20µl containing the appropriate buffer, 2µl of 1mg/ml BSA (Boehringer Mannheim) and 1-2 units of enzyme. The reaction volume was increased for the digestion of larger quantities of DNA. The reaction was then incubated at the appropriate temperature for 90 mins. When necessary the reaction was terminated by heating at
68°C or 80°C for 15 mins, according to the heat sensitivity of the enzyme. Double-digests, in which both enzymes can operate in the same buffer, were performed simultaneously. Otherwise, the DNA was purified between reactions as described in 2.2.2.

2.2.5 Dephosphorylation of 5' termini

To prevent the recircularisation of vectors during ligation reactions, calf intestinal phosphatase (CIP) was used to dephosphorylate the 5' ends of vectors before cloning. DNA was dephosphorylated with 1 unit of CIP (Boehringer Mannheim) in 50μl of 1x CIP buffer (50mM Tris-HCl, 0.1mM EDTA, pH 8.5) at 37°C for 30 mins. An additional unit of CIP was then added and reaction continued at 45°C for 45 mins. The reaction was terminated by adding a 1/10 volume of 200mM EGTA (Sigma) and heating to 65°C for 10 mins.

2.2.6 Phosphorylation of 5' termini

To allow the ligation of the K233/K232 adapter (chapter 5.2.1) into pSKMsx 2 it was necessary to phosphorylate the 5' ends of this adapter. The strands were annealed to one another by mixing equal amounts, heating to 65°C for 15 mins and then allowing to cool slowly to RT. 2.5μg of annealed adapter was then phosphorylated by 40 units of polynucleotide kinase (Boehringer Mannheim) in 80μl of 1mM ATP, 50mM Tris-HCl, 10mM MgCl₂, 0.1mM EDTA, 5mM dithiothreitol, 0.1mM spermidine, pH8.2 at 37°C for 30 mins. The phosphorylated DNA was then extracted with 1:1 phenol (Rathburn):chloroform (FS) and ethanol precipitated. The pellet was resuspended in 10μl of dH₂O.

2.2.7 DNA ligation

Insert and vector DNA were mixed at a ratio of 3:1 insert:vector, using 10ng of vector DNA. The DNA was ligated by 1 unit of T4 DNA ligase (Boehringer
Mannheim) in 20μl of 1x ligation buffer (66mM Tris-HCl, 5mM MgCl₂, 1mM dithioerythritol, 1mM ATP, pH7.5) at 16°C for 12-16 hrs.

2.3 DNA electrophoresis

2.3.1 Solutions

20X TBE: 1M Tris-HCl (pH8), 20mM EDTA, 1M boric acid (pH8.3)

10x DNA loading buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol (FS) in dH₂O

2.3.2 Agarose-gel electrophoresis

DNA molecules were separated according to their size on horizontal agarose gels. Flowgen agarose was routinely used and SeaKem GTG agarose (FMC Bio Products) used when DNA fragments were subsequently to be used for enzymatic manipulations or micro-injection. 0.8% to 2% agarose gels were used depending upon the size of the fragments being analysed. All gels were cast in 1x TBE buffer and contained 0.5μg/ml EtBr (BioRad). Loading buffer was added to the DNA sample, to give a final concentration of 1x, before being loaded into the gel. Gels were run in hybaid tanks containing 1x TBE at 50-100V. After electrophoresis, DNA fragments were visualised on a UV transilluminator and photographed using a video copy processor (Mitsubishi).

250ng of 1Kb DNA ladder (Boehringer Mannheim) was run on each gel to enable the size of DNA fragments to be determined approximately. The approximate concentration of DNA fragments was determined by comparing the intensity of each band to that of a known standard, routinely 250ng, 150ng, 100ng and 50ng of Hind III λ ladder (Boehringer Mannheim).
2.3.3 Purification of DNA from agarose gels

After electrophoresis, DNA fragments were visualised on a UV transilluminator and quickly excised using a sterile scalpel blade to avoid UV damage to the DNA. DNA was then isolated from the gel slice using a QIAEX Gel Extraction Kit (Qiagen) as per manufacturers instructions.

2.4 Transfer of DNA to membranes and hybridisation to radioactive probes

2.4.1 Solutions

Denaturing buffer: 0.5M NaOH, 1.5M NaCl
Neutralisation buffer: 1.5M NaCl, 0.5M Tris (pH 7.5)
20x SSC: 44.1g sodium citrate, 88.6g NaCl in 500 ml dH₂O
50x Denharts reagent: 5g ficoll, 5g polyvinyl pyrolidene, 5g BSA in 500ml dH₂O
Prehybridisation solution: 6x SSC, 5x Denharts reagent, 0.5% SDS, 100μg/ml salmon sperm DNA, 0.1% disodium pyrophosphate
Hybridisation solution: 6x SSC, 5x Denharts reagent, 0.5% SDS, 1mM EDTA, 0.1% disodium pyrophosphate
Wash solution (I): 2x SSC, 0.1% SDS
Wash solution (II): 1x SSC, 0.1% SDS

2.4.2 Southern transfer

DNA was transferred from agarose gels to nylon membranes (Hybond-N, Amersham) essentially as described by Sambrook et al. (1989). The DNA within the gel was denatured by agitating in an excess of denaturing buffer for 1 hr followed by a brief rinse in dH₂O. The gel was then neutralised in an excess of neutralisation buffer for 1 hr with agitation. A large strip of 3MM filter paper (Whatman) was soaked in 10x...
SSC and placed on a support. The ends of the paper were placed in a reservoir of 10x SSC, forming a wick. The gel was inverted and placed on top of this support, ensuring no air was trapped under the gel. Saran wrap (Dow Chemical Company) was used to form a barrier around the gel. The nylon membrane and 4 pieces of 3MM filter paper (Whatman), all pre-soaked in 2x SSC, were placed on top of the gel ensuring no air was trapped. A weighed stack of paper towels was then placed on top. Transfer of DNA by capillary action was complete after 16 hrs. The DNA was then bound to the membrane by baking for 2 hrs at 80°C.

2.4.3 Radioactive labelling of DNA

DNA fragments were radioactively labelled with [α-32P]-dCTP using the random prime reaction developed by Feinberg and Vogelstein, 1984. In this reaction random hexanucleotides prime polymerization along the DNA strand. The klenow fragment of E. Coli. polymerase 1 then catalysis polymerisation, incorporating a radiolabelled base at every C nucleotide.

25ng of DNA in 10μl of dH2O was denatured by heating to 100°C for 10 mins, then cooling on ice. Labelling was performed using a Random Prime Kit (Boehringer Mannheim). 1μl of each dATP, dTTP, dGTP solution, 2μl of reaction buffer, 1μl (2 units) of Klenow and 4μl of 10μCi/μl [α-32P]-dCTP (Amersham) were added to the denatured DNA. The reaction was then incubated at 37°C for 30 mins. The probe was then denatured by heating to 100°C for 10 mins and stored on ice before adding to the hybridisation buffer.

2.4.4 Hybridisation of radioactive DNA probes to membranes

All hybridisations were carried out in glass bottles (Hybaid), incubated in a rotating hybridisation oven (Hybaid). Membranes were prehybridised in prehybridisation solution for 1 hr at 57°C. The membranes were then placed in hybridisation buffer and hybridised to the denatured radiolabelled probe for 12-16 hrs at 57°C.
2.4.5 Post-hybridisation washes

The hybridisation solution was removed and membranes washed with 2x 5 min changes of wash solution (I) at 37°C in a rotary hybridisation oven (Hybaid). The filters were then removed and washed with 3x 30 min changes of wash solution (II) at ~65°C with agitation or until a desirable level of radioactivity was present. Membranes were then wrapped in Saran wrap (Dow Chemical Company).

2.4.6 Autoradiography

Membranes were placed in light-tight signal enhancing cassettes and exposed to X-OMAT x-ray film (Kodak). The exposure time was dependant upon the degree of radioactivity. The film was then developed in an automatic x-ray film processor RGII (Fuji).

2.5 DNA sequencing

Double-stranded DNA templates were sequenced using the dideoxy-sequencing method of Sanger et al. (1977) and the primers summarised in table 2.1. Both manual sequencing, using ³⁵S ATP, and automated sequencing, using fluorescent-dye-labelled dideoxynucleotides, were used.

2.5.1 Manual sequencing

2.5.1.1 Sequencing reaction

In a total volume of 16μl, double-stranded DNA and sequencing primer were mixed to give a final concentration of 0.16μg/μl and 30ng/μl respectively. 4μl of 1M NaOH was added and the mixture incubated at RT for 5 mins. 4μl of 2.5M ammonium acetate (adjusted to pH4.6 with acetic acid) was then added, followed by 55μl of ice
cold ethanol. The reaction was then precipitated on ice for 15 mins, pelleted by centrifugation for 15 mins at 4°C, washed in 1ml of cold 70% ethanol, dried under vacuum and resuspended in 8.75μl of dH2O.

The sequencing reaction was performed using a Sequenase Version 2.0 Kit (United States Biochemical). To the 8.75μl of template/primer, 1.25μl of DMSO, 2.5μl of sequenase reaction buffer, 1μl dithiothreitol, 2μl of diluted labelling mix (1:5), 0.5μl of [α-35S]-dATP (10μCi/μl) (Amersham) and 2μl of diluted sequenase (1:8) were added and the reaction incubated at RT for 5 mins. During this time each termination mix was diluted with DMSO to give a final concentration of 10% and 2.5μl of each aliquoted into separate wells of a 96 well plate (Sero well). This plate was then warmed to 37°C in a water bath and 3.5μl of the completed sequencing reaction added to each of the 4 wells. These samples were incubated in a 37°C water bath for 5 mins and then the reaction terminated by adding 4μl of stop solution.

2.5.1.2 Electrophoresis and detection of sequencing reactions

The products of the sequencing reaction were separated by electrophoresis on vertical polyacrylamide-gels using BioRad apparatus. Prior to assembling the gel apparatus, the glass plates and spacers were thoroughly cleaned and washed with ethanol. The plates were then sandwiched together, with the spacers maintaining a gap between the plates. The base was sealed by standing the glass plates in a tray containing 10ml of acrylamide (Severn Biotech), 50μl of TEMED (Gibco BRL) and 50μl of 25% ammonia persulphate (APS). This solution was drawn by capillary action into the space at the base of the plates before setting. The gel was prepared by mixing 60ml of acrylamide (Severn Biotech), 68.6μl of TEMED (Gibco BRL) and 68.6μl of APS. This was then poured between the two glass plates using a syringe and ensuring no air was trapped.

Before loading the samples, the gel was warmed to 50°C and the sequencing samples heated to 95°C for 3 mins and then cooled on ice. 2μl of each sample was then
loaded. The gel was run in TBE buffer, 1x in the bottom tray and 0.5x in the top, at 2.2KV. The gel was then removed from the plates, placed on 3MM filter paper (Whatman) and Saran wrap (Dow Chemical Company) placed on top. The gel was then dried at 60°C under vacuum on a Gel Dryer 583 (BioRad). The dried gel was then placed in a light-tight cassette with a signal enhancing screen and exposed to X-OMAT x-ray film (Kodak). Generally, a 12-16 hr exposure time was sufficient. Films were then developed in an automatic x-ray film processor RGII (Fuji).

2.5.2 Automated DNA sequencing

Double-stranded DNA was sequenced using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

250ng of template DNA, 19.2ng of primer (table 2.1) and 8μl of terminator ready reaction mix (Perkin Elmer) made up to 20μl in dH2O was covered with mineral oil (Sigma) and cycled through 94°C for 30 secs, 50°C for 15 secs and 60°C for 4 mins 25 times in a Hybaid Omnigene PCR machine. The product was then precipitated by adding 2μl of 3M NaAc (pH 7.2), 50μl of ethanol and incubating on ice for 15 mins. The precipitated DNA was then recovered by centrifugation for 20 mins at 4°C, washed in 70% ethanol and resuspended in 5μl of loading buffer (5:1 deionized formamide to 25mM EDTA (pH 8) containing 50mg/ml dextran blue) These samples were then analysed on an Applied Biosystems 373A DNA sequencer by Agnes Gallagher.

2.6 Isolation of DNA and RNA

2.6.1 Solutions

Tail-tip buffer: 100mM Tris (pH8.5), 5mM EDTA, 0.2% SDS, 200mM NaCl and 100μg/ml proteinase K (Boehringer Mannheim) added immediately before use.
2.6.2 DNA extraction from mouse tail-tips and embryonic yolk sacs

A small tail biopsy or embryonic yolk sac was placed in 0.5ml of tail-tip buffer and incubated at 55°C for 12-16 hrs. Samples were then vortexed and centrifuged for 10 mins. The supernatant was transferred to a clean eppendorf and the DNA precipitated by the addition of 0.5ml of isopropanol (FS). The DNA was pelleted by centrifugation for 15 mins, washed in 70% ethanol and resuspended in 0.5ml of TE.

2.6.3 Total RNA extraction from eukaryotic cell cultures and embryonic tissues

To prevent degradation of RNA by ribonucleases, sterile-disposable plastic wear was used throughout. Additionally, diethyl pyrocarbonate (DEPC; Sigma) was added to solutions to give a final concentration of 0.1% and incubated at 37°C for 12 hrs. Solutions were then autoclaved to remove any trace DEPC. Total RNA was isolated using either Total RNA Isolation Reagent or a RNeasy Kit.

2.6.3.1 Total RNA isolation reagent

Cell cultures were washed in PBS (Oxoid) and removed from the culture dish by treatment with 1:10 trypsin to versene (0.2% trypsin, 0.04% EDTA in Dulbecco 'A') at RT for 5 mins. The cells were then harvested by centrifugation at 550g for 5mins. 1ml of RNA Isolation Reagent (Advanced Biotechnologies Ltd) was added to the pelleted cells or embryonic tissue which was then homogenised by passing the lysate through increasingly narrower gauged hypodermic needles (Becton Dickinson). 0.2ml of chloroform (FS) per 1ml of RNA Isolation Reagent was added, the samples were then vortexed for 15 secs, incubated on ice of 5 mins and then centrifuged at 12000g for 15 mins at 4°C. After centrifugation the DNA and protein move to the lower organic phase, while RNA moves to the upper aqueous phase. The aqueous phase was then transferred to a fresh tube and the RNA precipitated by adding an equal
volume of isopropanol (FS), incubating at 4°C for 10 mins and then centrifugating at 12000g for 10 mins at 4°C. The RNA pellet was then washed twice in 75% ethanol, briefly dried under vacuum and resuspended in 50μl of DEPC-treated H₂O.

2.6.3.2 RNeasy kit method

Cultured cells were harvested as described in 2.6.3.1. Pelleted cells or embryonic tissues were then homogenised in lysis buffer (RNeasy Total RNA Kit; Qiagen) by passing the lysate through progressively narrower gauged hypodermic needles. Total RNA was then extracted from the lysates using a RNeasy Total RNA Kit (Qiagen) as per manufacturers instructions.

2.6.3.3 Quantification of total RNA

RNA concentration was determined by measuring the absorbance at 260nm in a spectrophotometer. An absorbance reading of 1 corresponds to 40μg of RNA per ml.

2.7 Polymerase chain reaction (PCR) protocols

2.7.1 Oligonucleotides

Oligonucleotides (described in table 2.1) were either synthesised by Agnes Gallagher as ammonium stocks on an Applied Biosystems 381A oligonucleotide synthesiser or supplied by Genysis as precipitates. Oligonucleotides were precipitated from ammonium stocks by adding a 1/10 volume of 3M NaAc (pH7), 2 volumes of 100% ethanol and incubating at -20°C for 30mins. The precipitate was collected by centrifugation for 10 mins, washed in 70% ethanol and resuspended in an appropriate volume of dH₂O. Oligonucleotides supplied as precipitates were resuspended in an appropriate volume of dH₂O.
Oligonucleotide concentration was determined by measuring the absorbance at 260nm in a spectrophotometer. An absorbance reading of 1 corresponds to approximately 20µg of oligonucleotide per ml.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence 5'-3'</th>
<th>Description</th>
<th>Use</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>J564</td>
<td>AAACCTGAAACAT</td>
<td>mMsx 1, downstream, 1253-1275</td>
<td>p</td>
<td>97°C 2min + 35x (94°C 1min, 55°C 1min, 72°C 1min) + 72°C 10min</td>
</tr>
<tr>
<td></td>
<td>CTTCCGACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J565</td>
<td>AAGATCTGGTTC</td>
<td>mMsx 1, upstream, 732-754</td>
<td>p</td>
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</tr>
<tr>
<td></td>
<td>CAGAACCCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J562</td>
<td>GAGCACAGGGTGC</td>
<td>mMsx 2, downstream, 1084-1104</td>
<td>p,s</td>
<td>97°C 2min + 33x (94°C 1min, 57°C 30sec, 72°C 1min) + 72°C 10min</td>
</tr>
<tr>
<td></td>
<td>TATGGAAGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J563</td>
<td>GGAGCACCCTGC</td>
<td>mMsx 2, upstream, 697-717</td>
<td>p,s</td>
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<tr>
<td></td>
<td>GATACAGGGAG</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>J525</td>
<td>TTTGAGACCTTC</td>
<td>mActin, upstream, 1214-1235</td>
<td>p</td>
<td>97°C 2min + 35x (94°C 1min, 55°C 1min, 72°C 1min) + 72°C 10min</td>
</tr>
<tr>
<td></td>
<td>AACACCCCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J253</td>
<td>ACTCTGCTTGC</td>
<td>mActin, downstream, 2568-2589</td>
<td>p</td>
<td>As above</td>
</tr>
<tr>
<td></td>
<td>TGATCCACTT</td>
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</tr>
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<td>J36</td>
<td>CAGTGAGGGCGG</td>
<td>cMsx 2, downstream, 664-681</td>
<td>p</td>
<td>97°C 2min + 30x (94°C 1min, 58°C 1min, 72°C 1min) + 72°C 10min</td>
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<tr>
<td></td>
<td>CCTGGAT</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>J37</td>
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<td>cMsx 2, upstream, 302-319</td>
<td>p</td>
<td>As above</td>
</tr>
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<td></td>
<td>ACGTCCCTT</td>
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<tr>
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<td>p</td>
<td>97°C 2min + 35x (94°C 1min, 50°C 1.5min, 72°C 2min) + 72°C 10min</td>
</tr>
<tr>
<td></td>
<td>GCCGCTCTACTCG</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGCAGTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L200</td>
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<td>cMsx 2, downstream, 922-941</td>
<td>P</td>
<td>As above</td>
</tr>
<tr>
<td></td>
<td>AGGGCTGGATGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GATCCACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacz-1</td>
<td>GATTACCGTTGA</td>
<td>βGal, upstream</td>
<td>p</td>
<td>94°C 2min + 30x (94°C 1min, 55°C 1min, 72°C 35 sec) + 72°C 5min</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lacz-2</td>
<td>AACCGTGATAT</td>
<td>βGal, downstream</td>
<td>p</td>
<td>As above</td>
</tr>
<tr>
<td></td>
<td>TCAGCCATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K232</td>
<td>AGCCATGCGATAC</td>
<td>Adapter, sense</td>
<td>c</td>
<td>N/A</td>
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<tr>
<td></td>
<td>GCGTAGATCTGT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGACTATCA</td>
<td></td>
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</tr>
</tbody>
</table>
Table 2.1 Summary of oligonucleotides. The sequence, description and use of each oligonucleotide is shown. p = PCR, s = sequencing, c = cloning.

2.7.2 PCR-based genotyping

Transgenic mice and embryos were identified by PCR, using primers for βGal (Lacz-1 and Lacz-2) on DNA extracted from tail biopsies or yolk sacs (2.6.2). Each PCR
reaction contained 2.5μl of PCR buffer (Perkin Elmer), 2.5μl of 25mM MgCl₂ (Perkin Elmer), 0.5μl of 100mM dNTPs (25mM each; Advanced Biotechnologies), 0.5μl of DNA template (2.6.2), 0.5μl of each primer (from a stock of 0.1μg/μl), 0.5μl of AmpliTaq (Perkin Elmer) and dH₂O up to 25μl. Reactions were overlaid with a drop of mineral oil (Sigma) and placed in an Hybaid Omnigene PCR machine under the appropriate conditions (table 2.1). The completed PCR reaction was then analysed by agarose-gel electrophoresis (2.3.2).

2.7.3 RT-PCR

2.7.3.1 DNase I treatment

Total RNA samples were treated with DNase I (Boehringer Mannheim) to remove any trace DNA contamination. 1.5μg (or the amount specified in the main text) of total RNA (2.6.3) was mixed with 8μl of 5x cDNA synthesis buffer (250mM Tris-HCl, 200mM KCl, 50mM MgCl₂, 50mM dithioerythritol, pH8.3; Boehringer Mannheim), 0.5μl (20 units) of RNase Inhibitor (Boehringer Mannheim), 2μl (20 units) of DNase I (Boehringer Mannheim) and made up to 40μl with DEPC-treated dH₂O. This reaction was incubated at 37°C for 30mins.

Total RNA was purified from the completed reaction using a RNeasy Kit as per manufacturers instructions (Qiagen). Purified RNA was eluted in 31.5μl of DEPC treated dH₂O.

2.7.3.2 First-strand cDNA synthesis

3μl of 100ng/μl oligo(dT-30mer) was added to the 31.5μl of purified total RNA (2.7.3.1) and heated at 65°C for 5 mins. The primer was then allowed to anneal to the RNA by placing the sample at RT for 10 mins. 10μl of 5x cDNA synthesis buffer (250mM Tris-HCl, 200mM KCl, 50mM MgCl₂, 50mM dithioerythritol, pH8.3; Boehringer Mannheim), 1μl (40 units) RNase Inhibitor (Boehringer Mannheim), 2μl
of 100mM dNTPs (25mM each; Advanced Biotechnologies) and 2.5μl (50 units) of Reverse transcriptase, M-MuLV (Boehringer Mannheim) was then added. The components were gently mixed and incubated at 37°C for 1 hr. The reaction was then terminated by heating at 90°C for 5 mins.

2.7.3.3 PCR amplification of first-strand cDNA

Each PCR reaction contained 10μl of 10x PCR buffer (Perkin Elmer), 10μl of 25mM MgCl₂ (Perkin Elmer), 0.8μl of 100mM dNTPs (25mM each; Advanced Biotechnologies), 3μl of first strand cDNA (2.7.3.2), 2μl of each primer (from a stock of 0.1μg/μl) [except for primers J36 and J37 when 7μl of each 0.1μg/μl primer was used], 0.5μl amplitaq (Perkin Elmer) and dH₂O up to 100μl. Reactions were overlaid with a drop of mineral oil (Sigma) and placed in a Hybaid Omnigene PCR machine under the appropriate conditions (refer to table 2.1). 25μl of the completed PCR reaction was then analysed by gel electrophoresis (2.3.2).

Adapted protocol for the amplification of full-length chick Msx 2: Each PCR reaction contained 10μl of 10x PCR buffer (New England Biolabs), 1mM MgSO₄, 0.8μl of 100mM dNTPs (25mM each; Advanced Biotechnologies), 2μl of cDNA (2.7.3.2), 4μl of each primer (from a stock of 0.1μg/μl), 0.5μl of Vent DNA Polymerase (New England Biolabs) and dH₂O up to 100μl. Reactions were overlaid with mineral oil (Sigma) and placed in a Hybaid Omnigene PCR machine under the conditions specified in table 2.1.

2.8 Transgenic methodology

2.8.1 Solutions

Micro-injection buffer: 10mM Tris, 0.1mM EDTA, pH7.5. Filter sterilised.
2.8.2 Methodology

The transgene was released from the vector by restriction digest (2.2.4) and isolated by gel electrophoresis (2.3.2 and 2.3.3). The transgene DNA, eluted in micro-injection buffer, was then passed through a Micropure 0.22 column (Amincon) by centrifugation at 5000rpm in a bench top centrifuge, diluted in micro-injection buffer to 2ng/μl and finally passed through two 0.22μm Millex-GV4 filters (Millipore) to remove any particles.

Mice were superovulated by staff at the transgenic facility and oocytes collected by either S. Jordan or L. McInnes DNA at 2ng/μl was injected into the pronucleus of collected oocytes. Following overnight culture, those which reached the two cell stage were transferred into pseudopregnant host mice. Micro-injections and embryo transfers were performed by S. Jordan and L. McInnes.

2.9 Analysis of mouse and chick embryos

2.9.1 Isolation of mouse and chick embryos

Mouse: The day of the vaginal plug, following mating, was designated E0.5. Pregnant females were killed by cervical dislocation and the embryos dissected from the uterus in ice cold PBS (Oxoid).

Chick: Fertilised Ross White eggs (Roslin, Edinburgh) were incubated on their sides in a 38°C humidified incubator, rotating by 180° once a day. Access to the embryo was achieved by puncturing the base of the egg, sealing the side with Sellotape and then cutting a window in the side of the egg. Chick embryos were staged according to Hamburger and Hamilton (1951). If further development was required, eggs were re-sealed with sellotape and returned to the incubator.
If embryos were to be used for tissue culture or *in situ* hybridisation; the work space, culled mother or egg were swabbed in 70% ethanol, dissection tools were flamed in 70% ethanol and all solutions were sterilised.

2.9.2 Whole-mount X-Gal staining of mouse embryos

2.9.2.1 Solutions

0.1M phosphate buffer: 126ml 0.1M NaH₂PO₄·2H₂O, 400ml 0.1M Na₂HPO₄ (pH7.3)

Fix: 2% formaldehyde (added as Millory's 10% neutral buffered formalin -10% formaldehyde in 0.1M NaH₂PO₄), 0.2% gluteraldehyde, 2mM MgCl₂, 5mM EGTA pH 8 in 0.1M phosphate buffer.

Detergent wash: 2mM MgCl₂, 0.1% sodium desoxycholate, 0.02% Nonidet P40 (ICN), 0.05% BSA (Sigma) in 0.1M phosphate buffer.

Stain solution: 0.085%NaCl, 5mM K₃Fe(CN)₆ (Sigma), 5mM K₄Fe(CN)₆ (Sigma), 0.1% X-gal (Boehringer Mannheim) in dimethyl formamide, made up in detergent wash.

4% PFA: made in PBS

2.9.2.2 Methodology

Isolated embryos were placed in fix for 1 hr at 4°C and then washed 3x 20 mins at RT in detergent wash. Embryos were then incubated for 3-6 hrs in stain solution at 37°C using glass containers. The embryos were then washed in 2x 20 mins changes of PBS and fixed in 4% PFA at 4°C for 12-16 hrs.
Embryos were analysed and photographed with a Wild Heerbrugg microscope, mounted with a Photoautomat (Wild Leitz) camera using 64 ASA colour film (Fuji).

2.9.3 Wax embedding and sectioning of embryos

Embryos were fixed for 12-16 hrs at 4°C in 4% PFA. For E10.5 to E13.5 mouse embryos (or equivalently sized chicks) embryos were washed in PBS at 4°C for 30 mins and then dehydrated by 40 min changes of 30%, 50% and 70% ethanol.

Embryos were then automatically processed in a Tissue-Tek VIP machine (Miles Inc) through 40 min changes of 85% and 95% ethanol, 2x 40 min changes of 100% ethanol, 2x 40 min changes of 100% xylene and 4x 40 min changes of paraffin wax at 60°C.

Embryos younger than E10.5 (or similarly sized chick embryos) were processed through the same solutions but with reduced incubation times (typically 20-30mins). To minimise tissue damage, these embryos were processed by hand.

After the final incubation in paraffin wax, embryos were embedded in fresh wax contained in a mould. The wax was set by floating the mould in a bath of cold water. Sterile and freshly made up solutions were used for the embedding of embryos for in situ hybridisation.

7μm sections were cut on a Wetzlar (Leitz) microtome. For in situ hybridisation the blade was swabbed with 70% ethanol. Sections were floated out in sterile water at 40°C and onto glass slides (Chance Propper). Slides were then incubated at 60°C overnight to seal on the sections. Slides were then stored in a sealed box with silica gel desiccant. For in situ hybridisation, sections were mounted on TESPA treated slides (see below).

TESPA slides: Glass slides were washed in 10% HCl in 70% ethanol for 20 secs, then washed in sterile dH2O for 20 secs and finally washed in 100% acetone (FS) for 20
secs. The slides were then air dried, washed in 2% TESPA (3-aminopropyl-triethoxy silane; Sigma) in acetone (FS) for 20 secs and washed in 2x 20 sec changes of 100% acetone (FS). Treated slides were then air dried and stored in a sealed box with silica desiccant.

2.9.4 Haematoxylin and eosin staining

Slides were dewaxed by placing in 2x 5 min changes of xylene, then rehydrating in 2x 5 min changes of 100% ethanol followed by 5 min changes of 90%, 70%, 50% and 30% ethanol. Slides were then washed for a few mins in water. Slides were placed in haematoxylin (Surgipath) for 4-5 mins, washed in running tap water and then differentiated in 1% HCl in 70% ethanol for a few secs. The slides were then washed in running tap water and transferred to lithium carbonate solution for a few secs. The slides were then washed in running tap water, stained in eosin (3 parts 1% aqueous eosin (Surgipath), 1 part 1% ethanol and 0.05% acetic acid) for 1-2 mins and rinsed in water. The slides were then processed as follows: 1x 15 secs in 100% ethanol, 2x 1 min in 100% ethanol and 1x 5 mins in 100% xylene. The slides were then transferred to fresh xylene and mounted with a glass coverslip (Chance Propper) in DPX.

Slides were analysed and photographed with a Ziess Axioplan 2, mounted with a Yashica 108 multiprogram camera using 64 ASA colour film (Fuji).

2.9.5 mRNA radioactive in situ hybridisation

2.9.5.1 Solutions

Proteinase K buffer: 50mM Tris, 5mM EDTA.
Hybridisation Mix: 50% formamide, 10% dextran sulphate, 1x denharts, 20mM Tris (pH8), 0.3M NaCl, 5mM EDTA, 10mM sodium phosphate, 0.5mg/ml tRNA and 50mM DTT (added immediately before use)
High Stringency Wash: 50% formamide, 2x SSC, 50mM DTT (added immediately before use)

NTE: 0.5mM NaCl, 10mM Tris, 5mM EDTA, pH7.5.

2.9.5.2 Radioactive labelling of riboprobes

The DNA template was linearised by restriction digest with the appropriate enzyme (refer to table 2.2 and chapter 2.2.4) and cleaned using a Gene Clean II Kit (Bio 101) as per manufacturers instructions, eluting in sdH₂O. Probes were prepared by transcription using T7 polymerase in the presence of³⁵SUTP as follows. 6μl of 10x transcription buffer (Boehringer Mannheim), 1μl of 10mM rATP, 1μl of 10mM rCTP, 1μl of 10mM rGTP, 1μl of 1M dithiothreitol (DTT), 3μl dH₂O, 12μl of³⁵SUTP (>1mMCi/100μl; Amersham), 5μl of linearised DNA template (0.5-1μg/5μl), 1.2μl of RNase Inhibitor (Boehringer Mannheim) and 0.8μl of T7 polymerase (Boehringer Mannheim) were combined in this order and incubated at 37°C for 25 mins. A further 0.8μl of T7 polymerase was then added and the reaction incubated at 37°C for a further 25 mins. 2μl of 10mg/ml tRNA and 1μl of DNase I (Boehringer Mannheim) was then added and the reaction incubated at 37°C for 10 mins. The reaction was stopped by adding 2μl of 100mM EDTA.

The riboprobe was purified by adding 100μl of TE with 50mM DTT, applying the mixture to a Microcon 30 filter (Amincon) and centrifuging for 15 mins in a bench top centrifuge. A further 100μl of TE with 50mM DTT was then spun through the column as before. 25μl of TE, 50mM DTT was then added to the microcon and the column incubated on ice for 20 mins. The microcon was then inverted into a fresh tube and the probe collected by centrifugation for 5 mins in a bench top centrifuge. This elution procedure was then repeated with a further 25μl of TE, 50mM DTT.

The incorporation efficiency and dpm/μl was determined by taking 1μl of probe and adding to 19μl of TE, 50mM DTT. This mix was added to two Whatman GF/B filters (10μl/filter) and one filter washed 3x in TCA (trichloroacetic acid solution; Sigma)
then once in 100% ethanol, drawn through under vacuum. The filters were then air
dried and both the washed and unwashed filters counted in separate scintillation vials
in 10ml of aquasol using a Packard Tri-Carb 1500 Liquid Scintillation Analyser. %
icorporation = (precipitated count)/(total count) x 100.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Enzymes for linearisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pTZλ2.2</td>
<td>Mouse Msx 1, 731-958</td>
<td>BssHII</td>
</tr>
<tr>
<td>pλ26AR3</td>
<td>Mouse Msx 2, 985-1371</td>
<td>Bam HI</td>
</tr>
<tr>
<td>GHox7sp</td>
<td>Chick Msx 1, 800bp Pst I/Eco RI fragment</td>
<td>Bam HI</td>
</tr>
<tr>
<td>G#8ps7</td>
<td>Chick Msx 2, 700bp Pst I fragment</td>
<td>Bam HI</td>
</tr>
</tbody>
</table>

Table 2.2 Summary of in situ hybridisation plasmids.

2.9.5.3 Prehybridisation

Slides were dewaxed and rehydrated as follows in batches of 20: 2x 5min in 100%
xylene, 2x 2 min in 100% ethanol, 2 min in 90%, 70%, 50%, 30% ethanol and finally
1x 2 min in PBS with agitation. The slides were then fixed in 4% PFA (pH7.3) for 10
min, split into two racks and washed twice in PBS for 2 min. The slides were then
incubated in proteinase K buffer with 20μg/ml Proteinase K (BCL Biochemicals) for
7.5 min at RT. The slides were then transferred to PBS for 1 min, 4% PFA for 2 min,
rinsed in sdH_{2}O for 10 sec, 0.1M triethanol amine (TEA) pH 8 for 30 sec, 2x 5 min
0.1M TEA containing 625μl/100mls of acetic anhydride (Sigma) with stirring, PBS
for 2mins and finally 0.85% NaCl for 2 mins. The slides were then dehydrated as
follows: 1 min changes in 30%, 50% 70%, 90% ethanol, then 3x 5 min changes of
100% ethanol. The slides were then air dried and stored in a sealed box with silica gel
dessicant.
2.9.5.4 Hybridisation

$^{35}$S riboprobe was diluted with TE, 50mM DTT so that when hybridisation mix was added at a ratio of 1:9 (probe to hybridisation mix), the final count was $1.1 \times 10^5$ dpm/µl. The probe/hybridisation mix was heated at 80°C for 2 min, rapidly cooled on ice and then ~50µl added to each slide. A glass coverslip was placed over each slide and the slides placed horizontally in a sealed box containing a tissue soaked in 5ml of 50% formamide, 5x SSC. The box was heat sealed inside two plastic bags and submerged in a water bath at 55°C for 16-18 hrs.

2.9.5.5 Post-hybridisation washes

Slides were removed from the hybridisation box and placed in 5x SSC, 10mM DTT for 20 min at 55°C, allowing the coverslips to be removed. The slides were then transferred to a Hybaid Omnislide Wash Module containing high stringency wash at 65°C for 30 min. The slides were then washed in 3x 10 min changes of NTE at 37°C before being incubated in NTE containing 20µg/ml of RNAase A for 30 min at 37°C. The slides were then washed in NTE at 37°C for 5 min, then transferred back to the wash module containing high stringency wash at 65°C for 30 min. The slides were then washed in 4x 10 min changes of 2x SSC at RT followed by 4x 5 min changes of 0.1x SSC at RT. Finally, the slides were dehydrated by 1 min changes in 30%, 50%, 70% and 90% ethanol followed by 2x 5 min changes of 100% ethanol. The slides were then air dried.

2.9.5.6 Autoradiography

Illuminated by a S902 safety filter lamp, slides were tipped (back to back) in 1:1 sdH$_2$O to Ilford K5 emulsion at 41°C. The slides were then allowed to dry slowly in a light tight box containing damp tissues for 12-16 hrs. The dried slides were then placed in a light-tight box containing silica desiccant and stored at 4°C. Slides were exposed to the film for 4-6 weeks and then developed by immersing in Kodak D19
developer for 4 min, washed in sdH$_2$O for 10 sec, fixed in a 1:3 dilution of AMFIX:sdH$_2$O for 5 mins and then rinsed in 2x 10min changes of water. The slides were then counter stained in 1% methyl green (Sigma), air dried and mounted with a glass coverslip in DPX.

Slides were analysed and photographed with a Zeiss Axioplan 2, mounted with a Yashica 108 multiprogram camera using 64 ASA colour film (Fuji).

2.9.6 Immunohistochemical staining of wax sections

Embryos were fixed in 4% PFA, embedded in wax and sectioned at 7μm as described in 2.9.3. Slides were then dewaxed by 2x 10 min changes in 100% xylene followed by 5 min in xylene. The sections were then rehydrated by placing in 2x 2 min changes of 100% ethanol, then passing through 90%, 70%, 50% and 30% ethanol, 2 min in each, and finally 5 min in sdH$_2$O. The slides were then placed in PBS for 15 min and then incubated in blocking serum, goat, (Vectastain Elite ABC Kit; Vector Laboratories), diluted as per manufacturers instructions, and containing 0.01% Triton X100 (Sigma) for 30 min. The serum was then removed and the sections incubated in the primary antibody, diluted in blocking solution (table 2.3), for 12-16 hrs. The primary antibody was then washed off by 3x 15 min washes in PBS. The sections were then re-blocked in diluted blocking serum, goat, (Vectastain Elite ABC Kit; Vector Laboratories) containing 0.01% Triton X 100 as per manufacturers instructions for 30 min. The serum was then removed and the sections incubated in biotinylated goat anti-mouse IgG secondary antibody (Vectastain Elite ABC Kit; Vector Laboratories) diluted in blocking solution as per manufacturers instructions for 1 hr. The slides were then washed in 3x 15 min changes of PBS and incubated in Vectastain Elite ABC reagent (Vector Laboratories) for 30 min, prepared as per manufacturers instruction. The sections were then washed in 3x 15 min changes of PBS, followed by a 5-10 min incubation in a peroxidase substrate solution (DAB Substrate Kit For Peroxidase; Vector Laboratories) prepared as per manufacturers instructions. The slides were then rinsed in running tap water, dehydrated by passing through 30%, 50%, 70% and 90%
ethanol, 1 min in each, followed by 2x 5min changes of 100% ethanol. Slides were then covered with a glass cover slip in DPX. To control against non-specific binding of the secondary antibody, sections were processed through the above procedure without adding primary antibody. No staining was observed for these controls.

Slides were analysed and photographed with a Ziess Axioplan 2, mounted with a Yashica 108 multiprogram camera using 64 ASA colour film (Fuji).

2.10 Eukaryotic cell culture

All media and reagents supplied by Gibco BRL unless otherwise stated.

2.10.1 Culture conditions for established cell lines

NIE-115: Mouse glioma cell line, cultured in DMEM, 10% FCS.

G7: Mouse myoblast cell line, cultured in DMEM, 10% horse serum, 10% FCS.

All cell lines were routinely grown in tissue-culture flasks (Nunc) in a humidified incubator at 37°C in 5% CO₂, changing the media every second day. All manipulations were performed in a laminar flow air cabinet. When cultures became confluent, they were subcultured by replacing the media with 1:10 trypsin to versene (0.2% trypsin, 0.04% EDTA in Dulbecco 'A') and incubating at RT with gentle agitation for 5 min or until all the cells had detached from the substrata. The cells were then transferred to a sterile tube and the trypsin inactivated by adding an equal volume of FCS. The cells were collected by centrifugation at 450g, washed in 5ml of the appropriated medium and then resuspended in the desired amount of the appropriated medium. Resuspended cells were then placed into fresh tissue culture flasks (Nunc).
2.10.2 Primary cultures of chick PRE

Fertilised Ross White eggs were incubated and embryos collected under sterile conditions as described in 2.9.1. Typically, twelve 5-day-old chicks were used per dissection. The embryos were placed in PBS and their heads removed using flamed forceps. The heads were then transferred to a fresh dish of PBS and the eyes removed using a flamed-tungsten needle and forceps under a Stemi 2000 (Zeiss) dissection microscope. Using a hypodermic needle to hold the eye so that the choriod fissure is upwards, the front of the eye was removed using a sterile scalpel blade. The back parts were collected and the inner neural retina removed with flamed forceps. If required, the neural retina was transferred to a sterile tube using a sterile 1ml pipette and stored on ice. The PRE and associated perioptic mesenchyme was then placed in a dish containing 7.5mg/ml of filter-sterilised collagenase A (Boehringer Mannheim) in PBS and incubated at 37°C for 5 min. The collagenase solution was then replaced with ice cold PBS. The PRE was then teased from the mesenchyme using flamed forceps and placed in a sterile tube, on ice, using a sterile flame-polished, glass pipette. If required, the mesenchyme was also collected.

In a laminar flow air cabinet the excess PBS was removed from the collected PRE, neural retina and mesenchyme. 2ml of trypsin/versene 1:10 (0.2% trypsin, 0.04% EDTA in Dulbecco 'A') was added and the cells incubated for 3 min at RT with gentle shaking. 2ml of FCS was then added and the cells dissociated by briefly sucking up and down in a sterile, flame-polished, glass pipette. The cells were then pelleted by centrifugation at 1200 rpm for 5 min, resuspended in 2ml of appropriate media, re-pelleted by centrifugation at 160g for 5 mins and then resuspended in the desired volume of medium and typically seeded into 4 wells of a 24-well tissue-culture plate (Linbro, ICN). Cells were grown at 37°C, 5% CO₂ in a humidified incubator, changing the medium every second day.
If required, cells were passaged by removing the medium and incubating in trypsin/versene 1:10 (0.2% trypsin, 0.04% EDTA in Dulbecco's 'A') at RT for 5 mins or until the majority of the cells had detached from the dish. The cells were then transferred to a sterile tube and the trypsin inactivated by adding an equal volume of FCS. The cells were then pelleted by centrifugation at 160g for 5 mins, resuspended in the appropriate medium, re-pelleted and finally resuspended in the desired volume of medium and seeded into fresh tissue-culture plates.

**Media:** EMEMF was used routinely.

<table>
<thead>
<tr>
<th>Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMEMF</td>
<td>EMEM, 8% FCS, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin.</td>
</tr>
<tr>
<td>NB27</td>
<td>Neurobasal, 1x B27, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin.</td>
</tr>
<tr>
<td>10,66F</td>
<td>10,66, 3% FCS, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin, 10ng/ml FGF 2 (R&amp;D Systems).</td>
</tr>
<tr>
<td>SFM1</td>
<td>10,66, 37.5µg/ml BSA, 40µg/ml insulin, 40µg/ml transferrine, 6.9µg/ml sodium selenite, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin, +/- 10ng/ml FGF 2 (R&amp;D Systems), +/- 10ng/ml Bmp 4 (Genetic Society).</td>
</tr>
<tr>
<td>SFM2</td>
<td>10,66, 1x N1 (Sigma), 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin, 2µg/ml dexamethasone (Sigma), 37.5µg/ml BSA, +/- 10ng/ml FGF 2 (R&amp;D Systems).</td>
</tr>
<tr>
<td>EDFM</td>
<td>10,66, 8%FCS, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin, 0.15mM ascorbic acid (Sigma).</td>
</tr>
<tr>
<td>EDFPH</td>
<td>10,66, 8% FCS, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin, 0.5mM 1-phenyl-2-thiourea (Sigma), 250U/ml testicular hyaluronidase (Boehringer Mannheim).</td>
</tr>
</tbody>
</table>
Substrata: Plastic was used routinely.

