FUNCTIONAL CHARACTERISATION OF TWO GENES EXPRESSED IN SUBSETS OF FOLLICLE CELLS DURING DROSOPHILA OOGENESIS

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DEDICATION

To my parents, for their never-ending love and support.
ACKNOWLEDGEMENTS

I would like to express my immeasurable gratitude to my supervisor, Professor Mary Bownes, for her help, guidance and encouragement during the course of my studies. Very special thanks to Dr. Debiao Zhao for his long-time help and advice in Shanghai as well as Edinburgh. Enormous thanks of course to past and present members of the group, namely Angela, Bryce, Claudia, Colin, Debbie, Diane, Dot, George, Janis, Kathleen, Neil, Simone, Stephen and Tanya. To Debbie, Tanya and Dot particularly, my sincere thanks for the happy time and cherished experiences together in and out of the laboratory. Special thanks also to Thomas who worked with me on the Broad-Complex during the summer of 1996.

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This study has been made possible only by the favours received from many different sources. I would like to acknowledge all those who sent me fly stocks and DNA clones, those who kindly assist me to use their experimental facilities, and those involved in washing-up and media preparation. Although the numbers are too great to mention here, I thank you all.

Finally, I would like to offer my sincere thanks to Ai Ying, for her continuous support and help, particularly during the strenuous thesis writing days. My special thanks also extends to my sister and my brother, for their understanding and support.
DECLARATION

I declare that this thesis was composed by myself, and that the work described is my own, unless otherwise stated.
The *Drosophila* egg is polarised along both the anterior/posterior (A/P) and dorsal/ventral (D/V) axes. To establish these two polarities, interactions between somatically derived follicle cells and germline cells are required during the different stages of oogenesis. It is, therefore, necessary to identify genes expressed in subsets of follicle cells, because these genes may be effectors of germline-soma signalling, or be directly involved in signal transduction, or they may be involved in sending signals to the oocyte.

413 enhancer-trap *Gal4* lines have been analysed for their reporter gene expression patterns in the ovaries. Among these, several lines that show reporter gene expression in subsets of follicle cells have been further analysed to identify their target genes. In this project it has been shown that *Myosin heavy chain at 95F (Mhc95F)* and the *Broad-Complex (BR-C)* are the target genes of two *Gal4* lines which show asymmetric follicle cell expression patterns.

*Mhc95F*, which encodes *Drosophila* myosin 95F, a class VI unconventional myosin, is expressed in the anterior follicle cells and the migrating border cells during oogenesis. Its expression is also detected in other migrating follicle cells, including the follicle cells undergoing centripetal migration and dorsal-anterior migration.

P-element mediated mutagenesis has been undertaken to create mutations in the *Mhc95F* gene. Several lines showing deletions in the chromosomal 95F region have been obtained. However, no strong evidence shows that the mutant phenotype of these lines and the *Mhc95F* gene are directly related. Additionally, targeted silencing technique has been used for the study of its function during development. Using a transformed fly line which contains the antisense *Mhc95F* gene downstream of the Gal4 binding sequences, UAS, to cross with a tissue-specific *Gal4* line, the progeny exhibit the malformed leg and unexpanded wing phenotype. Moreover, the female progeny from the cross have disrupted centripetal migration of the follicle cells, and degenerated egg chambers. These results indicate that myosin 95F is likely to be involved in cell shape change and cell migration during development.

The target gene of line C726b is the *BR-C*, which encodes a family of zinc-finger transcription factors and plays a key role in metamorphosis. During stage 10b of
oogenesis, BR-C mRNA is present in two groups of follicle cells over the lateral-dorsal-anterior oocyte. This expression domain is related to its function in dorsal appendage morphogenesis, and this is confirmed by studying the eggshell phenotype of partial "loss-of-function" BR-C mutants and heat-shock inducible BR-C transgenic fly lines.

Expression of the BR-C in the lateral-dorsal-anterior follicle cells is specified by the Gurken/Torpedo(DER) signalling pathway along the D/V axis in a dose-dependent manner. This expression domain appears to be further specified along the A/P axis by the Dpp signalling pathway which is initiated in the nearby follicle cells. It is proposed that the BR-C may provide a link between pattern formation and morphogenesis of the eggshell.
ABBREVIATIONS

amp  Ampicillin
ATP  Adenosine-5'-triphosphate
bp   Base pair
BSA  Bovine serum albumin
°C   Degrees Centigrade
cDNA Complementary deoxyribonucleic acid
DAB  3', 3'-Diaminobenzidine tetrahydrochloride
dATP Deoxyadenosine-5'-triphosphate
dCTP Deoxycytosine-5'-triphosphate
dGTP Deoxyguanosine-5'-triphosphate
dTTP Deoxypyrimidine-5'-triphosphate
ddATP 2' (3'-di) Deoxyadenosine-5'-triphosphate
ddCTP 2' (3'-di) Deoxycytosine-5'-triphosphate
ddGTP 2' (3'-di) Deoxyguanosine-5'-triphosphate
ddTTP 2' (3'-di) Deoxypyrimidine-5'-triphosphate
dNTP(s) deoxynucleotide-5'-triphosphate(s)
ddH2O Double distilled water
DNA  Deoxyribonucleic acid
DNase Deoxyribonuclease
EDTA Ethylenediamine-tetra-acetic acid
fs   Female sterile
g    Gram
HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HRP  Horse radish peroxidase
IPTG Isopropyl-β-D-thio-galactopyranoside
kb   Kilo-base pairs
kDa  Kilo-Daltons
L    Litre
M    Molar
MOPS Morpholinopropanesulphonic acid
mRNA messenger RNA
μg   microgram
μl   microlitres
μM   microMolar
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<tr>
<th>Symbol/Abbreviation</th>
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<tr>
<td>µmol</td>
<td>micromole</td>
<td></td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
<td></td>
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<tr>
<td>ml</td>
<td>millilitre</td>
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</tr>
<tr>
<td>mm</td>
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</tr>
<tr>
<td>mM</td>
<td>milliMolar</td>
<td></td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
<td></td>
</tr>
<tr>
<td>[^{32}P]</td>
<td>(\beta)-emitting isotope of phosphorus</td>
<td></td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
<td></td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>pH</td>
<td>-(\log_{10}) [hydrogen ion concentration]</td>
<td></td>
</tr>
<tr>
<td>psi</td>
<td>Pounds per square inch</td>
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</tr>
<tr>
<td>%</td>
<td>percentage</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
<td></td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)-amino-methane</td>
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<td>Tris-HCl</td>
<td>Tris solution, pH adjusted with HCl acid</td>
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<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-(\beta)-galactopyranoside</td>
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<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>TEMED</td>
<td>NNN'N'-tetra-methyl-1,2-diamino-ethane</td>
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<tr>
<td>U</td>
<td>Units</td>
<td></td>
</tr>
<tr>
<td>UV</td>
<td>Ultra-Violet</td>
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<tr>
<td>V</td>
<td>Volts</td>
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<tr>
<td>v/v</td>
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<tr>
<td>w/v</td>
<td>Weight per volume</td>
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<td>~</td>
<td>approximately</td>
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<tr>
<td>A</td>
<td>Alanine</td>
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<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>D</td>
<td>Aspartate</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>E</td>
<td>Glutamate</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>M</td>
<td>Methionine</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>V</td>
<td>Valine</td>
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CHAPTER 1

General Introduction: The Developmental Genetics Of Oogenesis
Oogenesis is a developmental stage highly adapted to produce eggs. In *Drosophila*, the mature egg carries not only nutrients to support subsequent embryogenesis, but also maternal signals to instruct embryonic pattern formation. The maternal signals are localised at subcellular regions within the oocyte (egg) to determine the two major axes, the anterior-posterior (AP) and dorsal-ventral (DV) axes (for review see St. Johnston and Nüsslein-Volhard, 1992). How polarities are initiated and established during oogenesis has been a topical question since the genetic hierarchy controlling embryonic segmentation was identified.

In addition to polarity formation, a number of developmentally important questions relating to oogenesis have been studied, and oogenesis has been used as a model system to study the cellular and molecular basis of developmental mechanisms. For example, how does one cyst cell (or cystocyte) within a 16-cell-germline cyst become determined to develop as the oocyte while its sister cells differentiate to become nurse cells in support of its development? How are the chromosomal structural changes in germline cells developmentally regulated? The egg chamber, which is the developmental unit of oogenesis, contains sixteen germline cells encapsulated by a sheet of epithelial follicle cells: how do the germline cells and somatic cells co-operate to form an egg chamber? Somatic follicle cell differentiation requires signals from the germline cells: what are they? Follicle cell and germline cell interactions are crucial in the polarity formation of both the embryo and the eggshell. However, some steps in the signalling pathways between these two cell types are missing. In addition, the cytoskeletal network appears to be involved in most of the developmental events in oogenesis. Oogenesis, therefore, provides a good model for studying developmental regulation of the cytoskeletal network. Moreover, oogenesis provides an ideal model for the study of cellular and molecular mechanisms underlying epithelial morphogenesis, a key area in development. Other branches of oogenesis studied include: programmed cell death; regulation of vitellogenesis; developmental control of the cell cycle; and hormonal regulation in development. All these suggest that oogenesis is not only a crucial developmental stage in the life cycle of *D. melanogaster*, but is also an ideal model system for studying a number of developmental questions (for reviews see Spradling, 1993; Lasko, 1994). In this chapter, I will discuss recent progress in the understanding of different aspects of oogenesis.
1.1. Morphology of oogenesis

The *Drosophila* female adult has a pair of ovaries, each of which contains 16-20 ovarioles that consist of developmentally ordered egg chambers (Figure 1.1A). Every egg chamber supports the development of a single oocyte/egg. The egg chambers, according to their size and morphology, can be divided into 14 developmental stages (Table 1.1 and Figure 1.1B) (for reviews see King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993; Lasko, 1994; Bownes, 1994A).

Oogenesis starts in the anterior compartment of the ovariole, the germarium (Figure 1.1C). The germline stem cells divide to produce a daughter stem cell and a cystoblast. The latter undergoes four mitotic divisions to form a 16-cell-cyst, one of which is determined to become the oocyte; the others become nurse cells. Cytokineses are incomplete at each of the cystoblast divisions, so the cells are connected by cytoplasmic bridges called ring canals. The germarium can be subdivided into four regions. The stem cell and the mitotically divided cystoblasts lie within germarium region 1, whereas newly formed 16-cell cysts are located in region 2a. In region 2b, the 16-cell cysts become lens-shaped with the pro-oocyte positioned at the centre of the cysts. By the time the cyst occupies germarium region 3, the oocyte is located at the posterior pole, where it will remain throughout the completion of oogenesis. Somatic stem cells are located at the border between germarium regions 1 and 2 (Margolis and Spradling, 1995). In region 2, follicle cells migrate from the wall of the germarium to encapsulate the 16-cell cysts. When they reach region 3, the cysts are surrounded by a single layer of follicle cells and referred to as stage-1 egg chambers (Figure 1.1C).

Stage-2 egg chambers bud from the germarium and enter previtellogenesis which lasts until stage 7. The developing egg chambers are connected by a stack of specialised interfollicular stalk cells (refer to Figure 1.1B and Figure 1.6B). The nurse cells and the oocyte are approximately the same size from stages 1 to 6. At stage 2, the oocyte nucleus, or germinal vesicle, is similar in size to the nurse cell nuclei. At about stage 3, the oocyte nucleus condenses to become a transcriptionally inactive karyosome. The germinal vesicle is positioned at the posterior of the oocyte up to stage 7. In early stage nurse cells, the banded polytene chromosomes progressively disperse to form large dispersed nucleoli, which are thought to facilitate the high levels of synthesis of ribosomes and other components required for oocyte growth. When egg chambers reach stage 7, they are no longer oval as in previous stages, but more elongated in shape.
Figure 1.1 *D. melanogaster* ovaries and the developmental sequence of oogenesis.

A. Dorsal view of the internal reproductive system of an adult female *D. melanogaster* (adapted from Mahowald and Kambysellis, 1980). Two ovarioles have been pulled loose from the right ovary.

B. Diagram of an adult wildtype ovariole (adapted from Peifer et al., 1993).

C. Drawing of a germarium (adapted from Mahowald and Kambysellis, 1980).

ant, anterior; bc, border cells; fc, follicle cells; nc, nurse cells; oc, oocyte; pc, polar cells; post, posterior; ppc, posterior polar cells; sc, stalk cells; st, stem cells; ST, stage.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Length</th>
<th>Average size</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10 hr</td>
<td></td>
<td>16 cell cyst within region 3 of germarium; oocyte and nurse cells similar in size up to stage 8</td>
</tr>
<tr>
<td>2</td>
<td>8 hr</td>
<td>25 x 25 μm</td>
<td>oocyte and nurse cell nuclei similar in size; nurse cell nuclei begin polyploidisation 8C</td>
</tr>
<tr>
<td>3</td>
<td>8 hr</td>
<td>35 x 35 μm</td>
<td>karyosome and endobody are visible in germinal vesicle</td>
</tr>
<tr>
<td>4</td>
<td>6 hr</td>
<td>40 x 50 μm</td>
<td>nurse cell nuclei contain similar amount of DNA and appear polypene</td>
</tr>
<tr>
<td>5</td>
<td>5 hr</td>
<td>55 x 75 μm</td>
<td>Nurse cell nuclei no longer polypene; posterior nurse cells have more DNA than anterior ones</td>
</tr>
<tr>
<td>6</td>
<td>3 hr</td>
<td>60 x 85 μm</td>
<td>egg chamber still oval; follicle cell divisions cease; nurse cell ploidy equal</td>
</tr>
<tr>
<td>7</td>
<td>6 hr</td>
<td>70 x 115 μm</td>
<td>egg chamber elongated; nurse cells have higher ploidy at posterior; no yolk visible in oocyte</td>
</tr>
<tr>
<td>8</td>
<td>6 hr</td>
<td>190 μm</td>
<td>yolk granules visible in oocyte; follicle cell layer still uniform</td>
</tr>
<tr>
<td>9</td>
<td>6 hr</td>
<td>275 μm</td>
<td>border cell migration in progress; most follicle cells migrate over oocyte; secretion of vitelline membrane begins; oocyte is about 1/3 of the size of egg chamber</td>
</tr>
<tr>
<td>10A</td>
<td>6 hr</td>
<td></td>
<td>follicle cells form columnar epithelium over oocyte; centripetal migration not visible; oocyte is 1/2 size of egg chamber</td>
</tr>
<tr>
<td>10B</td>
<td>4 hr</td>
<td>430 μm</td>
<td>centripetal migration in progress; dorsal follicle cells thicker than in ventral; vitelline membrane extends into opercular region</td>
</tr>
<tr>
<td>11</td>
<td>0.5 hr</td>
<td>490 μm</td>
<td>nurse cell dumping onset; oocyte larger than nurse cells</td>
</tr>
<tr>
<td>12</td>
<td>2 hr</td>
<td>540 μm</td>
<td>nurse cell dumping complete; 15 nurse cell nuclei remain at anterior; dorsal appendages not visible</td>
</tr>
<tr>
<td>13</td>
<td>1 hr</td>
<td>650 μm</td>
<td>gradual disappearance of nurse cell nuclei; dorsal appendages visible; oocyte elongates</td>
</tr>
<tr>
<td>14</td>
<td>&gt;2 hr</td>
<td>700 μm</td>
<td>no nurse cell nuclei remain; dorsal appendages complete their elongation</td>
</tr>
</tbody>
</table>

2. Average sizes of stage 2-7 egg chambers are based on Spradling (1993); From stage 8-14, only the average lengths of egg chambers are recorded (based on Mahowald and Kambysellis, 1980).
From stage 8 to stage 10B, the egg chambers grow quickly; the oocytes grow at a greater rate than the nurse cells as a consequence of the uptake of yolk proteins, which are synthesised in follicle cells and fat bodies. In a stage-10A egg chamber, the oocyte occupies about half of the egg chamber.

A sheet of somatic follicle cells is uniformly distributed over the germline cells from stage 1 to 8. However, starting at stage 9, a series of follicle cell migrations take place. The majority of follicle cells, which originally overlay the nurse cells, elongate and migrate posteriorly so that by stage 10A, the oocyte is covered by a sheet of thick columnar follicle cells, while only a thin layer of stretched cells are left covering the nurse cells. At the same time, a group of about 6 to 10 anterior follicle cells move through the nurse cell cluster to reach the nurse cell-oocyte border. During stage 10B, the anterior columnar follicle cells migrate centripetally along the DV axis to cover the anterior end of the oocyte. Up to this stage, the oocyte is surrounded by a layer of follicle cells, which secrete eggshell protein to protect the mature egg.

During stages 10B to 12, nurse cell cytoplasm is rapidly transferred through the ring canals into the oocyte. The transfer of nurse cell cytoplasm is quite rapid; most will be transported into the oocyte within 30 minutes. At stages 13 and 14, the nurse cells and follicle cells shrink and undergo apoptosis, leaving behind the mature egg, wrapped with a complete eggshell (chorion) and its specialised structures: a pair of dorsal appendages (filaments) at the anterior end of the egg that facilitate embryonic respiration; operculum (used for the larvae to hatch) and micropylar apparatus (for sperm entry).

1.2 The development of the ovary

1.2.1 Embryonic origin of the ovaries

The ovary contains germline cells and somatic cells. Germline cells are descendants of the pole cells, the earliest formed cells in the embryo. The differentiation of the pole cells is induced by a specialised cytoplasm, called pole plasm, which is located at the posterior pole of the egg and early embryo. The pole plasm contains maternal determinants that are associated with structures called polar granules and act to induce differentiation of the pole cells. After the 8th nuclear division of the syncytial blastoderm, 10 or more nuclei that are in contact with the pole plasm are the first to become cellularised. They then divide two or more times to produce a cluster of 40 to 60 pole cells.
The stepwise assembly of the pole plasm requires gene products synthesised in the nurse cells and translocated to the posterior pole of the oocyte during oogenesis. Genes such as oskar, vasa, valois, staufen, tudor, and mago nashi are required for polar granule assembly and are essential for pole cell specification (Ephrussi et al., 1991; Kim-Ha et al., 1991; Lasko and Ashburner, 1990; St. Johnston et al., 1991; Bardsley et al., 1993; Boswell et al., 1991). Since the maternal determinant for posterior segmentation, nanos, is also localised in the pole plasm, the pole granule assembly genes are also essential for posterior segmentation (Lehmann and Nüsslein-Volhard, 1991). Of these genes, oskar appears to play a central role in assembling the pole plasm. oskar mRNA and Staufen protein are the first molecules to be localised at the posterior pole in the developing oocyte, and an increase in oskar gene dosage increases the amount of Vasa protein and Staufen protein localised to the posterior pole (Breitwieser et al., 1996). That oskar seems to have a direct role in pole cell differentiation is shown by the phenotype of flies with altered oskar expression. A weak allele of oskar, osk^{201}, produces viable embryos with correct segmentation but no pole cells when raised at permissive temperature. More importantly, the number of maternal copies of the oskar gene directly controls the number of pole cells that form in the blastoderm embryo. Finally, anteriorly localised oskar mRNA leads to the formation of pole cells at the anterior end, as shown by the introduction of an oskar transgene fused to the 3' untranslated region of the bicoid gene (Ephrussi and Lehmann, 1992). These ectopic pole cells are functional germ cells when transplanted to the posterior of an agametic host. Formation of oskar-induced ectopic pole cells only requires the functions of the vasa and tudor genes of the pole plasm group. Other pole plasm genes have no effect. This suggests oskar, vasa and tudor may be crucial for pole cell formation, while the others may be involved in the posterior localisation of pole cell components (Ephrussi and Lehmann, 1992; for review see Lasko, 1994).

The product of germ cell-less (gcl), a cell-type specific nuclear pore-associated protein, is a pole plasm component that is required for specification of pole cells, but not posterior somatic cells. Mothers with reduced levels of gcl give rise to sterile adult progeny that lack germ cells. Mothers over-expressing gcl, on the other hand, produce progeny exhibiting a transient increase of the number of pole cells. It has been suggested that the gcl protein associates specifically with the nuclear pores of the pole cell nuclei to specify germ cell fate (Jongens et al., 1992; Jongens et al., 1994).
During gastrulation, the pole cells are carried into the embryo during midgut invagination. At 7 hours (25°C), the pole cells move between the cells of the future midgut and come to lie between the endodermal cells and the syncytial yolk sac membrane. Subsequently, most of the pole cells become associated with mesodermal cells on both sides of the embryonic gut to produce the gonads. These mesodermal cells are thought to be derived from the fifth abdominal segment (Mahowald and Kambysellis, 1980).

Not all of the pole cells are determined to become germ cells in *Drosophila*. Only 50-60% of the pole cells reach the gonads; the rest are lost during migration. A gene called *wunen* (*wun*) has been identified, which is involved in pole cell migration. Loss of *wun* function disrupts the orientation of germ cell movement causing the germ cells to disperse even though their normal target, the somatic gonad, is well formed (Zhang et al., 1996). Wun protein is similar to the enzyme type-2 phosphoric acid phosphatase (PAP2). It has two properties that allow it to use repulsion to guide the germ cells. Firstly, Wun can transform a permissive cellular environment into a repulsive one; secondly, Wun is expressed in the gut in a pattern that guides germ cells towards the mesoderm (Zhang et al., 1997).

Additionally, pole cell migration requires the function of *nanos*, a critical factor for abdomen formation. Although *nanos* is localised to the pole plasm during oogenesis, it is not involved in pole cell specification. However, pole cells lacking *nanos* activity fail to migrate into the gonad, and so do not become functional germ cells (Kobayashi et al., 1996).

Moreover, pole cell migration needs an untranslatable RNA, called *Polar granule component* (*Pgc*). Most pole cells in embryos produced by transgenic females expressing antisense *Pgc* RNA fail to complete migration and populate the embryonic gonad. Female adults that develop from these embryos often have agametic ovaries (Nakamura et al., 1996).

### 1.2.2 Larval and pupal ovaries

The ovaries are recognisable in the first instar larvae and, while they remain unchanged in organisation until the middle of the third instar, they increase in size throughout larval development. The larval ovary consists of two cell types: the germline derived oogonia, and the somatic mesodermal cells. The latter form the follicle cells, ovarian sheath, and other ovarian structures of the adult ovaries. Only a simple anterior-
posterior organisation is visible in the larval ovaries; the larger germline cells are located in the middle region, while smaller somatic cells occupy the two poles.

The transition from larval to adult ovaries starts during the middle of the third instar stage. This process is initiated by the formation of a two-dimensional array of cell stacks \textit{de novo} from a mesenchymal cell mass at the anterior end of the larval ovary (Godt and Laski, 1995). These cell stacks, called terminal filaments, are the primordial of the ovarioles. \textit{bric à brac (bab)} function is required for the formation of terminal filaments. In \textit{bab} mutants, terminal filament formation is disrupted, which leads to disruption of ovariole formation and female sterility (Godt and Laski, 1995). This therefore shows that terminal filament formation is essential for ovariole development. Underlying each terminal filament are germ cells that are subsequently incorporated into a developing ovariole. The ovarioles are bounded by a distinctive extracellular matrix called the tunica propria. The somatic cells at the posterior are thought to form the basal stalk region of the ovary. Basal stalk cells include those involved in joining to the oviduct and lysosome-rich cells assumed to function in recycling follicle cells sloughed off from mature egg chambers (Spradling 1993). The oviducts, accessory glands, uterus, vagina, and external genitalia of the adult, which are derived from the genital imaginal discs, are connected to the ovary during day 3 of pupation (Mahowald and Kambysellis, 1980).

1.3 Germline cell differentiation in early oogenesis

\textbf{Figure 1.2} Pattern of interconnections within a 16-cell cyst. One of the two cells with four ring canals, i.e. cell 1 or cell 2, always develops into the oocyte in a wildtype egg chamber (adapted from Spradling, 1993).
Oogenesis in adult flies commences when a germline stem cell asymmetrically divides into two daughter cells, one of which remains as a stem cell while the other becomes a cystoblast. The cystoblast immediately undergoes four synchronous rounds of divisions to form a cluster of sixteen cells, called cystocytes. Because cytokinesis is incomplete at each division, the cystocytes remain connected by specialised intercellular bridges known as ring canals. Following cyst formation, one of the cystoblasts develops into an oocyte while the other fifteen cells differentiate as nurse cells.

1.3.1 The fusome, a germline specific organelle

A germline specific organelle, called the fusome, plays a critical role in germ cell differentiation in Drosophila. It has been shown by electron micrographs that the fusome is a distinct region of cytoplasm rich in fibrils and vesicles but excluding mitochondria and most ribosomes (Storto and King 1989). It is derived from the spectrosome, a large sphere of fusomal material in stem cells and cystoblasts (Lin and Spradling, 1995). One of the major distinctions between spectrosomes and fusomes is the increased density of the tubule network in fusomes. Fusome growth is associated with the 16-cell cyst formation. During cystocyte division, one pole of each spindle associates with the fusome/spectrosome, and following each mitosis, as the spindle disaggregates, additional fusomal material accumulates in their place. By the fourth cystocyte division, the fusome forms an elongated and branched structure that extends through the ring canals to all the cells in the 16-cell cysts. Fusomes degenerate shortly after the formation of the 16-cell cyst (Lin and Spradling, 1995; for review see Mckearin, 1997).

The fusome contains a network of membrane tubules or reticulum. McKearin (1997) suggests that the fusome tubules might be a germ cell-specific modification of some intracellular membrane compartments, such as the trans-Golgi network, endosome, Golgi or endoplasmic reticulum. A few fusomal components have been identified. These include the membrane skeletal proteins $\alpha$-spectrin ($\alpha$-Spc), Hu-li tai shao (Hts), $\beta$-spectrin, ankyrin, and the mAb 1B1 antigen (de Cuevas et al., 1996). It has been shown that Hts is the Drosophila homologue of adducin, a mammalian skeletal protein that is enriched at sites of cell-cell contact (Yue and Spradling, 1992; Lin et al., 1994).

Ultrastructural and immunohistochemical studies show that the fusome/spectrosome localises to one pole of the mitotic spindle during cystocyte division (Storto and King, 1989; Lin and Spradling, 1995). At the first cystoblast
division, the spectrosome is associated with only one pole of the mitotic spindle, revealing that this division is asymmetric. During the subsequent three divisions, the growing fusome is always associated with the pole of each mitotic spindle that remained in the mother cell, and only extended through the newly formed ring canals after each division was completed (Lin and Spradling, 1995). These observations suggest that fusomes may play a role in establishing a system of directional transport between cystocytes that underlies oocyte determination.

1.3.2 Formation of germline 16-cell cysts

The 16-cell cysts are formed in an assembly-line fashion in the germarium. The first step towards this is the transition of the stem cell to the cystoblast. However, little is known about the mechanism by which this transition occurs. The analysis of a gene called \textit{bag-of marbles} (\textit{bam}) has shed some light on this process. \textit{bam} transcription is greatly induced in cystoblasts and the inactivation of \textit{bam} blocks cystoblast differentiation (McKearin and Spradling, 1990). In \textit{bam} mutant germ cells, the fusome reticulum is greatly reduced (McKearin and Ohlstein, 1995). Barn has been shown to be a fusome protein that co-localises with fusome-associated spectrin and Hts. In addition, it also accumulates in the cytoplasm of the mitotically active cystocytes in germarium region 1. The cytoplasmic Barn (\textit{BarnC}) protein could be critical for \textit{bam} function (McKearin and Ohlstein, 1995).

The cystoblast undergoes exactly four rounds of incomplete divisions to produce sixteen cystocytes. Therefore, a counting mechanism must exist to control these divisions. It appears that two fusomal proteins, Hts and \textit{\alpha}-spectrin, are required for the counting mechanism. Germ cell clones in female sterile mutations of \textit{hts} and \textit{\alpha-spc} form cysts that have four to eight interconnected nurse cells (Lin \textit{et al.}, 1994; Lin and Spradling, 1995; de Cuevas \textit{et al.}, 1996). Since the fusome is eliminated or reduced in these mutant germ cells, the assembly of the fusome could be essential for the completion of the four rounds of mitotic divisions. The link between mitotic counting and fusome function has been strengthened by the discovery of the accumulation of cyclin A in the fusome (Eberhart \textit{et al.}, 1996; McKearin, 1997). From these observations it can be seen that the fusome plays an active role in the mitotic counting mechanism in controlling the progression from the cystoblast to the 16-cell cyst.