Laminin: 1 µg of laminin (Sigma) per cm² of growth surface was added to each well and allowed to dry for 45 mins.

Matrigel protein coat: 20 µl of Matrigel Matrix (Becton Dickinson) was mixed with 0.5 ml of appropriate medium and added to culture dish. After 1 hr excess liquid was removed, wells rinsed in medium and cells seeded.

Matrigel thin gel: 50 µl of Matrigel Matrix (Becton Dickinson) per cm² of growth surface was added to each well and incubated at 37°C for 30 min. Cells were then seeded on top of this gel.

2.10.3 Transient transfection of eukaryotic cells

For 6-well plates: 1 µg of DNA was diluted in 100 µl of OptiMEM 1 with glutamax 1 (Gibco BRL) and 12 µl of Lipofectamine (Gibco BRL) mixed with 88 µl of OptiMEM 1 with glutamax 1 (Gibco BRL). The diluted DNA and Lipofectamine were then mixed together and incubated at RT for 30 min before adding 800 µl of OptiMEM 1 with glutamax 1 (Gibco BRL). The cells were then rinsed in OptiMEM 1 with glutamax 1 (Gibco BRL) and 1 ml of diluted DNA/Lipofectamine mix added to each well. The cells were then incubated with this mixture for 5 hrs in a 37°C, 5% CO₂ tissue-culture incubator before replacing with the appropriate medium.

For 24-well plates: 0.5 µg of DNA was diluted to 50 µl in OptiMEM 1 with glutamax 1 (Gibco BRL) and 6 µl of Lipofectamine (Gibco BRL) mixed with 44 µl of OptiMEM 1 with glutamax 1 (Gibco BRL). The diluted DNA and Lipofectamine were then mixed together and incubated at RT for 30 mins before adding 0.4 ml of OptiMEM 1 with glutamax 1 (Gibco BRL). The cells were rinsed in OptiMEM 1 with glutamax 1 (Gibco BRL) and 0.5 ml of diluted DNA/Lipofectamine mix added to each well. The cells were then incubated with this mixture for 5 hrs in a 37°C, 5% CO₂ incubator before replacing with the appropriate medium.
For 8-well chamber slides: 1μg of DNA was diluted in 100μl of Optimem 1 with glutamax 1 (Gibco BRL) and 12μl of Lipofectamine reagent mixed with 88μl of Optimem 1 with glutamax 1 (Gibco BRL). The diluted DNA and Lipofectamine were then mixed together and incubated at RT for 30 min before adding 1.6 ml of Optimem 1 with glutamax 1 (Gibco BRL). The cells were rinsed in Optimem 1 with glutamax 1 (Gibco BRL) and 150μl of diluted DNA/Lipofectamine mix added to each well. The cells were then incubated with this mixture for 5 hrs in a 37°C, 5%CO₂ incubator before replacing with the appropriate medium.

2.10.4 X-Gal staining of cultured cells

2.10.4.1 Solutions

Fix:  0.5ml formaldehyde (FS), 0.08ml 25% glutaraldehyde, 9.42ml PBS  
Stain:  0.02g potassium ferrocyanide (Sigma), 0.016g potassium ferricyanide (Sigma), 20μl 1M MgCl₂, 9.75ml PBS, 0.25ml 40mg/ml X-Gal (Boehringer Mannheim) in DMSO.

2.10.4.2 Methodology

Cultures were rinsed in PBS and fixed for 5 mins at 4°C in fix. The cells were then rinsed in PBS, covered in stain and incubated in the dark at 37°C for 3 hrs. The stain solution was then removed and the cells rinsed in PBS. If required, cells were then counter stained for up to 5 mins in Vector Nuclear Fast Red (Vector Laboratories) at RT, then rinsed in sdH₂O.

Stained cells were analysed and photographed on a Labovert FS (Leitz) inverted phase microscope mounted with a photoautomat (Wild Leitz) camera using 64 ASA colour film (Fuji). Cells were counted by recording the number of cells in a field of view at x10 magnification. The dish was then moved horizontally, allowing the
adjacent field to be viewed. This procedure was repeated until a sufficient number of cells had been analysed.

2.10.5 Extraction of unpolymerized cytoskeletal subunits

Cells were detergent extracted as described by Ferreira and Caceres (1992). Cells were rinsed twice in microtubule stabilising buffer (MTSB; 0.13M Hepes (pH 6.9) (Sigma), 2mM MgCl₂, 10mM EGTA; Sigma), then incubated in MTSB, 0.2% Triton X-100 (Sigma) for 4 min at RT. The cells were then rinsed in MTSB and fixed in -20°C methanol/acetone 1:1 for 10 min.

2.10.6 Immunofluorescent staining of cultured cells

Cells grown in 8-well, glass, chamber slides (Nunc) were rinsed in ice cold PBS. The chambers were then removed leaving the cells attached to the glass slide. The cells were then fixed with -20°C methanol (FS):acetone (FS), 1:1, for 10 min at RT, rehydrated for 20 min in PBS and then blocked in PBS containing 6.7% glycerol, 2% BSA (Sigma) and 0.2% Tween 20 (Sigma) for 1 hr. The primary antibodies (table 2.3) were then diluted to the optimal concentration in blocking solution. For dual labelling, primary antibodies were diluted together if raised in different species. The cells were incubated in the diluted primary antibodies for 1 hr at RT. The cells were then washed in 3x 5 min changes of PBS, 0.1% Tween 20 before being incubated for 1 hr in the dark at RT in fluorescein (FITC) and Texas-red conjugated secondary antibodies diluted in blocking solution (table 2.3). The slides were then washed in 3x 5 min changes of PBS, 0.1% Tween 20 and mounted with a glass coverslip in Vectashield mounting medium containing DAPI (Vector Laboratories). The edges of the slide were sealed in rubber solution (Pang) and observed with a fluorescence microscope.

To control for non-specific binding of the secondary antibodies in dual labelling experiments, cells were incubated with each of the primary antibodies separately then
incubated with the non-complementary secondary antibody (i.e. the mouse primary antibody was incubated with the anti-rabbit secondary antibody and vice versa). No stain was observed in these controls.

Images were captured on a Zeiss Axiosplan microscope with a Photometrics CCD camera and Digital Scientific Software.

<table>
<thead>
<tr>
<th>Ab</th>
<th>Description</th>
<th>Dilution used</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-TuJ1</td>
<td>Reacts with class III βtubulin</td>
<td>1:4000 and 1:10000 *1:500</td>
<td>Frankfurter</td>
</tr>
<tr>
<td>Mouse anti-Map2 (clone HM2)</td>
<td>Reacts with all three forms of Map 2</td>
<td>1:200 *1:200</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mouse anti-Gap43 (clone GAP 7B10)</td>
<td>Reacts with Gap 43</td>
<td>1:1000</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mouse anti-NF68 (clone NR4)</td>
<td>Reacts with NF68</td>
<td>1:200</td>
<td>Sigma</td>
</tr>
<tr>
<td>Mouse anti-Islet I (clone 40.2D6)</td>
<td>Reacts with Islet I</td>
<td>1:2000</td>
<td>NSHB</td>
</tr>
<tr>
<td>Rabbit anti-βGal</td>
<td>Reacts with E.coli βGalactosidase</td>
<td>1:200</td>
<td>5 Prime to 3 Prime</td>
</tr>
<tr>
<td>Donkey anti-rabbit IgG</td>
<td>Fluorescein (FITC) conjugated secondary Ab</td>
<td>1:200</td>
<td>Jackson ImmunoResearch laboratories</td>
</tr>
<tr>
<td>Donkey ant-mouse IgG</td>
<td>Texas Red conjugated secondary Ab</td>
<td>1:200</td>
<td>Jackson ImmunoResearch Laboratories</td>
</tr>
</tbody>
</table>

Table 2.3 Summary of antibodies used for immunofluorescent staining of cell cultures and immunohistochemical staining of wax sections. Dilutions marked with * correspond to the dilutions used for immunohistochemical staining of wax sections (2.9.6).
Chapter 3

Msx Expression During Mouse Eye Development

3.1 Introduction

Although the Msx genes are known to be essential for mouse eye development (Rauchman et al., 1997), their precise function is not clear. Elsewhere in the embryo it has been proposed Msx 1 and Msx 2 function during reciprocal signalling processes between tissues and are also associated with the suppression of myogenesis and chondrogenesis (chapter 1.3). To investigate their function in the developing eye, a detailed analysis of Msx 1 and Msx 2 expression in the eye was performed, linking domains of expression with biological processes.

The analysis was focused on eyes from E9.5 to E13.5 embryos since during this period of development numerous morphological and differentiation processes occur. As a result of these processes a uniform neuroepithelium, surface ectoderm and mesenchyme develop into the neural and pigmented cell types of the retina and the lens. Furthermore, preliminary analysis of Msx 1 and Msx 2 expression has shown that they are transcribed during this “event rich” period of eye development suggesting that they may function at this time (Monaghan et al., 1991).

Gene activity is routinely assayed in the embryo by in situ detection of either mRNA or protein. However, neither transcription nor translation are necessarily indicative of gene function, since post-translational modifications or the presence of certain co-factors may be required for function. Antibodies that specifically react with Msx 1 or Msx 2 have not been reported, while the similar structure of parts of these two proteins may make it difficult to generate specific antibodies. It was therefore decided to analyse Msx expression at the level of transcription.
To establish the precise expression pattern of *Msx 1* and *Msx 2* in developing eyes, embryos where isolated from Swiss mice at approximately 24 hr intervals between E9.5 and E13.5 and embedded in wax as described in chapter 2.9.3. Swiss mice are albino which aids analysis of gene expression in the pigmented retina epithelium. Embedded embryos were then serially sectioned at 7µm intervals and hybridised to either *Msx 1* or *Msx 2* 35S labelled riboprobes (chapter 2.9.5).

### 3.2 Msx 1 and Msx 2 riboprobes are specific

The specificity of the *Msx 1* and *Msx 2* probes was confirmed by identifying regions of the embryo that showed hybridisation to the *Msx 1*, but not *Msx 2* riboprobe and *vice versa*. For example the mesenchyme of the otic capsule at E13.5 showed strong hybridisation to the *Msx 1* probe but not the *Msx 2* probe, while the epithelium of the otic capsule hybridised only to the *Msx 2* probe (fig. 3.1). This pattern of labelling was constant throughout this structure, allowing the comparison of sections even though they may be from slightly different section planes. Other anatomical structures that exhibited specific hybridisation to one or other of the *Msx* probes are summarised in table 3.1.

<table>
<thead>
<tr>
<th>Age</th>
<th>Age</th>
<th>Anatomical structure</th>
<th>Hybridisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E12.5</td>
<td>Pituitary</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Nasal epithelium</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Pinna (mesenchymal component)</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Precartilage primordium of petrous part of temprol bone</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>E13.5</td>
<td>Lateral mesenchyme of maxillary upper molar tooth bud</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Pituitary</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Nasal epithelium</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Pinna</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Otic capsule: Mesenchyme</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td></td>
<td>Epithelium</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td></td>
<td>Peripheral cells of Meckel's cartilage</td>
<td>n</td>
<td>y</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of anatomical structures exhibiting unique hybridisation to either the *Msx 1* or *Msx 2* riboprobes, so confirming the specificity of these probes.
Fig. 3.1 *Msx 1* and *Msx 2* expression in the otic capsule of E13.5 mouse embryos. A) *Msx 1* expression was detected in the mesenchymal component of the otic capsule (asterisk), but not in the epithelial component (closed arrow), x100. B) *Msx 2* expression was detected in the epithelial component of the otic capsule (closed arrow), but not in the mesenchymal component (asterisk), x100. These observations confirm that the *Msx 1* and *Msx 2* probes do not cross-hybridise.
3.3 *Msx 1* and *Msx 2* expression in derivatives of the optic neuroepithelium

Fig. 3.2 Diagram of the axes of the eye. P = posterior, D = dorsal, V = ventral, A= anterior, Prox = proximal, Dist = distal.

*Msx 2* expression

At the earliest stage analysed (late E9.5), the distal-most region of the optic vesicle had started to thicken and invaginate forming the presumptive neural retina. As shown in fig. 3.3, the *Msx 2* probe only hybridised to the distal-most cells of the optic vesicle beneath the overlying surface ectoderm. *Msx 2* labelling of proximal cells of the optic vesicle, which were not adjacent to the surface ectoderm, was not observed (fig. 3.3).

Only the dorsal/posterior region of the distal optic vesicle (presumptive neural retina)
expressed *Msx 2* (fig. 3.3). Dorsally, expression ceased in the optic vesicle when it was not immediately beneath the surface ectoderm. Ventrally, expression decreased in a gradient, and could not be detected approximately midway along the dorsal/ventral axis. Along the posterior/anterior axis, labelling was detected in approximately the posterior-most 2/3 of the optic vesicle.

Approximately 24 hours later (E10.5), the optic vesicle had formed the characteristic bilayered optic cup and the lens vesicle had almost detached from the surface ectoderm. As shown in fig. 3.4, *Msx 2* labelling remained restricted to cells in the inner layer of the optic cup (presumptive neural retina), no labelling was detected in the outer layer (presumptive pigmented epithelium). Although *Msx 2*-expressing cells were located in the inner layer, the entire inner layer of the optic cup did not express *Msx 2*. Labelling was confined to the distal half of the cup, with the proximal expression boundary located opposite the back of the lens vesicle (fig. 3.4). Expression was still confined to the most dorsal/posterior quarter of the neural retina (fig. 3.4).

The *Msx 2* expression pattern observed in the retina of E10.5 mice was maintained in E11.5 embryos (fig. 3.5). By E12.5 the lens vesicle was almost entirely full of fibre cells. At this stage, the *Msx 2* probe continued to label cells at the distal margin of the retina (presumptive ciliary body), while the proximal neural retina and pigmented epithelium remained unlabelled. The proximal boundary, between *Msx 2*-labelled and unlabelled cells, was located opposite the lens equator. Distally, *Msx 2* label did not extend into the monolayered pigmented epithelium (fig. 3.6 and fig. 3.13). Expression in the distal margins of the neural retina was limited to the posterior half (fig. 3.6).

The most developmentally advanced stage analysed was E13.5. The expression of *Msx 2* in the retina at this stage was very similar to that observed at E12.5. Labelling was again confined to the posterior-distal margin of the neural retina (fig. 3.7 and fig. 3.13). During all the stages of retina development analysed, uniform *Msx 2* labelling was observed across the width of the neuroepithelium.
Fig. 3.3 Msx 2 expression in the optic neuroepithelium of an E9.5 mouse embryo. A) Transverse section through the posterior half of the eye, shown in (B), x20. Ai) Enlargement of the proximal part of the optic vesicle, shown in (A), x40. No Msx 2 expression was detected in posterior-proximal cells of the optic vesicle (open arrow head). Aii) Enlargement of the distal part of the optic vesicle, shown in (A), x40. For clarity panel (Aii) has been enlarged, see below. Msx 2 expression was detected in posterior cells of the optic vesicle beneath the surface ectoderm and lens pit (closed arrow). Dorsal and ventral boundaries of expression are marked by the broken lines. C) Transverse section through the anterior half of the eye, shown in (B), x20. Ci) Enlargement of the proximal part of the optic vesicle, shown in (C), x40. No Msx 2 expression was detected in the anterior-proximal cells of the optic vesicle (open arrowhead). Cii) Enlargement of the distal part of the optic vesicle, shown in (C), x40. No Msx 2 expression was detected in anterior-distal cells of the optic vesicle (open arrow). se = surface ectoderm, pnr = presumptive neural retina, pPRE = presumptive pigmented retina epithelium, lp = lens pit.
Fig. 3.4 *Msx 2* expression in the optic neuroepithelium of an E10.5 mouse embryo. A) Transverse section through the posterior half of the eye, shown in (B), x20. Ai) Enlargement of the dorsal part of the optic cup, shown in (A), x40. *Msx 2* expression was detected in dorsal-distal cells of the inner layer of the optic cup (closed arrow). Proximal and distal boundaries of expression are marked by broken lines. No *Msx 2* expression was detected in the outer layer of the optic cup (open arrowhead). Aii) Enlargement of the ventral part of the optic cup, shown in (A), x40. No *Msx 2* expression was detected in the posterior-ventral inner layer of the optic cup (open arrow). C) Transverse section through the anterior half of the eye, shown in (B), x20. Ci) Enlargement of the dorsal side of the optic cup, shown in (C), x40. No *Msx 2* expression was detected in the outer layer (open arrowhead) or inner layer (open arrow) of the anterior-dorsal optic cup. Cii) Enlargement of the ventral side of the optic cup, shown in (C), x40. No *Msx 2* expression was detected in the anterior-ventral inner layer of the optic cup (open arrow). pPRE = presumptive pigmented retina epithelium, pnr = presumptive neural retina, lv = lens vesicle, se = surface ectoderm.
A

pnr

lv

pPRE

se

proximal ← distal
dorsal

ventral
dorsal

1mm

A

(A)

pPRE

pnr

lv

se

B

C

Ci

Cii

112
Fig. 3.5 Msx 2 expression in the optic neuroepithelium of an E11.5 mouse embryo. A) Transverse section through the posterior-dorsal half of the eye, shown in (B), x20. Ai) Enlargement of the dorsal-anterior part of the retina, shown in (A), x40. Msx 2 expression was detected in distal cells of the dorsal-anterior neural retina (closed arrow). Proximal and distal boundaries of expression are marked by broken lines. No Msx 2 expression was detected in proximal cells of the neural retina (open arrow) or cells of the PRE (open arrowhead). Aii) Enlargement of the ventral-posterior side of the retina, shown in (A), x40. No Msx 2 expression was detected in the neural retina (open arrow) or the PRE (open arrowhead). C) Transverse section through the anterior-ventral half of the eye, shown in (B), x20. Ci) Enlargement of the dorsal-anterior side and Cii) enlargement of the ventral-posterior side of the retina shown in (C), x40. No Msx 2 expression was detected in the neural retina (open arrows) or the PRE (open arrowheads), in neither the ventro-posterior nor dorso-anterior side.

PRE = pigmented retina epithelium, nr = neural retina, lv = lens vesicle.
Fig. 3.6 Msx 2 expression in the optic neuroepithelium of an E12.5 mouse embryo. A) Transverse section through the posterior of the eye, shown in (B), x20. Ai) Enlargement of dorsal side and (Aii) enlargement of ventral side, shown in (A), x40. For clarity panels (Ai) and (Aii) have been enlarged, see below. Msx 2 expression was detected in posterior-distal cells of the neural retina on both ventral and dorsal sides (closed arrows). No Msx 2 expression was detected in posterior-proximal neural retina (open arrows) or in the PRE (open arrowheads). Proximal and distal boundaries of expression are marked by broken lines, refer also to fig. 3.13. C) Transverse section through the anterior of the eye, shown in (B), x20. Ci) Enlargement of dorsal side and (Cii) enlargement of ventral side, shown in (C), x40. Msx 2 expression was not detected in the anterior neural retina (open arrows) or anterior PRE (open arrowheads) on either the dorsal or ventral side. nr = neural retina, l = lens, se = surface ectoderm, PRE = pigmented retina epithelium.
Fig. 3.7 Msx 2 expression in the optic neuroepithelium of an E13.5 mouse embryo. A) Transverse section through the posterior-dorsal half of the eye, shown in (B), x20. Ai) Enlargement of dorsal-anterior side and (Aii) enlargement of ventral-posterior side, shown in (A), x40. Msx 2 expression was detected in distal cells of the posterior-dorsal neural retina on both dorso-anterior and ventro-posterior sides (closed arrows). Proximal boundaries of expression are marked by broken lines, refer also to fig. 3.13. No Msx 2 expression was detected in proximal cells of the posterior-dorsal neural retina (open arrows) or posterior-dorsal PRE (open arrowheads) in either the dorso-anterior or ventro-posterior side. C) Transverse section through the anterior-ventral half of the eye, shown in (B), x20. Ci) Enlargement of dorsal-anterior side and (Cii) enlargement of ventral-posterior side, shown in (C), x40. No Msx 2 expression was detected in anterior-ventral neural retina (open arrows) or PRE (open arrowheads) in either the dorso-anterior or ventro-posterior side. l = lens, se = surface ectoderm, nr = neural retina, PRE = pigmented retina epithelium.
**Msx 1 expression**

Msx 1 expression was not detected in the derivatives of the optic neuroepithelium until E11.5 (fig. 3.8 and 3.9). By this stage, the two layers of the optic cup could be distinguished; the lens vesicle had separated from the overlying surface ectoderm and had begun to produce primary lens fibres. In common with Msx 2 expression at this stage, Msx 1 labelling was confined to the distal margin of the inner layer of the optic cup. Likewise, the proximal boundary of Msx 1 expression in the inner layer lay opposite the equator of the lens vesicle. Distally, expression did not extend into the presumptive pigmented epithelium. The presumptive pigmented retina epithelium did not label with the Msx 1 probe (fig. 3.10). As illustrated in fig. 3.10, Msx 1 was also asymmetrically expressed around the rim of the optic cup. However, in contrast to Msx 2, which was confined to the dorsal/posterior quarter, Msx 1 was expressed around the dorsal half of the inner layer.

During the next 24 hours of eye development, Msx 1 expression was activated around the entire distal rim of the neural retina. At E12.5, it was possible to distinguish two distinct domains of Msx 1 expression. Within the dorsal half of the neural retina intense Msx 1 labelling was detected, while in the ventral half weaker labelling was detected (fig. 3.11). A sharp proximal boundary of expression lay opposite the lens equator, with a distal boundary at the junction between the pigmented epithelium and neural retina (fig 3.11 and fig. 3.13). The pigmented retina epithelium was not labelled by the Msx 1 probe at this stage (fig. 3.11).

By E13.5, the domain of strong Msx 1 labelling had extended further ventrally so that approximately the dorsal three quarters of the presumptive ciliary margin was now labelled strongly with the Msx 1 probe, while the remaining ventral one third was labelled only weakly (fig. 3.12). As shown in fig. 3.12, the pigmented epithelium remained negative for Msx 1, with the proximal and distal boundaries of expression within the neural retina remaining as they were at E12.5 (fig. 3.12 and fig. 3.13).
During all the stages of retina development analysed, *Msx I* labelling was uniform across the width of the neuroepithelium.
Fig. 3.8 *Msx 1* expression in the optic neuroepithelium of an E9.5 mouse embryo. A) Transverse section through the posterior of the eye, shown in (B), x20. Ai) Enlargement of proximal part and (Aii) enlargement of distal part of the posterior optic vesicle, shown in (A), x40. No *Msx 1* expression was detected in the posterior presumptive PRE (open arrowhead) or presumptive neural retina (open arrow). C) Transverse section through the anterior of the eye, shown in (B), x20. Ci) Enlargement of proximal part and (Cii) enlargement of distal part of the anterior optic vesicle, shown in (C), x40. No *Msx 1* expression was detected in the anterior presumptive PRE (open arrowhead) or presumptive neural retina (open arrow). pPRE = presumptive pigmented retina epithelium, pnr = presumptive neural retina, lp = lens pit.
Fig. 3.9 Msx 1 expression in the optic neuroepithelium of an E10.5 mouse embryo. A) Transverse section through the posterior of the eye, shown in (B), x20. Ai) Enlargement of dorsal side and Aii) enlargement of ventral side of the posterior optic cup, shown in (A), x40. No Msx 1 expression was detected in the posterior presumptive PRE (open arrowheads) or presumptive neural retina (open arrows) in either the dorsal or ventral side. Ai) Msx 1 expression was not detected in the surface ectoderm (asterisk). C) Transverse section through the anterior of the eye shown in (B), x20. Ci) Enlargement of dorsal side and (Cii) ventral side of the anterior optic cup, shown in (C), x40. No Msx 1 expression was detected in the anterior presumptive PRE (open arrowheads) or presumptive neural retina (open arrows) in either the dorsal or ventral side. pPRE = presumptive pigmented retina epithelium, pnr = presumptive neural retina, lv = lens vesicle, se = surface ectoderm.
Ac.

It.

PRE.

nr.

IV.

proximal → distal
dorsal

ventral
dorsal

ventral

1mm

(A)

D

P

V

A

•T.

Gil...r

se
dorsal

124
Fig. 3.10 Msx 1 expression in the optic neuroepithelium of an E11.5 mouse embryo. A) Transverse section through the posterior-dorsal side of the eye, shown in (B), x20. Ai) Enlargement of dorsal-anterior side of retina, shown in (A), x40. For clarity panel (Ai) has been enlarged, see below. Msx 1 label was detected in distal cells of the dorsal-anterior neural retina (closed arrow). Proximal and distal boundaries of expression are marked by broken lines. No expression was detected in the dorsal-anterior PRE (open arrowhead). Aii) Enlargement of ventral-posterior side of retina, shown in (A), x40. No Msx 1 expression was detected in the ventral-posterior neural retina (open arrow) or PRE (open arrowhead). C) Transverse section through the anterior-ventral side of the eye, shown in (B), x20. Ci) Enlargement of dorsal-anterior side of retina, shown in (C), x40. For clarity panel (Ci) has been enlarged, see below. Msx 1 expression was detected in distal cells of the dorsal-anterior neural retina (closed arrow). Proximal and distal boundaries of expression are marked by broken lines. No expression was detected in the dorsal-anterior PRE (open arrowheads). Cii) Enlargement of ventral-posterior side of the retina, shown in (C), x40. Msx 1 expression was not detected in the ventral-posterior neural retina (open arrows) or PRE (open arrowheads). PRE = pigmented retina epithelium, nr = neural retina, lv = lens vesicle, se = surface ectoderm.
Fig. 3.11 Msx 1 expression in the optic neuroepithelium of an E12.5 mouse embryo. A) Transverse section through the posterior of the eye, shown in (C), x20. Ai) Enlargement of dorsal side and (Aii) enlargement of ventral side of the posterior retina, shown in (A), x40. Msx 1 expression was detected in distal cells of the posterior neural retina, both dorsally and ventrally (closed arrows). The proximal boundaries of expression are marked by broken lines, refer also to fig. 3.13. Msx 1 expression was not detected in proximal cells of the neural retina (open arrow) or the PRE (open arrowheads) in either the dorsal or ventral side of the posterior retina. B) Transverse section through eye, as shown in (C), x20. Intense Msx 1 labelling was detected dorsally (closed arrow head) and weak labelling ventrally (open arrowhead). For clarity panel B has been enlarged, see below. D) Transverse section through the anterior of the eye, shown in (C), x20. Di) Enlargement of dorsal side and Dii) enlargement of ventral side, shown in (D), x40. Msx 1 expression was detected both dorsally and ventrally (closed arrows) in distal cells of the anterior neural retina. No expression was detected in anterior PRE (closed arrowheads) or proximal neural retina (open arrows), neither dorsally nor ventrally. PRE = pigmented retina epithelium, l = lens, nr = neural retina, se = surface ectoderm.
Fig. 3.12 *Msx* 1 expression in the optic neuroepithelium of an E13.5 mouse embryo. A) Transverse section through the posterior-dorsal side of the eye, shown in (C), x20. Ai) Enlargement of dorsal-anterior side and Aii) enlargement of ventral-posterior side of the retina, shown in (A), x40. *Msx* 1 expression was detected in distal cells of the posterior-dorsal neural retina (closed arrows), in both the dorso-anterior and ventro-posterior sides. Proximal boundaries of expression are marked by broken lines, also refer to fig. 3.13. No *Msx* 1 expression was detected in posterior-dorsal PRE (open arrowheads) or proximal neural retina (open arrows), in either the dorso-anterior or ventro-posterior side. B) Transverse section through eye, as shown in (C), x20. For clarity panel (B) has been enlarged, see below. Intense *Msx* 1 expression was detected in the dorso-anterior side (closed arrowhead) and weak expression in the ventro-posterior side (open arrowhead). D) Transverse section through the anterior-ventral side of the eye, shown in (C), x20. Di) Enlargement of dorsal-anterior side and Dii) ventral-posterior side of the retina, shown in (D), x40. *Msx* 1 expression was detected in distal cells of the anterior-ventral neural retina (closed arrows), in both the dorso-anterior and ventro-posterior sides. Proximal and distal boundaries of expression are marked by broken lines, also refer to fig. 3.13. *Msx* 1 expression was not detected in the anterior-ventral PRE (open arrowheads) or proximal neural retina (open arrows), in neither the dorso-anterior nor ventro-posterior side. PRE = pigmented retina epithelium, nr = neural retina, l = lens, se = surface ectoderm.

Enlargement of panel (B), shown opposite.
Fig. 3.13 Proximal-distal boundaries of *Msx* 1 and *Msx* 2 expression in the mouse neural retina, at E12.5 and E13.5. A and C) Transverse sections through E12.5 mouse eyes, at similar section planes, x40. B and D) Transverse sections through E13.5 mouse eyes, at similar section planes, x40. A and B) *Msx* 1 expression in distal cells of the neural retina (closed arrow). C and D) *Msx* 2 expression in distal cells of the neural retina (closed arrow). Proximal and distal boundaries of expression are marked by broken lines. nr = neural retina, l = lens.
3.4 *Msx* 1 and *Msx* 2 expression in derivatives of the surface ectoderm

At E9.5, the lens placode starts to invaginate forming the lens pit. No signal from the *Msx* 1 probe was detected in the lens pit at this stage. In contrast, *Msx* 2 expression was detected throughout the lens pit, with stronger expression in the posterior/dorsal part of the pit, adjacent to the region of the optic vesicle that expresses *Msx* 2 (fig. 3.14). Label extended out of the lens pit and into the adjacent surface ectoderm but poor histology of the surrounding surface ectoderm masked the boundary of this expression.

By E10.5, the lens vesicle has almost separated from the surface ectoderm. Again, *Msx* 1 was not detected in the lens vesicle or the surrounding surface ectoderm, while *Msx* 2 continued to be expressed at these sites. The entire lens vesicle was labelled by the *Msx* 2 probe, but stronger expression was again observed in regions adjacent to presumptive neural retina that was also expressing *Msx* 2 (fig. 3.14). The surrounding surface ectoderm was uniformly labelled for *Msx* 2, with no obvious boundary to this expression.

*Msx* 2 expression in the developing lens was maintained after the vesicle had separated from the overlying surface ectoderm at approximately E11.5. Stronger expression was still observed in regions adjacent to *Msx* 1- and *Msx* 2- expressing presumptive neural retina (fig. 3.14). Weak *Msx* 1 labelling was observed in the distal most part of the lens vesicle at E11.5, while the overlying surface ectoderm remained unlabelled (fig. 3.14). The *Msx* 2 probe continued to uniformly label the overlying surface ectoderm at this stage, with expression extending out of the eye into the surrounding ectoderm (fig. 3.14).

At E12.5 and E13.5, primary lens fibre cells have begun to differentiate. During this stage of lens development *Msx* 2 was expressed at, and distally to, the lens equator. It is interesting to note that the boundary of *Msx* 1 and *Msx* 2 expression in the neural retina, at this stage, was adjacent to the *Msx* 2-expressing lens equator (fig. 3.15).
Fig. 3.14 *Msx 1* and *Msx 2* expression in the surface ectoderm and lens vesicle of E9.5- E11.5 mouse embryos. A and B) Transverse sections through the centre of the lens pit of E9.5 embryos, x40. A) *Msx 1* expression was not detected in the lens pit (open arrow). B) *Msx 2* expression was detected in the lens pit (closed arrows), with stronger expression adjacent to *Msx 2* expressing-optic vesicle (closed arrow with asterisk). C and D) Transverse sections through the centre of the lens vesicle of E10.5 embryos, x40. C) *Msx 1* expression was not detected in the lens vesicle (open arrow) or the surface ectoderm (refer to fig. 3.9 A1 for better morphology). D) *Msx 2* expression was detected in the entire lens vesicle (closed arrows), with stronger expression adjacent to *Msx 2* expressing-presumptive neural retina (closed arrow with asterisk). *Msx 2* expression was also detected in the surface ectoderm (closed arrow head). E and F) Transverse sections through the centre of the lens vesicle of E11.5 embryos, x40. E) *Msx 1* expression was detected in distal cells of the lens vesicle (closed arrow), but not proximal cells (open arrow). *Msx 1* expression was not detected in the surface ectoderm (open arrow head). F) *Msx 2* expression was detected in the lens vesicle (closed arrows), with stronger expression adjacent to *Msx 2* expressing-neural retina (closed arrow with asterisk). *Msx 2* expression was also detected in the surface ectoderm (closed arrow head). pnr = presumptive neural retina, nr = neural retina, lp = lens pit, lv = lens vesicle, se = surface ectoderm.
Msx 1 labelling was detected in the anterior lens epithelium at E12.5, but no expression was observed at the lens equator (fig. 3.16). The surface ectoderm overlying the lens at E12.5 expressed Msx 2 but not Msx 1 (fig. 3.15 and fig. 3.16). The boundary between Msx 2-expressing and non-expressing surface ectoderm was located within the eye, as indicated in fig. 3.15.

3.5 Msx 1 and Msx 2 expression in the perioptic mesenchyme

At E9.5, the mesenchyme surrounding the entire optic vesicle was labelled by the Msx 1 probe (fig. 3.17). With the exception of a small population of mesenchymal cells located dorsally to the optic vesicle, perioptic mesenchyme did not label with the Msx 2 probe (fig. 3.17).

By 11.5d, Msx 1-labelled mesenchyme was limited to the distal half of the eye. Msx 1-expressing mesenchymal cells were closer to the retina distally compared to proximally (fig. 3.17). As shown in fig. 3.17, Msx 1-labelled cells were observed migrating into the optic cup. Msx 2-labelled perioptic mesenchyme was not observed at this stage or any of the later stages analysed (fig. 3.17 and fig. 3.15). However, it should be noted that the spreading of label from the surface ectoderm prevented any conclusion being drawn as to the state of Msx 2 expression in mesenchyme immediately beneath the surface ectoderm.

At E12.5, the Msx 1 probe labelled mesenchymal cells located around the distal tips of the retina. As can be seen in fig. 3.16, two distinct domains of expression could be distinguished; a domain of strong expression, distally, and a domain of weaker expression, proximally. The boundary between the heavily and lightly labelled cells lay approximately level with the boundary of expression within the neural retina (fig. 3.16). There was no clear proximal boundary between the lightly labelled mesenchyme and unlabelled mesenchyme, only a gradient of decreasing label (fig. 3.16). In front of the lens there was a population of weakly labelled Msx 1 cells with a band of unlabelled cells separating these cells from the overlying surface ectoderm (fig. 3.16).
In a similar manner there was also a band of unlabelled cells between the labelled mesenchyme immediately adjacent to the retina and the overlying surface ectoderm (fig. 3.16).
Fig. 3.15 Msx 2 expression in the surface ectoderm, lens and perioptic mesenchyme of E12.5-E13.5 mouse embryos. A) Transverse section through the centre of the lens of an E12.5 embryo, x20. Ai and Aii) Enlargements of (A), x40. Msx 2 expression was detected at the lens equator (closed arrows) and in anterior lens epithelium (open arrow). Note that the boundary of Msx 2 expression in the neural retina is adjacent to Msx 2 expression at the lens equator (broken line). Msx 2 expression was also detected in the surface ectoderm (closed arrowheads). The boundaries between Msx 2-expressing and non-expressing surface ectoderm cells are marked by solid lines. No Msx 2 expression was detected in the perioptic mesenchyme (asterisks). Note that possible spreading of label from the surface ectoderm prevented any conclusions being drawn as to the state of Msx 2 expression in mesenchyme immediately beneath the surface ectoderm. B) Transverse section through the centre of the lens of an E13.5 embryo, x20. Bi and Bii) Enlargements of (B), x40. Msx 2 expression was detected at the lens equator (closed arrows) and anterior lens epithelium (open arrow). The boundary of Msx 2 expression in the neural retina is adjacent to Msx 2 expression at the lens equator (broken line). Msx 2 expression was detected in the surface ectoderm (closed arrowhead). The boundaries between Msx 2-expressing and non-expressing surface ectoderm are marked by solid lines. No Msx 2 expression was detected in the perioptic mesenchyme (asterisks). nr = neural retina, le = lens equator, al = anterior lens epithelium, pm = perioptic mesenchyme, se = surface ectoderm.
Fig. 3.16 Msx 1 expression in surface ectoderm, lens and perioptic mesenchyme of E12.5 mouse embryos. A) Transverse section through the centre of the lens of an E12.5 mouse embryo, x20. Ai and Aii) Enlargements of (A), x40. Weak Msx 1 expression was detected in the anterior lens epithelium (closed arrow), but not the lens equator (open arrow). Msx 1 expression was not detected in the surface ectoderm (open arrow heads). Strong Msx 1 expression was detected in distal-perioptic mesenchyme (closed arrow heads with asterisks) and weaker expression proximally (closed arrow head). The boundary between strongly and weakly expressing mesenchyme is marked by a broken line. nr = neural retina, le = lens equator, al = anterior lens epithelium, se = surface ectoderm and pm = perioptic mesenchyme.
Fig. 3.17 Msx 1 and Msx 2 expression in the perioptic mesenchyme of E9.5-E11.5 mouse embryos. A and B) Transverse sections through the optic vesicle of E9.5 mouse embryos, x40. A) Msx 1 was expressed in the perioptic mesenchyme surrounding the entire optic vesicle (closed arrow). B) Msx 2 expression was only detected in a small group of perioptic mesenchymal cells dorsal of the optic vesicle (closed arrow). C and D) Transverse sections through the retina of E10.5 mouse embryos, showing the dorsal side of the eye, x40. C) Msx 1 expression was detected in the perioptic mesenchyme (closed arrow). D) Msx 2 expression was no longer detected in dorsal perioptic mesenchyme (open arrow). E and F) Transverse sections through the retina of E11.5 mouse embryos, showing the dorsal side. E) Msx 1 expression in the perioptic mesenchyme was limited to the distal half of the eye (closed arrows). Msx 1-expressing perioptic mesenchyme was closer to the retina distally (closed arrow with asterisk). Msx 1-expressing cells were observed migrating into the optic cup (closed arrowhead). F) Msx 2 expression was not detected in the perioptic mesenchyme of E11.5 eyes (open arrow). ov = optic vesicle, pnr = presumptive neural retina, lv = lens vesicle, nr = neural retina.
- Msx1
- Msx2
- E9.5
- E10.5
- E11.5
- dorsal
- ventral
- proximal
- distal
- ov
- pnr
- lv
- nr
- *
3.6 Discussion - correlation of Msx expression with developmental function

By understanding precisely which tissues express the Msx genes during eye development, it has been possible to speculate upon their function during the formation of this organ. The association of Msx 2 expression with cells of the optic vesicle, which are undergoing the morphological change from a monolayered to a multilayered neuroepithelium, which then gives rise to the neural retina, is consistent with Msx 2 functioning either to specify neural retina cell fate, or suppress pigmented epithelium fate. Indeed, the pigmented retina epithelium does not express Msx 2 or Msx 1 between E9.5 and E13.5. However, without cell fate markers or molecular markers that specifically label cells that will form the neural retina, it is not possible to state that only presumptive neural retina cells express Msx 2.

In fact at E9.5, only the posterior/dorsal two thirds of the distal optic vesicle expressed Msx 2, leaving approximately one third of the optic vesicle adjacent to the surface ectoderm non-expressing. Either this region is not destined to become neural retina or it had expressed Msx 2 earlier in development. Alternatively, Msx 2 may not be expressed in all presumptive neural retina cells and consequently Msx 2 function may not be required by all presumptive neural retina cells.

After the optic vesicle has formed a bilayered cup, Msx 2 expression is detected in the distal tips of the inner layer of the retina (presumptive ciliary margin) with Msx 1 expression becoming evident in this region at E11.5. This restricted expression may suggest that Msx expression is incompatible with neural retina differentiation. However, the last neural retina cells do not terminally differentiate until after birth and the proneural gene Mash 1 is expressed in neural retina progenitor cells until this time (chapter 1.5.2.3). Therefore, if the function of the Msx genes is to maintain an undifferentiated state, their expression might be predicted to persist within the central portion of the neural retina within these precursor cells like that of Mash 1. The neurogenic genes Notch and Delta suppress neural differentiation through lateral
inhibition, so maintaining a population of precursor cells (chapter 1.5.2.3). In mouse, Notch 2 is expressed in the presumptive ciliary margin like that of Msx 1 and Msx 2. It is therefore tempting to speculate that Notch 2 and perhaps the Msx genes function in this region to maintain a population of undifferentiated cells that, unlike precursor cells in the centre of the neural retina, are less committed to a particular lineage/s. Indeed, lower vertebrates contain a population of precursor cells located in the ciliary margin that are capable of generating all the cell types found in the retina (Wetts et al., 1989).

An alternative function for Msx 1 and Msx 2 in the presumptive ciliary margin could be to establish a boundary between pigmented retina epithelium and neural retina, or in the formation of components of the iris.

The in situ hybridisation data reveals that both Msx 1 and Msx 2 are asymmetrically expressed around the distal circumference of the neural retina. A precise comparison between the spatial and temporal distribution of these genes in this region cannot be accurately made, since eyes from individual embryos were hybridised to only one probe. Therefore, subtle differences in age, and errors incurred while normalising the plane of section, will have influenced the accuracy of these comparisons. Nonetheless, this data clearly shows that both genes are expressed in overlapping and distinct domains. It is not clear from the present data whether or not the initial domain of Msx 1 expression is wholly contained within the dorsal/posterior Msx 2 domain, but by E11.5 both genes overlap dorsal/posteriorly with Msx 1 expanding more anteriorly than Msx 2.

As the retina develops, the domain of Msx 2 expression expands anteriorly to occupy approximately the entire posterior half of the retina by E13.5. The expansion of Msx 1 expression is far more dramatic, by E12.5 the entire circumference of the presumptive ciliary margin expresses Msx 1, with a distinct domain of strong Msx 1 expression observed dorsally. This domain of strong Msx 1 expression subsequently expands ventrally over the next 24 hours, encompassing approximately the most dorsal three
quarters of the ciliary margin. The expansion of their expression around the circumference of the retina may reflect a wave of differentiation circling the retina at this time. However, this is unlikely since each gene apparently expands along different axes. This differential spread of expression together with the fact that Msx 2 is expressed long before Msx 1 suggests that either different factors control Msx 1 and Msx 2 expression or that these genes have a differential response to the same factors.

During tooth development Msx 1 activates Bmp 4. In turn, Bmp 4 then activates Msx 1 so creating a positive-feedback loop. This loop may amplify the Bmp 4 signal allowing it to spread rapidly across the dental mesenchyme (chapter 1.3.2). A similar mode of action could explain the rapid spread of Msx 1 expression, and perhaps even Msx 2, around the rim of the optic cup. The expression of Bmp 4 in the mouse eye has not been reported but Bmp 4 in Xenopus is expressed in the ciliary margin of the developing eye and, rather interestingly, is also restricted to the dorsal side of the retina (chapter 1.5.2.4).