The proper formation of the cysts also requires a locus called \textit{encore}. The mutant phenotype of \textit{encore} is unique because it results in precisely one extra round of mitosis in the germline. As a result, the \textit{encore} egg chambers contain twice the normal number of germline cells (Hawkins \textit{et al.}, 1996). In \textit{encore} mutant cysts, \textit{bam} expression is
expanded to the M4 cystocytes. Moreover, \textit{bam} null alleles acts as a dominant suppressor of the extra round of mitosis in \textit{encore} cysts. These observations suggest that \textit{bam} must be expressed for each of the cystocyte divisions. The clearance of the cytoplasmic Bam protein after the fourth mitotic division may serve as a signal for stopping the division cycles.

\subsection*{1.3.3 Oocyte specification}

In germarium region 2, cystocytes stop dividing after the formation of 16-cell cysts is completed. Chromosomes in the two oldest cystocytes form synaptonemal complexes; eventually complexes are only retained in one cell, which will become the oocyte. It seems that the germline cell fate has already been determined in region 2, as some mRNAs and proteins are selectively transported into one of the 16 cystocytes, the pro-oocyte.

How is one of the sixteen cystocytes selected to become the oocyte? Several models have been presented to answer this question. One of the simplest favour the existence of an "oocyte determination factor", which is asymmetrically distributed in each round of the mitotic division. However, this factor, if it exists, remains unidentified. One possibility is that the "oocyte determination factor" is actually a combination of factors and organelles, which is required in the initiation and maintenance of the asymmetry in the 16-cell cyst. Since the spectrosome/fusome is asymmetrically distributed during the cystoblast mitosis, it may serve as the oocyte determination factor. Furthermore, in the cystoblast, one of the two centrioles is associated with the spectrosome, and this marks the centriole of the oocyte microtubule organisation centre (MTOC), which is the only active MTOC within the 16-cell cyst. The oocyte MTOC has been shown to be critical in oocyte specification. Treating ovaries with microtubule-disrupting drugs prevents the formation of the oocyte and causes all cystocytes to differentiate into polyplloid nurse cells (Theurkauf \textit{et al.}, 1993). Strong mutants of \textit{Bicaudal-D (Bic-D)} and \textit{egalitarian (egl)} produce cysts of 16 nurse cells and no oocyte (Schüpbach and Wieschaus 1991; Suter \textit{et al.}, 1989; Suter and Steward, 1991). In \textit{Bic-D} and \textit{egl} mutations, the MTOC, which normally forms in the oocyte, is not detected and oocyte-specific accumulation of biomolecules is blocked. This indicates that \textit{Bic-D} and \textit{egl} probably play a role in microtubule-based transport early in oogenesis. Mach and Lehmann (1997) showed that Egl and Bic-D proteins are co-localised at all stages of oogenesis and form a protein complex. It is proposed that the Egl/Bic-D complex link microtubule polarity and RNA transport, and this in turn promotes oocyte differentiation during early stages of oogenesis.
Stonewall (stwI), which encodes a germ cell nuclear protein that has a helix-turn-helix DNA binding domain, is required for oocyte differentiation. Mutations in stonewall block proper oocyte differentiation and frequently cause the presumptive oocyte to develop as a nurse cell. Since the initial stages of cyst formation are not affected in stwI mutants, stwI transcriptional regulation is suggested to be essential for the maturation of cystocytes into specialised nurse cells and oocyte. It probably responds to oocyte determination signals and acts as a transcriptional regulator of genes required in nurse cells to promote nurse cell/oocyte differentiation. In the absence of stwI, oocyte differentiation cannot be maintained efficiently and the presumptive oocyte develops as a nurse cell; eventually the lack of Stwl-dependent gene expression causes all germ cells to activate apoptosis (Clark and McKearin, 1996).

1.3.4 Oocyte and the nurse cell differentiation

After the oocyte is specified, the germline cells further develop towards their different fates. One of the important aspects is the chromosomal structure, which becomes highly adapted to the specialised functions the cells perform during oogenesis. Oocyte chromosomes undergo synapsis and recombination during the early stages of cyst development. Then the nucleus condenses to become a transcriptionally inactive karyosome at about stage 3, and remains in meiotic prophase until just before the oocyte matures. As egg chambers develop, nurse cell chromosomes also undergo structural changes. The banded polytenic chromosomes in young nurse cells progressively disperse to form large dispersed nucleoli. These changes are thought to facilitate the high synthetic levels of ribosomes and other components required for oocyte growth.

Genes required for normal germ cell chromosomal development are required to sustain normal oocyte growth. Among these are ovarian tumor (otu), fs(2)cup (cup), and fs(2)B (Keyes and Spradling, 1997). otu and cup mutants have similar phenotypes: they disrupt the normal morphology of germ cell chromosomes. These two genes interact genetically, and both otu and cup encode cytoplasmic proteins (Sass et al., 1995; Keyes and Spradling, 1997). Thus their requirement for chromosomal structure changes is probably indirect. Another function of both cup and otu is in oocyte growth, as some alleles of these two genes affect the growth of the oocyte relative to the nurse cells during vitellogenesis. It is likely that this phenotype results from defects in transport from the nurse cells into the oocyte (Keyes and Spradling, 1997).

encore has been shown to be essential for cyst formation (Hawkins et al., 1996). In addition to this function, it is also required for oocyte differentiation. In wildtype egg chambers, the oocyte nucleus is arrested in meiosis-I with a DNA content.
of 4C, while the nurse cell nuclei begin endoreplication as the cyst exits the germarium. In *encore* mutants, the oocyte frequently partially acquires a nurse cell fate in that the oocyte nucleus undergoes endoreplication (Hawkins *et al.*, 1996). This phenotype is similar to some alleles of *otu* that allow formation of an oocyte/nurse cell syncytium. In *encore* mutants, an oocyte marker, *oskar* mRNA, is localised to the oocyte normally in the germarium and early egg chambers, but fails to be localised at slightly later stages (Hawkins *et al.*, 1996). This suggests that *encore* function is required for the maintenance of the oocyte fate once it has been determined.

### 1.4 Somatic cell differentiation in early oogenesis

#### 1.4.1 Somatic stem cells and non-dividing somatic cells

At germarium region 2, the 16-germ cell cysts are enveloped by a monolayer of epithelial follicle cells. These follicle cells are descendants of somatic stem cells. Using an FLP-catalysed mitotic recombination technique, Margolis and Spradling (1995) revealed that there are two somatic stem cells located near the border of germarium regions 2a and 2b (for germarium regions refer to Figure 1.1C). Each cyst at region 2b is covered by about 16 follicle cells, which are produced by one division of both somatic stem cells and four rounds of division of their progeny. These divisions require about 9.6 hours, which is consistent with the fact that new cysts bud from the germarium approximately every 10-12 hours (Margolis and Spradling, 1995). However, the division of the somatic stem cells and the germline stem cells are not co-ordinated. In agametic flies, somatic stem cells continue to divide in the absence of the germline cells. Therefore, a general co-ordination mechanism may exist to keep the balance between germline and somatic cell populations.

At the anterior-most tip of the germarium is the terminal filament containing a stack of 6-9 non-dividing somatic cells. The terminal filament is closely associated with another group of somatic cells called cap cells. Both the basal cells of the terminal filament and the cap cells are in close proximity to the germline stem cells. Lin and Spradling (1993) laser ablated the terminal filament in transplanted ovaries, and found that the rate of egg chamber production increased. Forbes *et al.* (1996A) showed that the terminal filament and cap cells express a cell-cell signalling molecule, Hedgehog (Hh), which acts as a morphogen in regulating cyst encapsulation in germarium region 2. This indicates that the terminal filament is the source of a signal which regulates follicle cell proliferation and differentiation in the germarium.
1.4.2 Cyst envelopment and follicle cell differentiation

Within germarium region 2a, the envelopment of the germline cysts starts when somatic follicle cells migrate from the wall of the germarium to surround the 16-cell cysts. This is the earliest migration these somatic cells perform during oogenesis. The individual cysts are not separated by the inwardly migrated follicle cells until the cysts reach germarium region 2b. Since the follicle cells do not attach to the 8-cell cysts, it is suggested that the association of the follicle cells with the 16-cell cysts is probably due to an inducing signal produced in the 16-cell cysts (Spradling, 1993).

Several signalling pathways appear to be involved in the regulation of cyst encapsulation. Hh is expressed in the terminal filament and cap cells. It acts as a morphogen source to regulate the proliferation and specification of somatic cells during germline cyst encapsulation in region 2b, which is about 2 to 5 cells away from the Hh expressing cells (Forbes et al., 1996B). Reducing Hh activity in germaria blocks cyst encapsulation, resulting in mis-incorporated egg chambers. Weakly affected germaria continue to produce large, abnormal egg chambers that contain more than 15 germline cells. Severely affected ovarioles are also produced that lack any budded egg chambers. Ectopic Hh expression in the ovary results in a dramatic increase in the number of somatic cells accumulating between the egg chambers. These interfollicular somatic cells fail to express a stalk cell marker, suggesting Hh activity is restricted in the proliferation of pre-follicle cells rather than stalk cells (Forbes et al., 1996B).

Some genes downstream of the Hh signalling pathway during embryonic and imaginal disc development, including patched (ptc) and cubitus interruptus (ci), are required for transmitting Hh signalling in somatic cell differentiation during oogenesis (Ingham, 1995). However, other effector genes such as decapentaplegic (dpp) and wingless (wg), are not regulated by Hh in oogenesis (Forbes et al., 1996B; Ingham, 1993; Ingham and Feitz, 1995). The function of the Hh signalling pathway in oogenesis has been shown by ectopic expression of Hh and loss of ptc function, which result in over-proliferation of the somatic cells. It has been suggested that Hh diffuses from the apical cells, including the cap cells, and regulates the proliferation of the somatic cells by antagonising the negative effects of ptc and ci activity in these cells (Forbes et al., 1996B).

Additionally, the Hh signalling pathway appears to be required for the differentiation of pairs of polar follicle cells, which are located at the anterior and posterior pole of each egg chamber (Forbes et al., 1996B). Polar cells are specified shortly after cyst encapsulation (Margolis and Spradling, 1995), and are required for
polarity organisation of the egg chambers. They specifically express Fascilin III and *neuralized* (Ruohola et al., 1991). FLP-catalysed mitotic recombination analysis reveals that the anterior and posterior polar cells are derived from the same cell lineage (Margolis and Spradling, 1995). They stop dividing long before those follicle cells around them. When Hh is ectopically expressed, extra polar cells are produced, which in turn disrupts egg chamber polarity (Forbes et al., 1996a).

Another signalling pathway, including neurogenic genes, *Notch* and *Delta*, is also essential for follicle cell differentiation and cyst encapsulation (Ruohola et al., 1991; Xu et al., 1992; Bender et al., 1993). Notch and Delta signalling has been shown to be involved in a number of developmental processes. They exhibit a lateral inhibition mechanism in specifying cell fate (Muskavitch, 1994). During early oogenesis, constitutively active *Notch* arrests follicle cells at a precursor stage, while the loss of *Notch* function eliminates this stage. Long interfollicular stalk structures are generated when constitutively active *Notch* is expressed in the germarium. On the contrary, "loss-of-function" alleles result in hyperplasia of stalk cells early in oogenesis and later, a loss of polar cells (Ruohola et al., 1991; Larkin et al., 1996). These observations indicate that *Notch* functions in holding the follicle cells in a precursor stage of development. Moreover, *Notch* is also required in the follicle cells for germ-line cyst envelopment. This has been shown by the analysis of some *Notch* mutant egg chambers that contain 32 germ cells, resulting from encapsulation of two germ-line cysts (Ruohola et al., 1991).

In addition, a novel signalling pathway, involving the neurogenic genes, *egghead* (*ego*) and *brainiac* (*bri*), is also critical for cyst encapsulation. Both *ego* and *bri* are required in germ cells. *ego* encodes a novel secreted or transmembrane protein, while *bri* encodes a novel, putative secreted protein (Goode et al., 1996A). *ego* and *bri* expression in germ-line cells correlates with follicular epithelium morphogenesis throughout oogenesis. In the absence of *ego* or *bri* function, formation of the follicular epithelium is less efficient. As a consequent, several cysts are wrapped in one chamber. Additionally, both genes are required for maintaining the follicular epithelium once the egg chamber is established. Mutations in both genes in the germ-line result in loss of apical-basal polarity and accumulation of follicle cells in multiple layers, particularly surrounding the oocyte. Furthermore, these two genes are required for follicle cell migration and dorsal-ventral polarity formation. It has been shown that *bri* cooperates with *torpedo*, a gene encoding the *Drosophila* EGF receptor, during follicle cell migration (Goode et al., 1995, 1996A, 1996B).
The maintenance of the follicular epithelium is also part of Notch’s function in oogenesis. In Notch mutants, a similar phenotype to mutants of both egh and brn is observed. This may suggest that brn and egh mediate Notch function, which is required in the somatic cells. However, neither brn nor egh is essential for polar/stalk cell fate specification, while Notch plays an active role in this function (Goode et al., 1996A).

1.4.3 Epithelial sheath

Epithelial sheath cells normally surround the entire ovariole; at the anterior, they completely envelop the germarium. These cells may interact with the germline stem cells. Margolis and Spradling (1995) suggested that the inner sheath cells might be involved in sending signal(s) to the germline cells to establish their sexual identity. This is based on the observations that these cells co-express markers in certain enhancer-trap line, and are absent in agametic ovaries. However, removal of sheath cells in transplanted germaria appears to have no effect on oogenesis (Lin and Spradling, 1991), although it is not known if the inner sheath cells are removed completely in this experiment.

1.5 Polarity formation during oogenesis

Systematic genetic analysis has identified four separate genetic hierarchies that control axis formation in embryos, three of which are designed for AP axis formation, while only one is used to define the DV axis. The stepwise establishment of polarities is initiated during oogenesis. In stage-10 oocyte, bicoid (bcd) and oskar (osk) mRNAs are localised at the anterior and posterior end respectively to define the AP axis of the future embryo, while gurken (grk) transcripts are at the dorsal-anterior corner to induce the DV polarity of the egg (for review see St Johnston and Nüsslein-Volhard, 1992). How the polarities are initiated and established are among the most important questions in oogenesis.

1.5.1 The origin of polarity

In the wildtype egg chamber, the oocyte is always located posterior to the nurse cells, which marks the AP polarity of the egg chamber. However, at germarium region 2, the pro-oocyte is located near to the centre of the lens-shaped 16-cell cyst. It moves posteriorward to make contact with the somatic follicle cells that have migrated from the germarium wall to envelop the cyst. At germarium region 3, the oocyte is positioned at the posterior of the newly formed stage-1 egg chamber. This movement appears to be
among the earliest morphological signs of polarity formation, and is a key step for axis establishment. In mutant egg chambers where the oocyte is positioned in the middle, localisation of \textit{bcd}, \textit{osk} and \textit{grk} mRNAs is disrupted, which in turn disrupts the two axes (Peifer \textit{et al.}, 1993; González-Reyes and St. Johnston, 1994).

The movement of the oocyte to the posterior requires the functions of several genes, including \textit{armadillo}, five \textit{spindle} genes (\textit{spindle A to E}), and \textit{dicephalic} (González-Reyes and St. Johnston; 1994). When one of these genes is mutated, the oocyte is usually mis-positioned in the egg chamber. \textit{armadillo} (\textit{arm}) is a segment polarity gene and encodes a \textit{Drosophila} homologue of adhesion junction components plakoglobin and \(\beta\)-catenin (Peifer, 1995). Germline \textit{arm} mutations disrupt the cell arrangement and cytoskeletal system, resulting in mis-positioning of the oocyte. Armadillo protein is distributed in the vicinity of cell-cell adhesive junctions in \textit{Drosophila} ovaries. In the gerarium, Armadillo is distributed at the posterior pole of the earliest egg chamber (Peifer \textit{et al.}, 1993). This is consistent with its proposed function in holding the oocyte at the posterior of the egg chamber. In \textit{arm} mutant egg chambers when the oocyte is positioned at the anterior end, maternal determinants appear to be localised correctly, although the AP polarity is inverted relative to that of the ovariole. However, when the oocyte is positioned at the middle in an \textit{arm} mutant egg chamber, the localisation of \textit{osk} and \textit{orb} mRNAs is disrupted (Peifer \textit{et al.}, 1993). These observations indicate that the establishment of polarity requires the oocyte to occupy one end of the egg chamber in order to make contact with the somatic follicle cells.

Genetic evidence suggests that the origin of oocyte polarity is directly linked to the initial cell fate determination that singles out the oocyte from its 15 sister cells. \textit{spindle} (\textit{spindle A-E}) genes are also required for oocyte specification. In some \textit{spindle} mutants, \textit{grk} and other RNAs are localised to the two cystocytes with four ring canals, indicating that two oocytes are specified in one 16-cell cyst (González-Reyes and St Johnston, personal communication). In these egg chambers, oocytes cannot be properly positioned at the posterior, which in turn disrupts polarity formation. Since the cloning of the \textit{spindle} genes has not been reported, it is unclear what roles they play in oocyte specification and positioning.

\textit{egl} and \textbf{Bic-D} activity is also required for both oocyte specification and the maintenance of polarised microtubules within the oocyte. In \textit{egl} and \textbf{Bic-D} mutant egg chambers, when no oocyte is specified, the polarisation process is also disrupted (Suter \textit{et al.}, 1989; Mach and Lehmann, 1997).
In summary, the origin of polarity within the egg chamber and the oocyte is strongly linked to the oocyte specification. Once the oocyte is singled out from its sister cells, a polarised cytoskeletal network is established in the germline cyst, which will lead to the posterior movement of the oocyte. The maintenance of the oocyte at the posterior pole probably requires the function of polar follicle cells. Disturbance of any of these steps will lead to the disruption of polarity formation (Figure 1.3 and 1.4).

1.5.2 AP axis formation

When the oocyte is correctly positioned at the posterior end of the egg chamber, it is in contact with the posterior follicle cells. A signal, which has been identified as the *Drosophila* transforming growth factor alpha (TGF-α) homologue, Gurken (Grk), is sent out and received by the adjacent posterior follicle cells (Neuman-Silberberg, 1993; González-Reyes *et al.*, 1995; Roth *et al.*, 1995). The Grk RNA and protein are localised at the posterior pole around the oocyte, in association with the posteriorly positioned germinal vesicle. It is proposed that the Grk protein is the ligand of Torpedo (Top), the *Drosophila* homologue of the epidermal growth factor receptor tyrosine kinase (EGF-R/DER), which is expressed in follicle cells (Roth *et al.*, 1995; González-Reyes *et al.*, 1995; Clifford and Schüpbach, 1992; Neuman-Silberberg and Schüpbach, 1993). The binding of Grk to Top/DER activates a signal transduction pathway, involving Ras, DRaf, and Dsor1, in adjacent follicle cells and induces these cells to adopt a posterior fate (Schnorr and Berg, 1996; Lee and Montell, 1997; Brand and Perrimon, 1994). In *grk* and *top* mutant egg chambers, follicle cells at the posterior pole express an anterior marker, indicating that they adopt the default anterior fate when Grk-DER signalling is disrupted (González-Reyes *et al.*, 1995; Figure 1.3 and 1.4).

Additionally, the differentiation of posterior follicle cells also requires signalling among the somatic cells. The pre-differentiation of the polar follicle cell fate is essential for this process. Therefore, signalling pathways required for polar cell differentiation, such as Notch-Delta and Hh signalling pathways, are also essential for axis formation.
Figure 1.3 Gurken signalling is required for both the anterior-posterior (AP) and dorsal-ventral (DV) axes determination during oogenesis. Arrows between the oocyte and the follicle cells show the direction of signalling. During stages 1-6, both the oocyte nucleus and the Gurken signal are located at the posterior pole of the oocyte. The Gurken signal causes the adjacent follicle cells to adopt a posterior fate. These follicle cells later send an unidentified signal back to the oocyte to re-orientate the microtubules in the germ cell cluster. This in turn leads to the localisation of the anterior determinant, \textit{bicoid}, at the anterior and the posterior determinant, \textit{oskar}, at the posterior of the oocyte. The re-orientation of the microtubules also leads to movement of the oocyte nucleus towards the anterior and localisation of the Gurken signal into the future dorsal-anterior corner during stage 8. The Gurken signal is again received by the adjacent follicle cells, and in turn it determines the dorsal-ventral axis of both the eggshell and the embryo.
Figure 1.4 A pathway of genes required for establishment of the AP and DV axes in *D. melanogaster* during oogenesis. A key step in this pathway is the re-orientation of the microtubules in the germ cell cluster during mid-oogenesis, which requires germline-soma cell-cell interactions. The microtubule re-orientation leads to localisation of morphogenetic determinants *bicoid* and *oskar* at both poles of the oocyte, which is crucial for establishment of the anterior/posterior axis. Re-orientation of the microtubules also leads to dorsal-anterior localisation of the Gurken mRNA and protein, which activates a number genes that are required for DV patterning of the eggshell and the embryo. For details see text.
The posterior follicle cells that have received the Grk signal from the oocyte are thought to send an unidentified signal back to the oocyte. Consequently, the microtubule network in the germline cells is re-arranged. The microtubules extend from the MTOC at the posterior end of the oocyte into the adjacent nurse cells during stages 1 through 6. This posterior MTOC degenerates during stages 7 and 8 and microtubules begin to associate with the anterior margins of the oocyte (Theurkauf et al., 1993; Theurkauf, 1994). This in turn leads to the establishment of AP polarity in the oocyte. Protein kinase A (PKA) may be required for the transmission of the signal from the posterior follicle cells to the oocyte, since the microtubule fails to be reorganised in a PKA germline mutant (Lane and Kalderon, 1994, 1995). Additionally, mago nashi appears to be required in the germline for the re-organisation of the microtubules (Micklem et al., 1997).

During early stages, microtubules are required for preferential accumulation of mRNAs and proteins in the previtellogenic oocyte, consistent with the idea that these molecules are transported by a microtubule-dependent mechanism to the oocyte (Pokrywka and Stephenson, 1995). When the microtubule polarity is re-oriented at stage 7, maternal determinants that are localised to the oocyte during early stages are re-distributed, as shown by the posterior localisation of osk RNA, anterior localisation of bcd RNA, and dorsal-anterior localisation of grk RNA. The central role played by microtubules in axis formation has been shown by using inhibitors of microtubule function to study the effect on maternal determinant localisation. Maternal determinants, such as bcd and osk, fail to be localised when the microtubule reorganisation is disrupted (Pokrywka and Stephenson, 1991, 1995; for reviews see Theurkauf, 1994; Cooley and Theurkauf, 1994; Pokrywka, 1995).

The anterior morphogen is bicoid (bcd), which encodes a transcription factor containing a homeodomain, which binds to DNA. bcd RNA is localised in the cytoplasm at the anterior pole of the egg, and is translated after fertilisation to produce an anterior to posterior gradient of Bcd protein that extends over two thirds of the embryo (Berleth et al., 1988; Frohnhofer and Nusslein-Volhard, 1986; Driever and Nusslein-Volhard, 1988). Some evidence demonstrates that the Bcd protein gradient is sufficient to determine the anterior polarity and pattern of the embryo (St Johnston et al., 1989). The anterior localisation of bcd mRNA is microtubule dependent: treating egg chambers with the microtubule depolymerizing agent, colchicine, disrupts its localisation (Pokrywka and Stephenson, 1991). In addition to the microtubules, three genes, exuperantia, swallow, and staufen, are thought to be directly involved in bcd RNA localisation (Marcey et al., 1991; Stephenson et al, 1988). From stage 9-10b, bcd
RNA accumulates in the apical regions of the nurse cells, a process that requires *exuperantia* function. From stage 10b-12, all of the *bicoid* RNA becomes localised to the anterior cortex of the oocyte. In a *swallow* mutant, this localisation is disrupted. It has been suggested that *swallow* encodes a component of the cytoskeleton that anchors *bcd* RNA at the anterior pole (Stephenson *et al.*, 1988). *staufen* is required for holding *bicoid* RNA at the anterior pole (St Johnston *et al.*, 1991; Ferrandon *et al.*, 1994). This is related in their anterior co-localisation. It has been shown that Staufen binds to a long stem-loop formed by three regions of the *bicoid* 3'UTR (Ferrandon *et al.*, 1994).

A greater number of genes have been characterised which are involved in posterior polarity determination. The localisation of maternal determinants to the posterior pole seems to be more complicated than for anterior localisation. A possible explanation for this is that all the components of pole plasm have to be localised, not only the posterior determinant, *nanos* RNA (Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). The first identified molecules to reach the posterior pole are Staufen protein and *osk* RNA. These are followed by Vasa protein, *cyclin B* RNA, and towards the end of oogenesis, *nanos* RNA (Ephrussi *et al.*, 1991; Kim-Ha *et al.*, 1991; Lasko and Ashburner, 1990; Whitfield *et al.*, 1989). It is a stepwise process, in which the components that localise early are required for the subsequent localisation of those that arrive later in oogenesis. However, it is unclear how the first molecule reaches the posterior pole. Several lines of evidence suggest that Staufen protein associates with *oskar* RNA at the anterior of the oocyte and the two are then transported around the cortex to the posterior pole as a complex, in a process requiring two other posterior group genes, *cappuccino* and *spire* (Manseau and Schüpbach, 1989; Theurkauf, 1994). The posterior localisation of these morphogenetic determinants is also microtubule dependent.

### 1.5.3 DV axis formation

The formation of the DV axis also requires an interaction between the germline and the follicle cells. First, a signal from the germline determines the dorsal follicle cell fate, and then, follicle cells signal back to pattern the DV axes of the egg/embryo (Schüpbach, 1987) (Figure 1.3 and 1.4).

During stage 8 of oogenesis, the germinal vesicle migrates from the posterior to the anterior margin of the oocyte. It appears that this movement is crucial for the establishment of DV polarity: in egg chambers with the germinal vesicle laser ablated, DV polarity fails to be established (Montell *et al.*, 1991). Germinal vesicle movement is
microtubule-dependent (Roth *et al*., 1995). The reorganisation of the germline microtubule network at stage 7, a key step in AP polarity formation, is the major force for germinal vesicle migration. This indicates that DV axis formation is dependent on the existing AP axis. As the germinal vesicle moves to the anterior margin, *grk* mRNA, which is associated with the germinal vesicle at the posterior pole during earlier stages, remains associated with the oocyte nucleus. As a result, *grk* mRNA and protein are localised at the dorsal-anterior corner of the oocyte (Figure 1.5), and this induces the follicle cells facing the germinal vesicle to adopt a dorsal fate. The receptor of the Grk signal is still Top/DER, which is used earlier in inducing the posterior follicle cell fate. Ras, Draf and Dsor1 are involved in transmitting the signal in the dorsal anterior follicle cells (Brand and Perrimon, 1994; Lu *et al*., 1994; Schnorr and Berg, 1996).

The localisation of *grk* RNA to the anterior corner surrounding the germinal vesicle requires the function of *fs(1)K10* (*K10*), a gene encoding a helix-loop-helix DNA binding protein (Wieschaus *et al*., 1978; Haenlin *et al*., 1987; Figure 1.5). *K10* RNA is transported to the anterior oocyte with its protein localised in the oocyte nucleus. In *fs(1)K10* mutant egg chambers, *grk* RNA is localised in the anterior apical region of the oocyte, which induces a ring of anterior follicle cells to become dorsalised. In addition to *K10*, a number of other genes also appear to be required in *grk* RNA localisation, such as *orb*, *Bic-D*, and *squid* (Christerson and McKearin, 1994; Lantz *et al*., 1992, 1994; Ran *et al*., 1994; Swan and Suter, 1996; Kelly, 1993).