If the activation of Msx around the rim of the retina is a consequence of a positive-feedback loop then why does this mechanism not also result in the expansion of Msx expression proximally? The answer to this question may perhaps be found by comparing the mode of action of Msx in the retina to their action in the developing neural tube. It has been proposed that Bmp 4 mediates a dorsalising signal in the neural tube and shown that Bmp 4 is co-expressed with Msx 1 at this site. This raises the possibility that the Bmp 4 signal is spread by a positive-feedback loop involving Msx 1 and Bmp 4. It might be predicted from this mechanism that the entire neural tube would be dorsalised. However, this does not happen due to an interplay between the dorsalising and ventralising signals. The ventralising signal within the neural tube may be mediated by Shh (chapter 1.3.5). An analogous mechanism may operate within the neural retina to prevent the spread of Msx 1 expression proximally. Indeed, Shh is expressed in the ganglion cell layer of the neural retina at E14.5 (chapter 1.5.2.3) and might block the spread of a Msx 1/Bmp 4 positive-feedback loop from the ciliary margin.
Numerous genes, like \textit{Msx}, are expressed asymmetrically during retina development and encode transcription factors, secreted proteins, components of the retinoic acid signalling system and cell surface receptor tyrosine kinases (chapter 1.5.2.4). These genes, in particular the receptor tyrosine kinases, are thought to pattern the neural retina to allow topographic mapping of neuron connections. In contrast to Msx, these molecules tend not to be restricted to the distal ciliary margin of the neural retina. Therefore, unless the establishment of these axes originates from the ciliary margin, it is unlikely that the \textit{Msx} genes play a role during these processes.

\textit{Msx 2} is expressed in the surface ectoderm before lens formation and becomes restricted to cells at, and in front of, the lens equator as the lens develops. In this region epithelial cells proliferate and differentiate into fibre cells. The function of Msx 2 in this region may therefore be to maintain a population of undifferentiated cells. Likewise, the surface ectoderm must be maintained in an uncommitted state to allow first the formation of the lens and subsequently the cornea. Indeed, \textit{Msx 2} is also expressed in the surface ectoderm after lens formation and may therefore function in the surface ectoderm to prevent the premature differentiation of this tissue. Msx 1 may have a different function from Msx 2 in the surface ectoderm derivatives since \textit{Msx 1} is not expressed in the surface ectoderm or the equatorial region of the lens. \textit{Msx 1} expression was only detected in the anterior lens epithelium after E11.5.

Within the developing lens vesicle, \textit{Msx 2} expression was stronger in regions of the vesicle adjacent to \textit{Msx 2}-expressing neuroepithelium. This observation might suggest that at both these sites, \textit{Msx 2} is controlled by the same secreted molecule and that cells of the lens vesicle adjacent to \textit{Msx 2}-expressing neuroepithelium are exposed to a higher concentration of this molecule than cells adjacent to non-expressing neuroepithelium. Similarly, the domain of \textit{Msx 1} expression within the anterior lens epithelium is immediately adjacent to \textit{Msx 1}-expressing mesenchyme. It is therefore possible that a secreted molecule capable of activating \textit{Msx 1}, and perhaps itself regulated by Msx 1, is produced in the mesenchyme, diffuses to the lens and activates
Msx 1 expression in the lens epithelium. Conversely, it is possible that Msx 1 function in the anterior lens epithelium results in Msx 1 expression in the mesenchyme. This is less likely since Msx 1 expression is detected in the mesenchyme before it is detected in the lens.

The function of Msx 1 in the perioptic mesenchyme is not clear. This tissue differentiates into the choroid and the sclera which surround the retina proximally and distally. Although Msx 1 is expressed in mesenchyme surrounding the entire optic vesicle, expression becomes confined to the distal portion of the eye at later stages. It is therefore not clear if Msx 1 plays a role in the differentiation of the sclera and choroid or if this gene has an alternative function at this site.

Mice null for both Msx 1 and Msx 2 exhibit arrested eye development at the optic vesicle stage, yet mice null for only one of these genes are reported to have normal eyes (chapter 1.5.1). This implies that these genes are functionally redundant. Msx function must therefore first be required prior to or at the optic vesicle stage, yet at this early stage of eye development these genes are not co-expressed, with the exception of a small population of mesenchymal cells located dorsally to the optic vesicle. Msx function may therefore only be essential within these cells for normal development. If this is not the case, then the apparent functional redundancy could be explained if both genes regulate the activity of the same diffusible molecule. The lack of production of such a molecule in one tissue could be compensated for by a neighbouring tissue. During the early stages of eye development, Msx 2 function in the surface ectoderm and the optic vesicle could be compensated for by Msx 1 function in distal perioptic mesenchyme and vice versa.

If it is assumed that Msx 1 and Msx 2 regulate secreted molecules with comparable activities, then the consequence of asymmetric expression of Msx 1 and Msx 2 in overlapping and distinct domains of the ciliary margin could be to establish differing concentrations of these secreted molecules. However, the fact that eye development apparently proceeds normally in mutants null for only one of the Msx genes raises the
possibility that differential expression of the Msx genes in the ciliary margin is not functionally significant.

3.7 Conclusion

The two most striking correlations between Msx 2 expression and the developmental processes occurring during eye development, are the expression of this gene in at least some cells of the optic vesicle that are presumed to give rise to the neural retina and, in common with Msx 1, the absence of expression in the pigmented retina epithelium. These observations suggest that Msx 2 functions to either specify neural retina cell fate or inhibit the differentiation of pigmented retina epithelium.

The differentiation of the optic vesicle into neural and pigmented retina is an attractive system in which to address Msx 2 function. Differentiated pigmented retina epithelium can transdifferentiate into cells of the neural retina following treatment with FGF 2 in culture (chapter 1.6). This demonstrates that cultured pigmented retina epithelium cells are able to undergo neuronal differentiation. This is perhaps not surprising since both the neural and pigmented retina are derived from the optic vesicle.

The development and characterisation of a pigmented retina epithelium (PRE) cell culture system is described in the next chapter.
Chapter 4

Chick Pigmented Retina Epithelium Cells in Culture

4.1 Introduction

The restricted expression of *Msx 2* mRNA in the presumptive neural retina of the optic vesicle and the subsequent absence of expression in the PRE (chapter 3), suggests that the encoded protein may function to specify neural retina cell fate. This proposed function was further investigated in cultured PRE cells. However, this culture system had first to be developed. This approach was chosen since PRE cells have several properties in culture that make them ideally suited to investigating Msx 2 function. Firstly, PRE and neural retina cells are both derived from the optic vesicle. This might suggest that PRE and neural retina cells have a similar intra-cellular environment and that consequently the PRE intra-cellular environment may be able to support Msx 2 function. Secondly, cultured PRE cells can transdifferentiate into neural retina cells in response to FGF 2 (chapter 1.6), demonstrating that they possess the ability to undergo neurogenesis which is very relevant to the proposed function of Msx 2. Finally, cultured PRE cells can dedifferentiate upon the loss of cell-cell contacts (chapter 1.6). It is tempting to speculate that the state of differentiation of dedifferentiated PRE cells is similar to that of optic vesicle cells. Again, this could be relevant to Msx 2 function given that *Msx 2* is expressed in optic vesicle cells.

Cultured PRE cells could be exploited in a variety of ways to explore Msx 2 function. One approach would be to investigate the possibility that transdifferentiation of PRE cells into neural retina, in response to FGF 2, or dedifferentiation, in response to disrupted cell-cell interactions, involves the activation of *Msx 2*. The association of *Msx 2* with either of these phenomena would be consistent with Msx 2 functioning *in vivo* during the differentiation of the neural retina. Alternatively, Msx 2 function could be addressed by ectopically expressing *Msx 2* in cultured PRE cells. If Msx 2 does
control the differentiation of neural retina cells, then ectopic expression in PRE cells may drive them towards a neural or dedifferentiated fate.

In this chapter the PRE cell culture system is characterised in terms of substrata since the mechanical properties of the substratum have been shown to affect the neural phenotype associated with FGF 2-induced transdifferentiation of PRE (Opas and Dziak, 1994), media and destabilisation of the differentiated state. Cultures of dissociated PRE were used, rather than explants, to ensure uniformity between experiments and to increase the number of experimental units per dissection. However, as discussed in chapter 1.6, dissociated and explant PRE cultures have different characteristics with respect to neural transdifferentiation. Consequently Msx 2 function in these different types of culture may differ.

Although, it was possible to isolate and culture PRE from E12-E15 CBA mouse eyes, the quality and quantity of cells limited any subsequent experimentation. This problem was caused by the difficulty associated with separating PRE from perioptic mesenchyme. Any contaminating mesenchyme then swamped the PRE in culture. To overcome this problem, it was decided to use chick eyes as the source of PRE since, at equivalent stages of eye development, the chick eye is several fold larger than that of mouse. PRE from stage 22-25 chicks was used, since PRE from chicks older than stage 27 fail to transdifferentiate in response to FGF 2 (chapter 1.6). It is therefore possible that Msx 2 function in PRE cells may also be age dependant.

To confirm that, as in mouse (chapter 3), Msx 1 and Msx 2 are not expressed in chick PRE, in situ hybridisation of probes for cMsx 1 and cMsx 2 was performed on stage 27 (5d), 29 (6d) and 31 (7d) chick eyes.
4.2  *cMsx 1* and *cMsx 2* are not expressed in chick pigmented retina epithelium

Stage 27-31 chick eyes were embedded in wax as described in chapter 2.9.3. Transverse sections were cut every 7μm and sections of the posterior, centre and anterior regions of the eye hybridised to either *cMsx 1* or *cMsx 2* 35S labelled riboprobes as described in chapter 2.9.5. The slides were exposed to film for 5 weeks to ensure maximum sensitivity.

As shown in fig. 4.1, both probes label the facial primordium of 6d (stage 29) chicks and weakly label the perioptic mesenchyme of 5d (stage 27), 6d (stage 29) and 7d (stage 31) chicks. The silver grains can be distinguished from the pigment granules since they are round and in a higher plane of focus than the rod shaped pigment granules, when observed at x100 magnification. The PRE was not labelled by *cMsx 1* or *cMsx 2* at any of the stages analysed. This confirms that like mouse PRE, chick PRE does not express *Msx 1* or *Msx 2* at levels that can be detected by *in situ* hybridisation of 35S labelled riboprobes.
Fig. 4.1 cMsx 1 and cMsx 2 expression in the PRE of 5-7 day old chick eyes. A and B) Transverse sections through the facial primordium of a 6d (stage 29) chick embryo, x40. A) cMsx 1 expression was detected in the facial primordium mesenchyme (closed arrow). B) cMsx 2 expression was detected in the facial primordium mesenchyme (closed arrow). C-H) Transverse sections through the PRE of 5d old chicks (C and D), 6d old chicks (E and F) and 7d old chicks (G and H), x100. C, E and G) Weak cMsx 1 expression was detected in the perioptic mesenchyme (closed arrows), no expression was detected in the PRE (open arrows). D, F and H) Weak cMsx 2 expression was detected in the perioptic mesenchyme (closed arrows), no expression was detected in the PRE (open arrows). Note that although silver grains can be distinguished from pigment granules, they are round and in a higher plane of focus than the rod shaped pigment granules, the large amount of pigment in (G) and (H) masks silver grains when photographed. fp = facial primordium, pm = perioptic mesenchyme, pre = pigmented retina epithelium, nr = neural retina.
4.3 Effect of substrata on PRE cell behaviour in culture

The aim of this experiment was to observe the growth of dissociated chick PRE cells on a range of substrata that differed in rigidity to determine an optimal substratum for future experiments. PRE from stage 23-24 chick embryos was dissociated and cultured in 10,66 medium, supplemented with 3% FCS, 0.3mg/ml glutamine, 70µg/ml penicillin, 0.13mg/ml streptomycin and 10 ng/ml FGF 2 (10,66F). These cells were grown on three types of substrata: plastic, plastic coated with matrigel protein and thin layered matrigel, decreasing respectively in their rigidity (chapter 2.10.2).

During the first three days, cells on both the protein coated and uncoated wells grew as monolayers with patches of small tightly packed pigmented cells at the centre, and large, poorly self-adhering non-pigmented cells at the periphery. Cells grown on the thin layered matrigel formed aggregates which developed into complex 3D structures. In common with the PRE monolayers, the 3D structures contained both pigmented and non-pigmented cells (fig. 4.2).

After 3 to 10 days in culture, cells grown on protein-coated plastic started to detach. This may have been due to the degradation of the protein coat itself. Cells grown on thin layered matrigel continued to grow in complex 3D structures, as previously described, for at least 20 days, while cells grown directly on plastic grew for more than 48 days.
Fig. 4.2 Effect of substrata on PRE cell behaviour after 3 days in culture. Dissociated PRE from stage 23-24 chick embryos was cultured in 10,66F on either plastic (A), x25, matrigel coated plastic (B), x10, or thin layered matrigel (C), x10. (A) Cells grown on plastic formed monolayers, with small pigmented cells at the centre (closed arrow) and large non-pigmented cells at the periphery (open arrow). B) Cells grown on the matrigel coated plastic detached after 3-10 days in culture (closed arrow). C) Cells grown on thin layered matrigel formed complex 3D structures containing both pigmented (closed arrow) and non-pigmented cells (open arrow).
4.4 Growth of chick PRE cells in a serum-free environment

A serum-free medium would be preferable for investigating the effect that FGF 2 has on Msx 2 expression in cultured PRE cells. This would be preferable because FCS may contain unknown growth factors which may include or mimic FGF 2. To establish if PRE cells can be cultured in a serum-free medium, PRE from stage 24 chick embryos was dissociated, plated out on plastic and grown in serum-free medium 1 (SFM1), 10,66 supplemented with 37.5\(\mu\)g/ml BSA, 40\(\mu\)g/ml insulin, 40\(\mu\)g/ml transferine, 6.9\(\mu\)g/ml sodium selenite, 0.3mg/ml glutamine, 70\(\mu\)g/ml penicillin and 0.13mg/ml streptomycin, with or without 10ng/ml of FGF 2 (chapter 2.10.2). These cultures were observed after 1, 2, 15 and 25 hrs in culture and then every 24 hrs thereafter.

As shown in fig. 4.3, cells grown in the presence or absence of FGF 2 form aggregates during the first hour. After 2 hours these aggregates begin to attach and spread over the substratum forming numerous discrete monolayers. These monolayers grew and merged after 15 hrs. Consistent with previous observations, cells towards the centre of the monolayer were pigmented, small and tightly packed, while cells towards the periphery were non-pigmented, large and loosely packed. The presence or absence of FGF 2 did not obviously affect this pattern of growth (fig. 4.3).
Fig. 4.3 Chick PRE cells grown in serum-free medium. Dissociated PRE from stage 24 chick embryos was cultured on plastic in serum-free medium 1, with (B, D and F) or without (A, C and E) 10ng/ml FGF 2. A and B) After 1 hr in culture, cells grown in the absence or presence of FGF 2 formed aggregates (closed arrows), x25. C and D) After 15 hrs in culture, aggregated PRE cells grown in the absence and presence of FGF 2 had attached to the plastic substratum forming numerous discrete monolayers (closed arrows), x10. E and F) After 3 days in culture, cells towards the centre of the monolayer were small and pigmented (closed arrows), while cells towards the periphery were large and non-pigmented (open arrows), x10. The presence or absence of FGF 2 did not affect these characteristics.
4.5 Destabilisation of the differentiated PRE state

Inadequate PRE-stabilising interactions at the periphery of PRE monolayers may explain why peripheral PRE cells had a different morphology to cells towards the centre.

To investigate destabilised PRE further, PRE was dedifferentiation as described by Itoh and Eguchi (1986) except 10,66 medium was used instead of Eagle's MEM. 8d (stage 34) chick PRE was dissociated, plated out directly onto the plastic of two 35mm wells and grown in EDFA (10,66 supplemented with 8% FCS, 0.3mg/ml glutamine, 70μg/ml penicillin, 0.13mg/ml streptomycin and 0.1mM ascorbic acid; chapter 2.10.2). This regime allowed the cells to recover from the dissection. After 5 days in this medium, the cells from one well were collected and replated in either EDFA or EDFPH (10,66 supplemented with 8% FCS, 0.3mg/ml glutamine, 70μg/ml penicillin, 0.13mg/ml streptomycin, 0.5mM 1phenyl-2-thiourea and 250U/ml testicular hyaluronidase). The cells were then grown for a further 7 days; during which time PRE cells in EDFA were not subcultivated and were allowed to reach a high density, while cells grown in EDFPH were subcultivated every second day replating at a low density.

These different cell culture routines had a dramatic effect on cell morphology (fig. 4.4). Cells forced into close proximity with one another were pigmented with a small hexagonal morphology. These cells were organised into a regular honeycomb pattern and were considered to be differentiated PRE cells. In contrast cells that were prevented from interacting with neighbouring cells by physical dissociation and the presence of phenythiourea and testicular hyaluronidase, thought to affect cell surface properties, therefore reducing cell-cell communication (Itoh and Eguchi, 1986), were unpigmented with a large irregular morphology. These cells were highly proliferative, as judged by the rapid increase in cell number, and appeared randomly organised. Cells with these attributes were considered to be dedifferentiated PRE.
Fig. 4.4 Destabilisation of the differentiated PRE state. Dissociated PRE cells from stage 34 chicks were cultured on plastic in EDFA, in two wells of a tissue culture plate. A) The cells in one well were not passaged. After 10 days in culture they were small, pigmented and arranged in a regular honeycomb pattern (closed arrow), x25. Such cells were considered differentiated. The cells in the second well were passaged after 5 days and replated in either EDFA or EDFPH. B) PRE cells replated in EDFA, after a total of 10 days in culture, x25. Cells towards the centre of the monolayer were small and pigmented (closed arrow), while cells towards the periphery were large and non-pigmented (open arrows). The cells replated in EDFPH were passaged every second day. C) PRE cells replated in EDFPH, after a total of 2 passages and 10 days in culture, x25. These cells had a large, irregular morphology, were non-pigmented and randomly organised (open arrow). Such cells were considered dedifferentiated. p0 = not passaged, p1 = passaged once and p2 = passaged twice.
4.6 Conclusion

The thin layered matrigel, a substratum with low rigidity, may allow cells to adopt a morphology more in keeping with their in vivo morphology. However, cells grown on these gels were difficult to observe and procedures such as DNA transfection and immunocytochemistry may be difficult to perform on these cells, since DNA or antibody penetration into the centre of the 3D structures may be difficult. In contrast, the 2 dimensional monolayers, formed when cells were grown on plastic and protein-coated plastic, were more amenable to observation and may be better suited to further experimental techniques. The relatively short life span of the matrigel protein-coated plastic reduces the practicality of this substratum.

The ability to grow PRE cells in serum-free medium means that any subsequent investigation into the effects FGF 2 has on PRE cells can be performed in a defined medium. This is important since FCS may contain unknown growth factors which may include or mimic FGF 2.

The observation that PRE cells at the periphery of monolayers, areas of low cell-cell contact, had characteristics associated with dedifferentiated cells, while cells at the centre of the monolayer, areas of abundant cell-cell contact, had characteristics associated with fully differentiated PRE supports the idea that cell-cell interactions are vital for maintaining a stable state of PRE differentiation. Furthermore, this effect was enhanced by growing cells in EDFPH with repeated passaging at low cell density which served to physically reduce cell-cell interactions. The ability to generate a large number of apparently equivalent cells from a relatively small number of cells is a major advantage of this cell culture system.

It was hypothesised that transdifferentiation of PRE cells into neural retina cells in response to FGF 2 or dedifferentiation in response to disrupted cell-cell interactions may involve the activation of cMsx 2. The involvement of cMsx 2 with these in vitro
phenomena would have been consistent with cMsx 2 functioning *in vivo* during the differentiation of the neural retina. However, preliminary experiments revealed no change in PRE cell morphology (such as the gain of neural processes) that might be indicative of PRE to neural retina transdifferentiation when dissociated cultures of stage 22-24 chick PRE are treated with FGF 2 (data not shown). In these same experiments, no activation of *Msx 2* expression was detected by RT-PCR (data not shown). The failure to observe the predicted FGF 2-induced transdifferentiation into neural retina and, perhaps also, the activation of *Msx 2* may be a consequence of using dissociated cultures (chapter 1.6). Whilst it is possible that the FGF 2 used in this preliminary study lacked biological activity, this is unlikely as the same batch of FGF 2 was shown by other workers to affect cell differentiation in other systems.

Preliminary analysis of *Msx 2* expression in dedifferentiated PRE cells by RT-PCR has also been uninformative due to technical difficulties. Rather than pursue these two approaches, a more direct approach was taken to investigate Msx 2 function in cultured PRE cells. It was decided to ectopically express *Msx 2* in PRE cells.
5.1 Introduction

In the experiments described in this chapter, Msx 2 function is investigated by ectopic expression in cultures of dissociated PRE. These cultures are described in chapter 4. Dedifferentiation, or transdifferentiation into neural retina cells, of Msx 2-expressing PRE cells would strongly suggest that Msx 2 functions in vivo to control the differentiation of neural retina cells.

To express Msx 2 in cultured PRE cells it was first necessary to generate an Msx 2 expression construct. A full-length chick Msx 2 cDNA was not readily available, therefore it was necessary to use a full-length mouse Msx 2 cDNA. The ectopic expression of a mouse protein in chick cells is not ideal. However, the amino acid sequence of the homeodomain of mMsx 2 and cMsx 2 is identical while there is also sequence identity in regions both 5' and 3' of the homeodomain, suggesting that they have a conserved function.

Transfected PRE cells could have been identified by the presence of mMsx 2, detected using mMsx 2-specific antibodies. However, there are no reports in the literature of an mMsx 2 specific antibody, while there is only a limited supply of a Msx homeobox-specific antibody. As an alternative approach to identifying transfected cells, an internal ribosomal entry site (IRES; Mountford and Smith, 1995) was cloned 3' to the mMsx 2 cDNA and 5' to βGeo (a reporter gene generated by fusing βGalactosidase with the neomycin resistance gene). This produced a polycistronic transcript that encoded both mMsx 2 and βGeo. Thus, cells with βGalactosidase activity will also express mMsx 2. Transcription of this construct was driven by the constitutively active enhancer/promoter from the human cytomegalovirus major immediate-early gene (CMV; Schmidt et al., 1990). This construct could be used to
generate cells that permanently express Msx 2, since transfected cells will be resistant to neomycin.

A control construct was also generated to enable any effect which the mMsx 2 expression construct might have on PRE cells to be directly attributed to mMsx 2 expression and not a side effect of the expression vector itself. The only difference between the control construct and the mMsx 2 expression construct was the absence of the mMsx 2 cDNA.

5.2 Generation of CMV expression constructs

5.2.1 Cloning strategy

mMsx 2 expression vector (pCiM2IβGeoSV40)

Step 1

Full-length mMsx 2 cDNA had previously been cloned (by Dr M. Collinson) into the Hind III and Eco RI sites of pBluescript II SK and termed pSK Msx 2. This cDNA contained 203 bp of unnecessary 3’ untranslated sequence and had no suitable restriction sites that would allow the cDNA to be recloned into the pCi expression vector. Therefore, pSK Msx 2 was cut with Bsm I and Eco RI, cleaving Msx 2 at 1263 and pSK at 701 respectively (chapter 2.2.4 and 2.3). This removed 108 bp from the most 3’ end of Msx 2 and allowed the insertion of a phosphorylated adapter (K233/K232) containing four desirable restriction sites (chapter 2.1 and 2.2.6). This new plasmid was termed pSK Msx 2a. A Sal I restriction site is present in the 3’ adapter and within the multiple cloning site of pSK (674), immediately 5’ of Msx 2. Digestion of pSK Msx 2a with Sal I released a fragment corresponding to the 1263 bp Msx 2 insert confirming the successful generation of pSK Msx 2a (chapter 2.2.4; fig. 5.1).
Fig. 5.1 Generation of the *mMsx 2* expression construct (pCiM2IβGeoSV40). A) Step 1, (Aii) the adapter K233/K232 was cloned into the Bsm I and Eco RI sites of pSKMsx 2 to generate pSKMsx 2a (sites indicated in **bold**) and destroying the Eco RI site (indicated by an asterisk). Sal I was predicted to digest pSKMsx 2a as shown in (Aii). Ai) Agarose-gel electrophoresis of the products from Sal I restriction digest analysis of 6 pSKMsx 2a clones. As expected Sal I released the *mMsx 2* insert (~1.3 kb band) from the pSK vector (~3 kb band). B) Step 2, (Bii) The *mMsx 2* cDNA was cloned into the Xho I and Mlu I sites of pCi to generate pCiM2SV40 (sites indicated in **bold**). Xho I/Mlu I were predicted to cleave pCiM2SV40 as shown in (Bii). Bi) Agarose-gel electrophoresis of the products from Xho I/Mlu I double restriction digest analysis of 3 pC1M2SV40 clones. As expected Xho I/Mlu I released the *mMsx 2* insert (~1.3 kb band) from the pCi vector (~4 kb band). C) Step 3, (Cii) An IRES-βGeo cassette was cloned into the Xba I site of pCiM2SV40 to generate pCiM2I3GeoSV40 (sites indicated in **bold**). Bam HI was predicted to digest pCiM2IβGeoSV40 as shown in (Cii). Ci) Agarose-gel electrophoresis of the products from Bam HI restriction digest analysis of 9 pCiM2IβGeoSV40 clones. Clones 1, 2, 5 and 7 released an approximately sized 1.2 kb band, predicted to contain SV40, from the vector. This confirmed that the IRES-βGeo cassette had been cloned in the correct orientation. M = 1 kb DNA ladder.
Step 2

The Msx 2 cDNA was removed from pSK Msx 2a by digestion with Xho I and Mlu I, which cut pSK 21 bp 5' of the Msx 2 insert, and the adapter 3' of Msx 2, respectively (chapter 2.2.4 and 2.3). This insert was cloned into the reciprocal sites (1058 and 1069) of the promega pCI mammalian expression vector to generate pCiM2SV40 (chapter 2.1). Restriction of pCiM2SV40 with Xho I and Mlu I released a fragment corresponding to the 1283 bp Msx 2 cDNA insert (chapter 2.2.4), confirming the successful generation of this construct (fig. 5.1).

This step places the Msx 2 cDNA downstream of the CMV promoter and a chimeric intron, composed of the 5' donor site from the first intron of the human β-globin gene and the branch and 3' acceptor site from the intron of an immunoglobin variable chain gene (Bothwell et al., 1981). An intron flanking a cDNA insert, has been shown to increase the levels of gene expression (Buchman and Berg, 1988; Evans and Scarpulla, 1989). An SV40 late polyadenylation signal is located below the Msx 2 insert. This causes the termination of transcription by RNA polymerase II and the addition of multiple adenosine residues to the 3' end of the transcript (Proudfoot, 1991). This increases the stability of the RNA and translation efficiency (Bernstein and Ross, 1989; Jackson and Standart, 1990).

Step 3

The IRES and reporter gene (βGeo) cassette was removed from Iββs (obtained from Dr M. Collinson) as an Xba I fragment (chapter 2.2.4 and 2.3) and cloned into the Xba I site of pCiM2SV40 (chapter 2.1), located 12 bp below the Msx 2 cDNA insert and upstream of the SV40 polyadenylation signal, to generate pCiM2IβGeoSV40. Clones which contained the Xba I insert in the correct orientation were identified by Bam HI digestion (chapter 2.2.4). As shown in fig. 5.1, there are two Bam HI restriction sites, one located 3' of the SV40 polyadenylation signal and the other
approximately 800 bp from the 3' end of the IRES/βGeo cassette. The presence of fragments corresponding approximately to the predicted 1062 bp and 10017 bp fragments following Bam HI digestion confirmed that the Xba I fragment had been successfully cloned in the correct orientation (fig. 5.1). The presence of fragments corresponding approximately to the predicted 5239 bp and 5840 bp fragments would have indicated that the Xba I fragment had been cloned in the wrong orientation.

Control expression construct (pCiIβGeoSV40)

The IRES/βGeo cassette, released from Iββs as an Xba I fragment (chapter 2.2.4 and 2.3), was cloned into the Xba I site of an empty pCi expression vector (chapter 2.1), placing it below the CMV promoter and chimeric intron, and above the SV40 polyadenylation signal, to generate pCiIβGeoSV40 (fig. 5.2). The orientation of the insert was again confirmed by Bam HI restriction digest (chapter 2.2.4; fig. 5.2). In the correct orientation, Bam HI digestion yielded fragments approximately corresponding to the predicted 1062 bp and 8723 bp fragments. In the wrong orientation bands corresponding to 4546 bp and 5239 bp fragments would have been observed. An Apa I digest of pCiIβGeoSV40 linearised the plasmid, further confirming the integrity of this control construct (fig. 5.2).
Fig. 5.2 Generation of the control expression construct (pCiβGeoSV40). B) An IRES-βGeo cassette was cloned into the Xba I site of the pCi expression construct to generate pCiβGeoSV40 (sites indicated in bold). Bam HI and Apa I were predicted to cleave pCiβGeoSV40 as indicated in (B). A) Agarose-gel electrophoresis of the products from Bam HI and Apa I restriction digest analysis of 2 pCiβGeoSV40 clones. As expected Bam HI released the SV40 fragment (~1.2 kb band) from the vector (>5 kb band) for both clones. This confirmed that the IRES-βGeo cassette had been cloned in the correct orientation. Note that the 1.2 kb band is very weak for clone 2. In addition, Apa I linearised both clones as predicted. M = 1 kb DNA ladder.
5.2.2 Restriction digest analysis of the \textit{mMsx 2} expression construct (pCiM2IβGeoSV40)

To confirm the integrity of the \textit{mMsx 2} expression construct (pCiM2IβGeoSV40) a variety of single- and double- digests were performed (chapter 2.2.4 and 2.3; fig. 5.3). These digests cleaved the construct into the predicted number of fragments which were approximately the predicted size confirming that all the inserts had been cloned intact, in the correct place, orientation and not as concatomers.
Fig. 5.3 Restriction digest analysis of the mMsx 2 expression construct (pCiM2IβGeoSV40). A) Agarose- (1%) gel electrophoresis of the products generated from digesting pCiM2IβGeoSV40 with the enzymes indicated in (A). B) Restriction digest map of pCiM2IβGeoSV40 showing the predicted locations of the restriction enzymes used in (A) and the sizes of the fragments expected following digestion. Sizes in brackets are the approximate size of the fragments actually observed. Sizes in bold correspond to broad bands which may contain 2 or more similarly sized fragments. M = 1 kb DNA ladder.
5.2.3 Sequence analysis of \textit{mMsx} 2 in the \textit{mMsx} 2 expression construct (pCiM2IβGeoSV40)

It was important to confirm that no mutations had been introduced into the \textit{mMsx} 2 cDNA, in particular the coding region, during the cloning processes which might alter the amino acid residue structure or result in a truncated protein being produced. A mutated protein may either not function or function abnormally. In either case, it would hinder the interpretation of the functional significance of any effect observed in PRE cells transfected with the \textit{mMsx} 2 expression construct. It was therefore decided to sequence both strands of the \textit{mMsx} 2 cDNA in the expression construct and compare these sequences to the published \textit{mMsx} 2 sequence (chapter 2.5).

The \textit{mMsx} 2 cDNA portion of the expression construct (pCiM2IβGeoSV40) was sequenced using the panel of primers described in chapter 2.7.1 and 2.5. Using these primers the entire \textit{mMsx} 2 anti-sense strand was sequenced and the sense strand partially sequenced (fig. 5.5). As can be seen in fig. 5.4, sequence was not obtained for the first 168 bp of 5' untranslated sequence, the last 39 bp of 3' untranslated sequence and a 120 bp region (529-649) within the coding region of the sense strand.

Differences between the published and expression construct \textit{mMsx} 2 sequence were disregarded and considered sequencing artefacts if corrected by an overlapping fragment or the complementary strand. A number of differences between the predicted and observed \textit{mMsx} 2 sequences could not be corrected for by overlapping fragments. An extra G was identified between residues 41 and 42 of the anti-sense strand in 2 independent sequencing reactions. In these same 2 reactions, residue 46 of the anti-sense strand, predicted to be a C, was a G in one reaction and a T in the other. The sequence of the complementary sense strand of this region was not obtained and it therefore remains possible that these alterations are artefacts. These possible mutations lie in the 5' untranslated region of \textit{mMsx} 2 and therefore would not effect the amino acid structure of Msx 2. Residue 1236 of the anti-sense strand,
predicted to be C, could not be identified. This region was only sequenced once and no sequence was obtained for the complementary sense strand. It is therefore possible that the observed change is merely a sequencing artefact. Even if this is not the case this mutation will have no effect on Msx 2 structure as it lies in the 3' untranslated region.

Two residues within the coding region were altered and would effect the amino acid structure of Msx 2. Residue 1152 of the sense strand, predicted to be C, was a G. This mutation was also found in the anti-sense strand and would result in amino acid 263 being converted from a I to M. Residue 1159 of the sense strand, predicted to be G, was a T, but in the anti-sense strand the complimentary C was a G. It therefore remains possible that this mutation is a sequencing artefact. If either of these two possible mutations of residue 1159 are true, then amino acid 266 would be converted from V to L. As shown in fig. 5.4(B), the amino acids of human, chick, quail and *Xenopus* Msx 2 that are equivalent to residues 263 and 266 in mouse Msx 2 are M and L, respectively. The equivalent residues in mouse, human and *Xenopus* Msx 1 are also M and L. This suggests that the published mouse Msx 2 sequence may be wrong and that residues 263 and 266, are indeed M and L, respectively. To confirm this suggestion it will be necessary to repeat the sequencing of this region.

The most 5' region of the *mMsx 2* insert was sequenced using the T7 primer which annealed in the neighbouring vector sequence. This primer site was located very close to the 5' cloning junction, so it was not possible to obtain sequence that spanned this junction. However, sequence from residue 4 onwards was obtained using this primer indicating that no gross alteration had occurred at the 5' cloning site. The sequence generated from primer M847 extends out of *mMsx 2*, through the 3' adapter and into neighbouring vector sequence. This sequence matched the predicted sequence, confirming that one copy of *mMsx 2* had been inserted with out any alteration at the 3' cloning site.
Fig. 5.4 Sequence analysis of the *mMsx 2* insert from the *mMsx 2* expression construct (pCiM2lβGeoSV40). A) Alignment of the fragments of sequence generated from each primer to the published *mMsx 2* sequence in the HGMP database. The position of residues that differ from the published sequence are indicated (refer to main text and fig. 5.5 for details). B) Comparison of amino acid sequences (229-267) of Msx 1 and Msx 2 in the higher vertebrates. Comparisons are based on a clustal multiple sequence alignment of predicted amino acid sequences published in the HGMP genome database. * corresponds to amino acid residues identical to the corresponding mouse gene. Residue 263 and 266 are highlighted in bold, note that mMsx 2 is the only protein predicted to have an I and V at these positions, respectively. Residues in brackets correspond to the predicted amino acid as determined from sequencing the *mMsx 2* expression construct. (M = mouse, H = human, C = chick, Q = quail and X = Xenopus)
Fig. 5.5 A) Comparison of the mMsx 2 anti-sense strand of the mMsx 2 expression construct (pCiM21βGeoSV40) to the published sequence in the HGMP genome database. B) Comparison of the mMsx 2 sense strand of pCiM21βGeoSV40 to the published sequence in the HGMP genome database. Expression construct sequence on bottom, published mMsx 2 sequence on top. Sequences were compared using the GAP program from GCG. Residues in bold mark the translation start and stop sites, N signifies an undetermined residue. "." signifies the insertion of a gap to maintain alignment.

A) Comparison of anti-sense mMsx 2 sequence.

```
1321 AATAACAAATCTCAGTATCAAGGTCGCCCTGTCGCTTGGGTGACAAT
1272
1296 .............................................. GAATTTTAGAGGTACCCNAT
1273
1271 GCAAGGTCGCAATTAGGAGCGAGACTTTGCACTCTCCAGGAGACAGG
1222
1272 GCAAGGTCGCAATTAGGAGCGAGACTTTGCACTCTCCAGGAGACAGG
1223
1221 GGGGGATGCTTTATGCAAACATCCATCCTGGAGTCTGCTCATTGC
1172
1222 GGGGGATGCTTTATGCAAACATCCATCCTGGAGTCTGCTCATTGC
1173
1171 TTCTTTAGGATACATGAGTGTCATCCATCAACCAGGCTGGCCATAGAT
1122
1172 TTCTTTAGGATACATGAGTGTCATCCATCAACCAGGCTGGCCATAGAT
1123
1121 CCAACAGGGGATGGGAGGACACAGGTCATGGAAGGGGTAGGAGCTCC
1072
1122 CCAACAGGGGATGGGAGGACACAGGTCATGGAAGGGGTAGGAGCTCC
1073
1071 GTATATGATGCTGTATGCTCCAGGGTGAGTATAGGAGGAGGAGCTCC
1022
1072 GTATATGATGCTGTATGCTCCAGGGTGAGTATAGGAGGAGGAGCTCC
1023
1021 AGCTTGAAGGGAGCATAGACAGGCTTTGCGCCATATTTTCACTTTTCAGCTTTCC
972
1022 AGCTTGAAGGGAGCATAGACAGGCTTTGCGCCATATTTTCACTTTTCAGCTTTCC
973
971 GCCTTTGAGCTTTTTCGCCCTTTAGGCTTTGGTTGGTCTCCGGTTCTTCT CCTAC
922
972 GCCTTTGAGCTTTTTCGCCCTTTAGGCTTTGGTTGGTCTCCGGTTCTTCT CCTAC
923
921 GACCTGGGTCTCTGCTAAGTGCTAGAGCTGAGAAGGGCGGCTTTCCGGCTCTG
872
922 GACCTGGGTCTCTGCTAAGTGCTAGAGCTGAGAAGGGCGGCTTTCCGGCTCTG
873
871 CTATGGCAGGTACTCCTGTGCTGCTGAGGGAGACTTTGAGGACTGAGCCGCTGAGAAC
822
872 CTATGGCAGGTACTCCTGTGCTGCTGAGGGAGACTTTGAGGACTGAGCCGCTGAGAAC
823
821 TGGGATGTGTGAAAGGGGTGGTGGCCTGCGCTTCCGGAACTTGGGGCTTTGACTCC
772
822 TGGGATGTGTGAAAGGGGTGGTGGCCTGCGCTTCCGGAACTTGGGGCTTTGACTCC
773
771 CAGGGTGCAGGGTGGGCTACTATGCTCTGGGGCCCAGGGAGATAGCTC
722
772 CAGGGTGCAGGGTGGGCTACTATGCTCTGGGGCCCAGGGAGATAGCTC
723
```
B) Comparison of sense mMx 2 sequence

151 CAAGCCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

953 .................... T

201 AGCAGGGCCCTCGGAGCTGTTAGGCTGGCCAGTCTCCACCTCCCCTCCTCA

911 AGCAGGGCCCTCCAGGATGTTAGGCTGGCCAGTCTCCACCTCCCCTCCTCA

251 GGACAGGCTGGCTCCACAGGCTGGAGCAGTCGCTGGCTCCAGAGCTACGC

301 GGACAGGGCTGGCTCCAGGCTGGAGCAGTCGCTGGCTCCAGAGCTACGC

351 GCCGAGAAAGCTATGGCTATCTCCGACTAAAGGCCTGACTTGTTTCGTC

401 GCCGAGAAAGCTATGGCTATCTCCGACTAAAGGCCTGACTTGTTTCGTC

451 GGAGCCGAGGGCAGCGCAGAGGAGCGCAGGGTCAAGGTCTCCAGCCTGCC

501 CTTCAGCGTGGAGGCGCTCATGTCCGACAGAGAGCCGCCCAGGAATCGC

551 CTTCAGCGTGGAGGCGCTCATGTCCGACAGAGAGCCGCCCAGGAATCGC

601 CTGCTGCCGGGACACGGCTTCCGGGACGCTCACAGTCCCGGGCCTCTCGT

575 ........................................................ T

651 CAAGCCTCTGGGACACGGCTTCCGGGACGCTCACAGTCCCGGGCCTCTCGT

574 CAAGCCTCTGGGACACGGCTTCCGGGACGCTCACAGTCCCGGGCCTCTCGT

651 CACCGTGGATACAGGAGCCCGGCAGATACTCCCCGCCGCCCAGACATATG

474 AGCCCCACACCTGCACCCAGCTAGGAACACACAGACCACCCGGAAGCAGAG

475 AGCCCCACACCTGCACCCAGCTAGGAACACACAGACCACCCGGAAGCAGAG

524 CACCGTGGATACAGGAGCCCGGCAGATACTCCCCGCCGCCCAGACATATG

575 CACCGTGGATACAGGAGCCCGGCAGATACTCCCCGCCGCCCAGACATATG

624 CACCGTGGATACAGGAGCCCGGCAGATACTCCCCGCCGCCCAGACATATG

675 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

724 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

775 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

824 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

875 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

924 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

975 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

1024 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

1074 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

1124 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG

1174 AGCCCGCTCTCCAGGAGACCCACATGCTGGCCGAGCTCCGGTGGTCAGG
5.2.4 Functional test of the \textit{mMsx2} expression construct (pCiM2IβGeoSV40) and control construct (pCiIβGeoSV40)

To confirm that the transcript produced from the \textit{mMsx2} expression construct encoded \textit{mMsx2} and \textit{βGal}, and that the transcript produced from the control construct encoded \textit{βGal}, the following experiment was performed; PRE from 6d chick embryos was dissociated and cultured for approximately 24 hrs in EMEMF (chapter 2.10.2) before being transiently transfected with either the \textit{mMsx2} expression construct (pCiM2IβGeoSV40) or the control construct (pCiIβGeoSV40) using lipofectamine (chapter 2.10.3). 3 days after transfection, total RNA was prepared from a proportion of these cells and the remainder stained for \textit{βGalactosidase} activity (chapter 2.10.4).

As can be seen in fig. 5.6, a large proportion of PRE cells exhibited \textit{βGalactosidase} activity 3 days after transfection. This confirmed that dissociated chick PRE cells can
be efficiently transfected using lipofectamine and that both constructs were transcribing \( \beta \text{Geo} \) as expected.