A small number of genes have been identified that are expressed in the follicle cells, downstream of the Grk-DER signalling pathway. *rhomboid*, the first to be identified, encodes a transmembrane protein (Bier *et al*., 1990). It shows an asymmetric expression pattern in the follicle cells along the DV axis. It has been suggested that it intensifies the action of Grk-DER signalling, as has been shown for some other developmental pathways (Ruohola-Baker *et al*., 1993). *pointed*, a gene encoding a transcription factor with ETS domains, is expressed in the dorsal midline follicle cells at stage 10A and is required for DV polarity formation in the eggshell (Klaes *et al*., 1994; Klämbt, 1993; Morimoto *et al*., 1996). It is expressed in response to Grk-DER signalling and is thought to down-regulate this signalling pathway in cells where it is expressed (Morimoto *et al*., 1996).

*CF2* is a zinc-finger transcription factor which is expressed in follicle cells except those in the dorsal-anterior region (Hsu *et al*., 1992). It is required for DV polarity formation of both the embryo and the eggshell. *CF2* is probably a mediator between the dorsal and ventral signals. Since the expression of *CF2* along the DV axis
is also specified by Grk-DER signalling, a gene must exist with an expression pattern which is dependent on Grk-DER signalling and which acts to suppress CF2 expression in the dorsal anterior follicle cells (Hsu et al., 1996).

In the absence of dorsal signals, follicle cells will follow a ventral fate, and contribute to a signal that is stored in the fluid between the oocyte and vitelline membrane. Twelve genes have been characterised in the signalling pathway from follicle cells to the oocyte. Mutations in these genes affect the DV axis of the embryo. But unlike the upstream genes, they have no effect on the eggshell. The ventral signal, which is thought to be produced by pipe, nudel or windbeutel, has not been characterised (Anderson et al., 1985A; Hong and Hashimoto, 1995). It is thought to be a ligand of Toll, a transmembrane protein that is evenly distributed on the oocyte membrane. This binding is critical to the final gradient distribution of the DV axis determinant, Dorsal protein, in the embryo (Figure 1.4; Anderson et al., 1985A, 1985B; Roth et al., 1989).

It is interesting that the position of the germinal vesicle and grk RNA is crucial to both AP and DV axes formation. González-Reyes et al. (1995) suggested that AP is the primary axis, with DV axis formation dependent on the prior establishment of the AP axis. The movement of the germinal vesicle to the anterior margin - the first step in DV axis formation - requires the already established AP polarity of the microtubule system within the oocyte, which is a result of the AP axis specification. In some mutant egg chambers, where the microtubule cytoskeleton develops a symmetric organisation, with 'minus' ends at both poles, the germinal vesicle can be localised at either end of the oocyte. As a result, grk RNA is mislocalised and the DV axis is disrupted.
Figure 1.5 Whole-mount *in situ* hybridisation to show grk mRNA localisation in the wildtype (A) and *fs(1)K10¹* (B,C) mutants during stages 8-10 of oogenesis. (A) In wildtype egg chambers, grk mRNA is localised at the dorsal-anterior corner of the oocyte during stages 8-10. This localisation is disrupted in *fs(1)K10¹* egg chambers. It is shown in (B) that grk mRNA is ectopically localised at the anterior end of the oocyte during stages 9-10A. However, the signal is stronger at the dorsal-anterior corner when compared to the rest of the anterior region (arrow). During stage 10B, ectopically localised grk mRNA nearly disappears, whilst strong signals remain at the dorsal-anterior corner of the oocyte (C).
1.6 Follicle cell migration and eggshell morphogenesis

Cell migration, which involves dynamic cell shape change and rearrangement of the cytoskeleton, is one of the major cellular activities in the morphogenesis of multicellular organisms. During oogenesis, follicle cells undergo a series of migrations in order to form egg chambers and specific structures of the eggshell. These epithelial cells provide an ideal model for the study of the molecular mechanisms regulating cell motility and morphogenesis.

During oogenesis, the first major follicle cell migration is that from the wall of the germarium to surround the 16-cell germinal cysts in germarium region 2. This is a significant step in the formation of egg chambers, which has been discussed in section 1.4.2. During middle and late oogenesis, a series of follicle cell migrations occur. These include: (1) During stage 9, a group of 6-10 follicle cells at the anterior tip of egg chambers migrate through the nurse cell cluster to the oocyte border; (2) At the same stage, the majority of the follicle cells move towards the posterior to form a columnar epithelium covering the oocyte, while the remaining follicle cells stretch to cover the nurse cell cluster; (3) During stage 10b, the anterior columnar cells migrate centripetally between the oocyte/nurse cell border to cover the anterior end of the oocyte; (4) Two groups of columnar cells at the dorsal-anterior region migrate anteriorly to produce a pair of dorsal appendages (filaments) (Spradling, 1993; Deng et al., 1997; Figure 1.6 and 1.7).

1.6.1 Border cell migration

Starting at stage 9, a group of 6-10 anterior cells delaminate from the follicle cell layer and migrate through the nurse cells (Figure 1.6D, 1.7B). These cells are so-called border cells because they complete their migration at the border between the nurse cells and the oocyte at stage 10. The border cells serve at least two functions during oogenesis: they participate in the formation of an anterior eggshell structure, the micropyle, thus maintaining an opening through which the sperm enters at fertilisation (Montell et al., 1992); they express Torso-Like (Tsl) which is required for embryonic terminal segmentation (Savan-Bhonsale and Montell, 1993).

The border cells are good models to study the temporal regulation of cell migration. The initiation of border cell migration is controlled by the slow border cell (slbo) locus (Montell et al., 1992). Weak slbo mutations result in retarded border cell migration, whereas stronger alleles cause complete failure of migration and female sterility. Null slbo alleles result in embryonic lethality. slbo encodes the Drosophila
homologue of C-EBP, a basic region/leucine zipper transcription factor (Friedman et al., 1989; Rørth and Montel, 1992; Rørth, 1994).

*breatless* (*btl*) is a key target gene of *sibo* in regulating border cell migration. During oogenesis, *btl* is specifically expressed in the border cells, and is dependent on *sibo*+ function. *sibo*-independent *btl* expression is able to rescue the migration defects in weak *sibo* mutants. *btl* mutations are dominant enhancers of weak *sibo* alleles. In the *btl* 5' regulatory region, there are eight *Drosophila* C/EBP binding sites, this therefore suggests that *btl* may be a direct target of *sibo* (Murphy et al., 1995).

*btl* encodes a homologue of vertebrate fibroblast growth factor receptor (FGFR), a membrane receptor tyrosine kinase (RTK). It has been found that heat-shock-induced expression of other RTKs, such as Sevenless, could partially rescue the migration defects in *sibo* weak alleles (Lee et al., 1996). These observations therefore indicate that RTK signalling pathways may play a key role in border cell migration. Lee et al. (1996) found that Ras, a common downstream effector for RTKs, is required for border cell migration. A dominant-negative Ras protein inhibits cell migration when it is expressed specifically in the border cells during the period when these cells normally migrate. When it is expressed prior to migration, the dominant-negative Ras promotes premature initiation of migration. Furthermore, reducing Ras activity in border cells prior to migration is able to rescue the migration delay in weak *sibo* alleles. These observations suggest that different levels of Ras activity are required for border cell migration at different stages. Moreover, it appears that the role Ras plays in regulating border cell migration acts via a Raf-independent pathway (Lee et al., 1996).

Non-muscle myosin-II could be a downstream target of Ras signalling in border cell migration, as in egg chambers with the light chain gene, *sqh*, is mutated, border cell migration is blocked (Edwards and Kiehart, 1996).

Border cells migrate along a track between nurse cells. This process requires properly built nurse cell intercellular bridges. The protein kinase A (PKA) catalytic subunit, DCO, seems to be involved in intercellular bridge formation in *Drosophila* oogenesis. Intercellular bridges in egg chambers from *PKA* deficient females are unstable, leading to the formation of multinucleate nurse cells by the fusion of adjacent cells. As a result, border cell migration is disrupted, implying that nurse cell junctions provide an essential path for border cell migrations. Furthermore, the highest levels of PKA catalytic subunit protein are associated with germ cell membranes, suggesting that
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Figure 1.6 β-Galactosidase staining shows subsets of follicle cells during oogenesis. A, B and D are \textit{Gal4} enhancer-trap lines screened in our lab which will be discussed in Chapter 3. C and E are \textit{lacZ} enhancer-trap lines.

A. Staining is observed in the polar follicle cells which are located at both the anterior and posterior poles of egg chambers.
B. The stalk cells interconnecting the egg chambers are stained.
C. The \textit{dpp-}lac\textit{Z} line is stained at the nurse cell associated follicle cells (arrowheads) and the centripetal cells (arrow) during stage 10.
D. The anterior polar follicle (arrow) cells and the migrating border cells show staining.
E. Shows staining in the follicle cells that centripetally migrate along the nurse cell-oocyte border.
the targets of PKA are associated with the membrane or membrane skeleton and contribute to the stabilisation of intercellular bridges (Lane and Kalderon, 1995).

Figure 1.7 Follicle cell migrations during mid-oogenesis. A. Shows a stage-8 egg chamber which has a monolayer of follicle cells over the germ cell cluster. During stage 9, a group of anterior follicle cells delaminate from the follicular epithelium and migrate through the nurse cell cluster (B, arrowhead). These cells stop migration at stage 10 and locate at the nurse cell-oocyte border (C). During stage 9, most follicle cells retract from the nurse cell cluster to cover only the oocyte (arrows in B), leaving only a few stretched cells to be associated with the nurse cells (B, C). During stage 10b, the anterior most columnar cells migrate centripetally along the nurse cell-oocyte border to cover the anterior end of the oocyte (arrows in C).
1.6.2 Migration over the oocyte

During stage 9, the majority of the 795 follicle cells begin to be displaced in a posterior direction (D. St Johnston, personal communication). Eventually more than 95% will stack up to form a columnar epithelium in contact with the oocyte, leaving only about 50 cells remaining over the nurse cells (Figure 1.7B). In order to cover the whole nurse cell cluster, the nurse cell associated follicle cells are stretched and very thin. The nuclei of these stretched cells are normally located at the small valleys created by the joining of two adjacent nurse cells. The function of these cells has not been fully characterised. They may be involved in sending signals to the nurse cells since a *Drosophila* homologue of TGF-β, *decapentaplegic* (*dpp*), is expressed in these cells (Figure 1.6C; Twombly et al., 1996).

The follicle cells that have migrated posteriorly to cover the oocyte are more columnar in shape. The migrating cells move only relative to their underlying substrates but not in relation to their immediate neighbours - they remain interconnected at all times. This is likely due to co-ordinated changes in the cytoskeleton within each follicle cell that are in conjunction with an anchoring point in the posterior pole of the oocyte. The oocyte probably has a signal on its surface at stage 8 to instruct this movement. However, little is known about the genes involved. A *tubulin-β3* gene may have a role in this process, since the follicle cells are the only adult tissue to express this gene, which has been shown to be involved in cell shape changes in some embryonic and pupal tissues (Kimble et al., 1989). However, the function of the *tubulin-β3* gene function in the follicle cells has not been characterised.

1.6.3 Centripetal migration

By stage 10B, when the majority of follicle cells originally covering the nurse cells have completed their migration towards the posterior, the columnar cells over the anterior of the oocyte start to migrate centripetally along the DV axis between the oocyte and the nurse cells (Figure 1.6E and 1.7C). These cells will secrete the anterior end of the eggshell, which along with the eggshell secreted by the columnar cells, will eventually cover the entire egg.

Mutations of a few genes have been identified that block centripetal migration and result in mature eggs with an opened anterior end (Schüpbach and Wieschaus, 1991). *fs(2)cup* is one of these genes. However, it seems that it is not directly involved in regulating centripetal migration. More likely, the failure of migration in *fs(2)cup* mutants is due to the growth of the oocyte being slower than in the wildtype. By stage
10, when centripetal cells start to migrate, the \( fs(2) \) \( cup \) mutant oocytes reach only 1/4 to 1/2 the size of corresponding wildtype oocytes, and occupy no more than 33% instead of 50% of the egg chamber. The under-sized oocyte seems to be too small to accommodate all the columnar cells, many of which are still in contact with the nurse cells. As a result, a centripetal migration never occurs in these egg chambers (Keyes and Spradling, 1997). These observations indicate that the initiation of the centripetal migration requires the anterior-most columnar cells to reach the border between the nurse cells and the oocyte. Thus, there may be a signal sent by the oocyte and received by the anterior-most columnar cells to initiate the migration.

Non-muscle myosin-II, a molecular motor encoded by \( zipper \) \( (zip) \), appears directly involved in centripetal migration. Antibody staining shows that the centripetal cells specifically accumulate this myosin at the edge of the apical (inner) surface that leads the penetration between the nurse cells and the oocyte. F-actin is also concentrated at this edge. The 'leading edge' of each centripetal cell joins to form a continuous band of actomyosin staining around the egg chamber, which decreases in diameter as the cells move centripetally (the leading edge is shown by \( \beta \)-Galactosidase staining in Figure 1.6E). Contraction of this actomyosin band could provide the force to pull the centripetal cells inwards. In egg chambers where the regulatory light chain (RLC) gene, \( sqh \), is mutated, centripetal cells fail to elongate. Consequently, all mature eggs display the 'open chorion' phenotype similar to that of \( fs(2) \) \( cup \) mutants (Edwards and Kiehart, 1996; Keyes and Spradling, 1997). However, since \( sqh \) mutants also produce undersized oocytes, it is not clear if the failure of centripetal migration is due to the relative position of the anterior-most columnar cells and the oocyte-nurse cell border. Furthermore, RLC may regulate other myosins. Thus the mutant phenotype caused by \( sqh \) could be due to the disruption of the organisation of a few myosins that may have a joint function in follicle cell migration.