Total RNA from these cells, along with control RNA was used to make first-strand cDNA as summarised in table 5.1 (chapter 2.6.3.2 and 2.7.3). RNA from 15d mouse limb buds and G7 myoblasts is known to contain \( mM\text{s}x\ 2 \) mRNA and consequently were used as positive controls. Untransfected chick PRE will not contain \( mM\text{s}x\ 2 \) mRNA and was used as a negative control. RNA from stage 24-25 chick limb buds is known to contain \( cMs\text{x} 2 \) mRNA and was used to test the species specificity of the mouse \( Ms\text{x} 2 \) primers. PCR was performed on each sample of first-strand cDNA, described in chapter 2.7, primed by either the mouse \( Ms\text{x} 2 \) primers (J562 and J563 which amplify a 407 bp fragment) or \( \beta \text{Actin} \) primers (J252 and J253 which amplify a 711 bp fragment). As can be seen in fig. 5.6, the mouse \( Ms\text{x} 2 \) primers did not amplify the endogenous chick \( Ms\text{x} 2 \) transcript in the chick limb bud sample, but strongly amplified the mouse \( Ms\text{x} 2 \) transcript in the mouse limb bud sample and chick PRE cells transfected with the \( mM\text{s}x\ 2 \) expression construct (pCiM2I\( \beta \text{Geo} \text{SV}40 \)). Weak bands of varying size were observed in the cDNA sample generated from PRE which was either transfected with the control construct or not transfected. This banding pattern probably reflects non-specific primed PCR rather than PCR contamination, since the control PCR reaction in which no cDNA was added was completely negative. This result confirmed that the \( mM\text{s}x\ 2 \) expression construct functions in PRE cells to produce \( mM\text{s}x\ 2 \) mRNA.
Fig. 5.6 Functional analysis of the mMsx 2 expression construct (pCiM2IβGeoSV40) and the control construct (pCiIβGeoSV40) in 6d chick PRE cells. A) RT-PCR on 6d PRE, transiently transfected with either the mMsx 2 construct, control construct or no DNA and cultured for a further 3 days, for mMsx 2 expression. mMsx 2 cDNA was detected by PCR using primers J562 and J563 (M2) which amplify a 407 bp fragment, and cβActin cDNA using primers J252 and J253 (AC) which amplify a 711 bp fragment. Two positive controls, G7 myoblasts and 15 d mouse limb bud RNA, were used. Stage 24-25 chick limb bud RNA served as a negative control. M = 1 kb DNA ladder. B and C) βGalactosidase histochemistry of 6d PRE, transiently transfected with either the mMsx 2 expression construct (B), x4, or the control construct (C), x4, and cultured for a further 3 days. The presence of blue cells confirmed that βGeo was being transcribed and translated from both constructs.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Construct</th>
<th>Amount of RNA used in cDNA synthesis (µg)</th>
<th>( m\text{Msx} ) 2 expression</th>
<th>( c\beta\text{Actin} ) expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRE</td>
<td>pCiM2IβGeoSV40</td>
<td>N/D</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>PRE</td>
<td>pCiβGeoSV40</td>
<td>N/D</td>
<td>?</td>
<td>y</td>
</tr>
<tr>
<td>PRE</td>
<td>no construct</td>
<td>0.2</td>
<td>?</td>
<td>y</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G7 myoblasts</td>
<td></td>
<td>1</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>Chick limb bud (stage 24-25)</td>
<td></td>
<td>1</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Mouse limb buds (E15)</td>
<td></td>
<td>1</td>
<td>y</td>
<td>y</td>
</tr>
</tbody>
</table>

Table 5.1 Summary of RT-PCR analysis of PRE cells transfected with either the \( m\text{Msx} \) 2 expression construct (pCiM2IβGeoSV40) or the control construct (pCiβGeoSV40) for \( m\text{Msx} \) 2 expression. 6d chick PRE was grown for 24 hrs before being transiently transfected by either pCiM2IβGeoSV40, pCiβGeoSV40 or no DNA. Total RNA was isolated from these cells 3 days after transfection and RT-PCR performed as described in the main text and fig. 5.6. ? refers to slight contamination or non-specific PCR.

5.3 \( m\text{Msx} \) 2 expressing PRE cells gain a neural phenotype

5.3.1 Description of \( m\text{Msx} \) 2 induced phenotype

To investigate the effect that ectopic \( m\text{Msx} \) 2 expression has on PRE cells, 6d chick PRE was dissociated and cultured for approximately 24 hours in EMEMF prior to transient transfection with either the \( m\text{Msx} \) 2 expression construct (pCiM2IβGeoSV40) or the control construct (pCiβGeoSV40; chapter 2.10.2 and 2.10.3). After an additional 3 days in culture, cells were fixed and stained for βGalactosidase activity (chapter 2.10.4). As can be seen in fig. 5.7, \( m\text{Msx} \) 2 transfected PRE cells generally extended longer processes than equivalent PRE cells transfected with the control construct. This general observation was analysed in more detail by counting the number of cells, transfected by each construct, that extended processes deemed to be approximately 10 times longer than wider. At the same time, the number of untransfected cells in each culture that extended such processes were also counted (table 5.2). This whole procedure was repeated independently 3 times.
and the results of all three experiments are summarised in graph 5.1. To avoid biasing the results, cultures were analysed without knowing which construct had been transfected.

It was observed that 2.76% of PRE cells, transfected with the control construct, extended processes that were 10 times longer than wide. In contrast, 11.09% of PRE cells, transfected with the mMsx 2 expression construct, extended processes that were judged to be 10 times longer than wide. 2.71% of neighbouring untransfected cells, within the mMsx 2 transfected culture, extended long processes. This is similar to the proportion of control transfected cells that extended long processes and implies that Msx 2 is having a morphological effect only on cells in which it is being expressed.

Graph 5.1 Percentage of mMsx 2 (pCiM2IβGeoSV40) and control (pCiIβGeoSV40) transfected, and neighbouring untransfected PRE cells, extending processes that are approximately 10 times longer than wide. In three independent experiments, 6d chick PRE was dissociated and grown for 24 hrs before being transiently transfected with either pCiM2IβGeoSV40 or pCiIβGeoSV40. After a further 3 days in culture, they were stained for βGalactosidase activity. For each culture the percentage of transfected and untransfected cells, extending processes judged to be 10 times longer than wide was determined (table 5.2). Error bars calculated as value +/- 1.96 x s.d.
Fig. 5.7. Phenotype associated with *mMsx* 2 transfected PRE cells. 6d chick PRE was dissociated, cultured for 24 hrs in EMEMF and then transiently transfected with either the *mMsx* 2 expression construct or the control construct. 3 days later the cells were stained for βGal activity. A) A proportion of *mMsx* 2 transfected cells extended processes that were approximately 10 times longer than wider (closed arrow), x4. B) Cells transfected with the control construct were generally rounded (open arrow), with a smaller proportion extending long processes, x4. C) A small proportion of *mMsx* 2 transfected cells had a distinct neural morphology (closed arrow), x4. Cells with this morphology were not observed in control transfected cultures (B). *mMsx* 2-induced neural cells had either two dendrites (D), closed arrow (x25), or several dendrites (F), open arrow (x25). Dendrites can be branched (F, open arrow heads) and appeared to make contacts with neighbouring cells (E, closed arrow heads, x25). Note that cells in (E) and (F) were counter stained with nuclear fast red. d = dendrites and cb = cell body.
Further analysis of PRE cultures transfected with the \( mMsx \, 2 \) expression construct revealed a subpopulation of transfected cells with a distinct neural morphology (fig. 5.7). These cells could be distinguished from other cells as they extended two or more long dendrites from well defined cell bodies. The dendrites were often branched and appeared, though not always, to make contacts with neighbouring cells.

An average taken from the three independent experiments suggests that this class of cell accounts for 0.98\% (\(+/-0.39\%\); value \(+/-1.96 \times \text{s.d.}\)) of \( mMsx \, 2 \) transfected cells and was never associated with untransfected cells (table 5.2). These cells were never observed in control transfected cultures implying that the occurrence of this cell type can be directly attributed to \( mMsx \, 2 \) expression.

<table>
<thead>
<tr>
<th>Ex</th>
<th>Construct Transfected</th>
<th>Transfected cells</th>
<th>Untransfected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total no.</td>
<td>No. with processes</td>
</tr>
<tr>
<td>1</td>
<td>pCiM2IβGeoSV40</td>
<td>1,397</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>pCiIβGeoSV40</td>
<td>1,001</td>
<td>24</td>
</tr>
<tr>
<td>2</td>
<td>pCiM2IβGeoSV40</td>
<td>417</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>pCiIβGeoSV40</td>
<td>474</td>
<td>15</td>
</tr>
<tr>
<td>3</td>
<td>pCiM2IβGeoSV40</td>
<td>621</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>pCiIβGeoSV40</td>
<td>297</td>
<td>10</td>
</tr>
</tbody>
</table>

Summary of above results

- pCiM2IβGeoSV40
  - Total no.: 2,435
  - No. with processes: 270
  - No. with neural morphology: 24
  - 11.09\% \( \pm 0.98\% \)
  - 2.71\% \( \pm 0\% \)

- pCiIβGeoSV40
  - Total no.: 1,772
  - No. with processes: 49
  - No. with neural morphology: 0
  - 2.76\% \( \pm 0\% \)
  - 3.65\% \( \pm 0\% \)

Table 5.2 Summary of three experiments investigating the effect \( mMsx \, 2 \) expression in PRE cells has on cell morphology. In each experiment 6d chick PRE was dissociated and cultured for approximately 24 hrs before being transiently transfected with either the \( mMsx \, 2 \) expression construct (pCiM2IβGeoSV40) or the control construct (pCiIβGeoSV40). Approximately 72 hrs after transfection, cells were fixed and stained for βGal activity. The percentage of transfected and untransfected cells with processes 10 times longer than wide were determined, as were the percentage of transfected and untransfected cells with neural a morphology.
The medium used during these experiments contained 8% FCS which may contain unknown factors. Although these factors are not sufficient to cause PRE cells to adopt a neural morphology (no neural-like cells were observed in control cultures) it is possible that they may be required to support Msx 2 function. In collaboration with C. Oram this possibility was tested by culturing dissociated 5d chick PRE in either serum-free medium (EMEM supplemented with N1 serum-free supplement, 0.3 mg/ml glutamine, 70μg/ml penicillin and 0.13 mg/ml streptomycin) or FCS-containing medium (EMEMF) for approximately 24 hrs (chapter 2.10.2). A proportion of these cells were then transfected with either the \textit{mMsx 2} expression construct or the control construct; cultured for a further 3 days and stained for β-Galactosidase activity (chapter 2.10.3 and 2.10.4). A similar proportion of \textit{mMsx 2} transfected PRE cells acquired a neural morphology irrespective of whether they had been grown in a serum-containing, or serum-free medium. No cells with a neural morphology were observed in cultures transfected with the control construct. This result confirms that the effect \textit{mMsx 2} expression has on PRE cells is not dependant on factors in the serum that may not be normally present in the developing eye (data not shown).

5.3.2 \textit{mMsx 2} expression does not induce a neural phenotype in perioptic mesenchyme

It is very probable that a small amount of perioptic mesenchyme will contaminate the chick PRE cultures. It may be this cell type that is responding to \textit{mMsx 2} rather than the PRE cells. This could explain why only a small proportion of the \textit{mMsx 2} transfected cells (0.98%) exhibit a neural morphology.

To investigate this possibility, 5d chick PRE and perioptic mesenchyme were isolated and dissociated (chapter 2.10.2). These cells were grown in EMEMF, in duplicate and at comparable densities, either separately or mixed together at an approximate ratio of 1:1. After approximately 24 hrs in culture, half the cultures were transfected with the
mMsx 2 expression construct (pCiM2IβGeoSV40) and half with the control construct (pCiβGeoSV40; chapter 2.10.3). Approximately 48 hrs after transfection, the cells were fixed and the presence of βGalactosidase protein detected by immunocytochemistry using a rabbit anti-βGal primary antibody and a fluorescein (FITC) conjugated donkey anti-rabbit secondary antibody (chapter 2.10.6).

No mMsx 2 transfected mesenchymal cells acquired a neural morphology (table 5.3) suggesting that perioptic mesenchyme cannot be converted to a neural phenotype upon transfection with the mMsx 2 expression construct. This implies that PRE cells are the cell type responding to mMsx 2 expression.

The mMsx 2 expression construct transfected 5% of PRE cells but only 0.86% of perioptic mesenchyme cells (table 5.3). Taking into account the fact that PRE cells are transfected 5.8 times more efficiently than mesenchyme cells, and assuming that the mesenchymal cells are not converted to a neural morphology by mMsx 2 expression, it might have been predicted from the number of neural cells in the mMsx 2 transfected culture (1.76%) that approximately 1.5% of the mMsx 2 transfected cells in the PRE/mesenchyme mixed culture would have a neural morphology. In actual fact only 0.84% of the mMsx 2-expressing cells had a neural morphology. This again confirmed that perioptic mesenchyme cannot respond to mMsx 2 expression by acquiring a neural phenotype. Furthermore, the presence of perioptic mesenchyme in the PRE cultures does not appear to affect the proportion of cells acquiring a neural phenotype following mMsx 2 expression.

Compared to previous experiments a higher proportion of mMsx 2 transfected cells acquired a neural morphology, 1.76% compared to 0.98%. This increase may be a consequence of using younger PRE cells and this is explored further in chapter 5.4.
<table>
<thead>
<tr>
<th></th>
<th>Cell type</th>
<th>PRE</th>
<th>PRE + mesenchyme</th>
<th>Mesenchyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfection efficiency</td>
<td>5</td>
<td>N/D</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Number of transfected</td>
<td>680</td>
<td>1,432</td>
<td>241</td>
<td></td>
</tr>
<tr>
<td>cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number with neural</td>
<td>12</td>
<td>12</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>morphology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of transfected cells</td>
<td>1.76</td>
<td>0.84</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>with neural morphology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3 Summary of the effect transfection of periopotic mesenchyme and PRE with the mMsx 2 expression construct (pCiM2IβGeoSV40) has on cell morphology. PRE and periopotic mesenchyme from 5d chick eyes were dissociated and cultured in duplicate, either separately or mixed together at a ratio of 1:1, for 24 hrs. Cultures were then transfected with either pCiM2IβGeoSV40 or pCiIβGeoSV40 and grown for a further 48 hrs before being fixed and stained for the presence of βGal using fluorescence based immunocytochemistry. The percentage of cells stained for βGal (transfection efficiency) and the percentage of βGal stained cells with a neural morphology in each culture was determined. N/D = not determined.

5.4 The effect of age on the competence of the PRE to respond to mMsx 2 expression

The previous experiment indicated that a slightly higher proportion of 5d mMsx 2 transfected PRE cells had gained a neuronal phenotype compared to transfected 6d PRE. This observation suggested that the ability of PRE cells to respond to mMsx 2 expression is dependant on the age or degree of commitment to the PRE lineage. Indeed, only PRE up to stage 27 (5d) responded to FGF 2 by transdifferentiating into
neural retina cell types (chapter 1.6). It is therefore possible that the ability of \textit{mMsx 2} expression to induce a phenotypic change in PRE is also limited by the age of this tissue.

This possibility was investigated further by setting up, in duplicate, dissociated PRE cultures from 4d, 5d and 6d chick embryos as described in chapter 2.10.2. These cultures were grown for 24 hrs in EMEMF, then half were transiently transfected with the \textit{mMsx 2} expression construct (pCiM2I\(\beta\)GeoSV40) and half with the control construct (pCiI\(\beta\)GeoSV40, chapter 2.10.3). Approximately 48 hrs after transfection, these cells were fixed and stained for \(\beta\)Galactosidase activity as described in chapter 2.10.4. The percentage of transfected cells with a neural morphology was then determined for each culture (graph 5.2 and table 5.4).

<table>
<thead>
<tr>
<th>PRE age</th>
<th>4d</th>
<th>5d</th>
<th>6d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of transfected cells</td>
<td>687</td>
<td>2,326</td>
<td>1,896</td>
</tr>
<tr>
<td>Number with neural morphology</td>
<td>10</td>
<td>37</td>
<td>8</td>
</tr>
<tr>
<td>% of transfected cells with neural morphology</td>
<td>1.45</td>
<td>1.59</td>
<td>0.42</td>
</tr>
</tbody>
</table>

| Number of transfected cells | 407  | 1,387 | 519 |
| Number with neural morphology | 0   | 0    | 0   |
| % of transfected cells with neural morphology | 0   | 0    | 0   |

Table 5.4 The effect of age on the competence of the PRE to respond to \textit{mMsx 2} expression. PRE cultures from 4d, 5d and 6d chicks were cultured in duplicate for 24 hrs, then transiently transfected with the \textit{mMsx 2} expression construct (pCiM2I\(\beta\)GeoSV40) or the control construct (pCiI\(\beta\)GeoSV40) and grown for a further 48 hrs. These cultures were then fixed, stained for \(\beta\)Gal activity and the percentage of transfected cells with a neural morphology determined.
Graph 5.2 Percentage of PRE cells, from differently aged donors, transfected with either the \( m\text{Msx}2 \) expression construct (pCiM2I/βGeoSV40) or the control construct (pCiIβGeoSV40) with a neural morphology. PRE cultures from 4d, 5d and 6d chicks were cultured in duplicate for 24 hrs, then transiently transfected with pCiM2IβGeoSV40 or pCiIβGeoSV40 and grown for a further 48 hrs. These cultures were then fixed, stained for βGal activity and the percentage of transfected cells with a neural morphology determined.

A similar proportion of \( m\text{Msx}2 \) transfected 4d and 5d chick PRE cells acquired a neural morphology, 1.45% and 1.59% respectively, while only 0.42% of \( m\text{Msx}2 \) transfected 6d PRE acquired a neural morphology. It has been reported that PRE from chicks aged 5 days and older fails to respond to FGF 2 by acquiring a neural phenotype (chapter 1.6). Therefore, the decrease in the number of cells being able to respond to \( m\text{Msx}2 \) expression, as the PRE matures, raises the possibility that the ability to acquire a neural phenotype in response to \( m\text{Msx}2 \) expression may be completely lost at later stages. However, this does not appear to be the case since C. Oram (personal communication) has demonstrated that PRE isolated from 9d chicks still possesses the ability to respond to \( m\text{Msx}2 \) expression by acquiring a neural phenotype. This was shown by culturing 9d PRE for 24 hrs before transiently transfecting with the \( m\text{Msx}2 \) expression construct (pCiM2IβGeoSV40). Approximately 0.2% of the transfected cells had acquired a neural phenotype after 48 hrs. Thus, there may be a reduction in the number of cells within PRE cultures that
are able to respond to *mMsx 2* expression as the starting tissue matures, but at least up until 9d, this competence is not completely lost.

5.5 *mMsx 2* expression in dedifferentiated PRE cells induces a neural morphology

Dedifferentiated PRE, described in chapter 4, has a different morphology and lacks pigment compared to differentiated PRE. These cells may therefore be more responsive to Msx 2 function than differentiated PRE. Alternatively, the ability to respond to *mMsx 2* by acquiring a neural phenotype may be lost during the prolonged dedifferentiation process. It was therefore decided to investigate Msx 2 function in dedifferentiated PRE cells.

6d and 4d chick PRE were dedifferentiated, under different regimes, before being transiently transfected with the *mMsx 2* expression construct and control construct. Dedifferentiation of 6d PRE was achieved by culturing at low density for 5 days in EMEMF, then passaged and grown for a further 2 days before being passaged for the final time (chapter 2.10.2). 24 hrs after the final passage the cells were transiently transfected with either the *mMsx 2* expression construct (pCiM2IβGeoSV40) or the control construct (pCiIβGeoSV40; chapter 2.10.3). 48 hrs after transfection the cells were fixed, stained for βGalactosidase activity (chapter 2.10.4) and the percentage of transfected and untransfected cells with a neural morphology determined for each culture (graph 5.3 and table 5.5).
Table 5.5 Summary of the effect mMsx 2 expression in 6d dedifferentiated PRE has on cell morphology. 6d chick PRE was dedifferentiated as described in the main text. Approximately 24 hrs after the final passage, the cells were transiently transfected with either the mMsx 2 expression construct (pCiM2IβGeoSV40) or the control construct (pCilβGeoSV40) and grown for a further 48 hrs. The cultures were then fixed and stained for βGal activity. The percentage of transfected and untransfected cells, in each culture, with a neural morphology was determined (refer to graph 5.3).

Dedifferentiation of 4d PRE was also achieved by culturing at low density in EMEMF for 4 days, then passaged, followed by a further 2 passages with 3 days between each passage (chapter 2.10.2). 24 hrs after the final passage a proportion of these cells (termed p3) were transiently transfected with either the mMsx 2 expression construct (pCiM2IβGeoSV40) or the control construct (pCilβGeoSV40) and the remaining cells (termed p4) grown for a further 3 days, passaged and then transiently transfected with the two constructs (chapter 2.10.3). All cultures were fixed and stained for βGalactosidase activity 48 hrs after transfection (chapter 2.10.4). The percentage of transfected cells with a neural morphology in each culture was determined (graph 5.3 and table 5.6).

<table>
<thead>
<tr>
<th>Construct transfected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>pCiM2IβGeoSV40</td>
</tr>
<tr>
<td>pCilβGeoSV40</td>
</tr>
<tr>
<td>Number of transfected cells</td>
</tr>
<tr>
<td>Number with neural morphology</td>
</tr>
<tr>
<td>% of transfected cells with neural</td>
</tr>
<tr>
<td>morphology</td>
</tr>
<tr>
<td>Number of untransfected cells</td>
</tr>
<tr>
<td>Number with neural morphology</td>
</tr>
<tr>
<td>% of untransfected cells with neural</td>
</tr>
<tr>
<td>morphology</td>
</tr>
</tbody>
</table>
Table 5.6 Summary of the effect \textit{mMsx 2} expression in 4d dedifferentiated PRE has on cell morphology. 4d chick PRE was dedifferentiated as described in the main text; either being passaged 3 times (p3) or 4 times (p4). After the final passage the cells were transiently transfected with either the \textit{mMsx 2} expression construct (pCiM2IβGeoSV40) or the control construct (pCilβGeoSV40) and grown for a further 48 hours. They were then fixed and stained for βGal activity. The percentage of transfected cells, in each culture, with a neural morphology was determined (refer to graph 5.3).

3.7 % of \textit{mMsx 2} transfected 4d (p4) PRE cells acquired a neural morphology, clearly demonstrating that dedifferentiated PRE cells maintain the ability to respond to \textit{mMsx 2} expression over a prolonged period, 14 days. 3.91 % of \textit{mMsx 2} transfected 6d dedifferentiated PRE gained a neural morphology. This is similar to the percentage of 4d dedifferentiated PRE cells that gained a neural morphology, 4.2 % and 3.7 % after 3 and 4 passages, respectively. Therefore, the effect that the age of the starting material had on the number of PRE cells capable of responding to \textit{mMsx 2} expression appears to be less critical if the cells are dedifferentiated.

A small number of cells with a neural morphology were identified in dedifferentiated PRE cultures transfected with the control construct; 0.13% of 6d dedifferentiated PRE cells and 0.6 % of 4d (p3) dedifferentiated PRE cells. Likewise, 0.22 % of untransfected 6d dedifferentiated PRE cells in the culture transfected by the \textit{mMsx}
2 expression construct also had a neural morphology. These observations imply that dedifferentiated PRE can spontaneously acquire a neural phenotype, though whether or not this mechanism is dependant on endogenous Msx 2 remains to be determined.

Graph 5.3 Effect of mMsx 2 expression in dedifferentiated PRE cells. A) 6d chick PRE was dedifferentiated as described in the main text. Approximately 24 hrs after the final passage the cells were transiently transfected with either the mMsx 2 expression construct.
(pCiM2IβGeoSV40) or the control construct (pCiβGeoSV40) and grown for a further 48 hrs. They were then fixed and stained for βGal activity. The percentage of transfected and untransfected cells with a neural morphology was determined in each culture (table 5.5). B) 4d chick PRE was dedifferentiated as described in the main text; either being passaged 3 times (p3) or 4 times (p4). After the final passage the cells were transiently transfected with either the mMsx 2 expression construct (pCiM2IβGeoSV40) or the control construct (pCiβGeoSV40) and grown for a further 48 hours. They were then fixed and stained for βGal activity. The percentage of transfected cells with a neural morphology was determined in each culture (table 5.6).

5.6 Time course analysis of neural phenotype following mMsx 2 expression

To investigate the appearance of neural-like cells in PRE cultures following mMsx 2 expression and to determine for how long these cells persist in the culture, 5d PRE was dissociated, plated out into several tissue culture plates and grown in EMEMF (chapter 2.10.2). Approximately 24 hrs later, these cultures were transiently transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40; chapter 2.10.3). At regular time intervals, 24 hrs, 48 hrs, 72 hrs and 96 hrs after transfection, a proportion of these transfected cells were fixed and stained for βGal activity (chapter 2.10.4). The percentage of transfected cells with a neural morphology was then determined (graph 5.4 and table 5.7).

<table>
<thead>
<tr>
<th>hrs after transfection</th>
<th>Number of transfected cells</th>
<th>Number with neural morphology</th>
<th>% of transfected cells with neural morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>460</td>
<td>1</td>
<td>0.22</td>
</tr>
<tr>
<td>48</td>
<td>645</td>
<td>16</td>
<td>2.48</td>
</tr>
<tr>
<td>72</td>
<td>570</td>
<td>6</td>
<td>1.05</td>
</tr>
<tr>
<td>96</td>
<td>403</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.7 Percentage of PRE cells transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40) with a neural morphology at regular time intervals after transfection. 5d PRE was dissociated and grown for approximately 24 hrs before being transiently transfected with pCiM2IβGeoSV40. Cells were fixed and stained for βGal activity at regular time intervals from the point of transfection. The percentage of transfected cells with a neural morphology was determined.

Graph 5.4 reveals that the maximum percentage of transfected cells with a neural morphology was observed 48 hours after transient transfection. This probably reflects
a period of maximal expression of the construct. In contrast, no neural-like cells were present 96 hrs after transient transfection, by which time expression of the construct may only be minimal. This suggests that the survival of these cells is dependant upon maintained $\text{mMsx}_2$ expression. Alternatively, the loss of these cells over time may not be directly due to the absence of $\text{mMsx}_2$, but rather the absence of specific factors required by neural cells in culture for their survival. In support of this idea, it has been possible to prolong the length of time, up to at least 6 days, for which $\text{mMsx}_2$ induced neural-like cells can be maintained in culture, by growing the cells on laminin in NB27 media designed to support neural cell growth (Neurobasal medium supplemented with B27 (Brewer et al., 1993) 0.3mg/ml glutamine, 70µg/ml penicillin and 0.13 mg/ml streptomycin; chapter 2.10.2), refer to fig. 6.5, fig. 6.7 and fig. 6.9.

Graph 5.4 Percentage of PRE cells transfected with the $\text{mMsx}_2$ expression construct (pCiM21βGeoSV40) with a neural morphology at regular time intervals after transfection. 5d PRE was dissociated and grown for approximately 24 hrs before being transiently transfected with pCiM21βGeoSV40. Cells were fixed and stained for βGal activity at regular time intervals from the point of transfection. The percentage of transfected cells with a neural morphology was determined (table 5.7).
The proposed function of Msx 2 during the differentiation of the optic vesicle into the neural retina was investigated by ectopically expressing mouse Msx 2 in cultured chick PRE cells. Forced Msx 2 expression in PRE cells, that in vivo would not normally express this gene, resulted in a small proportion of them acquiring a neural morphology, 0.98% of transfected 6d PRE cells.

This effect can be directly attributed to mMsx 2 expression since no neural-like cells were observed in equivalent cultures transfected with the control construct (pCIIβGeoSV40) which lacks the mMsx 2 insert. Restriction digest and sequencing analysis of the mMsx 2 insert in the expression vector confirmed that Msx 2 should be translated correctly. Thus, the effect observed is unlikely to be the consequence of aberrant Msx 2 function, due to a mutated protein. However, amino acid residue 263 of the Msx 2 protein produced from the mMsx 2 expression construct is predicted to be M, rather than the published I, and residue 266 is predicted to be a L, rather than the published V. The equivalent residues of Msx 2 in human, chick, quail and Xenopus are M and L, while the equivalent residues of human, mouse and Xenopus Msx 1 are also M and L. It is therefore probable that the published mouse Msx 2 sequence is incorrect. Even if this is not the case, the fact that these residues are present in the Msx 2 protein from other species, including chick, in a region that is highly conserved in mouse and chick suggests that they are unlikely to have an adverse effect on Msx 2 function.

It is possible that the effect of mMsx 2 expression on chick PRE morphology does not reflect the effect chick Msx 2 expression might have on chick PRE, or mouse Msx 2 expression might have on mouse PRE, but is an artefact due to the cross-species nature of this study. This question is addressed further in chapters 7 and 8. It is also possible that the effects of mMsx 2 expression are a consequence of abnormally high levels of mMsx 2 expression. This could be explored by expressing mMsx 2 from more physiologically relevant promoters, refer to chapter 8.2.4 and 8.3.4.
Only a very small proportion, 0.98%, of 6d mMsx 2 transfected PRE cells responded to mMsx 2 expression by acquiring a neural morphology. A larger proportion of these cells, 11.09%, exhibited abnormally long processes, implying that mMsx 2 expression is affecting more than 0.98% of the cells. Cells with abnormally long processes may represent immature neural-like cells. Nonetheless, it still necessary to account for approximately 90% of the transfected cells that appear normal. One possible explanation is that PRE cells do not respond to mMsx 2 expression by acquiring a neural morphology but that perioptic mesenchyme, which is likely to contaminate PRE cultures, does. However, this possibility is not supported by the fact that perioptic mesenchyme does not acquire a neural morphology when transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40).

Alternatively, a large amount of Msx 2 protein may be required before PRE cells can respond. It is likely that different cells will contain varying amounts of construct, so producing different amounts of Msx 2. This idea is supported by the observation that mMsx 2 transfected PRE cells that do acquire a neural morphology are intensely blue, indicating that they contain an abundant amount of βGalactosidase and hence also Msx 2. It is difficult, however, to correlate the amount of βGalactosidase activity with neural morphology since the cell volume of neural-like cells appears reduced, thus concentrating the βGalactosidase product. Furthermore, equally blue non-neural PRE cells can be identified, suggesting that high levels of Msx 2 may not, in itself, be sufficient to cause PRE cells to acquire a neural morphology.

Thus, it appears that properties of the PRE cells themselves govern whether or not they respond to mMsx 2 expression by acquiring a neural phenotype. Therefore, only a subpopulation of PRE cells, perhaps precursor cells or cells less committed to the PRE lineage, may be capable of responding to mMsx 2 expression. Indeed, approximately 1.5% of mMsx 2 transfected 4d and 5d chick PRE acquired a neural phenotype, a 1.5-fold increase from transfected 6d PRE. It is conceivable that younger PRE is either less committed to the PRE lineage or contains more precursor
cell types, so increasing the proportion of cells capable of responding to $mMsx\ 2$ expression.

PRE cells around the periphery of the expanding monolayer spontaneously dedifferentiate due to insufficient cell-cell contacts as described in chapter 4. It is possible that only this subpopulation of cells can respond to $mMsx\ 2$ expression. Indeed, transfection of either 4d or 6d dedifferentiated PRE does show an increase in the proportion of $mMsx\ 2$ transfected PRE cells that acquire a neural phenotype, an approximate 4-fold increase compared to transfected differentiated 6d PRE. However, there is not a sufficient increase in the proportion of transfected cells adopting a neural morphology to suggest that the cells ability to respond to $mMsx\ 2$ expression is solely dependant on their state of dedifferentiation. It is difficult to pinpoint why only a small proportion of presumed $mMsx\ 2$ expressing PRE cells acquire a neural morphology and this may be dependant upon several unknown factors.

It is perhaps not surprising that a small proportion (0.2%) of control transfected dedifferentiated PRE had a neural morphology since the expression of $FGF\ 2$, a molecule known to induce neural transdifferentiation, is known to be up-regulated in dedifferentiated cultures (chapter 1.6). Whether or not endogenous $cMsx\ 2$ is also up-regulated remains to be shown. However, there is no report of $FGF\ 2$ inducing transdifferentiation of dissociated PRE cells into neural retina which possibly casts doubts on this explanation. However, the observation that dedifferentiated PRE, which may endogenously express $FGF\ 2$, can spontaneously give rise to cells with neural morphology suggests that $FGF\ 2$ functions within the cell, or on the cell surface, and that dissociated PRE cells are unable to respond to exogenous $FGF\ 2$, perhaps due to the absence of specific cell surface proteins or interactions with neighbouring cells.

Although, mMsx 2-induced cells appeared morphologically neural and that their survival time could be increased by growing in media that supports neuronal cells,
more evidence is required before it can be concluded that these cells are neural. This issue is addressed in the next chapter.
Chapter 6

Molecular Characterisation of Neural-like Cells Induced From PRE by \textit{mMxs} 2 Expression

6.1 Introduction

To confirm that the cells with neural-like morphology which arise in chick PRE cultures following ectopic \textit{mMxs} 2 expression are neural, the expression of a range of neural cell-type specific markers was investigated. Given that \textit{Msx} 2 is normally expressed in cells of the mouse optic vesicle which are presumed to give rise to the neural retina (chapter 3), it is reasonable to speculate that the \textit{mMxs} 2-induced neural-like cells may have some of the characteristics of neural retina cells. Furthermore, \textit{in vitro}, PRE is capable of differentiating into neural retina cell types when treated with FGF 2. These neuronal cell types include ganglion, amacrine and photoreceptor cells (chapter 1.6). It was therefore decided to explore the possibility that \textit{mMxs} 2-induced neural-like cells have a neural retina cell type identity.

Ganglion cells are the first neural retina cell type to differentiate, withdrawing from the cell cycle between the second and third day of chick development (Kahn, 1973). Consequently, \textit{mMxs} 2-induced neural-like cells were first screened for markers of this cell type. Fluorescent immunocytochemistry was used to detect \textbeta Gal, present in the cytoplasm, and to test for the presence of the desired marker antigen in PRE cells transiently transfected with the \textit{mMxs} 2 expression construct (pCiM2I\textbeta GeoSV40). This enabled a precise correlation between the cell morphology and presence of marker antigen to be made in transfected cells.
6.2.1 Neuron-specific class III \( \beta \)Tubulin

Tubulin heterodimers are composed of approximately equal-sized subunits, termed \( \alpha \) and \( \beta \), and are a key component of microtubules. There are many isotypes of both subunits, either encoded by different genes or subject to different post-translational modifications (Lee et al., 1990). Class III \( \beta \)Tubulin can be detected by the monoclonal antibody TuJ1. Localisation of this protein in chick embryos using TuJ1, reveals that it is predominantly present in neurons and as such has been used by many investigators as a neuron-specific marker (Lee et al., 1990). Migrating neural crest and glial cells do not express this antigen (Trimmer et al., 1986; Lee et al., 1990). Within the eye of stage 22 chicks, TuJ1 immunoreactivity is associated with the ganglion cells of the retina and the lens vesicle (Lee et al., 1990). This marker is also induced in PRE which develops from optic vesicles cultured in the presence of FGF 2 (Pittack et al., 1997).

6.2.2 TuJ1 immunoreactivity in dedifferentiated PRE transfected with either the \( mMx \)2 expression construct (pCiM2I\( \beta \)GeoSV40) or the control construct (pCiI\( \beta \)GeoSV40)

To determine if the neural-like cells induced by ectopic expression of \( mMx \)2 in dedifferentiated PRE exhibit TuJ1 immunoreactivity, 5 day chick PRE was dissociated and cultured in EMEMF for 14 days during which time the cells were passaged 3 times (chapter 2.10.2). After the final passage, cells were plated out in 8-well, glass, chamber slides, grown overnight and transiently transfected with either the \( mMx \)2 expression construct (pCiM2I\( \beta \)GeoSV40) or the control construct (pCiI\( \beta \)GeoSV40; chapter 2.10.3). 48 hrs after transfection the cultures were fixed and fluorescent immunocytochemistry performed simultaneously for \( \beta \)Gal and class III \( \beta \)Tubulin (rabbit anti-\( \beta \)Gal, 1:200 dilution, and mouse anti-TuJ1, 1:4000 or 1:10000 dilution; as described in chapter 2.10.6 (fig. 6.1). For control purposes, neural retina from 6 and 7 day chick eyes were dissociated and grown overnight in EMEMF (chapter 2.10.2) before being processed for TuJ1 immunocytochemistry (fig. 6.1).
Fig. 6.1 (Shown opposite) TuJ1 and βGal immunoreactivity in dedifferentiated PRE transfected with the *mMsx* 2 expression construct. A-F) 5d chick PRE was dedifferentiated as described in the main text before being transiently transfected with the *mMsx* 2 expression construct (pCiM2IβGeoSV40). 48 hrs later the cells were stained for both βGal (rabbit anti-βGal, 1:200) and TuJ1 (mouse anti-TuJ1, (A and B) 1:4000, (C-F) 1:10000). For control purposes, dissociated neural retina (nr) from 6d (G) and 7d (H) chicks was cultured overnight and stained in parallel with the PRE cells for TuJ1 (mouse anti-TuJ1, (G) 1:4000, (H) 1:10000). Mouse anti-TuJ1 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse (1:200) and fluorescein (FITC) conjugated donkey anti-rabbit (1:200). A, C and E) Dapi and βGal. B, D and F) Dapi and TuJ1. A/B and C/D) βGal-expressing dedifferentiated PRE cells with a neural morphology and immunoreactive against TuJ1. E and F) βGal-expressing dedifferentiated PRE cell with no neural morphology and not labelled by TuJ1 (arrowheads). Arrows also indicate a non-βGal expressing cell that is labelled by TuJ1. A-H) x63 magnification.
As expected TuJ1 immunoreactivity was localised to the cytoplasm and axons of cultured 6 and 7 day neural retina at both the dilutions tested (1:4000 and 1:10000). A proportion of the neural retina cells were not labelled by TuJ1 which is consistent with TuJ1 being ganglion specific (fig. 6.1). Cells with a neural morphology were identified in dedifferentiated PRE cultures transfected with the mMsx 2 expression construct but not in cultures transfected with the control construct. In the majority of these cells, TuJ1 immunoreactivity was localised to the processes and cell bodies (fig. 6.1). Not all the neural-like cells were labelled by TuJ1, this observation is investigated in chapter 6.2.3.

A small number of βGal-negative cells in mMsx 2 transfected cultures were labelled by TuJ1, 8 out of 495 (fig. 6.1). A similar proportion of βGal-positive, non-neural cells were also labelled by TuJ1, though a precise count has not been performed. TuJ1-positive cells were also observed in control transfected cultures (27 out of 880) the proportion of which were βGal-positive was not recorded. These observations suggest that either a subpopulation of PRE cells are TuJ1-positive or that a cell type expressing this antigen is contaminating the cultures. These possibilities are addressed in more detail in the next section.

The presence of cells that were only labelled by anti-βGal or TuJ1 confirmed that there was no cross reactivity between rabbit anti-βGal and Texas-red conjugated donkey anti-mouse secondary antibody, or between mouse anti-TuJ1 and fluorescein (FITC) conjugated donkey anti-rabbit secondary antibody (fig. 6.1).

### 6.2.3 TuJ1 immunoreactivity in primary 5 day PRE and perioptic mesenchyme transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40)

It is possible that a small amount of perioptic mesenchyme may have contaminated the PRE cultures. To determine if this cell type is immunoreactive against TuJ1 and hence responsible for the small proportion of TuJ1-positive cells observed in control
transfected cultures, the following experiment was performed. PRE and perioptic mesenchyme from the same 5 day chick eyes were dissociated and cultured separately in EMEMF overnight in 8-well, glass, chamber slides (chapter 2.10.2). These cells were then transiently transfected with the \textit{mMsx} 2 expression construct (pCiM2IβGeoSV40; chapter 2.10.3), grown for a further 48 hrs and then processed for immunocytochemistry for βGal and TuJ1 (rabbit anti-βGal, 1:200, and mouse anti-TuJ1, 1:10000; chapter 2.10.6; fig. 6.2). For control purposes, dissociated neural retina from 7 day chick eyes was grown for 24hrs and stained for TuJ1 in parallel with the transfected PRE cells (fig. 6.2).

As previously observed, a small proportion of untransfected primary PRE cells were again stained by TuJ1 (fig. 6.2). Cultures of stage-matched perioptic mesenchyme, transfected with the \textit{mMsx} 2 expression construct, also contained TuJ1-positive cells (fig. 6.2). However, it is unlikely that mesenchymal contamination is the primary source of TuJ1-positive cells in PRE cultures since only 0.86% (n=928) of cells in the perioptic mesenchyme cultures were TuJ1-positive. No correlation between βGal-positive and TuJ1-positive mesenchymal cells was casually observed (fig. 6.2), while it has been previously demonstrated that ectopic expression of \textit{mMsx} 2 in perioptic mesenchyme does not induce a neural phenotype (chapter 5.3.2).

In cultures of 5d PRE, transfected with the \textit{mMsx} 2 expression construct, only 8 out of 12 βGal-positive, neural-like cells were labelled by TuJ1. Analysis of the experiment described in this section and in chapter 6.2.2 has revealed a combined total of 24 \textit{mMsx} 2-induced neural-like cells with a distinctive bipolar morphology. These cells had well defined processes, round, symmetric cell-bodies and were TuJ1-positive (fig. 6.2). A second class of neural-like cells has also been observed. These cells had multiple, branching processes and were either positive or negative for TuJ1 (fig. 6.2). Whether or not TuJ1-negative neural-like cells represent a unique population of cells, or are merely immature or dying cells remains to be investigated.
Fig. 6.2 (Shown opposite) TuJ1 and βGal immunoreactivity in primary 5d PRE and perioptic mesenchyme transfected with the mmMsx2 expression construct. PRE (A-F) and perioptic mesenchyme (G and H), isolated from the same 5d chick eyes, were cultured overnight, then transfected with the mmMsx2 expression construct (pCiM2IβGeoSV40). 48 hrs later the cells were stained for both βGal (rabbit anti-βGal, 1:200) and TuJ1 (mouse anti-TuJ1, 1:10000). I) For control purposes, neural retina from a 7d chick was cultured overnight and stained in parallel to the PRE cells for TuJ1 (mouse anti-TuJ1, 1:10000). Mouse anti-TuJ1 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse (1:200) and fluorescein (FITC) conjugated donkey anti-rabbit (1:200). A, C, E and G) Dapi and βGal. B, D, F, H and I) Dapi and TuJ1. A and B) Bipolar mmMsx2-induced neural-like cell labelled by TuJ1. C and D) Branching mmMsx2-induced neural-like cell not labelled by TuJ1. Asterisk in (D) indicates artefactual labelling due to autofluorescent debris, also observed in cultures in which the secondary antibody is omitted. E and F) Yellow arrow indicates a PRE cell in the mmMsx2 transfected culture that did not express βGal. This same cell was labelled by TuJ1. G and H) White arrow indicates a mesenchymal cell that is labelled by TuJ1 but not βGal. A-I) x63 magnification.
6.2.4 Intracellular localisation of TuJ1 in PRE cells transfected with the 
*mMsx 2* expression construct (pCiM2IβGeoSV40)

In cultures of rat brain neurons, the majority of class III βTubulin is not incorporated into microtubules during early axonal outgrowth, only becoming incorporated as these neurons differentiate (Ferreira and Caceres, 1992). It is therefore possible that the TuJ1 immunoreactivity observed in a small proportion of cultured PRE cells is associated with the cytosolic, rather than cytoskeletal class III βTubulin. This protein may be incorporated into microtubules when PRE cells are induced to form a neural morphology by *mMsx 2* expression.

To explore this possibility further, 5 day chick PRE was dissociated, plated out in 8-well, glass, chamber slides and cultured for approximately 24 hrs in EMEMF (chapter 2.10.2). These cells were then transiently transfected with the *mMsx 2* expression construct (pCiM2IβGeoSV40; chapter 2.10.3) and grown for a further 48 hrs. For control purposes, 7 day neural retina was dissociated, cultured for 24 hrs and stained for TuJ1 in parallel with the PRE cells (chapter 2.10.3 and 2.10.6). Prior to fixing, half the cells were treated with 0.2% Triton X-100 under microtubule-stabilising conditions (chapter 2.10.5). This treatment removes unpolymerized cytoskeletal subunits but preserves microtubules (Ferreira and Caceres, 1992). After extraction the cells were fixed and stained for both βGal and TuJ1 (1:200 and 1:4000, respectively; as described in chapter 2.10.6 (fig. 6.3).
Fig. 6.3 (Shown opposite) Intracellular localisation of TuJ1 to the microtubules of PRE and neural retina cells in culture. A, B, D and E) 5d chick PRE was dissociated, cultured overnight and then transiently transfected with the *mMsx* 2 expression construct (pCiM21βGeoSV40). 48 hrs later the cells were extracted (A and B) with Triton X-100 under microtubule-stabilising conditions before staining for TuJ1 (mouse anti-TuJ1, 1:4000) and βGal (rabbit anti-βGal, 1:200). D and E) Cells were not extracted before staining. Extracted cells only contain polymerised cytoskeletal proteins. C and F) For control purposes, neural retina from 7d chick eyes was cultured overnight and stained in parallel to the PRE cells for TuJ1 (mouse anti-TuJ1, 1:4000), (C) extracted, (F) not extracted prior to immunocytochemistry. Mouse anti-TuJ1 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse (1:200) and fluorescein (FITC) conjugated donkey anti-rabbit (1:200). α and D) Dapi and βGal. B, C, E and F) Dapi and TuJ1. A and B) βGal could not be detected in *mMsx* 2 transfected PRE cells confirming that cytosolic proteins had been extracted. Extracted PRE and neural retina (C) cultures contained cells that were immunoreactive against TuJ1. D and E) βGal was detected in non-extracted *mMsx* 2 transfected PRE cells. TuJ1-positive cells were observed in these cultures as well as in the non-extracted neural retina culture (F). A-F) x63 magnification.
As can be seen in fig. 6.3, βGal could not be detected in extracted cells confirming that the extraction procedure had been successful. TuJ1 staining of both PRE and control 7 day neural retina cultures was consistent with previous observations, irrespective of whether the cells were detergent extracted prior to immunocytochemistry (fig. 6.3). Thus, class III βtubulin is incorporated into the microtubules of cultured neural retina, PRE cells and presumably mMsx 2-induced neural-like PRE cells, though it was not possible to identify these cells since the βGal protein had been removed upon detergent extraction. Neural-like cells could be located in future experiments under phase contrast.

6.3.1 Microtubule associated protein 2 (Map 2)

Map 2 interacts with elements of the cytoskeleton and promotes microtubule assembly (Huber and Matus, 1984; Tucker et al., 1988). Map 2 is predominantly localised to the dendrites and cell bodies of neurons rather than the axons (Vallee, 1982; Frankfurter et al., 1983; Caceres et al., 1984). Glial cells of the brain are not labelled (Izant and McIntosh, 1980). Three forms of Map 2, termed a, b and c, have been identified which differ in size and temporal expression. Map 2b and Map 2c, but not Map 2a, are present in immature rat brain neurons. As these neurons mature the levels of Map 2c decrease with the appearance of Map 2a (Izant and McIntosh, 1980).

Mouse monoclonal anti-Map 2 (clone HIM 2) reacts against all three forms of Map 2, including chicken Map 2 (Tucker et al., 1988). Within the developing chick retina, this antibody specifically labels cells located in the ganglion cell layer, so is presumed to be a ganglion cell marker (chapter 6.7).

6.3.2 Map 2 immunoreactivity in PRE 48-72 hours after transfection with the mMsx 2 expression construct (pCiM2IβGeoSV40)

To determine if cells that gain a neural morphology following mMsx 2 expression express the neuronal marker Map 2, PRE from 5d chick eyes were dissociated and
cultured for approximately 24 hrs in EMEMF (chapter 2.10.2) before being transiently transfected with the \textit{mMsx} 2 expression construct (pCiM2I\(\beta\)GeoSV40; chapter 2.10.3). 72 hrs after transfection, the cultures were fixed and stained for both \(\beta\)Gal and Map 2 (rabbit anti-\(\beta\)Gal, 1:200 and mouse anti-Map 2, 1:200; as described in chapter 2.10.6 (fig. 6.4). As a positive control, 7 day chick neural retina was also dissociated, cultured for 24 hrs and stained in parallel for Map 2 (section 2.10.2 and 2.10.6; fig. 6.4). Anti-Map 2 intensely labelled a proportion of the cultured neural retina cells. Immunoreactivity was localised to the processes and a semi-circular domain adjacent to the nucleus of these cells (fig. 6.4). The presence of Map 2-negative cells in the neural retina culture confirms the specificity of this antibody and is consistent with the observation that only cells within the ganglion cell layer are immunoreactive against anti-Map 2 (chapter 6.7).

The intensity of Map 2 staining in \textit{mMsx} 2 transfected and untransfected cells was not significantly different from that of background staining. This intensity of staining was deemed to be negative (fig. 6.4). The intensity of Map 2 labelling in the neural-like cell shown in fig. 6.4(B) is slightly higher than in the untransfected neighbouring cell. The intensity of Map 2 labelling in the neural-like cell, after subtracting background label, is approximately 17-fold less than the intensity of labelling associated with the neural retina. Thus, this neural-like cell was judged to be negative for Map 2.

Analysis of Map 2 expression in PRE cells transiently transfected with the \textit{mMsx} 2 expression construct has been repeated a further two times. Both dedifferentiated and 5 day primary cultures were used and Map 2 expression assayed 48 hrs after transfection. These experiments were consistent with transfected and untransfected PRE cells not expressing Map 2. A minimum of 10 \textit{mMsx} 2-induced neural-like cells were observed that did not label with Map 2. No neural-like cells have been observed that label with Map 2.
Fig. 6.4 Map 2 and βGal immunoreactivity in PRE cultures 72 hrs after transfection with the mMsx 2 expression construct. A-D) PRE from 5d chick eyes was dissociated, cultured overnight and transiently transfected with the mMsx 2 expression construct (pCiM2βGeoSV40). 72 hrs later the cultures were stained for both βGal (rabbit anti-βGal, 1:200) and Map 2 (mouse anti-Map 2, 1:200). E) For control purposes, neural retina (nr) from 7d chicks was cultured overnight then stained in parallel to the PRE cells for Map 2 (mouse anti-Map 2, 1:200). Mouse anti-Map 2 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse and fluorescein (FITC) conjugated donkey anti-rabbit. A and C) Dapi and βGal. B, D and E) Dapi and Map 2. For all the panels, the Texas-red image was captured using the same exposure and camera settings. Therefore, the Texas-red pixel intensity value (figures in yellow) associated with a particular cell can be compared to another cell or area of the slide. E) Cells in the neural retina culture were strongly labelled by Map 2. A and B) The intensity of Map 2 label associated with the branched mMsx 2-induced neural-like cell was not significantly different from background labelling. C and D) This was also the case for βGal-positive, non-neural PRE cells and βGal-negative PRE cells. A-E) x63 magnification.
In common with TuJ 1, Map 2 may only label a proportion of the \( mMsx 2 \)-induced neural-like cells, perhaps only bipolar types. In the study described above, the morphology of the neural cells was not recorded. Consequently it is not possible to confidently conclude that bipolar cells with symmetric cell bodies are negative for Map 2. Nonetheless, at least two of the \( mMsx 2 \)-induced neural cells had a bipolar morphology and were negative for Map 2 (data not shown).

6.3.3 Map 2 immunoreactivity in PRE 6 days after transfection with the \( mMsx 2 \) expression construct (pCiM2I\( \beta \)GeoSV40)

Although a proportion of \( mMsx 2 \)-expressing PRE cells have a prominent neural morphology 72 hours after transfection, it is conceivable that these cells are not sufficiently differentiated to express Map 2 at levels that can be detected by immunocytochemistry. It was therefore decided to assay for Map 2 expression 6 days after transient transfection with the \( mMsx 2 \) expression construct (pCiM2I\( \beta \)GeoSV40).

PRE from 5 day chick embryos was dissociated and cultured for 1 day (chapter 2.10.2) before being transiently transfected with the \( mMsx 2 \) expression construct (chapter 2.10.3). To maximise the length of time the \( mMsx 2 \)-induced cells could be maintained in culture, cells were cultured in laminin-coated wells and in NB27 medium following transfection. 6 days after transfection the cells were fixed and stained for \( \beta \)Gal and Map 2 (rabbit anti-\( \beta \)Gal, 1:200 and mouse anti-Map 2, 1:200; as described in chapter 2.10.6 (fig. 6.5). As a positive control, 6 day chick neural retina was dissociated, cultured for 1 day on laminin in NB27 (chapter 2.10.2) and stained in parallel with the PRE cells for Map 2 (chapter 2.10.6).
Fig. 6.5 Map 2 and βGal immunoreactivity in PRE cultures 6 days after transfection with the mMsx 2 expression construct. A-D) PRE from 5d chick eyes was dissociated, cultured overnight and transiently transfected with the mMsx 2 expression construct (pCiM2βGeoSV40). The cells were grown on laminin in NB27 medium. 6 days after transfection the cultures were stained for both βGal (rabbit anti-βGal, 1:200) and Map 2 (mouse anti-Map 2, 1:200). E) For control purposes, neural retina (nr) from 6d chicks was cultured overnight then stained in parallel to the PRE cells for Map 2 (mouse anti-Map 2, 1:200). Mouse anti-Map 2 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse and fluorescein (FITC) conjugated donkey anti-rabbit, respectively. A and C) Dapi and βGal. B, D and E) Dapi and Map 2. For all the panels, the Texas-red image was captured using the same exposure and camera settings. Therefore, the Texas-red pixel intensity value (figures in yellow) associated with a particular cell can be compared to another cell or area of the slide. The pixel intensity values shown in (B) and (E) have been adjusted by subtracting the pixel intensity value associated with an area of the slide containing no cells. E) Cells in the neural retina culture were strongly labelled by Map 2. A and B) The intensity of Map 2 label associated with the branched mMsx 2-induced neural-like cell was not significantly different from the labelling associated with βGal-positive, non-neural PRE cells or βGal-negative PRE cells (C and D). Note that pixel intensity values are not shown for (D), since it is not possible to show the intensity of label associated with an area containing no cells. Background labelling can vary from one part of the slide to another. The labelling in (B) indicated by an asterisk lies outwith the neural-like cell and is considered an artifact since this structure also fluoresces at the dapi wavelength. A-E) x 63 magnification.
Anti-Map 2 labelled the 6 day neural retina as described for the 7 day neural retina (chapter 6.3.2; fig. 6.5). The intensity of Map 2 staining in \textit{mMsx} 2 transfected and untransfected cells was not significantly different from that of background staining. Equally, the intensity of Map 2 labelling associated with \textit{mMsx} 2-induced neural-like cells was not significantly greater than background labelling and was approximately 17-fold weaker than the intensity of stain associated with the neural retina (fig. 6.5). This degree of labelling was considered insignificant.

This experiment was repeated under the same conditions and on both occasions no \textit{mMcx} 2-induced neural-like cells were deemed to be Map 2-positive. However, on both occasions relatively few cells (~3) with a neural morphology were observed perhaps due to the prolonged period of time in culture. Given the small number of neural-like cells that were analysed for Map 2 immunoreactivity, it was not possible to reach a conclusion as to their state of Map 2 expression. Equally, it was not possible to determine whether or not \textit{mMsx} 2-induced neural-like cells with a bipolar morphology, which express TuJ1, were labelled with anti-Map 2.

### 6.4.1 Growth associated protein (GAP 43)

Gap 43 is a neuronal phosphoprotein which is associated with both axonal outgrowth and synaptic turnover (Levine \textit{et al.}, 1981; Benowitz \textit{et al.}, 1988). In cultured hippocampal neurons, the protein is predominately localised to axonal growth cones rather than dendritic cones (Goslin \textit{et al.}, 1988). During mouse retina development, Gap 43 mRNA is detected in ganglion cells (Capone \textit{et al.}, 1991). Mouse monoclonal anti-Gap 43 (clone GAP 7B10) specifically labels ganglion cells within the developing chick retina and has been used as marker of this cell type (Austin \textit{et al.}, 1995). This antibody recognises both the phosphorylated and dephosphorylated forms of Gap 43.
6.4.2 Gap 43 immunoreactivity in PRE 72 hours after transfection with the mMsx 2 expression construct (pCiM2IβGeoSV40)

To determine if mMsx 2-induced neural-like cells were immunoreactive against anti-Gap 43, PRE from 5 day chick embryos was dissociated and cultured for approximately one day in EMEMF (chapter 2.10.2) before being transiently transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40; chapter 2.10.3). 72 hours after transfection the cultures were fixed and stained for βGal and Gap 43 (rabbit anti-βGal, 1:200 and mouse anti-Gap 43, 1:1000; as described in chapter 2.10.6 (fig. 6.6). Neural retina from 6 day chick embryos was also dissociated, cultured overnight (chapter 2.10.2), stained for Gap 43 (chapter 2.10.6) and served as a positive control (fig. 6.6).

As can be observed in fig. 6.6 (E), a proportion of cells in the neural retina culture were intensely labelled by Gap 43. This is consistent with the antibody reacting only with ganglion cells and confirms its specificity. In those cells that were labelled, the protein was predominately localised to the periphery of the cell bodies.

PRE cells, irrespective of whether or not they were expressing βGal, showed only a very slight increase in the intensity of Gap 43 labelling compared to background levels. This is probably due to non-specific binding of the antibody. The labelling in these cells, after subtracting background label, was approximately 20-fold less than the intensity of label associated with the neural retina. Therefore, PRE cells were considered to be negative for Gap 43 (fig. 6.6).

In contrast, the intensity of Gap 43 labelling of the mMsx 2-induced neural-like cell shown in fig. 6.6 (B) was approximately 2 times greater than that of a neighbouring untransfected cell and 10 times less than that of cells in the neural retina culture, after subtracting the intensity of label associated with an area of the slide containing no cells. Gap 43 was localised uniformly across the cell body of the mMsx 2-induced neural cell, with weaker staining in the processes. Thus, this particular cell may
contain Gap 43, albeit at very low levels. The labelling observed in this instance is not due to cross reactivity between rabbit anti-βGal and Texas-red conjugated donkey anti-mouse, since βGal-expressing PRE exhibited equivalent intensities of Texas-red fluorescence to non-βGal expressing cells.

The above experiment was repeated under the same conditions a further 2 times. The combined observations revealed that all 17 mMsx 2-induced neural-like cells recorded were judged to be negative for Gap 43, with the exception of the cell shown in fig. 6.6. The weakly Gap 43-positive neural-like cell shown in fig. 6.6 has a bipolar morphology with a round symmetrical cell body. This type of cell was always labelled by TuJ1. It is therefore possible that the majority of the neural-like cells deemed to be negative for Gap 43 were not bipolar. Unfortunately, the morphology of these cells was not recorded since the significance of this morphological distinction was not appreciated when these observations were made, although it is known from recorded images that at least 2 of the 17 cells had a bipolar morphology and were judged to be Gap 43-negative. A more thorough analysis of Gap 43 expression in relation to neural cell morphology will need to be performed in the future. The conflicting observations, suggest that Gap 43 expression in these cells is near the limit of detection by fluorescent immunocytochemistry. Consequently, it is not possible from the present study to categorically conclude that mMsx 2-induced neural-like cells are either positive or negative for Gap 43.
Fig. 6.6 Gap 43 and βGal immunoreactivity in PRE cultures 72 hrs after transfection with the mMsx 2 expression construct. A-D) PRE from 5d chick eyes was dissociated, cultured overnight and transiently transfected with the mMsx 2 expression construct (pCiM2βGeoSV40). 72 hrs later the cultures were stained for both βGal (rabbit anti-βGal, 1:200) and Gap 43 (mouse anti-Gap 43, 1:1000). E) For control purposes, neural retina (nr) from 7d chicks was cultured overnight then stained in parallel to the PRE cells for Gap 43 (mouse anti-Gap 43, 1:1000). Mouse anti-Gap 43 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse and fluorescein (FITC) conjugated donkey anti-rabbit, respectively. A and C) Dapi and βGal. B, D and E) Dapi and Gap 43. For all the panels, the Texas-red image was captured using the same exposure and camera settings. Therefore, the Texas-red pixel intensity value (figures in yellow) associated with a particular cell can be compared to another cell or area of the slide. A and B) The intensity of Gap 43 label associated with the bipolar mMsx 2-induced neural-like cell is approximately 2 times that of the neighbouring βGal-negative cell and 10 times less than a labelled neural retina cell (E), after subtracting the intensity associated with an area of the slide containing no cells. A-E) x 63 magnification.
6.4.3 Gap 43 immunoreactivity in PRE 6 days after transfection with the \textit{mMsx} 2 expression construct (pCiM2IβGeoSV40)

Prolonged growth of PRE cells, following transfection with the \textit{mMsx} 2 expression construct (pCiM2IβGeoSV40) may allow the \textit{mMsx} 2-induced neural-like cells to reach a greater state of maturity. This may enable them to express the ganglion specific marker Gap 43. To test this idea, dedifferentiated 5 day PRE cells were plated out on laminin-coated 8-well, glass, chamber slides (chapter 2.10.2) and transiently transfected, the following day, with the \textit{mMsx} 2 expression construct (chapter 2.10.3). After transfection, the medium was changed from EMEMF to NB27, the latter supports the growth of neural cells. 6 days after transfection, the cultures were fixed and stained for βGal and Gap 43 (rabbit anti-βGal, 1:200 and mouse anit-Gap 43, 1:1000; as described in chapter 2.10.6 (fig. 6.7). For control purposes, neural retina from 5 day chick eyes was dissociated, cultured overnight on laminin in NB27 medium (chapter 2.10.2) and stained for Gap 43 in parallel with the PRE cells (chapter 2.10.6; fig. 6.7).

As can be observed in fig. 6.7 (E), the 5 day neural retina culture was labelled for Gap 43 in a similar manner to the 7 day neural retina culture described in chapter 6.4.2. Dedifferentiated PRE, irrespective of whether or not it expressed βGal, had a similar level of non-specific Gap 43 labelling which was considered insignificant in comparison with the intense label associated with the neural retina culture.
Fig. 6.7 Gap 43 and βGal immunoreactivity in PRE cultures 6 days after transfection with the mMsx 2 expression construct. A-D) Dedifferentiated PRE cells were transiently transfected with the mMsx 2 expression construct (pCiM21βGeoSV40) and cultured on laminin in NB27 medium. After 6 days the cells were stained for both βGal (rabbit anti-βGal, 1:200) and Gap 43 (mouse anti-Gap 43, 1:1000). E) For control purposes, neural retina (nr) from 5d chicks was cultured overnight then stained in parallel to the PRE cells for Gap 43 (mouse anti-Gap 43, 1:1000). Mouse anti-Gap 43 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse and fluorescein (FITC) conjugated donkey anti-rabbit, respectively. A and C) Dapi and βGal. B, D and E) Dapi and Gap 43. For all the panels, the Texas-red image was captured using the same exposure and camera settings. Therefore, the Texas-red pixel intensity value (figures in yellow) associated with a particular cell can be compared to another cell or area of the slide. A and B) The intensity of Gap 43 label in the cell body of the mMsx 2-induced neural-like cell is slightly higher than βGal-negative neighbouring cells and approximately 14 times weaker than labelled neural retina cells (E), after subtracting the intensity associated with an area of the slide containing no cells. A-E) x 63 magnification.
In common with the \textit{mMsx} 2-induced neural-like cell 72 hours after transfection (fig. 6.6), the neural-like cell shown in fig. 6.7 also had very weak Gap 43-labelling of the cell body. The intensity of this labelling, after subtracting background label, was approximately 14 times weaker than that associated with cells in the neural retina culture, but slightly stronger than surrounding untransfected PRE cells. A total of three \textit{mMsx} 2-induced neural-like cells were identified in this culture and all were associated with similar very weak, perhaps merely background, labelling with anti-Gap 43. Again, it is interesting to note that the neural-like cell shown in fig. 6.7 (A) has a round symmetrical cell body and two processes, though several very fine process are emanating from the cell body. Cells with this morphology are associated with TuJ1 expression. One of the three neural-like cells identified was not classed as a bipolar cell, but nonetheless was observed to have a weak staining. The morphology of the third neural-like cell was not recorded.

The intensity of Gap 43 labelling associated with \textit{mMsx} 2-induced neural-like cells is so close to the limit of detection that it is not possible to conclude that these cells are either positive or negative for this antigen. Equally, the small number (3) of \textit{mMsx} 2-induced neural-like cells observed prevents any meaningful correlation between morphology and Gap 43 expression from being made.

\textbf{6.5.1 Neurofilament 68 (NF 68)}

Neurofilaments are predominantly found in cells of neuronal origin and are a component of the cytoskeleton (Debus \textit{et al.}, 1983). NF 68 has the lowest molecular weight of the three known neurofilament proteins (Debus \textit{et al.}, 1983) and has been used as a marker of retinal ganglion cells in chick (Austin \textit{et al.}, 1995). Moreover, NF 68 is induced in cultured chick PRE explants treated with FGF 2 (Guillemot and Cepko, 1992).
6.5.2 NF 68 immunoreactivity in PRE 48-72 hours after transfection with the \textit{mMsx 2} expression construct (pCi\textit{M2Iβ}GeoSV40)

To determine if the neural-like cells that develop in PRE cultures transfected with the \textit{mMsx 2} expression construct (pCi\textit{M2Iβ}GeoSV40) are immunoreactive against NF 68, PRE from 5 day chick embryos was dissociated, cultured overnight in EMEMF (chapter 2.10.2) and then transiently transfected with the \textit{mMsx 2} expression construct (chapter 2.10.3). Approximately 72 hours after transfection, the cultures were fixed and stained for βGal and NF 68 (rabbit anti-βGal, 1:200 and monoclonal mouse anti-NF 68 (clone NR4) 1:200; as described in chapter 2.10.6 (fig. 6.8). As a positive control, neural retina from 7 day chick eyes was dissociated, cultured overnight in EMEMF (chapter 2.10.2) and stained in parallel for NF 68 (chapter 2.10.6; fig. 6.8).

As shown in fig. 6.8 (E), both the cell body and processes of neural retina cells were intensely labelled by anti-NF 68. However, not all the neural retina cells were labelled, which is consistent with the published observation that this antibody is ganglion specific (Austin \textit{et al.}, 1995).

There was no significant labelling of either βGal-expressing or non-expressing PRE cells above the background level. Similarly, no significant labelling of \textit{mMsx 2}-induced neural-like cells above the background level was observed, an example of which is shown in fig. 6.8 (B).
Fig. 6.8 NF 68 and βGal immunoreactivity in PRE cultures 72 hrs after transfection with the *mMsx* 2 expression construct. A-D) PRE from 5d chick eyes was dissociated, cultured overnight and transiently transfected with the *mMsx* 2 expression construct (pCiM21βGeoSV40). 72 hrs later the cultures were stained for both βGal (rabbit anti-βGal, 1:200) and NF 68 (mouse anti-NF 68, 1:200). E) For control purposes, neural retina (nr) from 7d chicks was cultured overnight then stained in parallel to the PRE cells for NF 68 (mouse anti-NF 68, 1:200). Mouse anti-NF 68 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse and fluorescein (FITC) conjugated donkey anti-rabbit, respectively. A and C) Dapi and βGal. B, D and E) Dapi and NF 68. For all the panels, the Texas-red image was captured using the same exposure and camera settings. Therefore, the Texas-red pixel intensity value (figures in yellow) associated with a particular cell can be compared to another cell or area of the slide. E) Cells in the neural retina culture were strongly labelled by NF 68. The intensity of NF 68 labelling in the *mMsx* 2-induced neural-like cell shown in (A) and (B) was not significantly different from label associated with an area of the slide containing no cells, βGal-expressing non-neural PRE cells or βGal-negative PRE cells (C and D). The staining in (B) marked by the asterisk also fluoresces under the dapi wavelength and is an artifact. A-E) x63 magnification.
The above experiment was repeated using dedifferentiated PRE cells, staining the cells 48 hrs after transient transfection. The combined data from these two experiments revealed that at least 13 \( m\text{Msx}\,2 \)-induced neural-like cells were negative for NF 68, whilst no neural-like cell, \( \beta\text{Gal} \)-positive or \( \beta\text{Gal} \)-negative, was positive for NF 68. The morphology of the neural-like cell shown in fig. 6.8 is associated with both TuJ1-positive and negative cells. While it is possible that some of the 13 neural-like cells observed in the present study had the bipolar morphology associated with TuJ1-expressing neural-like cells, no record of cell morphology was made. It can therefore not be concluded that bipolar neural-like cells with round, symmetrical cell bodies are not immunoreactive against NF 68. It will be important in the future to determine if such cells express NF 68.

6.5.3 NF 68 immunoreactivity in PRE 6 days after transfection with the \( m\text{Msx}\,2 \) expression construct (pCiM2\( \beta\text{GeoSV40} \))

Although NF 68 could not be detected in \( m\text{Msx}\,2 \)-induced neural-like cells 72 hrs after transfection, it is conceivable that this ganglion cell marker is only expressed after further differentiation. To test this idea, dedifferentiated PRE was plated out on laminin-coated 8-well, glass, chamber slides (chapter 2.10.2) and, 24 hours later, transiently transfected with the \( m\text{Msx}\,2 \) expression construct (pCiM2\( \beta\text{GeoSV40} \); chapter 2.10.3). After transfection, the medium was switched to NB27 which supports neural growth (chapter 2.10.2). 6 days after transfection, the cells were fixed and stained for \( \beta\text{Gal} \) and NF 68 (rabbit anti-\( \beta\text{Gal} \), 1:200 and mouse anti-NF 68, 1:200; as described in chapter 2.10.6; fig. 6.9). Dissociated 5-day-old embryonic chick neural retina was cultured overnight (chapter 2.10.2) and stained for NF 68 in parallel with the PRE cultures (chapter 2.10.6), thus serving as a positive control (fig. 6.9).
Fig. 6.9 NF 68 and βGal immunoreactivity in PRE cultures 6 days after transfection with the mMsx 2 expression construct. A-D) Dedifferentiated PRE cells were transiently transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40) and cultured on laminin in NB27 medium. After 6 days the cultures were stained for both βGal (rabbit anti-βGal, 1:200) and NF 68 (mouse anti-NF 68, 1:200). E) For control purposes, neural retina (nr) from 5d chicks was cultured overnight then stained in parallel to the PRE cells for NF 68 (mouse anti-NF 68, 1:200). Mouse anti-NF 68 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse and fluorescein (FITC) conjugated donkey anti-rabbit, respectively. A and C) Dapi and βGal. B, D and E) Dapi and NF 68. For all the panels, the Texas-red image was captured using the same exposure and camera settings. Therefore, the Texas-red pixel intensity value (figures in yellow) associated with a particular cell can be compared to another cell or area of the slide. E) Cells in the neural retina culture were strongly labelled by NF 68. A and B) The intensity of NF 68 label associated with the mMsx 2-induced neural-like cell shown in (A) and (B) was not significantly different from label associated with an area of the slide with no cells, βGal-expressing non-neural cells or βGal-negative PRE cells (C) and (D). A-E) x63 magnification.
As shown in fig. 6.9 (E), the 5 day neural retina culture stained for NF 68 in a similar manner to cultures of 7 day neural retina (fig. 6.8). There was no significant NF 68 labelling of either βGal-expressing or non-expressing dedifferentiated PRE above background levels. Equally, the mMsx 2-induced neural-like cell shown in fig. 6.9 (B) was not labelled by NF 68 above background levels. These cells are therefore considered to be negative for NF 68.

Only two mMsx 2-induced neural-like cells were observed in the present study, perhaps due to the prolonged period of time in culture following transfection. Consequently, further analysis will be required before it can be concluded that mMsx 2-induced neural-like cells, 6 days after transfection, are negative for NF 68. The morphology of the neural-like cell illustrated in fig. 6.9 (A) is associated with both TuJ1-positive and negative neural-like cells. It will be important to determine if mMsx 2-induced bipolar-neural cells are also negative for NF 68.

6.6.1 Islet 1

Islet 1 is a member of the Lim family of homeodomain-containing proteins. The gene is expressed in subsets of neurons including ganglion cells of the rat retina (Thor et al., 1991). Monoclonal mouse anti-Islet 1 (clone 40.2D6) recognises chick Islet 1 and labels ganglion cells within the developing chick neural retina (Austin et al., 1995).

6.6.2 Islet 1 immunoreactivity in PRE 48-72 hours after transfection with the mMsx 2 expression construct (pCiM2IβGeoSV40)

To test if the neural-like cells generated in PRE cultures transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40) express the ganglion marker Islet 1, PRE from 5 day chick embryos was dissociated, cultured for approximately 24 hours in EMEMF (chapter 2.10.2) and then transiently transfected with the mMsx 2 expression construct (chapter 2.10.3). Approximately 72 hours after transfection, the cultures were fixed and stained for βGal and Islet 1 (rabbit anti-βGal, 1:200 and mouse
anti-Islet 1 (40.2D6), 1:2000; as described in chapter 2.10.6; fig. 6.10). As a positive control, the neural retina from 7-day-old chick embryos was dissociated, cultured in EMEMF for 24 hours (chapter 2.10.2) and then stained for Islet 1 in parallel to the PRE cultures (chapter 2.10.6; fig. 6.10).

A proportion of the cells in the 7 day neural retina culture were intensely labelled by Islet 1. This is consistent with the antibody only reacting against ganglion cells of the retina and confirms its specificity. In those cells that were labelled, Islet 1 was localised to the nucleus with a 'speckled' pattern (fig. 6.10).

PRE cells, irrespective of whether or not transfected with the mMsx 2 expression construct did not exhibit any immunoreactivity against Islet 1 above background levels. Likewise, the mMsx 2-induced neural-like cell shown in fig. 6.10 was not labelled by anti-Islet 1. Thus, these cells are negative for Islet 1.

This analysis was repeated using primary PRE cultures from 6 day chick embryos and staining for Islet 1 48 hrs after transient transfection. At least, a total of 32 mMsx 2-induced neural-like cells were identified in these two independent studies which were all negative for Islet 1. At least 4 bipolar cells with round-symmetric cell bodies were recorded as being negative for Islet 1, none were positive. It is therefore likely that this type of mMsx 2-induced neural cell, which would be predicted to be labelled by TuJ 1, does not express the ganglion marker Islet 1.

Islet 1 might only be expressed in fully differentiated ganglion cells. It will therefore be important in future studies to determine if mMsx 2-induced neural-like cells remain negative for Islet 1 after a prolonged period of time in culture.
Fig. 6.10 Islet 1 and βGal immunoreactivity in PRE cultures 72 hrs after transfection with the mMsx 2 expression construct. A and B) PRE from 5d chick eyes was dissociated, cultured overnight and transiently transfected with the mMsx 2 expression construct (pCiM2IβGeoSV40). 72 hrs later the cultures were stained for both βGal (rabbit anti-βGal, 1:200) and Islet 1 (mouse anti-Islet 1, 1:2000). Mouse anti-Islet 1 and rabbit anti-βGal were detected using Texas-red conjugated donkey anti-mouse and fluorescein (FITC) conjugated donkey anti-rabbit, respectively. A) Dapi, βGal and Islet 1. B) βGal and Islet 1. C and D) For control purposes, neural retina (nr) from a 7d chick was cultured overnight and stained for Islet 1 (mouse anti-Islet 1, 1:2000). Note that in (C) and (D) mouse anti-Islet 1 was detected using fluorescein (FITC) conjugated donkey anti-mouse. Identical staining is observed when Texas-red conjugated donkey anti-mouse is used (data not shown). C) Dapi and Islet 1. D) Islet 1. C and D) Cells in the neural retina culture were strongly labelled by Islet 1. A and B) The mMsx 2-induced neural-like cell and βGal-negative neighbouring PRE cells were not labelled by Islet 1. A-D) x63 magnification.
6.7 *In vivo* immunolocalisation of TuJ1, Map 2, and Gap 43 in 7d chick neural retina

The localisation of Map 2 in embryonic chick retina is not described in the literature. It was therefore necessary to perform *in situ* immunohistochemistry for Map 2 on sections of chick retina, to determine which cell type expresses this antigen. It was also decided to determine if there were any subtle differences between the immunolocalisation of TuJ1, Map 2 and Gap 43 which could perhaps explain why a large proportion of *mMxx* 2-induced neural-like cells were labelled with TuJ1, but failed to react against other ganglion markers.

The eyes of 7-day-old chick embryos, which are known to express TuJ1, Map 2 and Gap 43 in culture, were fixed in 4% PFA, embedded in wax and 7μm-thick, transverse-serial sections cut (chapter 2.9.3). Immunohistochemistry was performed on the same eye for TuJ1, Map 2 and Gap 43 (mouse anti-TuJ1, 1:500, mouse anti-Map 2, 1:200 and mouse anti-Gap 43, 1:1000; as described in chapter 2.9.6; fig. 6.11).

Anti-Map 2 labelled only the ganglion cell layer of the retina, therefore is presumably ganglion specific in 7 day chick retinas. Likewise, Gap 43 which has previously been reported to be ganglion specific in both mouse and chick retina (Capone *et al.*, 1991; Austin *et al.*, 1995), also only labelled the ganglion cell layer, confirming the published observations (fig. 6.11).
Fig 6.11 Immunohistochemistry of 7 day chick retina for TuJ1, Map 2 and Gap 43. A 7 day chick eye was fixed in 4% PFA, embedded in wax and transverse serial-sections cut at 7 micron intervals. A) TuJ1 staining was detected in cells of the ganglion cell layer (closed arrow), a domain encompassing the inner plexiform layer and inner nuclear layer (open arrow), and cells beneath the PRE (closed arrowhead), x40. TuJ1-negative cells were observed within the inner nuclear layer (open arrowhead). B) Gap 43 staining was restricted to the ganglion cell layer (closed arrow), x40. C) Map 2 staining was restricted to the ganglion cell layer (closed arrow), x40. PRE = pigmented retina epithelium and nr = neural retina.
TuJ1 also strongly labelled the ganglion cell layer, but in contrast to Map 2 and Gap 43 also stained a region immediately adjacent to the ganglion layer (fig. 6.11). This domain of staining encompasses the inner plexiform layer and may also include some cells of the inner nuclear layer. Bipolar, horizontal and amacrine neuronal cells develop in this region as do glial Muller cells (Prada et al., 1991). A domain of the neural retina immediately below the PRE was also labelled by TuJ1, (fig. 6.11) a region in which photoreceptors will develop. The localisation of TuJ1 to cells other than ganglion cells in 7 day chick neural retina, together with the failure to observe labelling of mMx2-induced neural-like cells with a variety of ganglion markers, is consistent with the possibility that these cells may be equivalent to TuJ1-positive cells found outside the ganglion cell layer.

It was not possible to determine if the PRE expressed any of these antigens as the pigment masked the stain. However, the use of fluorescent secondary antibodies in future experiments should overcome this problem.

6.8 Conclusion

A small proportion (approximately 2%) of untransfected cells in PRE cultures transfected with either the mMx2 expression construct or the control construct were unexpectedly immunoreactive against the neuron-specific antigen TuJ1. Consequently, the subpopulation of TuJ1-positive/βGal-negative cells present in mMx2 transfected cultures cannot be due to an inefficiency of the IRES or factors released from transfected cells that effect untransfected neighbouring cells. Perioptic mesenchymal cells, which are likely to contaminate PRE cultures, are also unlikely to be the main TuJ1-positive cell type in these cultures since only a small proportion of cultured perioptic mesenchyme cells were positive for TuJ1 (0.86%). Thus, either PRE cells express this antigen in vivo at the time of dissection or it is induced in culture.
The PRE differentiated state is destabilised when placed in culture, as a result of inadequate cell-cell contacts (chapter 4). Under such circumstances it is conceivable that PRE cells could express the TuJ1 antigen which may not normally be expressed in vivo. Indeed, it has been casually observed that the number of TuJ1-positive cells in PRE cultures varies between experiments, suggesting that subtle differences in culture conditions, such as cell density, may influence TuJ1 immunoreactivity. Alternatively, factors such as FGF 2 which may be present in the crude FCS used in these experiments and possibly produced endogenously by PRE cells upon disruption of their cell-cell contacts (Bost et al., 1994) may induce TuJ1. In support of this idea, Pittack et al. (1997) have shown that PRE that develops from optic vesicles cultured in the presence of FGF 2 is immunoreactive against TuJ1.

It is conceivable that only TuJ1-positive PRE cells can respond to mMsx 2 expression and gain a neural morphology, since the majority of induced neural-like cells are TuJ1-positive. However, 4 out of 12 mMsx 2-induced neural-like cells are TuJ1-negative suggesting that TuJ1 expression is not essential for mMsx 2 to have an effect. However, it is possible that these negative cells were TuJ1-positive at an earlier stage of differentiation or will become positive at a later stage of differentiation. Providing that mMsx 2 does not preferentially stimulate the proliferation of TuJ1-positive PRE cells, further analysis of the number of TuJ1/βGal-positive PRE cells in cultures transfected with either the mMsx 2 expression construct or the control construct will determine whether or not mMsx 2 expression induces TuJ1. It would also be interesting to learn if TuJ1-positive PRE cells co-express endogenous cMsx 2.

The possibility that bipolar TuJ1-positive and TuJ1-negative neural-like cells are two distinct cell types, was explored by investigating the distribution of TuJ1 immunoreactivity in the chick retina. TuJ1 labelled all the domains of the neural retina in which neuronal cell types develop. Negative cells were located in the inner nuclear layer. The location of these negative cells, together with the report that glial cells are not immunoreactive against TuJ1 (Trimmer et al., 1986) suggests that they, and perhaps the TuJ1-negative neural-like cells produced in PRE cultures following
ectopic $mMsx\,2$ expression, may be glial cell types. The vertebrate retina contains two classes of glial cells, Muller cells and astrocytes. In mouse, astrocytes differentiate from the optic stalk at post-conceptional day 17 and migrate into the retina (Huxlin et al., 1992). This class of glial cell is labelled by Glial fibrillary acidic protein (GFAP), a major component of glial cell intermediate filaments (Sarthy et al., 1991). In contrast, Muller cells develop directly from the neural retina and in chick are immunoreactive against 5E10 which binds Filamin, an actin-binding protein (Lemmon, 1986).

PRE cultures transfected with the $mMsx\,2$ expression construct have been stained for GFAP using polyclonal rabbit anti-GFAP (clone 20334) but this antibody failed to react with cultured neural retina preventing any conclusions from being made. It will be important, in the future, to determine if the TuJ1-negative $mMsx\,2$-induced neural-like cells are glial cell types, perhaps using anti-Filamin (5E10).

Cells with a neural morphology, that arise in PRE cultures 48-72 hours after transfection with $mMsx\,2$, were negative for the retinal ganglion markers Map 2, NF 68 and Islet 1. It was not possible to determine if these cells were either positive or negative for the ganglion marker Gap 43, since the labelling was at the limit of detection. Analysis of these antigens was performed before TuJ1, consequently the significance of neural cell morphology was not appreciated at the time of analysis. Therefore, there is an element of doubt as to whether sufficient bipolar cells, which are TuJ1 positive, were observed to conclude that this cell type is negative for Map 2 and NF 68. It will be necessary to confirm these results before a confident conclusion can be drawn. However, it is possible to state that $mMsx\,2$-induced bipolar neural cells are negative for Islet 1 without the need to confirm this result.

It is surprising that neural-like cells labelled by TuJ1 did not express other ganglion markers. However, TuJ1 labels neuronal precursors of the CNS before or during their terminal division and therefore is a very early neuronal marker (Memberg and Hall, 1994). It is possible that $mMsx\,2$-induced neural-like cells have not yet reached a state
of differentiation which permits the expression of Map 2, NF 68 and Islet 1. In an attempt to address this possibility transfected cells were cultured for 6 days before being stained for some of these markers. It was postulated that this extended time in culture would allow mMsx 2-induced neural-like cells to undergo further differentiation. Although no positive Map 2 or NF 68 neural-like cells were observed 6 days after transfection, there were not enough of these cells to conclude with confidence that all mMsx 2-induced neural-like cells would be negative for these markers. To reach a confident conclusion it will be necessary to observe a greater number of cells. This could be achieved by analysing the cultures between 72 hours and 6 days when a larger number of neural-like cells may be present. It is important to bear in mind that increased time in culture is not necessarily linked to increased differentiation. It is conceivable that the absence of factors essential for complete ganglion differentiation are missing from this in vitro system.

Alternatively, the fact that mMsx 2-induced neural-like cells are TuJ1-positive but negative for ganglion markers suggests that they are not ganglion cells. The localisation of TuJ1 to regions of the neural retina in which horizontal, bipolar, amacrine and photoreceptor cells develop is consistent with this idea. In rat, horizontal cells are immunoreactive against B-1 (Drager et al., 1984; Hofstein et al., 1985), bipolar cells are immunoreactive against RET B2 which binds an uncharacterised cell surface antigen (Akagawa and Barnstable, 1986), amacrine cells from both rat and chick are immunoreactive against HPC 1 which binds the cell surface protein Syntaxin (Barnstable et al., 1985; Guillemot and Cepko, 1992) and rod photoreceptors are immunoreactive against rho 1D4 which binds pig, dog, cat, rat and rabbit Rhodopsin (MacKenzie et al., 1984). Preliminary analysis showed that 7 day chick neural retina failed to react with HPC 1 and rho 1D4 preventing any further analysis of mMsx 2 transfected PRE cells. In the future, it will be important to determine which of the retina neuron-specific antigens are expressed by mMsx 2-induced neural-like cells.
In summary, ectopic expression of *mMsx* 2 in dissociated cultures of chick PRE induces a small population of cells to gain a neural morphology. In turn, a proportion of these cells are immunoreactive against TuJ1 which is consistent with them being neuronal. It remains to be shown exactly to which type of retina cell type, if any, these cells are equivalent, although it appears that they may not be ganglion cells. The presence of *mMsx* 2-induced neural-like cells that are negative for TuJ1 suggests a mixed population of neural cells is being generated. Although not proven, the immunolocalisation of TuJ1 in the chick neural retina perhaps suggests that neural-like TuJ1-negative cells induced in PRE cultures by *mMsx* 2 are glial. If this is the case, it may imply that ectopic expression of *mMsx* 2 in chick PRE generates a neural precursor which is at least bipotent differentiating into both glial and neuronal cell types. The analysis of glial-specific antigens in *mMsx* 2-induced neural-like cells will help understand the differences between TuJ1-positive and negative neural-like cells.
Investigation into the Conservation of Function of Mouse and Chick Msx and of Msx 1 and Msx 2

7.1 Introduction

The eyes of mice null for either Msx 1 or Msx 2 are reported to develop normally, while the eyes of mice null for both these genes exhibit eye defects ranging from microphthalmia to anophthalmia (chapter 1.5.1). These observations suggest that Msx 1 and Msx 2 have a conserved function during eye development and that the absence of one protein can be substituted for by the other. In this chapter, the possibility that mMmsx 1 and mMmsx 2 have a conserved function is explored further by comparing the effect ectopic mMmsx 1 expression has on cultured chick PRE, with the effect ectopic mMmsx 2 expression has on these cells.