Centripetal cells might be used as a signal source for the patterning of the columnar cells, because the signalling molecule, Dpp, is also expressed in these follicle cells (Figure 1.6C). A decrease in the expression level of Dpp in these cells results in a diminished operculum (Twombly, 1996; and see Chapter 6). It is hypothesised that the Dpp signal is involved in the AP patterning of the eggshell (Deng and Bownes, 1997; and see Chapter 6).
1.6.4 Dorsal-anterior follicle cell migration and dorsal appendage formation

At the dorsal-anterior region of the mature egg of D. melanogaster, lies a pair of chorionic appendages, also called the dorsal appendages or dorsal filaments. Each dorsal filament is formed by a group of follicle cells that start migrating over the anterior part of the oocyte at stage 11. Spradling (1993) suggests that about 150 follicle cells form each dorsal appendage. This number may be exaggerated. As shown by staining of a dorsal appendage marker, the Broad-Complex (BR-C), there are about 55-65 follicle cells in each group (Deng and Bownes, 1997; and see Chapter 6). Filament-producing cells begin to secrete the filament bases to attach to the main body of the eggshell before they begin migrating towards the anterior. As cells migrate past the growing end, they join a cylinder of cells secreting chorion proteins and commence secretion themselves. The dorsal appendages complete their elongation at stage 14.

The production of the dorsal appendages is dependent upon the Grk-DER signalling pathway which induces dorsal-ventral polarity of the eggshell and the embryo. Thus, the regulation of appendage formation provides a good model for the study of signal instructed morphogenesis. In strong grk mutants, the dorsal appendages are absent. In fs(1)K10° mutant egg chambers, grk RNA is ectopically localised to form an anterior ring (Figure 1.5B), which induces the dorsal appendages to fuse at the ventral side. The downstream effector gene that directly regulates dorsal appendage formation is likely to be the Broad-Complex, a gene complex encoding a family of zinc-finger transcription factors (see Chapter 6). In addition, non-muscle myosin-II and unconventional myosin 95F seems to be downstream of Grk-DER signalling and are likely to be required for dorsal follicle cell migration (Edwards and Kiehart, 1996; and see Chapter 5).

1.6.5 Summary of follicle cell origin, migration and function

The follicle cells eventually synthesise the eggshell for protecting the egg. The five shell layers of the oocyte, moving outward are: the vitelline membrane, the wax layer, the inner chorionic layer (ICL), the endochorion, and the exochorion. The vitelline membrane is a very firm structure that is able to maintain the egg shape even after the outer layers having been removed. The wax-layer and the thin ICL are essential for waterproofing the egg. The endochorion maintains air passageways encircling the egg. These air passageways open to the outside through areopyles. In Drosophila, a few small aeropyles are located in a region of specialised chorions at the posterior pole and
in two major structures: the dorsal respiratory horn and dorsal appendages (for review see Spradling, 1993).

Figure 1.8 Origin, division, migration and function of the follicle cells of *D. melanogaster*. (ST=stage; Modified from Mahowald and Kambysellis, 1980)
The eggshell is built by correctly positioning subsets of follicle cells that are responsible for individual portions of the structure. For example, the micropyle is produced by a small group of border cells that migrate from the anterior pole of the egg chamber to reach the anterior oocyte at stage 10. The dorsal appendages are progressively produced, from base to tip, by much larger groups of anteriorly migrating follicle cells. The follicle cells therefore produce the eggshell by precisely migrating, changing cell shapes, integrating their structural gene expression. These changes require interactions between individual follicle cells and between follicle cell layer and the underlying germline cells.

The origin, division, migration, and function of the follicle cells are summarised in Figure 1.8.

1.7 Nurse cell-oocyte transport

Most of the cytoplasm in the oocyte is provided by the nurse cells through the interconnecting ring canals. During oogenesis, the oocyte nucleus enters meiosis and is thought to be transcriptionally quiescent, whereas the nurse cell nuclei are synthetically active. The nurse cells produce messenger RNA (mRNA), proteins, metabolic precursors, and organelles, which are transported to the oocyte. As described earlier, during early oogenesis, some mRNAs and proteins are selectively transported into the oocyte. These include morphogenetic determinants, such as osk. There is also nurse cell cytoplasm flowing into the oocyte gradually through the ring canals, supporting the steady growth of the oocyte. Late in oogenesis, the nurse cell cytoplasm is dumped rapidly into the oocyte through the ring canals, resulting in the regression of the nurse cells and a doubling of the oocyte volume in about 30 minutes. All nurse cell cytoplasm is transported into the oocyte by stage 14. The residual nurse cells then undergo apoptosis, leaving only the mature egg.

1.7.1 Ring canals and components

The ring canals are the end products of a complex elaboration of the arrested mitotic cleavage furrows that form during the cystoblast divisions. The channel of the ring canal is about 1μm in diameter. Three components of the ring canals that have been identified are filamentous actin and the protein products of hu-li tai shao (hts) and kelch (Yue and Spradling, 1992; Xue and Cooley, 1993)
In germarium region 2a, both filamentous actin and Hts protein are added to the ring canals. Hts is specific to ring canals, whereas actin filaments also occur in the cytoplasm. Hts protein is thought to assemble or stabilise the actin at the rim of the ring canal, as actin and Kelch protein cannot be detected in hts mutant egg chambers. Hts is a homologue of adducin, a mammalian membrane skeleton protein that is enriched at sites of cell-cell contact. Adducin dimers are known to bind actin and stabilise associations of actin and spectrin (Yue and Spradling, 1992).

The Kelch proteins are specifically localised to the ring canals beginning at stage 2 (Xue and Cooley, 1993). In kelch mutant ovaries, the transport of the nurse cell cytoplasm to the oocyte never reaches completion, which results in undersized eggs. However, in early oogenesis, the actin filaments and ring canals show no obvious structural defects, as Hts and actin are localised to the rim of the ring canals as normal. In later stages, actin and Hts extend into the channel and block the ring canals, suggesting that kelch is required to maintain the ring canal structure as they grow in diameter (Xue and Cooley, 1993; for review see Cooley and Theurkauf, 1994).

Ring canals are required not only in transport of materials from the nurse cells to the oocyte, but also in oocyte specification. Within the wildtype 16-cell cyst, it is always one of the cystocytes with four ring canals that becomes the oocyte. In some mutant egg chambers, such as encore and stonewall, where the germline cyst undergoes an extra round of division, one of the cystocytes with five ring canals is determined to be the oocyte (Hawkins et al., 1996; Clark and McKearin, 1996). This suggests that one of the two cystocytes with the maximum number of ring canals has the potential to become the oocyte.

1.7.2 Selective transport during early stages

As described earlier, from the germarium to stage 6 of oogenesis, a number of mRNAs and proteins synthesised in the nurse cells are selectively transported into the oocyte. Among these are factors involved in oocyte differentiation, maternal determinants to instruct the embryonic pattern formation, and factors involved in their localisation within the ooplasm.

The earliest transport occurs at germarium region 2, along with the specification of the oocyte. The only microtubule organising centre (MTOC) located at the future oocyte MTOC is thought to act as a centre of attraction for mRNAs and proteins from the nurse cells. The selective transport is microtubule dependent: treating egg chambers
with microtubule de-stabilisation drugs leads to the disruption of the localisation of the RNAs in the oocyte (for reviews see Ding and Lipshitz, 1993; St Johnston, 1995).

1.7.3 Transport during late stages

At stage 11 of oogenesis, the transport of the cytoplasm from the nurse cells to the oocyte is very rapid. This leads to the rapid growth of the oocyte and the complete regression of the nurse cells. Before the onset of nurse cell regression, the nurse cell nuclei become permeable to allow the transfer of the karyoplasm into the oocyte. The remnants of the nuclei, including the membrane and the condensed chromosome, are excluded from the flow of cytoplasm through the ring canals.

Just prior to this rapid cytoplasmic transferral, stage-10 egg chambers undergo cytoskeletal rearrangements. In the nurse cells, an extensive array of cytoplasmic actin filament bundles are formed, with one end anchored in the plasma membrane and the other embedded in the nuclear membrane. This holds the nurse cell nuclei in place while the cytoplasm and karyoplasm are able to flow through the ring canals. In mutants the rearrangement of the actin cytoskeleton is disrupted: the nurse cell nuclei flow towards the ring canals when the final cytoplasmic transfer begins and block the channels, which results in the “dumpless” phenotype and small eggs.

Three “dumpless” genes, chickadee, singed and quail, have been identified (Cooley et al., 1992; Cant et al., 1994; Mahajanmiklos and Cooley, 1994). They all encode proteins homologous to actin-binding proteins. The Chickadee protein is a Drosophila homologue of profilin, a small monomer actin binding protein (Cooley et al., 1992). Profilin is involved in the polymerisation of actin filament and it may also involved in signal transduction. singed encodes a protein homologous to sea urchin fascin, and quail encodes a villin-like protein. Both of these are actin filament crosslinking proteins. There is no functional redundancy between these two genes. Thus, they may play similar but distinct roles in bundling the actin filaments (for review see Cooley and Theurkauf, 1994).

It seems that non-muscle myosin-II is also involved in the transport of cytoplasm from the nurse cell to the oocyte. This has been illustrated by using mutants of the X-linked gene spaghetti squash (sqh), which encodes the regulatory light chain of non-muscle myosin-II, to assess the requirement for myosin-II in oogenesis and early embryogenesis (Karess et al., 1991). Homozygous germline clones of the hypomorphic mutation sqh<sup>l</sup> were induced in otherwise heterozygous mothers. Consequently, developing oocytes in such sqh<sup>l</sup> germline clones often fail to reach full size due to a
defect in 'dumping'. In contrast to other "dumpless" mutants described above, sqh' egg chambers show no evidence of ring canal obstruction, and no obvious alteration in the actin network. However the distribution of myosin-II is abnormal. This has led to the suggestion that the molecular motor responsible for cytoplasmic dumping is supplied largely, if not exclusively, by nurse cell myosin-II and that the regulation of myosin activity is one means by which cytoplasmic transport may be controlled during oocyte development (Wheatley et al, 1995).

1.8 Vitellogenesis and regulation

1.8.1 Yolk proteins and yp genes

Vitellogenesis, a process during which yolk is accumulated in the developing oocyte, starts at stage 8 and ends at around stage 11. Yolk proteins are important for embryogenesis not only as a nutritional supply but also as a carrier for ecdysteroid. The Yolk Proteins (YPs) are produced in the female fat body and in stage 8-11 follicle cells. The proteins produced in the fat body are secreted into the haemolymph and those in the follicle cells are secreted to the oocyte surface. The YPs in the haemolymph are selectively accumulated in the oocyte by receptor-mediated endocytosis where they are assembled as yolk granules. The yolk proteins are available during all stages of oogenesis, but the uptake only commences at stage 8 of oogenesis. It is continued until the vitelline membrane and chorion assemble and yolk can no longer reach the oocyte surface.

The small gene family of YPs has three members, which are similar in molecular weight and encoded by three single-copy yolk protein genes (yps). All three are located on the X-chromosome. The yp1 and yp2 genes are located at 9A and are divergently transcribed, whereas the third gene, yp3, is located at 1213C. The three genes are coordinate transcribed in both the fat body and the follicle cells (for review see Bownes, 1994B).

It has been shown that the YPs have sequence similarity to the vertebrate lipase (Bownes, 1992). Although the YPs have no lipase activity, the conserved region does contain a lipid binding domain. This has been suggested to bind steroid hormones conjugated to fatty acids in an active storage form. Indeed, when YPs are purified and digested with proteases, a material that reacts with ecdysteroid antibodies is released. 3-
D structures of YPs will be helpful in fully understanding their function in storing ecdysteroid.

**1.8.2 Regulation of yp gene expression**

The yolk proteins are expressed in a sex-, tissue- and temporally specific way. Regulation of expression occurs at different levels, providing a good model to study eukaryotic gene regulation.

Many physical factors, including juvenile hormone (JH) and the moulting hormone ecdysone, have been identified that are involved in the regulation of yolk protein expression. JH seems to be required for yp gene regulation in both the fat body and the ovary. It is also essential for yolk uptake. However, there is no evidence that JH interacts directly with the yp genes to stimulate their transcription. 20-hydroxyecdysone (20-HE, the active form of ecdysone) seems to be required only in fat body regulation. The 20-HE regulation of yp gene expression can be mapped to cis-acting DNA sequences (Bownes *et al.*, 1996). Injection of 20-HE into the male body stimulates transient expression of yp genes in the fat body, but not any other tissues. This may suggest that the sexual differences in yp gene expression are related to different titres of hormones in males and females. However, there seems to be similar levels of ecdysone in both females and males (Bownes, 1989), which may contradict this hypothesis.

In addition, there is also a nutritional effect on yolk protein expression. Starvation of female flies leads to rapid cessation of vitellogenesis, where both the levels of yolk protein transcripts in the fat body and ovarian yolk uptake are affected. It is hypothesised that the nutritional effect is initiated by an unidentified gut hormone (Bownes, 1994B).

Yolk protein expression is sexually regulated. The female, but not the male, fat body can express YPs. Intersexual flies always express yolk proteins in their fat bodies, but pseudomales (chromosomal females that appear to be morphologically male) do not. A good way to illustrate the control of yp genes by sex genes is to use a temperature-sensitive mutant, *transformer-2* (tra-2*). XX tra-2* flies are females at 16°C but pseudomales when reared at 29°C. When the flies are reared at 16°C, YPs are expressed in their fat body, but expression is repressed when shifted to 29°C. Returning the flies to 16°C leads to their expression again.

Ronaldson and Bownes (1995) reported that a 747 bp promoter region of *yp3* is sufficient to direct sex-specific expression in the female fat body and both the temporal-
and cell-type-specific expression during oogenesis. Two elements that independently
govern yp3 transcription in these tissues have been separated and no other sequences in
the upstream, downstream, or coding regions have been identified that are
autonomously involved in yp3 expression. Interestingly, the regulatory region of yp3
genes from housefly Musca domestica and blowfly Cauliflora, can only drive the
reporter gene expression tissue specifically but not sex specifically, suggesting that sex
determination regulation of vitellogenesis is not well conserved during evolution
(Tortigolione and Bownes, 1997).

The ovary expresses yolk proteins in a temporal- and cell-type-specific way. If
an ovary is implanted into a male, or to a larval body, vitellogenesis and yp gene
expression continue in the follicle cells. Therefore, the sex of the surrounding tissue and
the hormone environment have little effect on ovarian yolk protein expression. This has
been demonstrated in tra-2
n flies. When reared at 16°C and shifted to 29°C after
eclosion, follicle cell expression of YPs continues, although fat body expression of YPs
stops.

1.8.3 Regulation of yolk protein uptake

Yolk protein uptake is essential for oocyte maturation. YPs are selectively
endocytosed by a receptor-mediated mechanism. The yolk protein receptor has been
cloned, and is encoded by the yolless (yl) locus. yl mutants fail in the uptake of yolk
proteins. Yl protein is a member of the low-density lipoprotein receptor (LDLR) family
(Schonbaum et al., 1995). It has been shown that yl transcripts are selectively enriched
in the oocyte starting at stage 2. At stages 8-10, yl transcripts are abundant in the oocyte
(Deng and Bownes, unpublished data, see Chapter 4).

The uptake of yolk proteins is also regulated by juvenile hormone. Starved flies
and isolated abdomens, which have ovaries that do not take up yolk proteins, can be
induced to do so after treatment with the JH analogue, ZR515. Mutant flies with
reduced levels of JH fail to undergo vitellogenesis, while yolk uptake can be stimulated
by ZR515 (Redfern and Bownes, 1982). However, the circulating levels of YPs are
normal in these flies. It is likely that JH functions in changing the protein trafficking
activities near the oocyte surface so that yolk proteins are able to be taken up.
1.9 Methods for studying Drosophila oogenesis

1.9.1 Genetic methods

The advantages of choosing Drosophila melanogaster as a model animal to study genetics and development include: (1) it has a relatively short life cycle; (2) there are only three major chromosomes that are polyploid in some tissues, which facilitates cytogenetic mapping; (3) many genetic tools can be used to modify its genome; (4) large collections of mutations and chromosomal rearrangements are available; and (5) it has better studied genetic background than any other animal.

Oogenesis occurs in the adult stage; special mutants are, therefore, required for the study of this developmental process, as lethal mutations die earlier than oogenesis can be analysed. Female sterile mutations are probably the simplest and most direct source of potentially important genes required for oogenesis. Systematic screens for female sterile mutations have been carried out in a number of laboratories in the 1970s and 1980s (summarised in Spradling, 1993). These efforts have greatly promoted our understanding of the molecular genetics of oogenesis. More than 1300 female sterile mutations have been analysed; most fall into one of the phenotypic categories listed in table 1.3 (Spradling, 1993; Lasko, 1994).

Many genes have essential functions prior to adult development. Flies with null mutations in such genes usually die before their functions in oogenesis can be analysed. The easiest way to analyse the function of vital genes in oogenesis has been through the analysis of hypomorphic or conditional alleles. However, these mutants are not always available, and their residual activities sometimes obscure the analysis of their function. Fortunately, a number of techniques have been developed to overcome the difficulties of studying null-mutation phenotype of vital genes.

Pole cell transplantation provides a means of generating egg chambers with a mutant germline. An easier way to produce mosaic egg chambers is through the dominant female sterile technique (Wieschaus et al., 1981; Perrimon and Gans, 1983). The ovo allele is an ideal dominant female sterile mutation because it arrests oogenesis relatively early and there is no leaky expression (Perrimon et al., 1989). It has been widely used to study X-linked genes because ovo is located on the X chromosome. More recently, the generation of autosomal ovo transformed fly lines has allowed the study of genes on other chromosomes (Chou and Perrimon, 1992; Chou et al., 1993).
<table>
<thead>
<tr>
<th><strong>Category</strong></th>
<th><strong>Phenotype</strong></th>
<th><strong>Examples</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>rudimentary</td>
<td>very small ovaries; germaria</td>
<td>stonewall</td>
</tr>
<tr>
<td></td>
<td>difficult to identify; some lacking in germline cells</td>
<td></td>
</tr>
<tr>
<td>tumorous</td>
<td>ovarioles contain an excess of small, disorganised cells in the tip region</td>
<td>orb, ovarian tumor, fs(2)cup</td>
</tr>
<tr>
<td>degenerating</td>
<td>egg chambers are formed but arrested at a characteristic stage before undergoing degeneration</td>
<td>egalitarian</td>
</tr>
<tr>
<td>small egg</td>
<td>produce small round eggs no larger than stage-10 oocytes</td>
<td>kugeli</td>
</tr>
<tr>
<td>dumpless</td>
<td>nurse cell dumping is blocked</td>
<td>chickadee, singed</td>
</tr>
<tr>
<td>ventralised</td>
<td>lack dorsal appendages</td>
<td>gurken, torpedo</td>
</tr>
<tr>
<td>dorsalised</td>
<td>dorsal appendages expanded and fused at ventral side</td>
<td>fs(1)K10, squid</td>
</tr>
<tr>
<td>cup-shaped</td>
<td>eggs lack anterior chorion</td>
<td>fs(2)cup</td>
</tr>
<tr>
<td>fused filament</td>
<td>only a single dorsal appendage produced</td>
<td>rhino, torpedo</td>
</tr>
<tr>
<td>thin chorion</td>
<td>much less chorion than the wildtype</td>
<td>chiffon</td>
</tr>
<tr>
<td>collapsed egg</td>
<td>normal-looking stage-14 eggs but collapse when held in the ovary or following oviposition</td>
<td>Nasrat</td>
</tr>
<tr>
<td>maternal effect</td>
<td>gene products only function after fertilisation</td>
<td>bicoid, nanos</td>
</tr>
<tr>
<td>lethal</td>
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*Based on Spradling (1993).
In the case of follicle cells, it is difficult to generate a useful frequency of large mosaics within the follicle cells by normal somatic recombination, since multiple stem cells contribute to the follicle cells surrounding each egg chamber. This problem has been solved by using high frequency recombination catalysed by the yeast FLP gene and its FRT target sequences (Xu and Rubin, 1994).

An alternative way to study the function of vital genes in oogenesis is to rescue the lethal phenotype in early developmental stages through generating heat-shock inducible transgenic flies. This method is also useful when a gene has multiple functions in different stages of oogenesis. For example, Bic-D is essential both in the germarium and in mid-oogenesis. Through heat-shock rescue of null mutations in the germarium stage, its function in RNA localisation during later stages has been revealed (Swan and Suter, 1996).

1.9.2 General molecular method

When a suitable mutation is available, genetic analysis allows a gene to be placed in a developmental pathway. In order to understand its function in more detail, molecular techniques are indispensable. Cloning, sequencing, and expression analysis allow the gene to be understood at the molecular level, which obviously helps to characterise the role a gene plays in different developmental stages, including oogenesis.

One of the powerful molecular methods available is the detection of gene expression in situ. Based on their interesting expression pattern in oogenesis, candidate genes are studied in further detail. For example, Drosophila myosin-V has been cloned and characterised in our lab, because it has a localised expression pattern in the oocyte (MacIver, McCormack and Bownes, personal communication). In addition, “differential display” allows genes uniquely expressed in oogenesis to be identified. Furthermore, enhancer-trap techniques, with the help of P-element constructs, is also based on the analysis of expression patterns, but uses a reporter gene to facilitate large and fast screening. This will be discussed in detail in 1.9.3 and Chapter 3.

Increasing knowledge of the biochemical functions of gene products also helps to identify more essential genes in oogenesis. For example, the knowledge that a gene required for oogenesis belongs to a particular signalling pathway allows people to test the involvement of its downstream and upstream genes in oogenesis. Additionally, cytoskeletal components also attract a lot of interest, since the crucial role the cytoskeletal network plays in oogenesis has been discovered. Moreover, developmentally important genes characterised in other organisms provide a source for
the identification of homologous genes in *Drosophila*, although the use of fly genes to find homologues in other species has been more widely applied.

### 1.9.3 P-element and derived techniques

*Drosophila* has long been a favourite organism for the study of genetics and developmental biology. However, the full potential is achieved through the use of a transposable element, called the P-element. Most of the P-element related techniques can be used to study oogenesis. Through the use of these techniques, knowledge of oogenesis has been greatly increased.

The autonomous P-element is a 2907-bp sequence with 31-bp terminal inverted repeats and an 11-bp subterminal inverted repeat (O’Hare and Rubin, 1983). These repeats are required in *cis* for efficient transposition, but are not sufficient for it. Internally, there is a transposase gene, which contains four exons and is required in *trans* for transposition. Since functional transposase is only produced in the germline cells, the integration of the transposon into the chromosome only happens there.

Among all the P-element techniques, germline mediated transformation, a method introduced by Rubin and Spradling (1980), is probably the most useful and basic technique. The modified P-element has its transposase gene removed and replaced by a selectable marker gene. Its transposition into the *Drosophila* genome requires transposase provided by a genomic source or a helper plasmid. The helper plasmid itself is not able to transpose due to the lack of inverted terminal sequences. Co-injection of the helper plasmid with the modified P-element allows the P-element to be inserted into the germ cell chromosome. Flies emerging from the embryo that have incorporated into the nuclei of one or more pole cells produce eggs or sperm carrying a transformed chromosome (G0 generation). Therefore, the next generation (G1 generation) will contain a few flies with a transformed genome. If a *white* (*w*) gene is used as the marker gene, red eyed flies can be found in the G1 flies in a *w* background.

The germline transformation technique has been widely used to rescue mutant phenotypes, or to increase gene copies to study dosage effect. This technique is also used for transgene expression by fusing the gene to a specific promoter. For example, transformed fly lines carrying a fusion gene with a heat-shock inducible promoter leads to strong expression of this gene under heat-shock conditions. The Gal4-UAS dual element combination allows a given gene to be expressed in a wide variety of patterns (Brand and Perrimon, 1994; Deng *et al.*, 1997; also see Chapter 3).
A problem with classical genetic methods has long been how to get molecular information for a gene only known by the phenotype of mutants. The reverse problem, how to obtain mutations of a gene only known for its DNA sequence, is becoming more and more common. Interestingly, both problems can be solved in *Drosophila* by obtaining a P-element insertion in the gene. In the former case, the P-insertion allows cloning by transposon tagging. In the latter, P-element insertion mutants can be identified by using PCR or Southern hybridisation techniques.

Once a P-element insertion has been identified close to or within a gene, mutants of the gene can be created by mobilising the P-element; occasionally the transposon excision is imprecise so that the flanking genomic DNA is also excised, causing a deletion mutation. Alternatively, the P-element tends to jump to a local position at a high frequency, thus new insertional mutations can also be created (Tower *et al.*, 1993; Zhang and Spradling, 1993). Recently, a P-element mediated male recombination technique has been developed. This leads to a large deletion of the flanking sequence to the P-element insertion (Preston, 1996a, 1996b).
1.10 Aims of this project

Increasing evidence suggests a crucial role is played by the somatically derived follicle cells in polarity formation, and oocyte differentiation and maturation. It is, therefore, necessary to identify more genes expressed in follicle cells that are involved in these functions. This project was designed to identify and characterise genes expressed in subsets of follicle cells based on the enhancer-trap technique (Chapters 3 and 4), and to study their functions during oogenesis through molecular and genetic analysis (Chapters 5 and 6).

The follicular epithelium also provides an ideal model to study cell-cell interaction, cell adhesion and migration, execution of inductive signals, and epithelial morphogenesis. Through the study of follicle cell expressed genes, the molecular mechanisms involved in these cellular activities can also be explored.
CHAPTER 2
Materials and Methods
2.1 Suppliers and solutions

2.1.1 Suppliers

Chemicals were purchased from Sigma, BDH, Aldrich and Fison.

Restriction enzymes, modification enzymes, polymerases and reverse transcriptase were supplied by Boehringer Mannheim, Gibco BRL, NBL, NEB, Pharmacia, Promega or USB.

Radioisotopes $^{32}\text{P}d\text{CTP}$ and $^{35}\text{S}d\text{ATP}$, Hybond-N, Hybond-N+, Hybond-C were obtained from Amersham.

Digoxigenin labelling and detection kit was purchased from Boehringer Mannheim.

2.1.2 Preparation of solutions and sterilisation

Typically, buffers and solutions were prepared with double distilled water (ddH$_2$O). Sterilisation was achieved either by autoclaving (15psi for 15min) or by passing through a 0.22μm pore sized filter.

All necessary plasticware was sterilised by autoclaving and dried at 37°C.

Solutions required to be RNAase free were prepared by supplementing with 0.05% (v/v) DEPC (Diethyl pyrocarbonate [BDH]), incubating overnight at 37°C and then autoclaving to remove the DEPC. Chemicals reactive to DEPC were dissolved in RNAase free ddH$_2$O and then autoclaved as per normal. Glassware to be used in the manipulation of RNA was baked at 180°C for 8 hours. Plasticware to be used in RNA work was incubated in 0.1N HCl at room temperature for overnight, followed by rinsing with RNAase free ddH$_2$O.