The expression of mMmsx 2 in the presumptive neural retina of the mouse optic vesicle/cup suggested that the gene may play a role in controlling the differentiation of the neural retina. In support of this hypothesis, ectopic expression of mouse Msx 2 in chick PRE cells resulted in a small proportion acquiring a neural identity. These observations also suggest that Msx 2 function might be conserved between chick and mouse. This idea is explored by investigating the expression of cMsx 1 and cMsx 2 in the developing chick retina and establishing if ectopic expression of these genes in chick PRE has an effect similar to the ectopic expression of mouse Msx 2 in these cells.

Exploring the effect that ectopic expression of mouse Msx 1 and chick Msx has on cultured chick PRE cells will help assess whether or not the effects observed when these cells were transiently transfected with the mouse Msx 2 expression construct
reflects normal \textit{in vivo} Msx function. This issue is also addressed in chapter 8 which describes the approach taken to misexpress mouse \textit{Msx 2} in mouse PRE, \textit{in vivo}.

### 7.2 Ectopic mouse \textit{Msx 1} expression in chick PRE cultures

Mouse Msx 1 function was investigated by ectopically expressing the encoding gene in both primary and dedifferentiated cultures of chick PRE. PRE from 5 day chick eyes was dissociated and cultured for 24 hrs in EMEMF, then transiently transfected with either the \textit{mMsx 2} expression construct (pCiM2IβGeoSV40), control construct (pCiβGeoSV40) or the \textit{mMsx 1} expression construct (pCM1IβGeoiSV40; chapter 2.10.1 and 2.10.3). The \textit{mMsx 1} expression construct, driven by CMV, contained an IRES, below which was cloned the βGeo reporter gene (illustrated in fig. 7.1 and supplied by Dr. S. Thomson). 72 hours after transient transfection the cells were fixed, stained for βGal activity and the percentage of βGal expressing cells with a neural morphology determined (table 7.1; chapter 2.10.4).

<table>
<thead>
<tr>
<th>Construct</th>
<th>Number of βGal\textsuperscript{m} cells</th>
<th>Number of βGal\textsuperscript{m} cells with a neural morphology</th>
<th>% of βGal\textsuperscript{m} cells with a neural morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCiM2IβGeoSV40</td>
<td>1,141</td>
<td>14</td>
<td>1.2</td>
</tr>
<tr>
<td>pCM1IβGeoiSV40</td>
<td>1,266</td>
<td>3</td>
<td>0.2</td>
</tr>
<tr>
<td>pCiβGeoSV40</td>
<td>781</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7.1 Percentage of PRE cells, transfected with either the \textit{mMsx 2} expression construct (pCiM2IβGeoSV40), \textit{mMsx 1} expression construct (pCM1IβGeoiSV40) or control construct (pCiβGeoSV40) with a neural morphology. PRE from 5 day chick eyes were cultured overnight before being transfected with either the \textit{mMsx 1}, \textit{mMsx 2} or control expression construct. Cultures were stained for βGal activity 72 hours after transfection.
As shown in fig. 7.2, βGal-expressing cells with a neural morphology were observed in cultures transfected with either the \( m\text{Msx} \, 2 \) expression construct or \( m\text{Msx} \, 1 \) expression construct. Cells with this morphology were not observed in the control transfected culture (table 7.1). \( m\text{Msx} \, 1 \)-induced neural cells had two to three unbranched processes (fig. 7.2). Cells with this morphology were also observed in cultures transfected with the \( m\text{Msx} \, 2 \) expression construct. In addition cells with several branching processes were observed in \( m\text{Msx} \, 2 \) transfected cultures but not in \( m\text{Msx} \, 1 \) transfected cultures. The efficiency of neural induction by transfection with the \( m\text{Msx} \, 1 \) expression construct and \( m\text{Msx} \, 2 \) expression construct also differed; 0.2 % of \( m\text{Msx} \, 1 \) transfected PRE cells acquired a neural phenotype compared to 1.2 % of \( m\text{Msx} \, 2 \) transfected cells. These differences in morphology and efficiency of neural induction have also been observed by other investigators (Dr. S. Thomson and C. Oram, personal communications).

Dedifferentiated PRE cells also responded to ectopic \( m\text{Msx} \, 1 \) expression by acquiring a neural phenotype 48 hrs after transfection (fig. 7.2). Again transfected cells with complex-branching processes were absent from these cultures.
Fig. 7.2 Neural morphology associated with primary and dedifferentiated PRE transfected with either the *mMsx* 2 expression construct (pCiM2IβGeoSV40) or the *mMsx* 1 expression construct (pCM1IβGeoSV40). A, C and E) Dissociated PRE from 5 day chick eyes was cultured overnight before being transfected with either the *mMsx* 1 or *mMsx* 2 expression construct. The cultures were stained for βGal activity 72 hrs later. B and D) 5 day chick PRE was dedifferentiated by passaging at low density 5 times over 14 days. These cells were then transfected with either the *mMsx* 1 or the *mMsx* 2 expression construct. 48 hrs later the cells were stained for βGal activity. *mMsx* 1-induced neural cells were both bipolar, (B) closed arrow, x25, and tripolar, (A) closed arrow, x25, but did not exhibit branching processes. *mMsx* 2-induced neural cells were also bipolar, (C) closed arrow, x25, tripolar, (E) closed arrow, x25, and in contrast to *mMsx* 1-induced neural cells can have branching processes, (D) closed arrows, x4.
7.3 Comparison of mouse and chick Msx expression in the developing eye

The expression pattern of the chick Msx genes during eye development has not been documented. Therefore, before investigating the function of these genes by ectopic expression in chick PRE cultures, a preliminary analysis of their expression in the eye was performed. Although similar morphological processes take place during chick and mouse eye development, the timing and rate of development of different components of the eye may differ between chick and mouse. This may make it difficult to accurately compare chick and mouse eyes at equivalent stages of development.

During the second day of chick development the optic vesicle invaginates forming the bilayered retina, by the third day lens fibres have started to differentiated within the lens vesicle. These stages of development correspond to the stages of mouse eye development analysed in chapter 3. Therefore, 2-3d chick embryos were wax embedded, serially sectioned at 7 μm and then hybridised to either cMsx 1 or cMsx 2 35S labelled riboprobes as described in chapter 2.9.3 and 2.9.5. An exposure time of 6 weeks ensured maximum sensitivity.

At the earliest stage of chick eye development analysed, the optic vesicle had not yet started to invaginate and there was no visible sign of lens pit formation. cMsx 2 expression could not be detected in either the optic vesicle, overlying ectoderm or the perioptic mesenchyme at this stage (data not shown). No results were obtained for cMsx 1 expression at this stage. At the earliest stage of mouse eye development analysed, the optic vesicle already showed signs of invagination, and so was considered to be more developmentally advanced than the chick optic vesicle analysed in this study. Nonetheless, mMx 2 was detected in cells of the dorsal/posterior region of the optic vesicle that underlie the surface ectoderm in the mouse eye (chapter 3.3). Expression was also observed in the lens pit, surface ectoderm and a small population of mesenchyme cells located dorsally to the mouse optic vesicle (chapter 3.4 and 3.5).

As eye development progresses, the optic vesicle invaginates to form a bilayered optic cup and the lens placode invaginates to form the lens pit. In chick, at this stage of eye
development *Msx 2* was expressed in the surface ectoderm adjacent to the lens pit which itself was negative for *Msx 2* (fig. 7.3). No results were obtained for *cMsx 1* at this stage. In an equivalent staged mouse (~E10.5), *Msx 2* was also expressed in the surface ectoderm and, in contrast to chick, the lens pit too (chapter 3.4). In common to both chick and mouse, the perioptic mesenchyme was labelled by the *Msx 2* probe at the lens pit stage (fig. 7.3 and chapter 3.5). At this stage, *Msx 2* expression could not be detected in any derivatives of the chick optic vesicle (fig. 7.3). In contrast, the distal-posterior/dorsal quarter of the mouse neural retina expressed *Msx 2*.

At the oldest stage of chick eye development analysed, the lens vesicle had completely detached from the surface ectoderm but did not contain differentiating lens fibre cells. The chick lens, at this stage, was not labelled by the *cMsx 2* probe (fig. 7.6) In contrast, the mouse lens, at this stage, expressed *Msx 2* (chapter 3.4). Labelling of the anterior lens epithelium of chick by the *cMsx 1* probe was close to background levels (data not shown). It is therefore impossible to conclude that this tissue is either positive or negative for *cMsx 1*. Interestingly, the same anterior lens epithelium in mouse was weakly labelled by *Msx 1* (chapter 3.4). The surface ectoderm overlying the mouse lens vesicle at this stage of eye development uniformly expressed *mMsx 2* (chapter 3.4). In contrast, *cMsx 2* expression in the surface ectoderm was only detected in the dorsal half of the chick eye (fig. 7.6). In common to both chick and mouse, *Msx 1* expression was not detected in the surface ectoderm at this stage (fig. 7.6 and chapter 3.4). Both *cMsx 1* and *cMsx 2* expression was detected in chick perioptic mesenchyme at the lens vesicle stage (fig. 7.6). *cMsx 2* was dorsally restricted while *cMsx 1* was more broadly expressed in this tissue. In an equivalent staged mouse, only *Msx 1* was expressed in the peri-optic mesenchyme (chapter 3.5). Expression of *Msx 2* in the retina of both mouse and chick at the lens vesicle stage was asymmetrically restricted to the dorsal/posterior side of the presumptive ciliary margin (fig. 7.4 and chapter 3.3). However, in mouse expression was restricted to the inner layer of the ciliary margin while in chick expression was restricted to the outer layer (fig. 7.4 and chapter 3.3). It is at this stage of eye development in mouse that *Msx 1* expression was first detected in the dorsal half of the retina, confined to the
distal tips of the inner layer of the optic cup (chapter 3.3). $cMsx\,1$ expression could not be detected at this stage of chick retina development (fig. 7.5). As previously mentioned, it is possible that the state of retina development was not precisely matched between chick and mouse at this stage. It will therefore be important to investigate $cMsx\,1$ expression at slightly later stages of chick development.
Fig. 7.3 cMxx 2 expression in the chick eye at the lens pit stage. A) Transverse section through a chick eye at the lens pit stage, x40. Ai) Enlargement of the proximal part of the eye shown in (A), x100. cMxx 2 expression was not observed in the presumptive PRE (open arrow with asterisk) or in the presumptive neural retina (open arrow). cMxx 2 expression was detected in peroptic mesenchyme (closed arrowhead). Aii) Enlargement of the distal-dorsal part of the eye shown in (A), x100. cMxx 2 expression was detected in the surface ectoderm adjacent to the lens pit (closed arrow). cMxx 2 expression was not detected in the presumptive neural retina (open arrow) or in the lens pit (open arrowhead). B) Transverse section through the same eye as shown in (A), but at a different level, x100. cMxx 2 expression was not detected in the lens pit (open arrowhead). pm = perioptic mesenchyme, lp = lens pit, pPRE = presumptive pigmented retina epithelium, pnr = presumptive neural retina, se = surface ectoderm.
Fig. 7.4 *cMsx* 2 expression in the chick retina at the lens vesicle stage. A) Transverse section through the posterior half of a chick eye at the lens vesicle stage, x20. Ai) Enlargement of the dorsal side of the retina shown in (A), x40. For clarity panel (Ai) has been enlarged, see below. *cMsx* 2 expression was detected in the outer layer of the dorsal presumptive ciliary margin (closed arrow). The proximal and distal boundaries of expression are marked by the broken lines. *cMsx* 2 expression was not detected in either the inner layer of the presumptive ciliary margin (open arrowhead with asterisk) or in the proximal neural retina (open arrowhead). Proximal PRE did not express *cMsx* 2 (closed arrowhead). Aii) Enlargement of the ventral side of the retina shown in (A), x40. *cMsx* 2 expression was not detected in either the outer (open arrow) or inner (arrowhead with asterisk) layer of the posterior ventral presumptive ciliary margin. *cMsx* 2 expression was not detected in either proximal neural retina (open arrowhead) or PRE (closed arrow). B) Transverse section through the anterior half of a chick eye at the lens vesicle stage, x20. Bi) Enlargement of the dorsal side and (Bii) ventral side of the retina shown in (B), x40. *cMsx* 2 expression was not detected in either the inner layer (arrowheads with asterisks) or outer layer (open arrows) of the anterior presumptive ciliary margin, both dorsally and ventrally. Anterior neural retina (open arrowheads) and PRE (closed arrowhead) were also not labelled for *cMsx* 2, dorsally and ventrally. pcm = presumptive ciliary margin, PRE = pigmented retina epithelium, nr = neural retina, se = surface ectoderm, l = lens vesicle.
Fig. 7.5 *cMsx I* expression in the chick retina at the lens vesicle stage. A) Transverse section through the posterior half of a chick eye at the lens vesicle stage, x20. Ai) Enlargement of the dorsal side, and (Aii) ventral side, of the retina shown in (A), x40. *cMsx I* expression was not detected in either the outer (open arrows) or inner (arrowhead with asterisk) layer of the presumptive ciliary margin, both dorsally and ventrally. *cMsx I* expression was not detected in proximal neural retina (open arrowheads) or PRE (closed arrowheads), neither dorsally nor ventrally. Note that the eye shown in this figure was serially sectioned. No *cMsx I* was detected in the retina of other sections. pcm = presumptive ciliary margin, nr = neural retina, PRE = pigmented retina epithelium.
Enlargement of panel (B) shown opposite.

Enlargement of panel (C) shown opposite.
Fig. 7.6 cMtx 1 and cMtx 2 expression in the chick lens, surface ectoderm and perioptic mesenchyme at the lens vesicle stage. A and C) Transverse sections, at a similar plane, through the centre of the chick lens vesicle, x40. A) cMtx 1 expression was not detected in the surface ectoderm (open arrows). cMtx 1 expression was not detected in the lens vesicle (open arrowhead), though as described in the main text it was not possible to conclude that the anterior lens epithelium was negative for cMtx 1. C) cMtx 2 expression was detected in dorsal surface ectoderm (closed arrow), but not ventral surface ectoderm (open arrow). cMtx 2 expression was not detected in the lens vesicle (open arrowhead). B and D) Transverse sections, at a similar plane, through the dorsal side of the chick eye at the lens vesicle stage, x40. B) cMtx 1 expression was detected in the perioptic mesenchyme (closed arrow). D) cMtx 2 expression was also detected in the perioptic mesenchyme (closed arrow). For clarity panels (B) and (C) have been enlarged, see opposite. l = lens vesicle, se = surface ectoderm, pm = perioptic mesenchyme, r = retina.
7.4 Attempted cloning of full-length chick \textit{Msx} 2 cDNA

In order to ectopically express chick \textit{Msx} 2 in chick PRE cultures it was first necessary to isolate a full-length \textit{cMsx} 2 cDNA and generate a \textit{cMsx} 2 expression construct. The published \textit{cMsx} 2 sequence has a coding region of only 780 bp (Coelho \textit{et al.}, 1991). It was therefore decided to generate \textit{cMsx} 2 cDNA by RT-PCR of RNA from \textit{cMsx} 2-expressing tissue. The upstream primer was designed to contain a Xho I site and the downstream primer a Nsi I site. The introduction of these sites to the amplified \textit{cMsx} 2 cDNA would enable it to be directly cloned into the reciprocal sites of pCiM2IβGeoSV40, replacing the pre-existing mouse \textit{Msx} 2 insert.

7.4.1 Part 1: RT-PCR of \textit{cMsx} 2

Total RNA was isolated from 4d chick heads and 4d chick limb buds, 1.5μg of which was treated with DNase I and used to generate first-strand cDNA, primed by oligo-T (chapter 2.6.3.2 and 2.7.3). For each RNA population a control reaction was performed in which reverse transcriptase was omitted from the reaction.

Amplification of full-length \textit{cMsx} 2 was performed using the upstream primer L199 (11-30) which anneals 91 bp above the translation start site, and the downstream primer L200 (922-941) which anneals 62 bp below the translation stop site. These primers were predicted to amplify a 955 bp fragment (chapter 2.7.1 and fig. 7.7A). To reduce the chance of introducing a mutation into the amplified product, PCR was performed with Vent\textsubscript{R} DNA polymerase which possesses 3′ to 5′ proof reading exonuclease activity (chapter 2.7.3.3). Products were not amplified from the minus reverse transcriptase control samples, confirming that there was no DNA contamination (data not shown). The limb bud cDNA failed to give a satisfactory PCR with L199 and L200 (data not shown), whilst head cDNA yielded bands of varying sizes (fig. 7.7B). The reaction could not be optimised to yield a single band. It was therefore decided to isolate both the 900 bp and 1kb band, which were of similar size to the predicted product, by gel purification as described in chapter 2.3.
Fig. 7.7 Attempted cloning of *cMsx 2*. B) Agarose-gel electrophoresis of the PCR products generated by PCR of first-strand 4d chick head cDNA using chick *Msx 2* primers, L199 and L200 (position shown in (A)). C) The 1 kb and 900 bp bands, in (B), were purified and reamplified under three different conditions. The annealing temperature for the first five cycles of all the reactions was 50°C. The remaining 30 cycles were then performed with an annealing temperature of either 50°C (a), 52°C (b) or 54°C (c). D) To test if the 900 bp PCR product was indeed *cMsx 2* it was digested with Bam HI and Eco RI. The predicted digestion products are summarised in (A). The enzymes linearised pSK, but did not cleave the 900 bp product as expected (closed arrow). E) To confirm that the 900 bp PCR product was not *cMsx 2*, the agarose-gel shown in (D) was Southern blotted and hybridised to the α32P CTP probe shown in (A). The autoradiograph in (E) shows that this probe hybridised to linearised pG#8ps7 (*cMsx 2* in situ plasmid) and to a lesser extent pGHox7sp (*cMsx 1* in situ plasmid). The 900 bp PCR product did not hybridise to the probe (closed arrow). M = 1 kb DNA ladder.
7.4.2 Part 2: Southern blot analysis of amplified products

To increase the amount of each product, a small amount of the purified 900 bp and 1 kb product was reamplified. The primers (L199 and L200) were annealed at 50 °C, the temperature used in the initial reaction, for the first 5 cycles and then either 50 °C, 52 °C or 54 °C for the remaining 30 cycles (chapter 2.7.3.3 and fig. 7.7C). As shown in fig. 7.7C, the 900 bp fragment was amplified under all conditions, though the 1 kb fragment failed to be reamplified. Consequently, the 1 kb fragment was not analysed further. In addition to the 900 bp fragment, a 500 bp fragment was produced in all reactions, except in the minus-template control. This may be due to non-specific annealing of the primers to the template.

The 900 bp fragment was isolated from the 500 bp band by gel electrophoresis, chapter 2.3, and digested with either Eco RI or Bam HI (section 2.2.4). Eco RI was predicted to cut \( cMsx \) at 597, cleaving the 955 bp product into 598 bp and 357 bp fragments. Bam HI was predicted to cut \( cMsx \) at 421, cleaving the 955 bp band into 422 bp and 533 bp fragments. As shown in fig. 7.7D, neither Eco RI nor Bam HI digested the 955 bp band as predicted, but did successfully linearise the plasmid pSK demonstrating that the enzymes were active. The amplified 955 bp product was therefore not \( cMsx \) as expected.

This was further confirmed by Southern blotting the Eco RI and Bam HI digested PCR product and hybridising it to a 205 bp \( cMsx \) probe (679-884; fig. 7.7A). This probe was prepared by digesting pG#8ps7 (\( cMsx \) in situ probe plasmid) with Pst I and isolating the 400 bp band which was then further digested with Sau 3A to remove the L200 primer site. The probe was then random prime-labelled with \( \alpha^{32}\text{P} \) CTP (chapter 2.4).

As shown in fig. 7.7E, linearised pG#8ps7 was intensely labelled by the probe. The extreme intensity of the signal was due to the excessive amount of linearised pG#8ps7 loaded on the gel. The pSK vector and linearised \( cMsx \) in situ hybridisation plasmid
(pGHox7sp) were weakly labelled. This suggests that the hybridisation stringency was insufficient. However, no signal was detected for the digested and uncut PCR products confirming that the 955 bp PCR product was not cMsx 2.

The PCR-based approach to cloning cMsx 2 proved unsuccessful. Rather than refining this approach or embarking on a different strategy, e.g. screening cDNA libraries, it was decided to focus on generating a cMsx 1 expression construct. The availability of a full-length cMsx 1 cDNA and the evidence that mMsx 1 and mMsx 2 have a conserved function made this a viable proposition.

7.5 Generation of a cMsx 1 expression construct (pCicM11βGeoSV40)

To explore cMsx 1 function by ectopic expression in cultured chick PRE cells, it was necessary to generate a cMsx 1 expression construct.

7.5.1 Step 1: generation of pCicM1SV40

Full-length chick Msx 1 cDNA was obtained from Dr. S. Wedden, cloned into the Eco RI site of pKS. This insert, approximately 1.3 kb, was released by Eco RI digestion and ligated into the Eco RI site (1063) of the promega pC1 mammalian expression vector to generate pCIcM1SV40 (chapter 2.1 and 2.2). This places the insert under the control of the CMV promoter, downstream of a chimeric intron and upstream of a SV40 late polyadenylation signal. As shown in fig. 7.8A, both clones 14 and 16 released a fragment approximately corresponding to expected 1.3 kb cMsx 1 insert upon digestion with Eco RI but only clone 16 yielded fragments approximately corresponding to the expected 1003 bp, 427 bp and 3878 bp fragments upon Pst I digestion. Thus, the cMsx 1 insert was in the wrong orientation in clone 14 and in the correct orientation in clone 16.
Fig. 7.8 Generation of the chick Msx 1 expression construct (pCicM1IβGeoSV40). Aii) Step 1, full-length chick Msx 1 was cloned into the Eco RI site of the pCi expression vector to generate pCicM1SV40 (sites indicated in bold). Eco RI and Pst I were predicted to cleave pCicM1SV40 as indicated in (Aii). Agarose-gel electrophoresis of the products from Eco RI and Pst I restriction digest analysis of 2 pCicM1SV40 clones. As expected Eco RI released cMsx 1 (~1.3 kb) from the vector for both clones 14 and 16. Only clone 16 was digested by Pst I to give fragments corresponding to the expected 1003 bp, 427 bp, and >4000 bp fragments (open arrowheads). Thus, cMsx 1 is in the wrong orientation in clone 14 and in the correct orientation in clone 16. Bii) Step 2, an IRES-βGeo cassette was cloned into the Xba I sites of pCicM1SV40 to generate pCicM1IβGeoSV40 (sites indicated in bold). Bam HI was predicted to cleave pCicM1IβGeoSV40 as indicated in (Bii). Agarose-gel electrophoresis of the products from Bam HI restriction digest analysis of 18 pCicM1IβGeoSV40 clones. Digestion of clones 1, 4, 6, 10 and 16 with Bam HI released the SV40 fragment (~1.2 kb band) from the vector (>4 kb band) as predicted. This confirmed that the IRES-βGeo cassette was in the correct orientation in these clones. M = 1 kb DNA ladder.
7.5.2 Step 2: generation of pCicM1IβGeoSV40

The IRES/βGeo cassette was released from pKSCiM2IβGeoSV40 as an Xba I fragment and cloned into the Xba I site of pCicM1SV40, 18 bp below the cMxx I insert and 30 bp above the SV40 polyadenylation signal, to generate pCicM1IβGeoSV40. The orientation of this insert was tested by Bam HI digestion which yielded fragments approximately corresponding to the predicted size, 1062 bp and 10023 bp (fig. 7.8B).

7.5.3 Restriction digest analysis of the cMxx I expression construct (pCicM1IβGeoSV40)

To confirm the integrity of the cMxx I expression construct a variety of restriction digests were performed on this construct (fig. 7.9; chapter 2.2.4). These digests cleaved the construct into the predicted number of fragments which were approximately the predicted size, confirming that the inserts had been cloned in the correct location, orientation and not as concatamers. The orientation of the cMxx I insert was further confirmed by sequence analysis (chapter 7.5.4).
Fig. 7.9 Restriction digest analysis of the cMx 1 expression construct (pCicM11βGeoSV40).
A) Agarose-gel (1%) electrophoresis of the products generated from digesting pCicM11βGeoSV40 with the enzymes indicated in (A). B) Restriction digest map of pCicM11βGeoSV40 showing the predicted locations of the restriction sites for the enzymes used in (A) and the sizes of the fragments expected following digestion. Sizes in brackets are the approximate size of the fragments actually observed. Sizes in bold correspond to broad bands which may contain two or more similarly sized fragments. Asterisks correspond to fragments that were too small to be detected in this experiment. M = 1 kb DNA ladder.
7.5.4 Preliminary sequence analysis of \(c\text{Msx 1}\) in the \(c\text{Msx 1}\) expression construct (pCicM1\(\beta\)GeoSV40)

The integrity of the \(c\text{Msx 1}\) insert was investigated further by sequence analysis. As summarised in fig. 7.10, the sense and anti-sense strands of the \(c\text{Msx 1}\) insert in the \(c\text{Msx 1}\) expression construct have only been partially sequenced using a panel of primers described in chapter 2.7.1 and 2.5. The T7 primer annealed approximately 30 bp upstream of the 5' end of the \(c\text{Msx 1}\) cDNA. The sequence generated from this primer was complementary to the published 5' end of the sense strand of \(c\text{Msx 1}\) (Suzuki et al., 1991) and included the translation start site at nucleotide 87 (fig. 7.10 and fig. 7.11A) confirming that \(c\text{Msx 1}\) had been cloned in the correct orientation. The sequence of the sense strand, generated from primer M849 which annealed downstream of \(c\text{Msx 1}\) and within the IRES, partially matched the predicted vector sequence (1065-1081) and the 3' end of \(c\text{Lsx 1}\). Again this verified the orientation of \(c\text{Msx 1}\) within the \(c\text{Msx 1}\) expression construct (fig. 7.10 and fig. 7.11B).

Both strands of \(c\text{Msx 1}\) have been sequenced between 641 and the translation stop codon (929). Discrepancies between the published and expression construct sequence were disregarded if corrected by another overlapping fragment or by sequence generated from the complementary strand. However, two discrepancies could not be corrected for in this way. Residue 701 was a T in the sense strand and an A in the anti-sense strand, as opposed to the published C and G, respectively (fig. 7.10 and fig. 7.11C,D). Residue 791 of the anti-sense strand was an A as opposed to the published G, while the complementary C of the sense strand was identified as a T in one fragment and a G in another. These possible mutations at 701 and 791 lie within the coding region of \(c\text{Msx 1}\) but would not effect the final amino acid structure.

A 209 bp region (254-463) of \(c\text{Msx 1}\) in the expression construct remains to be sequenced, while between 87-254 and 463-641 only one strand has been sequenced. Until sequence is obtained for these regions it will not be possible to exclude the
possibility that a base pair change may have occurred during the cloning of the cMsx 1 expression construct that could affect the amino acid structure of cMsx 1.

Fig. 7.10 Preliminary sequence analysis of the cMsx 1 insert in pCicM11βGeoSV40. Schematic alignment of the fragments of sequence generated from each primer to the published cMsx 1 sequence in the HGMP database. The positions of residues that differ from the published sequence are indicated (refer to main text and fig. 7.11 for details).

Fig. 7.11 A) Comparison of the anti-sense sequence of cMsx 1 in the expression construct (pCicM11βGeoSV40), generated with the T7 primer, to the published cMsx 1 sequence in the HGMP genome database. B) Comparison of the sense sequence of cMsx 1 in the expression construct, generated with the M849 primer, to the published cMsx 1 sequence in the HGMP genome database. The underlined nucleotides match the predicted sequence of pCic (1065-1081). C) Comparison of the compiled anti-sense sequence of cMsx 1 (641-929) in the expression construct to the published cMsx 1 sequence in the HGMP genome database. D) Comparison of the compiled sense sequence of cMsx 1 (641-929) in the expression construct to the published sequence in the HGMP genome database. A-D) Expression construct sequence is on the top and the published cMsx 1 sequence on the bottom. Sequences were compared using the gcg "Gap" program. Nucleotides in bold mark the translation start and stop sites, N corresponds to an undetermined nucleotide, "." signifies the insertion of a gap to maintain alignment (refer to main text for details).

A) Comparison of anti-sense cMsx 1 sequence generated with the T7 primer.

177 ....GGCCATGAGCGATTTCCACGCTGAAGGGCAACGGGGAAGGGGACACG 132
258 GTTCCCGCCATGAGCGCTTCCACGCTGAAGGGCAGCGGGGAAGGGGACACC 209
131 TAGNNTTTGTCGCTNTCCTCNTCGCCGCCCATACCCGCCGCGACGGGGAG 82
208 TTGGGTTTGGCTCTCTCCTCGCCGCCCGCATCGGCCGCCGCGACGGGGAG 159
81 ACCGCCACCGGAGCTGNTGAGCCNGAGGCGGGCGGCTGTCGATGCGGA 32
158 GCCCGCGCCGGGCTTCTGCTGAGCGCGAGGCGGGCGGCTGTCGCTGCGGA 109
B) Comparison of sense cMsx 1 sequence, spanning the 3' junction between cMsx 1 and the vector.

C) Comparison of compiled anti-sense cMsx 1.
D) Comparison of compiled sense cMsx 1 sequence.

7.6 Ectopic expression of chick Msx 1 in chick PRE cultures

cMsx 1 function was explored by ectopic expression in chick PRE cultures. PRE was isolated from 5d chick embryos, dissociated, plated out in multiple wells and cultured for approximately 24 hrs in EMEMF (chapter 2.10.2). These cultures were then transiently transfected with either the mMsx 2 expression construct (pCiM2βGeoSV40), cMsx 1 expression construct (pCicM1βGeoSV40) or the control construct (pCiβGeoSV40) and cultured for a further 72 hours (chapter 2.10.3). They were then fixed and stained for βGal activity (chapter 2.10.4; fig. 7.12). The number of βGal-positive cells with a neural morphology was then determined (table 7.2).
Fig. 7.12 Primary and dedifferentiated PRE cultures stained for βGal activity after transient transfection with either the *cMsx* 1 (pCiM1lβGeoSV40), *mMsx* 2 (pCiM2lβGeoSV40) or control (pCilβGeoSV40) expression construct. A, C and E) 5 day chick PRE was dissociated, cultured overnight in EMEMF and then transiently transfected with either *cMsx* 1 (A), *mMsx* 2 (C) or the control construct (E), x4. These constructs successfully transfected these cells as judged by βGal staining 72 hrs after transfection. As summarised in table 7.2, no *cMsx* 1 transfected primary PRE cells had a neural morphology, though cells with a neural morphology were observed in *mMsx* 2 transfected cultures. B and D) 5 day old chick PRE was dedifferentiated as described in the main text, then transiently transfected with either *cMsx* 1 (B), *mMsx* 2 (D) or the control construct (data not shown), x25. These cultures were stained for βGal 48 hrs later. B) Only one *cMsx* 1 transfected cell out of 769 analysed was judged to have a neural morphology (closed arrow). D) *mMsx* 2 transfected cells with a neural morphology could be observed (closed arrow). Also refer to table 7.3.
1.2% of PRE cells transfected with the mouse \( Msx \, 2 \) expression construct acquired a neural phenotype demonstrating that the PRE cells used in this particular experiment could respond to Msx function. The chick \( Msx \, 1 \) expression construct successfully transfected PRE cells, judged by the number of \( \beta \text{Gal} \)-positive cells, but none of the 1796 transfected cells had a neural morphology 72 hrs after transfection (table 7.2 and fig. 7.12).

Similarly, there was no convincing evidence that the chick \( Msx \, 1 \) expression construct was capable of inducing a neural phenotype in cultures of dedifferentiated chick PRE. PRE was isolated from 5d chicks, dissociated and cultured in EMEMF for 14 days. During this period the cells were dedifferentiated by passaging 5 times; 3 days lapsed between the first 3 passages, then 4 days and finally 1 day (chapter 2.10.2). 24 hrs after the final passage the cultures were transiently transfected with either the \( mMsx \, 2 \) expression construct (pCiM2\( \beta \)GeoSV40), \( cMsx \, 1 \) expression construct (pCicM1\( \beta \)GeoSV40) or control construct (pCil\( \beta \)GeoSV40) and cultured for a further 48 hours before being stained for \( \beta \text{Gal} \) activity (chapter 2.10.3 and 2.10.4). The number of \( \beta \text{Gal} \)-positive cells with a neural morphology was then determined (table 7.3).

<table>
<thead>
<tr>
<th>Number of ( \beta \text{Gal} ) Cells</th>
<th>Number of ( \beta \text{Gal} ) Cells with a neural morphology</th>
<th>% of ( \beta \text{Gal} ) Cells with a neural morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCiM2( \beta )GeoSV40</td>
<td>1,141</td>
<td>14</td>
</tr>
<tr>
<td>pCicM1( \beta )GeoSV40</td>
<td>1,796</td>
<td>0</td>
</tr>
<tr>
<td>pCil( \beta )GeoSV40</td>
<td>781</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 7.2 Percentage of PRE cells transfected with either the \( mMsx \, 2 \) expression construct (pCiM2\( \beta \)GeoSV40), \( cMsx \, 1 \) expression construct (pCicM1\( \beta \)GeoSV40) or control construct (pCil\( \beta \)GeoSV40) with a neural morphology (see text for details).
Number of $\beta$Gal$^{\text{ve}}$ cells | Number of $\beta$Gal$^{\text{ve}}$ cells with a neural morphology | % of $\beta$Gal$^{\text{ve}}$ cells with a neural morphology
--- | --- | ---
pCiM2I$\beta$GeoSV40 | 619 | 13 | 2.1
pCicM1I$\beta$GeoSV40 | 769 | 1 | 0.13
pCil$\beta$GeoSV40 | 97 | 0 | 0

Table 7.3 Percentage of dedifferentiated PRE cells transfected with either the $mMsx\,2$ expression construct (pCiM2I$\beta$GeoSV40), $cMsx\,1$ expression construct (pCicM1I$\beta$GeoSV40) or control construct (pCil$\beta$GeoSV40), with a neural morphology (see text for details).

The observation that 2.1% of dedifferentiated PRE cells transfected with the $mMsx\,2$ expression construct had acquired a neural phenotype after 48 hours, confirmed that these cells could respond to Msx function. Only one dedifferentiated PRE cell transfected with the $cMsx\,1$ expression construct was judged to have neural a morphology (bipolar) out of the 769 analysed (fig. 7.12, B). Cells with a neural morphology spontaneously arise in dedifferentiated cultures of chick PRE at an incidence of 0.1-0.5% of all cells (chapter 5.5). Thus, the observed 0.13% of $cMsx\,1$-transfected dedifferentiated PRE cells with a neural morphology is similar to the proportion of cells that spontaneously acquired a neural morphology. Therefore, there is no evidence that the chick $Msx\,1$ expression construct increases the proportion of PRE cells with a neural morphology.

7.7 Conclusion

The observation that ectopic expression of $mMsx\,1$ in chick PRE cells had a similar effect to ectopic $mMsx\,2$ is consistent with these genes having a conserved function. Furthermore, the presence of eye defects in mice null for both $Msx\,1$ and $Msx\,2$, but not mice null for $Msx\,1$ or $Msx\,2$ alone (chapter 1.5.1), suggests that the $Msx$ genes are functionally redundant during eye development. The $Msx$ double-knockout phenotype suggests that $Msx$ function is required prior to or at the optic vesicle stage. However, $Msx\,1$ and $Msx\,2$ are only co-expressed at later stages of retina development, after E11.5, in the presumptive ciliary margin zone (chapter 3.3). $Msx\,1$ is however expressed in the perioptic mesenchyme at the optic vesicle stage. If both
Msx genes regulate the same diffusible molecule, then the absence of Msx 2 function in the optic vesicle could be substituted for by Msx 1 function in the neighbouring mesenchyme. Equally, the absence of Msx 1 function in the perioptic mesenchyme could be overcome by Msx 2 function in the neighbouring optic vesicle.

Ectopic expression of mMsx 1 in chick PRE cultures resulted in a smaller proportion (0.2%) of transfected cells acquiring a neural phenotype compared to equivalent cells expressing mMsx 2 (1.2%). Likewise, the morphology of mMsx 1-induced neural cells was less extreme in comparison to mMsx 2-induced neural cells. Cells with complex branched processes were not observed in mMsx 1 transfected cultures. These observations might suggest that Msx 1 is not as active as Msx 2. Alternatively, these differences may merely reflect differences in the efficiency of the expression constructs used. The major difference between these two constructs was in the position of the intron. The intron was located between the CMV promoter and the mMsx 2 cDNA in the mMsx 2 expression construct, but was downstream of the βGeo cassette in the mMsx 1 expression construct. It is reported that an intron placed in the 5' untranslated region of a reporter gene can greatly increase expression levels compared to when placed 3' (Evans and Scarpulla, 1989).

During mouse retina development, both Msx 1 and Msx 2 become confined to the presumptive ciliary margin zone. Given that mMsx 1, which is not expressed at the earlier stages of retina formation, has a similar effect to mMsx 2 when ectopically expressed in chick PRE, suggests that the Msx genes may be performing a comparable role in the presumptive ciliary margin zone as Msx 2 in the presumptive neural retina of the optic vesicle/cup (refer to discussion 9.2.1). Indeed, cMsx 2 is expressed in the ciliary margin of the chick suggesting that expression at this site is functionally significant. In both species, Msx 2 is confined to the dorsal/posterior side of the distal retina. However, in mouse expression is restricted to the inner layer of the ciliary margin zone, whereas in chick expression was restricted to the outer layer. Whether or not this reflects a functional difference between chick and mouse at this site remains to be investigated.
In other regions of the eye, the expression patterns for *Msx 1* and *Msx 2* also had similarities and differences between chick and mouse. *Msx 2* is expressed in the surface ectoderm of the developing eye in both chick and mouse. Such domains of *Msx* expression, common to both chick and mouse, may correspond to sites in which expression is functionally significant. Conversely, there may be no selective pressures to maintain or prevent expression in areas in which *Msx* has no function. For example, *Msx 2* is expressed in the lens pit and lens of the mouse eye, but expression of this gene is absent from these structures in chick. This might imply that *Msx 2* has no significant function in the developing lens.

If *Msx 2* has an important role in controlling the differentiation of neural retina cells, as suggested by its expression in presumptive neural retina cells of the mouse optic vesicle/cup and its ability to cause cultured chick PRE cells to undergo neuronal differentiation, then it might be predicted that *cMsx 2* would also be expressed in the presumptive neural retina of chick. However, *cMsx 2* expression could not be detected in the chick optic vesicle prior to invagination. It is possible that an equivalently staged mouse optic vesicle might not express *mMsx 2* either, but only late mouse optic vesicles have been studied. Later in development, expression could not be detected in chick presumptive neural retina at the lens pit stage though in mouse this tissue expresses *mMsx 2*.

The absence of *cMsx 2* expression in the optic vesicle and early optic cup of chick, in stark contrast to mouse, might suggest that chicks have evolved to use a different protein in place of Msx 2. Structurally this protein cannot be significantly different from mouse Msx 2 since the mouse protein is able to function within the chick environment to drive neural differentiation of chick PRE cells. Msx 1 is structurally similar to Msx 2, consequently mouse Msx 1 had the same effect as mouse Msx 2 on the differentiation of PRE cells. Thus in chick, *cMsx 1* is a good candidate for functioning in the optic vesicle in place of Msx 2. In order to support this it will be necessary to determine if *cMsx 1* is expressed in the chick optic vesicle.
It was intended to compare the function of chick and mouse \textit{Msx} genes by ectopically expressing both \textit{cMsx 1} and \textit{cMsx 2} in cultured chick PRE. It was not possible to clone a \textit{cMsx 2} cDNA, but a \textit{cMsx 1} expression construct was generated. Cells with a neural morphology were not observed in primary chick PRE cultures following transient transfection with this construct, while transfection of dedifferentiated PRE cultures failed to increase the number of cells with a neural morphology above background levels. The presence of βGal-expressing cells in these cultures confirmed that the cells had been successfully transfected. However, it is possible that the construct was unable to produce functional \textit{cMsx 1}. Restriction digest analysis confirmed that the gross structure of the \textit{cMsx 1} cDNA was normal, while preliminary sequence analysis confirmed that the cDNA insert was in the correct orientation to produce functional Msx 1. The first 554 bp's of the \textit{cMsx 1} cDNA remain to be sequenced. It is therefore possible that a mutation lies in this region that either alters the amino acid sequence or results in the production of a truncated protein. Either one of these eventualities could result in the production of a non-functional protein. It will therefore be necessary to eliminate these possibilities by sequencing this region and demonstrating that \textit{cMsx 1} can be produced from this construct before it can be concluded that \textit{cMsx 1} is not capable of driving neural differentiation in chick PRE cultures.

The apparent inefficiency of mouse \textit{Msx 1}, compared to mouse \textit{Msx 2}, at inducing neural differentiation in chick PRE cultures might suggest that this function is primarily associated with Msx 2. In chick, \textit{Msx 1} may be too divergent to perform the same function as \textit{Msx 2}. Therefore, in light of the inability of the \textit{cMsx 1} expression construct to drive neural differentiation in PRE cultures, it will be important in the future, to investigate the consequence of ectopic \textit{cMsx 2} expression in chick PRE cultures.

Finally, it is possible that chick \textit{Msx} genes will have no affect on PRE cells and that the effect mouse \textit{Msx} genes have is a consequence of expressing mouse genes in chick...
cells. Although chick and mouse $Msx$ genes are structurally very similar, they do contain subtle differences. For instance, the nine amino acids between residues 28-36 in mouse Msx 1 are absent from chick Msx 1, while the 12 amino acids between residues 21-32 in mouse Msx 2 are absent from chick Msx 2. Such structural differences might result in mouse Msx proteins affecting cellular processes that are not influenced by chick Msx.
Chapter 8

Ectopic Expression of Mouse Msx 2 in the PRE of Transgenic Mice

8.1 Introduction

Ectopic expression of *mMsx* 2 in chick PRE cultures, by transient transfection with constitutively active expression constructs, leads to a small proportion of transfected cells acquiring a neural phenotype. This effect is consistent with *Msx* 2 expression in cells of the mouse optic vesicle and cup, that are presumed to give rise to the neural retina. These observations suggest that during normal retina development *Msx* 2 functions to either specify neural or suppress PRE differentiation amongst apparently equivalent cells of the optic vesicle. However, this proposal is based on the function of mouse *Msx* 2 in chick PRE cells. The intra- and extra-cellular environments of PRE may be different between mouse and chick. Consequently, mouse *Msx* 2 function in one species may not reflect the function in the other. In addition, structural differences between chick and mouse *Msx* 2 could result in *mMsx* 2 affecting chick PRE but not mouse PRE.

To investigate whether *mMsx* 2 function in chick PRE reflects the function of this gene in mouse PRE, an attempt was made to ectopically express *mMsx* 2 in cultured E12-E15 mouse PRE. This approach proved impractical since the quality and quantity of material limited the number of transfected cells available for assay. An alternative approach was therefore taken; transgenic mice were generated which would express *mMsx* 2 in the PRE during normal retina development. In contrast to cell culture, the PRE *in vivo* maintains its normal cell-cell contacts and is surrounded by its natural *in vivo* environment. Thus, a combination of *in vivo* and cell culture approaches to studying Msx function in PRE cells will help determine the extent to which the *in vivo* PRE environment effects *mMsx* 2 function.
During the synthesis of pigment within the PRE, Trp 2 converts DOPAchrome into 5, 6-dihydroxyindole 2-carboxylic acid (DHICA; Mackenzie et al., 1997b), whilst Trp 1 converts DHICA into 5, 6-quinone 2-carboxylic acid (Kobayashi et al., 1994). Mice null for Trp 1, brown mutation, (Shibahara et al., 1991; Jackson, personal communication) have normal, pigmented eyes demonstrating that this gene is not essential for pigmentation of the eye. Eyes of Trp 2 mutant mice are also pigmented. However it is not known if this is a null mutation and consequently whether Trp 2 is essential for the production of pigment in the eye (Budd and Jackson, 1995). 1.4kb of Trp 1 promoter, extending from -1334 to 107 and containing part of the first exon, directs Lac Z expression to the PRE from E11 onwards (Raymond and Jackson, 1995). 3.6 kb of Trp 2 promoter, extending from 454 to -3181, directs Lac Z expression to the presumptive PRE from late E9.5 onwards (MacKenzie et al., 1997b).

1.8 kb of the Trp 1 and 3.6 kb of the Trp 2 promoters were used in this study to drive Msx 2 expression in the PRE. To monitor transgene expression, an IRES/βGeo cassette was cloned downstream of the mMx 2 cDNA. The study described in this chapter was performed in collaboration with S. Jordan and L. McInnes, where indicated in the main text, and is only a preliminary investigation. Consequently, further research will be required to fully interpret the results of this chapter.

### 8.2 Msx 2 expression driven by 1.8 kb of the Trp 1 promoter

#### 8.2.1 Generation of a Trp1-driven Msx 2 expression construct

(pTrp1iM2IβGeoSV40)

**Step 1**

The multiple cloning site of pSK was modified by digesting pSK Msx 2a (chapter 5.2.1) with Sca I (cuts pSK at 2526) and Nsi I (cleaves within the 3' adapter of pSK Msx 2a; chapter 2.2.4 and 2.3). This removed the 1272 bp Msx 2 insert, along with
1123 bp of vector sequence, leaving the Mlu I, Bgl II and Sal I sites of the adapter in place. This plasmid was circularised with a 1145 bp fragment generated by digesting pSK with Sca I (position 2526) and Pst I (position 711) to give a modified pSK plasmid that contains a Bgl II site (pSKBgIII; chapter 2.1, 2.2.7 and 2.3).

Step 2

The CMV promoter, intron, Msx 2 cDNA, IRES/βGeo cassette and SV40 polyadenylation signals were transferred in two parts from pCiM2IβGeoSV40 (chapter 5.2.1) into the Bgl II/Bam HI sites of pSKBgII. pCiM2IβGeoSV40 was digested with Bgl II (cleaves 5 bp upstream of CMV) and Bam HI (cleaves 398 bp downstream of βGeo and 11 bp downstream of SV40) to produce a 6984 bp fragment comprising of the CMV promoter, intron, Msx 2 cDNA and the IRES/βGeo cassette, a 1062 bp fragment containing the SV40 polyadenylation signals and a 3691 bp fragment of unwanted vector (chapter 2.2.4 and 2.3). The Bgl II/Bam HI 6948 bp fragment was cloned into the Bgl II/Bam HI sites of pSKBgIII (chapter 2.1 and 2.2.7). Clones in which the Bgl II end of the insert had ligated to the reciprocal Bgl II site of pSKBgIII were selected if they released a fragment approximately corresponding to the predicted 6948 bp fragment upon digestion with Bgl II and Bam HI (chapter 2.2.4 and 2.3; fig. 8.1). Clones in which the insert had been ligated in the opposite orientation would not have released the insert upon digestion with these enzymes. A selected clone was then digested with Bam HI (chapter 2.2.4) and the 1062 bp Bam HI fragment, containing the SV40 polyadenylation signal, inserted (chapter 2.1 and 2.2.7). The orientation of this insert was determined by digesting the clones with Xba I (chapter 2.2.4 and 2.3). This enzyme was predicted to cleave 22 bp downstream of Msx 2, 30 bp upstream of SV40 and 23 bp downstream of SV40, to yield 5777, 274 and 5330 bp fragments if the insert was in the correct orientation. Clones displaying this pattern of digestion were termed pSKCiM2IβGeoSV40 (fig. 8.1).
Fig. 8.1 Step 2, generation of the Trp 1-driven Msx 2 expression construct

(pTrp1iM2lβGeoSV40). Ai) A 6948 bp Bgl II/Bam HI fragment comprising of
CMV-intron-mMsx 2- IRES-βGeo was cloned into the Bgl II and Bam HI sites of pSKBgl II
to generate pSKCiM2lβGeo (sites indicated in bold). Aii) Agarose-gel electrophoresis of the
products from Bgl II/Bam HI restriction analysis of 10 pSKCiM2lβGeo clones. As predicted
clones 4, 7 and 9 released the >6 kb insert from the 3 kb vector upon digestion with these
enzymes, confirming that the insert had been cloned in the correct orientation. Bi) A Bam HI
fragment, approximately 1 kb in size and comprising the SV40 polyadenylation signal, was
cloned into the Bam HI site of pSKCiM2lβGeo to generate pSKCiM2lβGeoSV40 (sites
indicated in bold). Xba I was predicted to cleave pSKCiM2lβGeoSV40 as indicated in (Bi).
Bii) Agarose-gel electrophoresis of the products from Xba I restriction digest analysis of 2
pSKCiM2lβGeoSV40 clones. Only clone 1 gave expected digestion pattern, confirming the
orientation of the SV40 polyadenylation signal. The broad band >3 kb, marked by an asterisk,
corresponds to the expected 5.7 and 5.3 kb fragments. M = 1 kb DNA ladder.
Step 3

The 4.5 kb Trp I promoter, as described in 8.1, was released from pBFLP.Trp I (gift from S. Jordan) as a Bam HI fragment (chapter 2.2.4 and 2.3). This fragment was cloned into the Bam HI site (719) of pSK (chapter 2.1 and 2.2.7) and the orientation of the insert determined by Sac I single-digests and Sac I/Bgl II double-digests (chapter 2.2.4 and 2.3). Clones were selected in which the Pst I site of pSK lay 3' of the promoter and were termed pSKTrpI. These clones digested to give fragments approximately corresponding to the predicted 2140 bp and 5360 bp fragments upon Sac I digestion and 2100 bp, 600 bp and 4720 bp fragments upon Sac I and Bgl II digestion (fig. 8.2). A 1.8 kb portion of the Trp 1 promoter, extending from 107 to the Bgl II site, was removed from pSKTrpI as a Bgl II/Pst I fragment (chapter 2.2.4 and 2.3) and ligated into the reciprocal sites of pCiM2SV40 (chapter 5.2.1; chapter 2.1 and 2.2.7). This replaced the CMV promoter with the 1.8 kb Trp 1 promoter in the correct orientation to drive Msx 2 expression. This cloning step was verified by digesting clones with Bgl II and Pst I, which released the 1.8 kb promoter from the vector, and with Xba I which yielded fragments corresponding approximately to the predicted 3314 and 2942 bp fragments (chapter 2.2.4 and 2.3; fig. 8.2). This construct, termed pTrp1iM2SV40, contains a chimeric intron between the promoter and cDNA, and SV40 polyadenylation signals below the cDNA insert.

Step 4

The 1.8 kb Trp 1 promoter, intron and Msx 2 cDNA were removed from pTrp1iM2SV40 as a Bgl II/Nsi I fragment (chapter 2.2.4 and 2.3). This fragment was cloned into the reciprocal Bgl II/Nsi I sites of pSKCiM2IβGeoSV40, replacing CMV-intron-Msx 2 with Trp 1-intron-Msx 2, thus generating pTrp1iM2IβGeoSV40 (chapter 2.1 and 2.2.7). A thorough restriction digest analysis confirmed the success of this cloning step (chapter 8.2.2).
Fig. 8.2 Step 3, generation of the Trp 1-driven Msx 2 expression construct (pTrp1iM2IβGeoSV40). Ai) The 4.5 kb Trp 1 promoter was cloned into the Bam HI site of pSK to generate pSKTrp1 (sites indicated in bold). The predicted locations of Sac I and Bgl II restriction sites are indicated in (Ai). Aii) Agarose-gel electrophoresis of the products from either a single Sac I, or double Sac I and Bgl II, restriction digest analysis of 7 pSKTrp1 clones. Only clone 2 and 3 yielded the expected >5 kb and approximate 2 kb fragments upon Sac I digestion, and the expected approximately sized 5 kb, 2 kb and 600 bp fragments upon Sac I/Bgl II double digestion. This confirmed that for clones 2 and 3 the Trp 1 promoter had been cloned in the correct orientation. Bi) 1.8 kb of the Trp 1 promoter was released from pSKTrp1 by Bgl II/Pst I digestion and cloned into the reciprocal sites of pCiM2SV40 to generate pTrp1iM2SV40 (cloning sites indicated in bold). Xba I was predicted to cleave pTrp1iM2SV40 as indicated in (Bi). Bii) Agarose-gel electrophoresis of the products from a single Xba I, or double Bgl II/Pst I restriction analysis of 6 pTrp1iM2SV40 clones. All clones, with the exception of clone 1, yielded the expected 4.5 kb and 1.8 kb fragments upon Bgl II/Pst I digestion, and the expected 2.9 kb and 3.3 kb fragments upon Xba I digestion. This confirmed that the 1.8 kb Trp 1 promoter had been cloned in the correct orientation. M = 1 kb DNA ladder.
In summary, the Trp 1-driven mouse Msx 2 expression construct
(pTrp1iM2IβGeoSV40) possesses 1.8 kb of Trp 1 promoter upstream of a chimeric
intron (Bothwell et al., 1981) and mouse Msx 2. An IRES/βGeo cassette has been
cloned downstream of Msx 2, followed by the SV40 late polyadenylation signal. The
entire 9387 bp transgene can be released by Bgl II/Spe I digestion with no vector
sequence 5' and only 17 bp 3'.

8.2.2 Restriction digest analysis of the Trp 1-driven Msx 2 expression
construct (pTrp1iM2IβGeoSV40)

The integrity of the Trp 1-driven Msx 2 expression construct was confirmed by
restriction digest analysis as summarised in fig. 8.3 (chapter 2.2.4 and 2.3). These
enzymes cleaved the construct into the predicted number of fragments which were
approximately the predicted size. This confirmed that the inserts had been successfully
cloned in the correct location, orientation and not as concatamers.
Fig. 8.3 Restriction digest analysis of the Trp1-driven M\textit{sx} 2 expression construct (pTrp1iM2I\(\beta\)GeoSV40). B) Agarose-gel (1\%) electrophoresis of the products generated from digesting pTrp1iM2I\(\beta\)GeoSV40 with the enzymes indicated in (A). A) Restriction digest map of pTrp1iM2I\(\beta\)GeoSV40 showing the predicted locations of the restriction sites for the enzymes used in (B) and the sizes of the fragments expected following digestion. Sizes in brackets are the approximate size of the fragments actually observed. Sizes in bold correspond to broad bands which may contain two or more similarly sized fragments. Asterisks correspond to fragments that were too small to be detected in this experiment. M = 1 kb DNA ladder.
8.2.3 Sequence analysis of the Trp 1-driven Msx 2 expression construct (pTrp1iM2IβGeoSV40)

The orientation of the Trp 1 promoter within the Trp 1-driven mMsx 2 expression construct (pTrp1iM2IβGeoSV40) was confirmed by sequence analysis (chapter 2.5). The primer, K665, anneals approximately 31 bp downstream of the Trp 1 promoter of this construct. As illustrated in fig. 8.4 and 8.5, sense-sequence generated from this primer aligned against the predicted 3' end of the Trp 1 promoter (107 to -27). This confirmed that the Trp 1 promoter was present in the correct orientation to drive mMsx 2 expression.

Fig. 8.4 Summary of the sequence analysis of the Trp 1 promoter within the Trp 1-driven Msx 2 expression construct (pTrp1iM2IβGeoSV40). Schematic alignment of the sequence generated from K665 to the published Trp1 sequence in the HGMP database (refer to main text and fig. 8.5).
Fig. 8.5 Sequence comparison of the 3' end of the Trp 1 promoter (107 to -27) in pTrp1iM2IβGeoSV40 with the published sequence in the HGMP genome database. The sense-strand was sequenced using the primer K665. pTrp2iM2IβGeoSV40 sequence is above the published sequence. Sequences were compared using the gcg "Gap" program. "N" corresponds to an undetermined nucleotide and "." represents the insertion of a gap to maintain alignment. Note that this data is from a single sequencing reaction. Therefore it is not possible to comment on the mismatches.

136 ................................................. GTTATA 131
-70 ATCACAAAGGAAAACCAGTGGGAGGGAGTCATGTGCTGCCTAGTAGTTAAA -21
130 GGCAAGAGNAATTCAGTGCGAGGNNANNNTAGTGNAGCTGGANGA 81
-20 GGCAAGGA.AAATTCGCTGAGAAGGGATTAGTGAGAGCTGGAAG. 27
80 NNAGGGCCAGCCCCTCCCAATGNTGGGAGTTGGATGCCTGGGAAG. 31
28 AGAGGACCCCATCCCAATGCTGGCTTGGGATTTACTGTC 77
30 TGNCCGAAAATCTCTTCGGCAATTAACAG 1 3'
78 TGGCAGAAAATCTCTTCGGGCAATTAACAGCTGGCATCAGGGGAAAAGCA 127 3'

8.2.4 In vitro functional test of the Trp 1-driven Msx 2 expression construct (pTrp1iM2IβGeoSV40)

Prior to generating transgenic mice carrying the Trp 1-driven mMsx 2 transgene (Trp1iM2IβGeoSV40), chick PRE cells were transiently transfected with this construct to confirm that it produced a transcript encoding mMsx 2 and βGal. PRE from 6d chick eyes was dissociated, split between 6 wells (area/well ~2 cm²) and cultured in EMEMF for approximately 24 hours (chapter 2.10.2). These cultures were then transiently transfected, in duplicate, with either the Trp 1-driven mMsx 2 expression construct, the control construct (pCiIβGeoSV40) or with no DNA (chapter 2.10.3). 3 days after transfection, total RNA was prepared from one set of cultures and the other stained for βGal activity (chapter 2.6.3.2 and 2.10.4).

First-strand cDNA was prepared from total RNA isolated from mMsx 2 and control transfected PRE cultures, along with total RNA prepared from G7 myoblast cells, 15d mouse limb buds and stage 24-25 chick limb buds. Myoblast and mouse limb bud
RNA served as positive controls while chick limb bud RNA served as a negative control for \(m\text{Msx} 2\) (refer to table 8.1 and chapter 2.7.3). PCR was performed on each cDNA sample primed either by the mouse \(M\text{sx} 2\) primers (J562 and J563) which amplify a 407 bp fragment, or \(\beta\text{Actin}\) primers (J252 and J253) which amplify a 711 bp fragment (chapter 2.7.3.3). The \(\beta\text{Actin}\) primers amplified the predicted 711 bp band from all cDNA samples confirming that cDNA had been successfully generated from each RNA population (fig. 8.6 and table 8.1). The mouse \(M\text{sx} 2\) primers only amplified the predicted 407 bp fragment from cDNA prepared from \(m\text{Msx} 2\) transfected PRE, G7 cells and mouse limb bud (fig. 8.6 and table 8.1). The mouse \(M\text{sx} 2\) primers were unable to anneal to chick \(M\text{sx} 2\) cDNA (fig. 8.6 and table 8.1). These results demonstrated that the 1.8 kb Trp 1 promoter was active in chick PRE and that the Trp 1-driven \(m\text{Msx} 2\) expression construct could produce \(m\text{Msx} 2\) mRNA in these cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Construct</th>
<th>Amount of RNA used in cDNA synthesis ((\mu\text{g}))</th>
<th>(m\text{Msx} 2) expression</th>
<th>(\beta\text{Actin}) expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>6d PRE</td>
<td>pTrpl(\text{iM2})(\beta)GeoSV40</td>
<td>0.25</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>6d PRE</td>
<td>pCI(\beta)GeoSV40</td>
<td>N/D</td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td>n</td>
<td>y</td>
</tr>
<tr>
<td>Chick limb bud (stage 24-25)</td>
<td></td>
<td></td>
<td>1</td>
<td>y</td>
</tr>
<tr>
<td>Mouse limb bud (E15)</td>
<td></td>
<td></td>
<td>1</td>
<td>y</td>
</tr>
</tbody>
</table>

Table 8.1 Summary of RT-PCR analysis of PRE cells, transfected with either the Trp 1-driven \(m\text{Msx} 2\) expression construct or the control construct, for \(m\text{Msx} 2\) expression. 6d chick PRE was grown for 24 hrs before being transiently transfected with either the Trp 1-driven \(M\text{sx} 2\) expression construct (pTrpl\(\text{iM2}\)\(\beta\)GeoSV40) or the control construct (pCI\(\beta\)GeoSV40). 3 days after transfection total RNA was isolated and RT-PCR performed as described in the main text and fig. 8.6. N/D = not determined.

As shown in fig. 8.6, a large proportion of pTrpl\(\text{iM2}\)\(\beta\)GeoSV40 transfected cells exhibited \(\beta\text{Gal}\) activity 3 days after transfection. This again confirmed that the 1.8 kb Trp 1 promoter was active in chick PRE cells and demonstrated that this construct could produce \(\beta\text{Geo}\) efficiently.
Fig. 8.6 Functional analysis of the Trp 1-driven Msx 2 expression construct in cultured 6d chick PRE cells. A) RT-PCR for Msx 2 expression in 6d chick PRE cultures, 3 days after transfection with either the Trp1-driven Msx 2 expression construct (pTrp1im2lβGeoSV40) or the control construct (pCiλβGeoSV40). cDNA was prepared from these cultures, G7 myoblasts, E15 mouse limb buds and stage 24-25 chick limb buds as described in the main text. Msx 2 cDNA was detected by PCR using primers J562 and J563 (M2) which amplify a 407 bp fragment and βActin cDNA using primers J252 and J253 (AC) which amplify a 711 bp fragment. M = 1 kb DNA ladder. B) βGal histochemistry of an equivalent pTrp1im2lβGeoSV40 transfected culture, x25. C) A pTrp1im2lβGeoSV40 transfected cell with a neural morphology (closed arrow), x25.
Only one out of 1500 pTrp1iM2IβGeoSV40 transfected 6d PRE cells observed was judged to have a neural morphology (shown in fig. 8.6C), no cells with a neural morphology were observed in the control-transfected cultures (n=1223). In an independent experiment, dissociated 5d chick PRE was grown in EMEMF for 24 hrs (chapter 2.10.2), then transiently transfected with either the Trp 1-driven mMsx 2 expression construct or the control construct (chapter 2.10.3). Two days later these cultures were stained for βGal activity (chapter 2.10.4). None of the 1195 mMsx 2-transfected cells analysed had a neural morphology. This was also the case for the 1504 control-transfected cells. In both sets of experiments, the ability of the cells to respond to Msx 2 expression by acquiring a neural phenotype was not confirmed by transfecting equivalent cells with the CMV-driven mMsx 2 expression construct (pCiM2IβGeoSV40). It will therefore be necessary to repeat these experiments before a confident conclusion can be made upon the effect that the Trp 1-driven Msx 2 expression construct has on the morphology of chick PRE cells.

8.2.5 Generation and analysis of Trp 1-driven Msx 2 transgenic mouse lines

The 9387 bp Trp1iM2IβGeoSV40 transgene was released from pSK by digestion with Bgl II and Spe I, cleaned and diluted to 2 ng/μl as described in chapter 2.8. This DNA was then micro-injected into the pronucleus of CBA x C57BL/6 F1 fertilised eggs. 225 of the 239 micro-injected eggs survived to the 2 cell stage. These embryos were then re-implanted into 9 pseudopregnant CD1 females. The micro-injection and re-implantation procedures were performed by S. Jordan.

Hosts were allowed to go to term, giving birth to 41 pups. Transgenic founders were identified by PCR of DNA, prepared from tail-tip biopsies, for βGeo using primers lacZ-1 and lacZ-2 which amplify a 370 bp fragment (chapter 2.7.2) As shown in fig. 8.7 five mice were positive for βGeo. The general behaviour and overall eye morphology of these mice appeared normal.
Fig. 8.7 Identification of Trp1-driven Msx 2 transgenic mice. DNA was extracted from tail biopsies of the offspring of CD1 mice implanted with Trp1iM21βGeoSV40 injected CBA x C57BL/6 F1 fertilised eggs. βGeo was detected by PCR using primers lacz-1 and lacz-2 which amplify a 370 bp fragment. PCR products were observed by electrophoresis on a 1.2% agarose-gel containing EtBr. M = 1 kb DNA ladder.
Two of the founders (A57.3 and A56) were female, so were crossed with male CBA x C57BL/6 F1 mice to generate transgenic males. The offspring of A57.3 (7 females and 9 males) did not carry the transgene, determined by PCR for βGeo (data not shown) demonstrating that this line did not transmit the transgene. Consequently this line was not analysed further. The offspring of A56 did include transgenic males, two of which were termed B294 and B300.1.

As summarised in table 8.2 the three male founders (A57, A57.1 and A57.2) and first generation A56 males (B294 and B300.1) were crossed with CD1 females and the embryos analysed at a variety of stages (E10.5-E15) for the presence of the transgene, gross eye abnormalities and expression of the transgene by βGal histochemistry (chapter 2.7.2 and 2.9.2). Transgenic embryos were identified by PCR analysis of DNA extracted from embryonic yolk sacs for βGeo using the primers lacZ-1 and lacZ-2 (chapter 2.7.2). The percentage of embryos carrying βGeo was near the predicted 50% for all four lines demonstrating that the transgene was being transmitted through the germ line. The gross eye morphology of transgenic embryos from all four lines was indistinguishable from wild type siblings in whole-mounts (data not shown). For each of the four lines, embryos carrying the transgene showed no βGal staining at any of the stages analysed (data not shown). The possible reasons for this are discussed further in chapter 8.4. In light of these observations no further analysis of the four Trp 1-driven Mx2 transgenic lines was performed. Transgenic embryos from lines A57, A57.1 and A57.2 were frozen down by B. Doe in anticipation of their use in future studies.

<table>
<thead>
<tr>
<th>Line</th>
<th>No. of embryos analysed at each stage</th>
<th>Percentage of embryos that are transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E10.5</td>
<td>E11.5</td>
</tr>
<tr>
<td>A57</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>A57.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A57.2</td>
<td>15</td>
<td>-</td>
</tr>
<tr>
<td>A56</td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 8.2 Summary for each Trp1-driven Mx2 line of the number of embryos analysed at each stage and the percentage of which were transgenic (refer to main text for details).
8.3  Msx 2 expression driven by 3.6 kb of the Trp 2 promoter

8.3.1  Generation of a Trp 2-driven Msx 2 expression construct
(pTrp2iM2IβGeoSV40)

Step 1

Full-length mMsx 2 cDNA was released from pSK Msx 2a (chapter 5.2.1) by Sal I which cleaves pSK at 674, 15 bp 5' to the Msx 2 insert, and Mlu I which cleaves 3' to Msx 2 within the adapter (chapter 5.2.1; chapter 2.2.4 and 2.3). This 1288 bp fragment was cloned into the Xho I (1058) and Mlu I (1069) sites of the promega pCI mammalian expression vector (chapter 2.2.7 and 2.1). This places Msx 2 downstream of the CMV promoter and a chimeric intron, and upstream of the SV40 polyadenylation signal to generate pCi-xM2SV40. Clones were analysed by Xho I/Mlu I digestion (chapter 2.2.4 and 2.3) which linearised the 5285 bp plasmid, confirming that the Xho I site had been destroyed by ligation with the Sal I end of Msx 2 (fig. 8.8A). The presence of Msx 2 cDNA was further confirmed by an Apa I/Nhe I double-digest (chapter 2.2.4 and 2.3) which yielded fragments approximately corresponding to the predicted 170 bp, 265 bp and 4850 bp fragments (fig 8.8A).

The CMV-intron-Msx 2 sequences were removed from pCi-xM2SV40 by digestion with Bgl II, which cleaves 5 bp upstream of CMV, and Xba I, which cleaves 22 bp downstream of Msx 2 (chapter 2.2.4 and 2.3). This 2363 bp fragment was cloned into the reciprocal Bgl II and Xba I sites of pSKBglII (chapter 8.2.1) to produce pSKCiM2 (chapter 2.2.7 and 2.1). The success of this cloning step was confirmed by digesting clones with Bgl II and Xba I (chapter 2.2.4 and 2.3) which yielded fragments corresponding to the predicted 2363 bp and 2939 bp fragments (fig 8.8B). In addition, digestion with Xho I (chapter 2.2.4 and 2.3) linearised the 5302 bp plasmid (fig 8.8B).
Fig. 8.8 Step 1, generation of the Trp 2-driven \( Msx \) 2 expression construct (pTrp2iM2IβGeoSV40). Ai) A 1.3 kb Sal I/Mlu I fragment containing full-length \( mMsx \) 2 cDNA was cloned into the Xho I/Mlu I sites of the Promega pCi expression vector to generate pCi-xM2SV40 (sites indicated in \textbf{bold}, asterisk indicates that the Xho I and Sal I sites were destroyed upon ligation). The predicted locations of Nhe I and Apa I restriction sites are indicated in (Ai). Aii) Agarose-gel electrophoresis of the products from either Apa I/Nhe I or Xho I/Mlu I restriction digest analysis of 4 pCi-xM2SV40 clones. For clones 1-3 Xho I/Mlu I digestion linearised the plasmid, confirming that the Xho I site had been destroyed. Apa I/Nhe I digestion of clones 1-3 released the expected fragments. Bi) The CMV-intron-\( Msx \) 2 sequences from pCi-xM2SV40 were cloned into the Bgl II/Xba I sites of pSKBgl II to generate pSKCiM2 (cloning sites indicated in \textbf{bold}). The predicted location of the Xho I restriction site is indicated in (Bi). Bii) Agarose-gel electrophoresis of the products from either Bgl II/Xba I or Xho I restriction analysis of 4 pSKCiM2 clones. For all clones the predicted 3 kb and 2.5 kb fragments were generated upon digestion with Bgl II/Xba I, whilst Xho I linearised the 5 kb plasmid as expected. Ci) The CMV-intron-\( Msx \) 2 sequences from pCi-xM2SV40 were also cloned into the Bgl II/Nsi I sites of pSKCiM2IβGeoSV40 to generate pSKCi-xM2IβGeoSV40 (cloning sites indicated in \textbf{bold}). Cii) Agarose-gel electrophoresis of the products from Nsi I/Bgl II restriction analysis of 4 pSKCi-xM2IβGeoSV40 clones. All 4 clones generated the expected 2.5 kb and >5 kb fragments upon Nsi I/Bgl II digestion. M = 1 kb DNA ladder.
The CMV-intron-\textit{Msx} 2 sequences were also removed from pCi-xM2SV40 by digestion with Bgl II, which cleaves 5 bp upstream of CMV, and Nsi I, which cleaves 9 bp downstream of \textit{Msx} 2 (chapter 2.2.4 and 2.3). This 2350 bp insert was ligated into the reciprocal Bgl II and Nsi I sites of pSKCiM2I\textbeta GeoSV40 (chapter 8.2.1), replacing the CMV-intron-\textit{Msx} 2 sequences with the modified version which does not contain a Xho I site between the intron and \textit{Msx} 2 cDNA (chapter 2.2.7 and 2.1). This modified construct was termed pSKCi-xM2I\textbeta GeoSV40 and the success of this cloning step confirmed by digesting clones with Bgl II and Nsi I (chapter 2.2.4 and 2.3) which released the predicted 2350 bp insert from the 9004 bp of IRES/\textbeta Geo-SV40-vector sequence (fig 8.8C).

Step 2

The 3634 bp Trp 2 promoter, extending from 454 to -3181, was removed from pPB2 (gift from S. Jordan) as a Xho I/Cla I fragment (chapter 2.2.4 and 2.3) and re-cloned into the Xho I (position 668) and Cla I (position 684) sites of pSK (chapter 2.2.7 and 2.1). Digestion of pSKTrp2 with Xho I and Cla I (chapter 2.2.4 and 2.3), yielded fragments approximately corresponding to the predicted 3634 bp insert and 2944 bp of vector confirming the success of this cloning step (fig. 8.9A).

The Trp 2 promoter was then released from pSKTrp2 by digesting with Xho I and Pst I (chapter 2.2.4 and 2.3). This 3661 bp fragment was then cloned into the Xho I and Pst I sites of pSKCiM2 (chapter 2.2.7 and 2.1), replacing the CMV promoter with the Trp 2 promoter to generate pSKTrp2iM2. This cloning step was confirmed by digesting with Xho I and Pst I (chapter 2.2.4 and 2.3), which yielded fragments approximately corresponding to the predicted 3661 bp and 4425 bp fragments (fig. 8.9B).
Fig. 8.9 Step 2, generation of the Trp 2-driven Msx 2 expression construct (pTrp2iM2IβGeoSV40). Ai) 3.6 kb of Trp 2 promoter was cloned into the Xho I/Cla I sites of pSK to generate pSKTrp2 (cloning sites indicated in **bold**). Aii) Agarose-gel electrophoresis of the products from Xho I/Cla I restriction digest analysis of pSKTrp2. As predicted, a 3 kb and 4 kb fragment was generated upon Xho I/Cla I digestion. Bi) The Trp 2 promoter from pSKTrp2 was cloned into the Xho I/Pst I sites of pSKCiM2 to generate pSKTrp2iM2 (cloning sites in **bold**). Bii) Agarose-gel electrophoresis of the products from Xho I/Pst I restriction digest analysis of 5 pSKTrp2iM2 clones. As predicted, 3.8 kb and 4.5 kb fragments were generated upon Pst I/Xho I digestion. M = 1 kb DNA ladder.
Ai

200 bp

pTrp 2

3634 2944

Xho I  Cla I

Aii

Xho I/Cla I

Bi

280 bp

pTrp 2

3661

Xho I

Pst I

Bii

Pst I/Xho I

1 2 3 4 5

M

bp

4.5kb

3.8kb

- 5090

- 4072

- 3054
Finally, the Trp 2-intron portion was removed from pSKTrp2iM2 by Xho I and Nhe I digestion (chapter 2.2.4 and 2.3). This 3883 bp fragment was then cloned into the Xho I and Nhe I sites of pSKCi-xM2IββGeoSV40 (chapter 2.2.7 and 2.1), replacing CMV with the Trp 2 promoter to generate pTrpM2IββGeoSV40. A thorough restriction digest analysis confirmed the success of this cloning step (refer to chapter 8.3.2).

In summary, the Trp 2-driven mouse Msx 2 expression construct (pTrp2iM2IββGeoSV40) possesses 3634 bp of Trp 2 promoter, upstream of a chimeric intron and mouse Msx 2. An IRES/βGeo cassette is located 3' of the Msx 2 cDNA and a SV40 late polyadenylation signal, 3' of the IRES/βGeo cassette. The entire 11234 bp transgene can be removed intact from the vector by digestion with Xho I and Spe I, leaving only 11 bp of vector sequence 5' and 10 bp of vector sequence 3'.

8.3.2 Restriction digest analysis of the Trp 2-driven Msx 2 expression construct (pTrp2iM2IββGeoSV40)

The integrity of the Trp 2-driven mMsx 2 expression construct was confirmed by restriction digest analysis as summarised in fig. 8.10 (chapter 2.2.4 and 2.3). These enzymes cleaved the construct into the predicted number of fragments which were approximately the predicted size. This confirmed that the inserts had been successfully cloned in the correct location, orientation and not as concatamers.
Fig. 8.10 Restriction digest analysis of the Trp 2-driven Msx 2 expression construct (pTrp2iM2IβGeoSV40). B) Agarose-gel (1%) electrophoresis of the products generated from digesting pTrp2iM2IβGeoSV40 with the enzymes indicated in (A). A) Restriction digest map of pTrp2iM2IβGeoSV40 showing the predicted locations of the restriction sites for the enzymes used in (B) and the sizes of the fragments expected following digestion. Sizes in brackets are the approximate size of the fragments actually observed. Asterisks correspond to fragments that were too small to be detected in this experiment. M = 1kb DNA ladder.
8.3.3 Sequence analysis of the Trp 2-driven Msx 2 expression construct (pTrp2iM2IβGeoSV40)

The orientation and gross structure of the mMsx 2 cDNA in the Trp 2-driven mMsx 2 expression construct (pTrp2iM2IβGeoSV40) was further confirmed by sequence analysis. The same mMsx 2 cDNA was sequenced during the construction of the CMV-driven mMsx 2 expression construct (chapter 5.2.3) and did not contain any mutations that were predicted to affect Msx 2 function. It is therefore unlikely that mutations have been introduced during the generation of the Trp 2-driven mMsx 2 expression construct. Nonetheless, as summarised in fig. 8.11 both strands of the Msx 2 cDNA insert have been partially sequenced (chapter 2.5).

Primer M859 annealed approximately 65 bp upstream of the Msx 2 insert. Anti-sense sequence generated using this primer spanned the junction between the vector and the 5' end of Msx 2. This sequence matched both vector and 5' Msx 2 sequence, confirming that the Msx 2 insert was in the correct orientation to produce Msx 2 (fig. 8.11 and fig. 8.12).

Within the 5' untranslated region an extra G was identified between nucleotides 41 and 42 of the anti-sense strand, whilst nucleotide 46 of the anti-sense strand, predicted to be a C, was sequenced as a G. The complementary strand to this region has not been sequenced, so it is possible that these changes are merely sequencing artefacts. However, it is interesting to note that these discrepancies were also observed when sequencing the Msx 2 insert of the CMV-driven Msx 2 expression construct (pCiM2IβGeoSV40; chapter 5.2.3). Nonetheless, these possible mutations lie 5' to the translation start site, so would not be predicted to affect the structure of Msx 2.

Both strands of Msx 2 have been sequenced between nucleotides 50 and 781, with the exception of a 41 bp region between 360 and 401 for which only the sense-strand has been sequenced. However, this 41 bp sequence precisely matched the published
sequence. In the adjacent regions no discrepancies between the published and Trp 2-driven mMsx 2 sequence were observed that were not correct in an overlapping fragment or the opposite strand.

382 bp of Msx 2, extending from 781 to the stop codon at 1165, has only been sequenced in one direction. Until the complementary sense-strand is sequenced, it will not be possible to exclude the possibility that a base pair change may have occurred in this region during the cloning of the Trp 2-driven Msx 2 expression construct that could render Msx 2 non-functional. However, this is unlikely as no mutations predicted to affect Msx 2 function were found in the same cDNA used in the CMV-driven mMsx 2 expression construct (chapter 5.2.3).

Fig. 8.11 Sequence analysis of Msx 2 within the Trp 2-driven Msx 2 expression construct (pTrp2iM2IβGeoSV40). Schematic alignment of the fragments of sequence generated from each primer with the published Msx 2 sequence in the HGMP database. The positions of residues which differ from the published sequence are only indicated for region A, since only one strand of region B has been sequenced (refer to main text and fig. 8.12).
Fig. 8.12 A) Comparison of pTrp2iM2IβGeoSV40 anti-sense Msx 2 sequence (0-1165) with the published sequence in the HGMP genome database. The underlined nucleotides correspond to the adjacent vector. B) Comparison of pTrp2iM2IβGeoSV40 sense Msx 2 sequence (50-781) with the published sequence in the HGMP genome database. The sequence in bold mark the translation start and stop codons, "N" corresponds to an undetermined nucleotide and "." signifies the insertion of a gap to maintain alignment.

**A) Comparison of compiled anti-sense Msx 2**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>1121</th>
<th>1166</th>
<th>1211</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence</td>
<td>TTAGGATAGATGTCACAAGCCATATCCAACNGNTGGCATAGAGT</td>
<td>CCAACAGGCGGGATGGGGAGCACAGGTCTATGGGGTAGGATGCGCC</td>
<td>CCAACAGGCGGGATGGGGAGCACAGGTCTATGGGGTAGGATGCGCC</td>
</tr>
<tr>
<td>Start codon</td>
<td>1171</td>
<td>1211</td>
<td>1211</td>
</tr>
<tr>
<td>Sequence</td>
<td>TTAGGATAGATGTCACAAGCCATATCCAAC</td>
<td>CCAACAGGCGGGATGGGGAGCACAGGTCTATGGGGTAGGATGCGCC</td>
<td>CCAACAGGCGGGATGGGGAGCACAGGTCTATGGGGTAGGATGCGCC</td>
</tr>
<tr>
<td>Stop codon</td>
<td>1171</td>
<td>1211</td>
<td>1211</td>
</tr>
<tr>
<td>Sequence</td>
<td>TTAGGATAGATGTCACAAGCCATATCCAAC</td>
<td>CCAACAGGCGGGATGGGGAGCACAGGTCTATGGGGTAGGATGCGCC</td>
<td>CCAACAGGCGGGATGGGGAGCACAGGTCTATGGGGTAGGATGCGCC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sequences were compared using the gcg "Gap" program. Nucleotides in bold mark the translation start and stop codons, "N" corresponds to an undetermined nucleotide and "." signifies the insertion of a gap to maintain alignment.
B) Comparison of compiled sense Msx 2.

```
732 .................................................. T 732
1 AAGCTCCCTCTTTAACAATCGGCTTTAATTACGGCTTGTTTGGTT 50
731 TGGGCGATTATACCCCTTGAGGGATCGCTAATTAAAGAATCCTGGCTGACTG 682
51 TGGGCGATTATACCCCTTGAGGGATCGCTAATTAAAGAATCCTGGCTGACTG 100
681 CTCCCTGTAATTACCTCTTAATTTTTTCTTCAACGGGGGAGAGGCCC 632
101 CTCCCTGTAATTACCTCTTAATTTTTTCTTCAACGGGGGAGAGGCCC 150
631 CAGCGGCTGCCAGATGAGAGTTGCCAGCGGTGTCGCGCGCCGACAGCTACGC 532
201 CAGCGGCTGCCAGATGAGAGTTGCCAGCGGTGTCGCGCGCCGACAGCTACGC 250
531 GACACGCTGCGCTCCAGGGACTCCAGCGGTGGCGAGGTCCGTGGCGTCAGG 482
251 GACACGCTGCGCTCCAGGGACTCCAGCGGTGGCGAGGTCCGTGGCGTCAGG 300
481 GAACTCTTCTGCTGAGAGTTGCCAGCGGTGGCGAGGTCCGTGGCGTCAGG 432
301 GAACTCTTCTGCTGAGAGTTGCCAGCGGTGGCGAGGTCCGTGGCGTCAGG 350
431 GACCAGGAAAGTGCTGCTCTTGACTAAAGGCGGTGACCTGCTTTTCGCT 382
351 GACCAGGAAAGTGCTGCTCTTGACTAAAGGCGGTGACCTGCTTTTCGCT 400
381 CGGATGAGGAGGGCCCCGCGGTACTGGCCGGCCCGGGTCCTGGGCTGGA 332
401 CGGATGAGGAGGGCCCCGCGGTACTGGCCGGCCCGGGTCCTGGGCTGGA 450
331 GAGGACGCGAGGGCGCAGCGCAGAGGAGCGCAGGGTCAAGGTCTCCAGCCCTGCC 282
451 GAGGACGCGAGGGCGCAGCGCAGAGGAGCGCAGGGTCAAGGTCTCCAGCCCTGCC 500
281 CTTCAGCCTGGAGGCGGTATACGTCAGAAGAAGCGCCTCAAGAGATCGC 232
501 CTTCAGCCTGGAGGCGGTATACGTCAGAAGAAGCGCCTCAAGAGATCGC 550
231 CGGCCGTCGCCACCGGACTGCGTCCTGCCCTGCTGHTCCGTGGGCTGCGT 182
551 CGGCCGTCGCCACCGGACTGCGTCCTGCCCTGCTGHTCCGTGGGCTGCGT 600
181 CTGCTGCGCGACGAGCAGCGGTTCGCTGCTGACTCCGCGGCTCTGCTC 132
601 CTGCTGCGCGACGAGCAGCGGTTCGCTGCTGACTCCGCGGCTCTGCTC 650
131 CAAAGGCTCTTCAGGAGCCGCTCGCAAGCAGATCGGAAAATCTCGAAGAGCAGGAG 82
651 CAAAGGCTCTTCAGGAGCCGCTCGCAAGCAGATCGGAAAATCTCGAAGAGCAGGAG 700
```
8.3.4 \textit{In vitro} functional test of the Trp 2-driven \textit{Msx 2} expression construct (pTrp2iM2IβGeoSV40)

Prior to generating transgenic mice carrying the Trp 2-driven \textit{mMsx 2} transgene (Trp2iM2IβGeoSV40), chick PRE cells were transfected to confirm that the construct could produce βGeo. PRE from 5d chick embryos was dissociated and cultured in EMEMF for approximately 24 hrs before being transiently transfected with either the Trp 2-driven \textit{Msx 2} expression construct (pTrp2iM2IβGeoSV40) or the control construct (pCiIβGeoSV40; chapter 2.10.2 and 2.10.3). These cells were then grown for a further 48 hrs in NB27 medium, then stained for βGal activity (chapter 2.10.4).

As shown in fig. 8.13, a large proportion of the cells in cultures transfected with the Trp 2-driven \textit{mMsx 2} expression construct exhibited βGal activity. This confirmed that the 3.6 kb Trp 2 promoter was active in chick PRE and demonstrated that the construct was capable of producing βGeo. No transfected PRE cells had a neural morphology. However, equivalent cells were not transfected with the CMV-driven \textit{Msx 2} expression construct (pCiM2IβGeoSV40) to demonstrate that the cells could respond to \textit{Msx 2} expression. A more thorough investigation into the effects transfection of the Trp 2-driven \textit{Msx 2} expression construct has on PRE cell morphology will be required before any conclusions can be made.
Fig. 8.13 Functional analysis of the Trp 2-driven Msx 2 expression construct in cultured 5d chick PRE cells. 5d chick PRE was dissociated, cultured for 24 hrs and transfected with the Trp 2-driven Msx 2 expression construct (pTrp2iM2IβGeoSV40). 2 days later cells were stained for βGal activity (refer to main text for details). x4 magnification.
8.3.5 Generation and analysis of Trp 2-driven *Msx 2* transgenic mouse lines

The 11234 bp Trp 2-driven *mMsx 2* (Trp2iM2IβGeoSV40) transgene was removed from the vector by Xho I and Spe I digestion, cleaned and diluted to 2 ng/μl as described in chapter 2.8. The transgene was then micro-injected into the pronucleus of CBA x C57BL/6 F1 fertilised eggs, 325 of which developed to the two cell stage. These embryos were then re-implanted into 11 pseudopregnant CD1 females. The pronuclear micro-injections and re-implantation of embryos were performed by L. McInnes.

Sixty-five offspring were screened for the presence of the transgene by PCR of DNA, extracted from tail biopsies, for βGeo using primers lacZ-1 and lacZ-2 which amplify a 307 bp fragment of the gene (fig. 8.14; chapter 2.6.2 and 2.7.2). Seven founders were identified (3 male and 4 females) whose general behaviour and overall eye morphology appeared normal. The four female founders (A80.1, A81.3, A81 and A88) were crossed with CBA x C57BL/6 F1 males to generate transgenic males.

As summarised in table 8.3, the 3 male founders (A80, A81.1 and A82.2) and next generation A80.1 (B562), A81.3 (B561, B561.3) and A81 (B581, B581.1) males were crossed with CBA x C57BL/6 F1 females. The embryos from these matings were analysed at a variety of stages (E9.5-E13.5) for the presence of the transgene (chapter 2.6.2 and 2.7.2), gross eye abnormalities and transgene expression by staining for βGal activity (chapter 2.9.2). A transgenic male was obtained from the A88 x (CBA x C57BL/6) F1 mating and termed B684 but this line has not yet been analysed.
Fig. 8.14 Identification of Trp 2-driven Msx 2 transgenic mice. DNA was extracted from tail biopsies of offspring from pseudopregnant CD1 mice implanted with Trp2iM2iβGeoSV40 injected CBA x C57BL/6 F1 fertilised eggs. βGeo was detected by PCR using primers lacZ-1 and lacZ-2 which amplify a 370 bp fragment. PCR products were observed by electrophoresis on a 1.2% agarose-gel containing EtBr. Only a selection of the 65 tail biopsies are shown. Tail biopsy 177 from founder mouse A81.1 is not shown.
Table 8.3 Summary for each Trp 2-driven mMsx 2 transgenic line, of the number of embryos analysed at each stage and the percentage of which were transgenic. Line A81 exhibited βGal activity at all stages analysed (refer to main text for details).

All embryos generated from A80, A81.1 and A81.2 lines did not carry the transgene, judged by PCR analysis of DNA extracted from embryonic yolk sacs for βGeo using the primers lacz-1 and lacz-2 (chapter 2.6.2 and 2.7.2). Embryos from these lines did not stain for βGal and their gross eye morphology was indistinguishable from wild type siblings (data not shown). This demonstrated that for A80, A81.1 and A81.2 the transgene (Trp2iM2IβGeoSV40) was not being transmitted through the germ line.

The predicted number of embryos, 50%, were transgenic for lines A80.1 and A81.3 demonstrating that at least the βGeo portion of the Trp2iM2IβGeoSV40 transgene was being transmitted through the germ line. However, transgenic embryos from these lines, at the stages summarised in table 8.3, exhibited no βGal activity and had a gross eye morphology indistinguishable from wild type siblings at all the stages analysed (data not shown). Consequently, no further analysis was performed on these lines.

Analysis of line A81

38% of A81 embryos carried the transgene demonstrating that the transgene could be transmitted through the germ line (chapter 2.6.2 and 2.7.2). Transgenic embryos at all the stages analysed (E9.5, E11.5 and E13.5) exhibited βGal staining in the eye.
confirming that the transgene was being expressed (chapter 2.9.2). Whole-mount staining of E9.5 embryos for βGal showed very weak staining in the dorsal side of the optic vesicle (fig. 8.15). At E11.5 and E13.5, patches of βGal-positive cells were observed in the PRE, often arranged in columns along the distal/proximal axis (fig. 8.15). Outwith the eye, cells in the telencephalon and trunk adjacent to the hind limb were also stained for βGal (data not shown). These sites are also stained in transgenic mice which only express βGal from the same 3.6 kb Trp 2 promoter (MacKenzie et al., 1997b).

To analyse βGal activity in A81 embryos in more detail, whole-mount stained embryos were fixed, embedded in wax, serially sectioned at 7 μm and counter-stained with haematoxylin and eosin (chapter 2.9.2, 2.9.3 and 2.9.4). The weak βGal stain observed in whole-mount E9.5 embryos could not be detected after sectioning. Sections through the eyes of E11.5 and E13.5 βGal-stained transgenic embryos revealed that βGal-positive cells were located in the PRE and, in E11.5 transgenic eyes, also the non-pigmented inner layer of the presumptive ciliary margin. For unknown reasons the βGal stain leached out of these sections before they could be photographed. However, C. Oram has since confirmed these observations and shown that in the eyes of E10.5 A81 embryos βGal-positive cells are also present in the PRE and inner layer of the presumptive ciliary margin (fig. 8.16; personal communication).

The gross morphology and degree of pigmentation of A81 transgenic E9.5, E11.5 and E13.5 eyes was indistinguishable from wild type siblings in whole-mounts and in sections (fig. 8.15 and fig. 8.17).
Fig. 8.15 Whole-mount staining of A81 embryos for βGal activity. A and B) E9.5. C and D) E11.5. E and F) E13.5. A) Weak staining in the dorsal optic vesicle at E9.5 is indicated by the arrow. B, D and F) Comparison of the gross morphology of A81 transgenic eyes with the eyes of wild type siblings. Embryos were obtained by mating either B581 or B581.1 with CBA x C57BL/6 F1 females.
Fig. 8.16 A section through the eye of a βGal stained E10.5 A81 embryo. This embryo was obtained by crossing B581 with CBA x C57BL/6 F1 females. Embryos were stained for βGal, fixed and embedded in wax. Sections were cut at 7 microns. βGal-positive cells were detected in the PRE (arrow) and in cells of the inner layer of the presumptive ciliary margin (arrowhead). nr = neural retina, PRE = pigmented retina epithelium, lv = lens vesicle. x20 magnification. Photograph provided by C. Oram.
Fig. 8.17 Histological comparison of A81 eye morphology between transgenic and wild type siblings. A and B) E9.5, x20. C and D) E11.5, x20. E and F) E13.5, x10. A, C and E) Transgenic. B, D and F) Wild type. Embryos were obtained from male B581 or B581.1 X (CBA x C57BL/6) F1 female matings. Embryos were stained for βGal, post fixed in 4% PFA and wax embedded. 7 micron sections were then counter stained with haematoxylin and eosin. Note that the βGal stain leached out of these sections before being photographed. No differences between the morphology of transgenic eyes and wild type eyes were observed at any of the stages analysed. ov = optic vesicle, lv = lens vesicle, nr = neural retina, PRE = pigmented retina epithelium and l = lens.
8.4 Conclusion

Only one transgenic line (A81) exhibited transgene activity, as judged by βGal activity, out of the four Trp 1-driven Msx 2 lines and three Trp 2-driven Msx 2 lines that transmitted the transgene. Therefore, it will be necessary to generate additional Trp 2-driven Msx 2 lines in the future to recapitulate the effects observed in A81 mice. The Trp 1-driven transgene was expected to be active in the PRE from E11.5 onwards (Raymond and Jackson, 1995) and the Trp 2-driven transgene in the presumptive PRE from E9.5 onwards (MacKenzie et al., 1997b). It is not yet clear why such a high proportion of Trp-driven Msx 2 lines failed to exhibit transgene activity at the stages analysed.

Chick PRE cells transfected with either the Trp 1- or Trp 2-driven Msx 2 expression construct produced βGeo confirming that both the Trp 1 and Trp 2 promoters used were active in at least chick PRE and that IRES mediated translation of βGeo was functional. Furthermore, mouse Msx 2 mRNA could be detected by RT-PCR in chick PRE cells transfected with the Trp 1-driven expression construct. Translation of βGal in transgenic mouse embryos, mediated by IRES sequences has been described (Kim et al., 1992; Mountford et al., 1994). Thus, there are no reasons to suspect that the apparent lack of transgene activity in the majority of the transgenic lines was due to the design or construction of these constructs.

It is possible that rearrangements of the transgenes may have occurred upon integration into the genome, therefore disrupting their function, or that these lines may express Msx 2 but not βGeo. Both these scenarios could account for the apparent lack of transgene activity but have not yet been investigated. Alternatively, the lack of transgene activity may be due to so-called 'position effects' in which the transgene is thought be silenced at the chromatin level in a position-dependant fashion. In particular transgenes comprising of mammalian cDNAs or prokaryotic reporter genes are particularly prone to repressive position effects, indeed it has been suggested that such sequences serve as active foci for gene silencing (Clark et al., 1997). This type
of silencing may account for the high proportion of Trp-driven Msx 2 transgenic lines with no transgene activity, since both transgenes contain Msx 2 cDNA and the large prokaryotic βGeo reporter gene.

Preliminary analysis of A81 transgenic embryos, suggests that the Trp 2-driven transgene is only active in small patches of PRE. In contrast, Trp 2-LacZ transgenic mice express Lac Z uniformly through out the PRE (MacKenzie et al., 1997b). Again the reasons for this apparent difference in promoter activity is not yet known. The patches of βGal-positive cells may represent clones of cells, that arose during development from a single PRE precursor cell in which the suppression of the transgene was lost before proliferation ceased. Alternatively, the transgene may be active in all cells of the PRE, but for some unknown reason IRES mediated translation of βGeo only occurs in a small proportion of these cells. To establish whether or not the transgene is active in all cells of the PRE it will be important to perform in situ hybridisation on A81 embryonic eyes for Msx 2 expression.

The absence of expression in most lines and the patchy expression of the transgene in the PRE of A81 may be a consequence of Msx 2 function. It was predicted that ectopic expression of Msx 2 in the PRE of mice would drive neurogenesis. This in turn might imply that the pathways controlling PRE differentiation would be down-regulated in response to ectopic Msx 2 expression, consequently resulting in the loss of Trp-driven transgene expression. To explore this possibility further it would be necessary to confirm that Msx 2 is indeed being ectopically expressed in these mice and to generate control transgenic mice in which the Msx 2 cDNA has been mutated.

Ectopic expression of mouse Msx 2 in chick PRE cells resulted in a small proportion acquiring a neural phenotype. However, the eyes of transgenic A81 embryos at all the stages analysed were indistinguishable from wild type siblings. This may suggest that mouse Msx 2 does not have the same effect in mouse PRE cells in vivo. However, it will not be possible to reach this conclusion until it has been confirmed that Msx 2 is
indeed being ectopically expressed in the PRE of these mice by *in situ* hybridisation. The *Msx 2* insert of pTrp2iM21\(\beta\)GeoSV40 has not been fully sequenced. It is therefore also possible that a mutation may have occurred during the generation of this construct that affects *Msx 2* function. However, this is unlikely since no alterations that are predicted to affect *Msx 2* function were identified when the same cDNA was sequenced in the CMV-driven *mMsx 2* expression construct (chapter 5.2.3).

It is conceivable that PRE cells may only be able to respond to *Msx 2* expression when removed from their *in vivo* environment. Thus, the maintenance of direct cell-cell contacts and *in vivo* factors in A81 transgenic mice, as opposed to dissociated cultures, may prevent or limit the ability of PRE to respond to *Msx 2*. This idea could be further investigated by dissociating and culturing PRE from A81 transgenic embryos. These cells, when removed from their *in vivo* environment, may then respond by acquiring a neural phenotype. Although PRE cells ectopically expressing *Msx 2* *in vivo* may not exhibit a neural morphology, they may nonetheless express neural cell-type specific markers. It will be very interesting to learn if any neural markers are mis-expressed in the PRE of these mice. In particular it would be important to establish if the neuronal marker TuJ1 is expressed in the PRE of A81 transgenic embryos, since approximately three quarters of the neural cells induced in chick PRE cultures by *mMsx 2* express this marker.

The observation that \(\beta\)Gal-positive cells were observed in the non-pigmented inner layer of the ciliary margin in A81 transgenic embryos might be relevant to *Msx 2* function. PRE cells at the distal tip of the retina ectopically expressing *Msx 2* may acquire a neural fate in accordance with the proposed function of *Msx 2*. By virtue of their position, these cells may then be able to contribute to the inner layer of the retina (presumptive neural retina). This observation will have to be further investigated by comparing the position of \(\beta\)Gal-positive cells in A81 transgenic embryos with embryos expressing only \(\beta\)Geo from the same Trp 2 promoter, or transgenic embryos
in which the *Msx 2* insert of the Trp 2-driven *Msx 2* expression construct has been mutated.
Chapter 9

Discussion

9.1  *Msx* function in chick PRE cultures

The differentiated state of PRE is maintained *in vivo* and *in vitro* by direct cell-cell interactions. Disruption of these interactions, either by repeatedly passaging cultured PRE cells at a low density or by treating with phenylthiourea and testicular hyaluronidase, which affect cell surface properties, results in dedifferentiation associated with the down-regulation of PRE markers and an increase in proliferation (Itoh and Eguchi, 1986; Grisanti and Guidry, 1995; chapter 4.5).

In common with PRE, the differentiated state of chick Muller cells is also maintained by direct contact with neighbouring cells, in particular neurons. Again, physical disruption of these contacts leads to the dedifferentiation of Muller cells into a population of cells with similar characteristics to dedifferentiated PRE (Moscona, 1986). Both dedifferentiated PRE and dedifferentiated neural retina cells can differentiate into lens-like cells or PRE cells depending upon culture conditions (Itoh and Eguchi, 1986; Moscona, 1986; Agata *et al.*, 1993; Turque *et al.*, 1996). These shared properties and characteristics suggest that dedifferentiated PRE and dedifferentiated neural retina cells are equivalent in terms of their potential for differentiation. The common developmental origin of PRE and neural retina might suggest that dedifferentiated neural retina and dedifferentiated PRE are comparable to uncommitted optic vesicle cells.

*In vivo*, PRE and neural retina are both derived from the optic vesicle, and *in vitro* treatment of PRE with FGF 2 can drive transdifferentiation into neural retina (chapter 1.6). Thus, the very small proportion of PRE cells in dedifferentiated cultures, but not in primary differentiated cultures, that spontaneously acquire a neural morphology (chapter 5.5) may be neural retinal cell types. These observations suggest that destabilization of the differentiated PRE state enables PRE cells to enter a default
neurogenic pathway or to undergo neurogenesis as a consequence of the culture conditions. Indeed, it has been shown that disruption of cell-cell interactions leads to an up-regulation of FGF 2 in PRE cultures (Bost et al., 1994). This gene encodes a protein that is capable of transdifferentiating PRE explants to neural retina (Pittack et al., 1991; Guillemot and Cepko, 1992). Thus, endogenous FGF 2 expression in dedifferentiated PRE cells may cause them to undergo neural differentiation. Ectopic expression of either mouse Msx 1 or Msx 2 in dedifferentiated PRE cultures significantly increases the proportion of PRE cells acquiring a neural phenotype, whilst ectopic Msx expression in primary cultures induces neural cells that would not otherwise be produced (chapter 5). Msx could therefore function in PRE cultures to drive or enhance the dedifferentiation of PRE, which indirectly results in the up-regulation of FGF 2 and neural differentiation. Alternatively, Msx could play a more direct role in driving neurogenesis in PRE cells than merely destabilising the PRE state of differentiation.

Preliminary observations suggest that ectopic Msx 2 expression in chick PRE cultures results in the production of at least two distinct neural cell types. One class has a bipolar morphology and expresses the neuronal marker TuJ1, whilst the other has several processes and is either positive or negative for TuJ1 (chapter 6.2). TuJ1 is a very early neuronal marker and may be expressed by all retinal neurons (chapter 6.7). It will therefore be interesting to learn precisely what types of retinal neurons are generated by Msx 2. It has been reported that TuJ1 does not label glial cells (Trimmer et al., 1986). This raises the possibility that TuJ1-negative, Msx 2-induced, neural cells are Muller cells (Trimmer et al., 1986; Lee et al., 1990). Whether these different cell types arise from a single PRE cell, or from different PRE cells that each give rise to only one neural cell type, remains to be established. Thus, Msx 2 may function in one of two ways; either to generate multipotent precursors or to directly drive the differentiation of several different neural cell types (fig. 9.1). To address this important question, time-lapse analysis of cultures transfected with a Msx 2 expression construct containing the Green fluorescent protein reporter gene (GFP) could be performed followed by immunocytochemistry for neural retina cell-type
specific markers. GFP can be detected in living cells (Pines, 1995) making it possible to trace the lineage of any GFP-expressing cell.

During retina development, Msx 2 is expressed in undifferentiated cells of the optic vesicle. Therefore, Msx 2 may only be able to function in dedifferentiated PRE cells which, as previously discussed, may be similar to optic vesicle cells. However, it has not been possible to investigate this, since even in primary differentiated PRE cultures, cells towards the periphery rapidly dedifferentiate (chapter 4). Therefore, it is possible that ectopic Msx expression in such cultures only affects dedifferentiated cells at the periphery. Indeed, Msx-induced neural-like cells are located at the periphery of monolayers. However, neural-like cells may require a low cell density environment in which to develop such as that found at the periphery of monolayers. Therefore, the distribution of neural-like cells may reflect either cell density or the distribution of dedifferentiated PRE cells.

FGF 2 can drive neurogenesis in PRE explants (chapter 1.6), perhaps suggesting that there is no requirement to lose PRE identity in order to undergo FGF 2-driven neural differentiation. This may also be the case for Msx-induced neurogenesis. However, FGF 2 has been shown to dedifferentiate dissociated PRE cells (Pittack et al., 1991; Opas and Dziak, 1994). We cannot, therefore, be sure that FGF 2-induced neural cells have not passed through a dedifferentiated state. Further analysis of the transgenic line A81, engineered to express Msx 2 in differentiated PRE under the control of the Trp 2 promoter, may help address this issue (chapter 8.3).
Fig. 9.1 Speculative models for Msx function in chick PRE cultures. A) Msx may function in PRE cells to directly drive the differentiation of individual PRE cells into different neural cell types. B) Alternatively, Msx may function in PRE cells to generate multipotent precursors capable of differentiating into different neural cell types. It is not possible to distinguish Msx function in differentiated PRE cells from Msx function in dedifferentiated PRE cells (refer to text for details).

The reports that FGF 2, like Msx expression, drives neurogenesis in PRE raises the possibility that FGF 2 and Msx function in a common pathway. Indeed, there are aspects of Msx and FGF 2 function that are shared. Rat PRE explants treated with FGF 2 give rise to several distinct neural retina cell types; amacrine, photoreceptors and ganglion cells (Zhao et al., 1995). Thus, perhaps in common with Msx function, FGF 2 functions to either generate multipotent retinal precursors or drive the differentiation of several different neural cell types. FGF 2-induced neural differentiation is not affected by the presence of mitomycin C which inhibits cell proliferation (Zhao et al., 1995). This may argue against FGF 2 generating a precursor population. However, it was not shown that all the normal retinal cell types were generated or that the correct proportions of these cell types were present in
mitomycin C treated cultures. It would be interesting to determine if Msx function is impaired by blocking cell proliferation.

While there are indeed aspects of Msx and FGF 2 function in PRE cells that are shared, there are also notable differences. Firstly, there is no report that exogenous FGF 2 can drive neurogenesis in dissociated PRE cultures. This suggests that cell-cell interactions, only found in explant cultures, could be required for exogenous FGF 2-induced neural differentiation. This is not the case for Msx-induced neural differentiation. Secondly, exogenous FGF 2-induced neural differentiation is age dependant. PRE explants from chicks aged over stage 27 fail to acquire a neural fate upon treatment with FGF 2 (Pittack et al., 1991), whilst Msx 2 can induce neural differentiation in PRE from chicks aged at least stage 35 (chapter 5.4).

It has been suggested in this thesis that endogenous FGF 2 expression in dedifferentiated PRE cells could account for the neural-like cells that spontaneously arise in dedifferentiated cultures. However, endogenous FGF 2-induced neural differentiation does not appear to require an explant environment or to be dependant on PRE age.

It is conceivable that endogenous FGF 2 expression or FGF 2 treatment of PRE explants could affect Msx 2 expression. It will therefore be important to investigate Msx and FGF 2 expression in neural cells that arise spontaneously from dedifferentiated PRE. It will also be interesting to investigate the expression of Msx in explants treated with FGF 2 and the expression of FGF 2 in dissociated PRE cells ectopically expressing Msx. This will determine whether or not these genes function in a common pathway and if so, their hierarchical order within that pathway.

During the initial stages of tooth development, the dental epithelium signals, via Bmp 4, to the underlying mesenchyme which responds by expressing numerous genes including Msx 1 (Chen et al., 1996). Msx 1 in turn activates Bmp 4 expression within the mesenchyme (Chen et al., 1996). This mesenchymal Bmp 4 may have two
functions; firstly, to act on the dental epithelium in a reciprocal fashion and secondly, to induce Msx 1 expression in neighbouring mesenchyme cells thus establishing a positive-feedback loop. This would allow a rapid spread of the Bmp 4 signal through the mesenchyme over greater distances than diffusion alone would permit.

Throughout development co-expression of Bmp 4 and Msx 1 is observed in a variety of other tissues, suggesting that a positive-feedback loop could be a common feature of Msx 1 function (chapter 1.3). Whether Msx 2 functions in an equivalent manner remains to be established. If Msx 1 and Msx 2 function in the eye is associated with a positive-feedback loop, using a shared secreted protein, it could explain the apparent functional redundancy indicated by the failure of eye development in mice null for both Msx 1 and Msx 2 (Rauchman et al., 1997). In such a scenario the absence of gene function in one tissue could be compensated for by the action of the other in a neighbouring tissue. In support of this idea Msx 1 expression in mouse perioptic mesenchyme neighbours Msx 2 expression in derivatives of the neuroepithelium and surface ectoderm (chapter 3). Contrary to this hypothesis neither ectopic Msx 1 nor Msx 2 expression in primary dissociated PRE cultures resulted in neighbouring, non-expressing cells acquiring a neural fate. In common with the apparent failure of dissociated PRE cells to respond to exogenous FGF 2, disruption of cell-cell interactions may also render them non-responsive to the hypothesised Msx-regulated secreted protein. This observation suggests that a feedback loop may not be essential for Msx function in vitro.

9.2 Msx function in the developing retina

9.2.1 Recruitment of retinal precursors

To help understand how the function of Msx 2 in PRE cultures might reflect its function during retina development, it is worth briefly considering the events that occur during retinogenesis. In mouse, cells of the forebrain are recruited to a retinal fate by the action of genes such as Rx. Mice null for Rx fail to form optic sulci, while
ectopic over-expression of *Xenopus Rx* in 4-8 cell *Xenopus* embryos results in ectopic retinal tissue (Mathers *et al.*, 1997). In wild type mice, *Rx* is expressed in the optic sulci, vesicle and across all layers of the neural retina. The gene is down-regulated as each cell type terminally differentiates (Mathers *et al.*, 1997). *Pax 6* and *Lhx 2* may have a comparable role to *Rx*; they too are expressed in the developing optic vesicle, becoming down-regulated as the neural retina differentiates. Mice null for either of these genes exhibit arrested development of the retina at the optic vesicle stage (Grindley *et al.*, 1995; Porter *et al.*, 1997). This could be due to a failure in the recruitment of retinal precursor cells or in their subsequent differentiation.

The differentiation of these precursors is, partially at least, controlled by lateral inhibition, involving the function of the neurogenic genes *Notch* and *Delta* (chapter 1.5.2.3). Delta is expressed on the surface of differentiating cells and activates Notch on neighbouring cells which in turn inhibits the expression of proneural genes such as *Mash 1* (Pompa *et al.*, 1997). *Mash 1* functions to promote the differentiation of already committed precursor cells but is not involved in the specification of these cells (Tomita *et al.*, 1996a).

Analysis of cell lineage in the developing rat retina has demonstrated that a single neural retina precursor can give rise to both glial and neuronal cell types (Turner and Cepko, 1987). The specification of these multipotent precursors to particular lineages appears to be controlled by environmental factors. For example, CNTF or a related cytokine stimulates neural precursors to differentiate into bipolar cells rather than photoreceptors (Ezzeddine *et al.*, 1997).

The effect Msx 2 has on PRE cultures is consistent with Msx 2 functioning during retina development as a proneural gene, like that of *Mash 1*. However, the expression pattern of *Msx 2* in the retina, and the eye phenotype associated with *Msx 1/Msx 2*-null mice, do not support this function. *Mash 1* is expressed in proliferating progenitor cells located throughout the neural retina, expression ceasing with the terminal differentiation of each cell type (Guillemot and Joyner, 1993a; Tomita *et al.*, 1996a).
1996a). In contrast, Msx 2 is only transiently expressed in retinal progenitor cells in the optic vesicle, with expression quickly becoming confined to the ciliary margin long before all the progenitor cells of the neural retina have differentiated (chapter 3.3). Retinogenesis in mice null for Mash 1 proceeds normally, though delayed, whereas mice null for both Msx 1 and Msx 2 exhibit arrested eye development at the optic vesicle stage (Tomita et al., 1996a; Rauchman et al., 1997).

Alternatively, Msx 2 may function during retinogenesis to recruit neural retina cells in a similar manner to Rx (fig. 9.2). This is not only consistent with the effect Msx 2 has on cultured PRE cells, but also arrested development of the optic vesicles in Msx double-knockout mice and the expression of Msx 2 in cells of the optic vesicle that are destined to give rise to the neural retina.

Although Msx 2 expression was predominantly associated with cells of the optic vesicle that are predicted to give rise to the neural retina, without cell fate or molecular markers that specifically label presumptive PRE cells the possibility cannot be excluded that some cells of the optic vesicle destined to become PRE also express Msx 2. Therefore, it is tempting to speculate that Msx 2 may function to recruit both neural retina and PRE cells. This is supported by the observation that ectopic expression of Myc in cultured quail neural retina enhanced its transdifferentiation into PRE, while ectopic expression in PRE enhanced its transdifferentiation into neural retina (Plaza et al., 1995; Turque et al., 1996). This paradox could be explained if the function of Myc in both PRE and neural retina is to generate a population of retinal precursors capable of differentiating into both PRE and neural retina cell types. It will therefore be important to establish if ectopic Msx 2 expression in neural retina cultures results in PRE differentiation. If so, this might explain why only a small proportion of cells ectopically expressing Msx 2 in PRE cultures gain a neural phenotype, since Msx 2-expressing precursors may also differentiate into PRE cells.
Speculative model for Msx 2 function in the retina

Msx 2 may function in the developing optic vesicle to recruit cells to a neural fate. These cells are multipotent, giving rise to Müller glial cells and all 5 neuronal cell types of the neural retina. Msx 2 may function earlier in retinogenesis in generating retinal precursors that contribute to both pigmented and neural retina (refer to text for details).

In higher and lower vertebrates, neurogenesis commences in the centre of the developing optic cup and spreads to the periphery. This involves the sequential withdrawal of cells from the cell cycle and their subsequent differentiation (Jacobson, 1968; Young, 1985; Prada et al., 1991). In lower vertebrates, e.g. Xenopus, retinal growth is maintained at the periphery of the retina (Hollyfield, 1971; Straznicky and Gaze, 1971). The Xenopus ciliary margin contains a highly proliferative population of cells that is the most likely source of cells required for the radially growing retina (Dorsky, 1995). Indeed, lineage analysis of cells from this region has shown that they can contribute to the PRE and all the cell types of the neural retina (Wetts, 1989).
There is no evidence that higher vertebrates retain the ability to generate retinal cells from the ciliary margin. However, it is interesting to note that Notch is expressed in the ciliary margin of both Xenopus and mouse, Xotch and Notch 2 respectively (Dorsky et al., 1995; Williams et al., 1995). This might suggest that in this region developmental processes requiring Notch function are shared between higher and lower vertebrates. Notch functions during retinogenesis to maintain cells in an undifferentiated state (chapter 1.5.2.3) and therefore may function in the Xenopus, and possibly mouse, ciliary margin to maintain retinal precursor cells.

If similar events occur in the mouse ciliary margin as they do in the Xenopus ciliary margin, then Msx 1 and Msx 2 which are expressed in this region (chapter 3.3) may function to recruit or maintain retinal precursor cells. In support of Msx 1 functioning in this process, Msx 1 can drive neurogenesis in cultured PRE cells. Thus the Msx genes may have a universal function throughout retina development in generating or maintaining retinal precursors. Msx 2 expression in the outer layer of the chick ciliary margin and inner layer of the mouse ciliary margin, might reflect differences between these two species in the location of the precursor cells within the ciliary margin.

It is conceivable that ciliary margin precursor cells may also generate cells required for the formation of the iris. By injecting replication-incompetent retroviral vectors, expressing a reporter gene, into the ciliary margin of cultured mouse embryos it should be possible to determine if cells of the ciliary margin contribute to the formation of both the iris and retina, or only one of these structures.

9.2.2 Establishment of positional information

Retinal cells require positional information in order to make specific connections with cells in the optic tectum (Sperry, 1963). Numerous genes have been identified that are asymmetrically expressed during retinogenesis, reviewed in chapter 1.5.2.4, and as such may function to establish polarity within the retina. At E9.5, the earliest stage of
mouse development analysed, Msx 2 is only expressed in the most posterior/dorsal two thirds of the optic vesicle. Asymmetric expression raises the possibility that Msx 2 function is not required by all neural retina cells. Thus, neural retina cells derived from precursor cells that expressed Msx 2 in the optic vesicle may be directly imprinted with different positional information from those that did not express Msx 2. Alternatively, non-expressing optic vesicle cells may have expressed Msx 2 earlier in development or are not destined to give rise to the neural retina.

At later stages of mouse eye development, Msx 2 is asymmetrically expressed in the posterior half of the ciliary margin. Msx 1 expression is initially restricted to the dorsal half of the mouse ciliary margin at E11.5, before expanding around the entire circumference of the retina. In chick, Msx 2 is also asymmetrically expressed in the ciliary margin at the lens vesicle stage. If it is shown that a population of retinal precursor cells resides in the ciliary margin of higher vertebrates, then it is possible that retinal cells derived from precursor cells expressing different combinations of the Msx genes could also be directly imprinted with different positional information.

Alternatively, if the ciliary margin of mouse does not contain a population of multipotent retinal precursors, different combinations of Msx 1 and Msx 2 expression in this region may function indirectly to establish retinal polarity. A clue to the mechanism of this indirect method of establishing retinal polarity, can be gained from the Xenopus gene Xbr1. This gene has many similarities with mouse Msx in terms of structure, expression and, possibly, function. Xbr1, like the Msx genes, is a homeobox-containing gene showing 57% amino acid identity to Msx 1 and 2 within the homeodomain. In common with Msx 2, Xbr1 is asymmetrically expressed in the ciliary margin (Papalopulu and Kintner, 1996). In addition to its possible function in the development of progenitor cells within this region, it has also been suggested that this gene is indirectly involved in the establishment of retinal polarity (Papalopulu and Kintner, 1996). This proposed function is based on the co-expression of Bmp 4 which encodes a secreted protein (Papalopulu and Kintner, 1996). The asymmetric diffusion of such a molecule could provide retinal cells with a gradient of positional
information. However, it is not clear if such a molecule would be able to act over large distances.

In a similar manner to *Xbrl*, different combinations of *Msx* expression in the mouse ciliary margin could establish polarity by regulating the production of diffusible proteins. In common with *Xenopus*, this molecule may be Bmp 4 since *Msx 1* has been shown to regulate *Bmp 4* during tooth development in mouse (chapter 1.3.2). It will be interesting to learn if *Bmp 4* is also expressed in the ciliary margin of mice, whether or not higher vertebrates have an *Xbrl* homologue and if so the functional relationship with *Msx 1* and *Msx 2* in the ciliary margin.

It has already been suggested (chapter 9.1) that *Msx 1* and *Msx 2* may be functionally redundant during eye development, though not initially co-expressed, by regulating the production of a common secreted molecule. However, it is difficult to envisage how such a mechanism could operate if asymmetric expression of *Msx 2* in the optic vesicle and differential expression of *Msx 1* and *Msx 2* in the ciliary margin establishes retinal polarity. This may suggest that *Msx 1* and *Msx 2* are not essential for the establishment of retinal polarity. Alternatively, retinal polarity may be disrupted in either *Msx 1*- or *Msx 2*-null mice but the associated defects have not yet been identified.

The establishment of polarity by the *Msx* genes may not be confined to the retina. Within the neural tube, the expression of *Msx 1*, *Msx 2* and *Bmp 4* are dorsally restricted (chapter 1.3.5). This may suggest that they play a role in establishing a dorsal-ventral axis of polarity within the neural tube. Interestingly, *Xbrl*, in *Xenopus*, is also expressed in the dorsal neural tube (Papalopulu and Kintner, 1996).
9.3 A universal function for Msx in recruiting and maintaining precursor cells?

The correlation of Msx expression with populations of undifferentiated cells is not limited to the neuroepithelial components of the eye. Msx 2 is expressed in the lens placode and later in the surface ectoderm overlying the lens, which gives rise to the lens and cornea respectively (chapter 3.4). In the developing lens, Msx 2 expression becomes restricted to the lens equator, a region in which epithelial cells proliferate and begin to differentiate into fibre cells. Thus, the expression of Msx 2 in derivatives of the surface ectoderm is consistent with this gene functioning to recruit lens precursor cells from the surface ectoderm and subsequently maintaining a population of precursors at the lens equator. Similarly, the later expression of Msx 2 in the surface ectoderm overlying the lens is consistent with Msx 2 functioning in the recruitment of cornea precursors.

Msx has also been implicated in establishing precursor cells during the development of non-eye structures. Muscle precursor cells (myoblasts) of the limb are derived from the lateral dermomyotome and migrate to their target sites in the limb before differentiating (Cossu et al., 1996a). Expression of Msx 1 in the lateral dermomyotome is consistent with this gene functioning during the recruitment of limb myoblasts (MacKenzie et al., 1997a). Myoblast differentiation is inhibited by signals originating from the lateral mesoderm (Cossu et al., 1996b). Bmp 4 is expressed in the lateral mesoderm and can mimic the effects this tissue has on muscle differentiation. This suggests that the inhibiting signal may be mediated by Bmp 4 (Pourquie et al., 1996). Given that Bmp 4 has been demonstrated to up-regulate Msx 1 expression during tooth development, it raises the possibility that Bmp 4 function during myogenesis may be mediated by Msx 1. Indeed, Msx 1 inhibits the expression of MyoD in 10T1/2 cells whilst, in the chick limb, the onset of muscle differentiation and the expression of Msx 1 are mutually exclusive (Wang and Sassoon, 1995; Woloshin et al., 1995). Thus, during limb myogenesis Msx 1 may not only function to recruit
myoblasts from the lateral dermomyotome, but may also maintain these precursors by
suppressing further muscle differentiation until they reach their target sites.

*Msx* genes may play a comparable role during chondrogenesis in the dorsal
components of vertebrae, limbs, facial primordia and calvaria (chapter 1.3.7). The
spinous process differentiates from mesenchymal cells located between the neural tube
neural tube, or implanting beads soaked in Bmp 4, at a position lateral to the neural
tube, *Msx 2* expression is induced. This is also associated with the formation of
ectopic cartilage (Takahashi *et al.*, 1992; Monsoro-Burq *et al.*, 1996; Watanabe and
Le Douarin, 1996). *Msx 2* is normally expressed in mesenchymal cells that are thought
to give rise to spinous process, while *Bmp 4* is expressed in the dorsal neural tube
(Liem *et al.*, 1995; Monsoro-Burq *et al.*, 1995). Thus the expression of these genes,
together with the consequence of ectopic *Bmp 4* expression, is consistent with them
playing a role in recruiting bone precursors (osteoblasts).

The *Msx* genes may play an additional role in preventing the differentiation of
osteoblasts. This is supported by the observation that the expression of *Msx 1* and
*Msx 2* is mutually exclusive with the onset of chondrogenesis in both spinous process
and limb (Ferrari *et al.*, 1995; Monsoro-Burq *et al.*, 1996). Furthermore, treatment of
stage 23 chick mandibular arches with anti-sense *Msx 2* oligonucleotides enhances
chondrogenesis (Mina *et al.*, 1996). Msx-binding sites have been identified in several
bone specific genes (Hoffmann *et al.*, 1996; Yang and Gerstenfeld, 1997). Binding of
Msx to the promoter of one of these genes, *Osteocalcin*, is predicted to suppress
transcription (Hoffmann *et al.*, 1996; chapter 1.3.7). Thus, at some chondrogenic sites
Msx may function to recruit osteoblasts and subsequently maintain them by
suppressing chondrogenesis.

At least superficially, *Msx* function during neurogenesis and myogenesis appears to be
evolutionary conserved. The *Drosophila* homologue of vertebrate *Msx* genes, *Msh*, is
expressed in neural progenitors (neuroblasts) that form in the dorsal portion of the
neuroectoderm (Isshiki et al., 1997). In Drosophila Msh loss-of-function mutants, all neuroblasts form normally but dorsal neuroblasts fail to differentiate into the appropriate cell types. Ectopic Msh expression in ventral neuroblasts, that would not normally express Msh, disrupts their differentiation (Isshiki et al., 1997). Similarly, during Drosophila myogenesis Msh is expressed in dorsal muscle progenitors (D’Alessio and Frasch, 1996; Nose et al., 1998). The initial formation of dorsal muscle progenitors proceeds normally in Msh loss-of-function mutants, but the subsequent production of specific muscle cell types is affected (Nose et al., 1998). Ectopic expression of Msh in the entire mesoderm prevents the proper development of normally Msh non-expressing muscle progenitors (Nose et al., 1998).

Thus during both Drosophila neurogenesis and myogenesis, Msh is not required for the initial formation of progenitors but is required for the recruitment of these progenitor cells into specific lineages. It is therefore apparent that the Msx genes have an ancient function in recruiting specific precursor cells to particular fates during myogenesis and neurogenesis. It will be interesting to learn if Msx has a similar function at other sites of expression.

9.4 Suggested direction of future research

Recruitment of neural retina cells can perhaps be considered in three parts. Firstly, neural retina precursor cells are selected from the optic vesicle. Secondly, recruited precursors, or their daughter cells, are specified to one of at least seven possible fates. In addition to cell-type specification, neural retina cells may also be imprinted with a spatial identity. Thirdly, once specified to a particular lineage, retinal cells differentiate. This is perhaps an over simplification and in reality it is more likely that retinoblasts progressively become biased towards a particular fate. Future research into Msx function during retinogenesis will contribute to our understanding of these three aspects of cell recruitment.
Recently published work, Hyer et al. (1998), has suggested a mechanism by which surface ectoderm-derived FGF 1 selects neural retinal precursor cells from the underlying optic vesicle. Removal of the surface ectoderm overlying the chick optic vesicle results in optic vesicle cells differentiating in an unorganised fashion. They fail to form a bilayered cup and have the pigmented phenotype of PRE and the morphology of early neural retina cells (Hyer et al., 1998). This phenotype can be rescued by implanting FGF 1-producing fibroblasts distal of the optic vesicle; non-pigmented cells were observed closest to the implant (Hyer et al., 1998). During normal development, at the time the optic vesicle makes contact with the surface ectoderm FGF 1 is expressed in the surface ectoderm and the receptor, FGFR 1, is expressed throughout the optic vesicle (Wanaka et al., 1991; Delong and McAvoy, 1993). As discussed in chapter 9.2.1, Msx 2 may function in the optic vesicle to recruit neural retina cells. Thus, surface ectoderm-derived FGF 1 could recruit optic vesicle cells to a neural retina fate by regulating the expression of Msx 2 in the optic vesicle. This possibility could be explored further in vivo by testing whether Msx 2 expression in the optic vesicle is affected by removal of the surface ectoderm, whether the rescue of optic vesicle patterning by FGF 1 is associated with up-regulation of Msx 2 and whether forced Msx 2 expression in the optic vesicle can rescue optic vesicle patterning in chicks lacking surface ectoderm. In vitro, the Msx 2 functional assay, described in this thesis, could easily be used to investigate whether FGF 1 functions upstream of Msx 2. Chick PRE could be grown in the presence of FGF 1, whilst monitoring Msx 2 expression and the acquisition of neural phenotype. It has been suggested that exogenous FGF 2 may be unable to drive neural differentiation in dissociated PRE cells (chapter 1.6). It may therefore be necessary to investigate the effects of FGF 1 in explant PRE cultures.

Msx 2 has been proposed to function in PRE cultures to generate multipotent precursors (fig. 9.1). Indeed, neural retina precursor cells are multipotent and may be specified to particular fates by the actions of environmental cues (Turner and Cepko, 1987). CNTF has been shown to promote the differentiation of bipolar cells (Ezzeddine et al., 1997). The role this cytokine, and others, may have in specifying
neural retina precursors to particular fates could be further explored by determining the proportions of each retinal cell type present in Msx 2-transfected PRE cultures grown in the presence or absence of cytokines.

The Msx-PRE functional assay could be used to screen for genes functioning downstream of Msx during recruitment of retinal cells. Msx 2 is a transcription factor, therefore differences in gene expression between PRE cells transfected with an Msx 2 expression construct and those transfected with a control construct would correspond to downstream genes. Differences in gene expression could be detected by in situ hybridisation of probes for candidate downstream genes. Genes likely to function during the recruitment of retinal cells are ideal candidates (reviewed in chapter 1.5.2.1 and 1.5.2.2), as are genes that have been associated with Msx function at other sites of the embryo (reviewed in chapter 1.3). Alternatively, differences in gene expression could be detected by differential display reverse transcription PCR (DDRT-PCR; Bauer et al., 1993). The identification of novel genes would increase our understanding of the genes involved in the recruitment of neural retina cell types, whilst the identification of known genes would draw together existing pathways and may provide clues as to the molecular mechanisms required for the recruitment of neural retina cells. The same pathways and mechanisms may also control cell recruitment at other sites of Msx function, aspects of which may even be universal to cell recruitment.

Differentiated PRE cells express PRE genes such as Mitf (Mochii et al., 1998) and can be considered committed, since in vivo they do not give rise to other cell types. However, when the extracellular environment cannot support PRE differentiation, PRE cells dedifferentiate, lose the ability to express Mitf (Mochii et al., 1998) and can give rise to both PRE and lens-like cells (chapter 1.6). Consequently, dedifferentiated PRE can be considered uncommitted. It is not known whether Msx can recruit differentiated (committed) PRE cells to a neural fate, or only dedifferentiated (uncommitted) PRE cells.
This will be an important issue to address in the future as it may contribute to our general understanding of cell commitment. For instance, can a cell simultaneously express two differentiation pathways or does one pathway suppress the other? If the latter, it would be interesting to investigate the mechanism by which a whole pathway can be suppressed.

The transgenic mice engineered to ectopically express $Msx\ 2$ in the PRE under the control of the $Trp\ 2$ promoter (chapter 8.3) may provide an excellent system in which to explore these questions. The $in\ vivo$ environment may maintain the differentiated state of PRE cells, making it possible to determine if $Msx\ 2$ can function in differentiated (committed) PRE cells. The analysis of PRE- and neural retina-markers in the PRE of these mice may establish if both PRE and neural differentiation pathways can operate simultaneously within the same cell.
References


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