2.1.2 General solutions and buffers

General solutions and buffers used are listed in Table 2.1:

All solutions were stored at room temperature unless otherwise stated.
## Table 2.1. List of general solutions and their composition

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O (RNAase free)</td>
<td>Add 0.05% (v/v) DEPC (Diethyl Pyrocarbonate) to sterile ddH₂O and incubate overnight at 37°C, then autoclave to remove the DEPC</td>
</tr>
<tr>
<td>Denaturing solution (for Southern blotting)</td>
<td>1.5M NaCl, 0.5M NaOH</td>
</tr>
<tr>
<td>100 x Denhardt’s solution</td>
<td>2% (w/v) Bovine Serum Albumin, 2% (w/v) Ficoll\textsuperscript{TM}, 2% (w/v) Polyvinylpyrrolidone (ml. wt. 400,000)</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>0.5M Diaminoethanetetra-acetic acid, adjust to pH 8.0</td>
</tr>
<tr>
<td>70% (v/v) Ethanol</td>
<td>70 ml Ethanol made up to 100 ml with sterile ddH₂O</td>
</tr>
<tr>
<td>Ethidium Bromide</td>
<td>10 mg/ml in sterile ddH₂O</td>
</tr>
<tr>
<td>10 x loading buffer (for DNA)</td>
<td>0.25% (w/v) Bromophenol Blue, 0.25% (w/v) Xylene Cyanol FF, 30% (v/v) Glycerol</td>
</tr>
<tr>
<td>10 x loading buffer (for RNA)</td>
<td>0.25% (w/v) Bromophenol Blue, 0.25% (w/v) Xylene Cyanol FF, 1mM EDTA (pH8.0), 50% (v/v) Glycerol</td>
</tr>
<tr>
<td>MOPS (for RNA agarose gels)</td>
<td>20mM Na-MOPS pH 7.0, 50mM sodium acetate, 10mM EDTA</td>
</tr>
<tr>
<td>Neutralising solution</td>
<td>1.5M NaCl, 0.5M Tris-HCl pH 7.2, 0.001M EDTA</td>
</tr>
<tr>
<td>1 x PBS (phosphate buffered saline):</td>
<td>8g NaCl, 0.2g KCl, 1.44g Na₂HPO₄, 0.24g KH₂PO₄ dissolved in 800ml water, pH adjusted to 7.4, made to 1 litre</td>
</tr>
<tr>
<td>Phenol/Chloroform</td>
<td>Re-distilled and pre-equilibrated (in 100mM Tris-HCl pH8.0) phenol was purchased from Sigma and mixed with Chloroform and Isoamyl Alcohol in the ratio 25:24:1 respectively. To prevent oxidation, 8-Hydroxyquinoline was added to 0.1% (w/v) and the solution was stored at 4°C</td>
</tr>
<tr>
<td>Pre-hybridisation solution</td>
<td>5x SSPE, 5x Denhardt’s, 0.5% (w/v) SDS</td>
</tr>
<tr>
<td>10% SDS</td>
<td>10% Sodium Dodecyl Sulphate in sterile ddH₂O</td>
</tr>
<tr>
<td>20 x SSC</td>
<td>3M NaCl, 0.3M Tri-sodium Citrate, adjust to pH 7.0</td>
</tr>
<tr>
<td>20 x SSPE</td>
<td>3.6M NaCl, 0.2M sodium phosphate, 0.02M EDTA pH7.7</td>
</tr>
</tbody>
</table>
Materials and Methods

| 10 x TAE: | 0.4M Tris-acetate, 10mM EDTA pH 7.8 |
| 10 x TBE: | 0.89M Tris-HCl, 0.89M boric acid, 10mM EDTA |
| 1 x TE: | 10mM Tris-HCl, 1mM EDTA, pH 8.0 |
| Ribonuclease A (RNAase A) | 20 mg/ml in 50% Glycerol, boiled for 5 min to inactivate contaminating DNAases, and stored at -20°C |
| Deoxyribonuclease I (DNAase I) | 20 mg/ml in 50% (v/v) Glycerol, stored at -20°C |
| Proteinase K | 20 mg/ml in 50% (v/v) Glycerol, stored at -20°C |

2.2 Fly stocks and maintenance

2.2.1 Drosophila melanogaster stocks and strains

The wildtype strain used in this work was Drosophila melanogaster Oregon R (OrR) (Lindsay and Zimm, 1992). Other strains of flies used in this work are listed in Table 2.2.

Table 2.2. Lists of Drosophila melanogaster strains used in this work

<table>
<thead>
<tr>
<th>Strain class</th>
<th>Designation</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BR-C (broad)</td>
<td>br¹</td>
<td>Viable</td>
<td>Kiss et al., (1988)</td>
</tr>
<tr>
<td></td>
<td>br⁵</td>
<td>Lethal</td>
<td>Belyaeva et al., (1980)</td>
</tr>
<tr>
<td></td>
<td>br⁶</td>
<td>In(1)2B3-4;3C1</td>
<td>Belyaeva et al., (1980)</td>
</tr>
<tr>
<td>BR-C (rbp)</td>
<td>rbp¹</td>
<td>Lethal</td>
<td>Belyaeva et al., (1980)</td>
</tr>
<tr>
<td>BR-C (2Bc)</td>
<td>l(l)2Bc¹</td>
<td>Lethal</td>
<td>Belyaeva et al., (1980)</td>
</tr>
<tr>
<td>BR-C (npr)</td>
<td>npr⁶</td>
<td>Lethal</td>
<td>Belyaeva et al., (1980)</td>
</tr>
<tr>
<td>Deficiencies</td>
<td>Df(1)S39</td>
<td>Df(1)1E1-2; 2B5-6</td>
<td>Belyaeva et al., (1980)</td>
</tr>
<tr>
<td></td>
<td>y²Y67g19.1</td>
<td>Dp(1;Y)1A; 2B17-18</td>
<td>Belyaeva et al., (1980)</td>
</tr>
<tr>
<td></td>
<td>Df(3R)crb87-4</td>
<td>Df(3R)95E8-F1; 95F15</td>
<td>Flybase</td>
</tr>
</tbody>
</table>
### Materials and Methods

<table>
<thead>
<tr>
<th><strong>Gal4 lines</strong></th>
<th><strong>Gal4-55B</strong></th>
<th><strong>Df(3R)95F7; 96A17-18</strong></th>
<th><strong>Brand &amp; Perrimon (1993)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C865</strong></td>
<td><strong>Gal4-MHC95F</strong></td>
<td><strong>Deng et al., (1997)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>C726b</strong></td>
<td><strong>Gal4-BR-C</strong></td>
<td><strong>Deng et al., (1997)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>C826</strong></td>
<td></td>
<td></td>
<td><strong>Deng et al., (1997)</strong></td>
</tr>
<tr>
<td><strong>133Y</strong></td>
<td></td>
<td></td>
<td><strong>Deng et al., (1997)</strong></td>
</tr>
<tr>
<td><strong>C532</strong></td>
<td></td>
<td></td>
<td><strong>Deng et al., (1997)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>UAS lines</strong></th>
<th><strong>UAS-ΔDraf</strong></th>
<th><strong>Constitutively activated Draf gene</strong></th>
<th><strong>Brand and Perrimon, (1993)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>UAS-dpp</strong></td>
<td><strong>P_{w^{+mc}} = UAS-dpp.S}42B.4</strong></td>
<td><strong>Staehling-Hampton et al., (1994)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>UAS-pntP1</strong></td>
<td></td>
<td><strong>Morimoto et al. (1996)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>UAS-pntP2</strong></td>
<td></td>
<td><strong>Morimoto et al. (1996)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>UAS-lacZ</strong></td>
<td></td>
<td><strong>Brand and Perrimon, (1993)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>UAS-GFP</strong></td>
<td></td>
<td><strong>Flybase</strong></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>dpp</strong></th>
<th><strong>dpp^{gr52}</strong></th>
<th><strong>Twombly et al., (1996)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>dpp^{gr87}</strong></td>
<td><strong>Twombly et al., (1996)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>gurken</strong> (grk)</th>
<th><strong>P'[grk', w']</strong></th>
<th><strong>X7; 28-20/TM3</strong></th>
<th><strong>Neuman-Silberberg &amp; Schüpbach, (1994)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>P'[grk', w']</strong></td>
<td>2 insertions on the</td>
<td><strong>Neuman-Silberberg &amp; Schüpbach, (1994)</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>X chromosome</td>
<td></td>
</tr>
<tr>
<td><strong>grk^{WG}</strong></td>
<td></td>
<td></td>
<td><strong>Schüpbach &amp; Wieschaus, (1991)</strong></td>
</tr>
<tr>
<td><strong>grk^{HK}</strong></td>
<td></td>
<td></td>
<td><strong>Schüpbach &amp; Wieschaus, (1991)</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>torpedo</strong></th>
<th><strong>top^{QYI}</strong></th>
<th><strong>Schüpbach &amp; Wieschaus, (1991)</strong></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th><strong>balancers</strong></th>
<th><strong>X chromosome</strong></th>
<th><strong>w-/w-,FM6; +/-; +/-</strong></th>
<th><strong>A. Jarman (personal communication)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>balancers</strong></td>
<td><strong>2nd chromosome</strong></td>
<td><strong>w-; Pin/Cyo; +/-</strong></td>
<td><strong>A. Jarman (personal communication)</strong></td>
</tr>
<tr>
<td><strong>balancers</strong></td>
<td><strong>3rd chromosome</strong></td>
<td><strong>w-; +/-; TM3,Sb/TM6,Tb</strong></td>
<td><strong>A. Jarman (personal communication)</strong></td>
</tr>
</tbody>
</table>
2.2.2 Fly food and the maintenance of fly stocks

All fly stocks were maintained at 18°C on Staffan cornmeal food (250g cornflour, 500g sugar, 175g yeast pellets and 100g agar dissolved in 10 litres distilled water). The food was boiled and cooled to approximately 40°C before being poured into bottles or vials. A fungicide, Nipagin, was added to a final concentration of 4.5 μg/L and occasionally antibiotics such as Gentamycin (40μg/l) were added. In the event of mite infections, strips of Whatman filter 3M paper soaked in 3% (v/v) Benzyl Benzoate (in Ethanol) and air dried were placed on top of the fly food.

2.3 Collection of developmental staged flies

2.3.1 Stages of *Drosophila melanogaster* development at 25°C

Table 2.3 lists the developmental stages of wild-type *Drosophila melanogaster* OrR at 25°C.

**Table 2.3 Life Cycle of *Drosophila melanogaster***

*(Based on Bownes and Dale, 1982)*

<table>
<thead>
<tr>
<th>HOURS</th>
<th>DAYS</th>
<th>DEVELOPMENTAL STAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>Fertilisation and fusion of pronuclei</td>
</tr>
<tr>
<td>1.5</td>
<td>0</td>
<td>Preblastoderm stage. Migration of cleavage nuclei and pole cell formation</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>Blastoderm stage. Migrated nuclei form cells in the previously syncytial blastoderm</td>
</tr>
<tr>
<td>3.5</td>
<td>0</td>
<td>Gastrulation begins</td>
</tr>
<tr>
<td>6 - 8</td>
<td>0</td>
<td>Segmentation visible</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>Larval differentiation nearly complete</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>Hatching from egg. Onset of first larval instar</td>
</tr>
<tr>
<td>48</td>
<td>2</td>
<td>First moult. Second larval instar begins</td>
</tr>
<tr>
<td>72</td>
<td>3</td>
<td>Second moult. Third larval instar begins</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>Puparium formation with white puparium</td>
</tr>
<tr>
<td>122</td>
<td>5</td>
<td>Puparium darkens</td>
</tr>
<tr>
<td>124</td>
<td>5</td>
<td>Puparium moult</td>
</tr>
<tr>
<td>132</td>
<td>5</td>
<td>Pupation. Eversion of imaginal discs</td>
</tr>
<tr>
<td>216 - 240</td>
<td>9-10</td>
<td>Eclosion of adult from pupal case</td>
</tr>
</tbody>
</table>
2.3.2 Collection of staged eggs

About 300-500 flies (3-4 days after eclosion) were transferred into population cages which was covered with petri dish containing apple juice food\(^1\) or Adh food\(^2\) with the yeast paste smeared in the middle. Eggs were washed off from the petri dish and collected in a sieve and rinsed thoroughly using distilled water. The eggs were then subjected to subsequent treatment.

2.3.3 Collection of early and late third instar larvae, pupae and sexed adults

Egg laying flies were placed in fresh food bottles for 4 hours and then removed. The bottle was then incubated at 25\(^\circ\)C for 72 hours, 96 hours and 120-140 hours. Early third instar larvae were collected after 72 hours by floating them out of the food using a saturated sucrose solution, rinsed with distilled water and frozen in liquid nitrogen. Late third instar larvae were picked with a paintbrush as they crawled up the walls of the bottles after incubated for 96 hours, placed in microfuge tube, frozen in liquid nitrogen or dry ice/ethanol bath and stored at -70\(^\circ\)C.

Pupae were collected by picking them off the inside of the food bottles after 120-140 hours incubation, placed in microfuge tube and frozen in liquid nitrogen or dry ice/ethanol bath, and stored at -70\(^\circ\)C.

Adult flies were collected ranging in age from newly eclosed to 10 days old flies. The flies were anaesthetised with di-ethyl ether, sexed, placed in microfuge tube, frozen in liquid nitrogen or dry ice/ethanol bath and stored at -70\(^\circ\)C.

2.3.4 Dissecting of ovaries

Flies were transferred to fresh cornmeal food which was topped with dry yeast, and incubated at 25\(^\circ\)C for 2-3 days before dissection. The temperature sensitive flies incubated at 18\(^\circ\)C were shifted to 29\(^\circ\)C after eclosion and were fed with yeast for 2-3 days before dissection.

---

\(^1\) Apple juice plates: Mix 9 g of agar and 10 g of sugar with 300 ml of water and bring to boil. When the foam is reduced add 100 ml of apple juice, bring to boil and add 10 ml of 10% Nipagin. Cool to 60\(^\circ\)C and pour into Petri dishes.

\(^2\) Adh food: 100 g dried flake yeast, 100 g brown sugar, 16 g agar in one litre and Nipagen at final concentration of 4.5 \(\mu\)g/L.
Flies to be dissected were anaesthetised with di-ethyl ether and sexed. Female flies were placed on a convex slide with Ringer’s solution or 1 x PBS and dissected under dissecting microscope to take out the ovaries. Ovaries were subjected to subsequent treatment.

2.3.5 Collection of virgin flies and crosses

Virgin flies were collected by either of the following two ways: (1) Black pupae were picked out with a brush, sexed and placed into vials with fresh food individually. (2) Vials or bottles were emptied of flies, and the newly hatched flies (every 6 hours at room temperature) were anaesthetised and sexed. Same sexed flies were placed together in vials containing fresh fly food.

Crosses of flies were generally carried out by placing 2-3 virgin female flies and 2-3 male flies into the same vial containing fresh fly food. They were incubated at 25°C or 18°C depending upon the genotypes of the flies.

2.4 Bacteria and bacteriophage

2.4.1 Media preparation

All appropriate media for bacteria and bacteriophage growth are listed in Table 2.4. They were prepared in distilled water and sterilised by autoclaving (15 psi/15 min). All media were stored at room temperature.

Antibiotics were added to the media at the relevant concentration if necessary (Table 2.5). All antibiotics were stored at −20°C.
Table 2.4. Media for bacteria growth

<table>
<thead>
<tr>
<th>Media type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB Broth</td>
<td>Per litre: 10 g Bactotryptone, 5 g Yeast extract, 10 g NaCl, adjust to pH 7.5 with NaOH</td>
</tr>
<tr>
<td>LB plates</td>
<td>Add 15 g Difco agar/litre LB broth, autoclave and pour. Allow ~80 ml/150 mm plate</td>
</tr>
<tr>
<td>LB Top agar</td>
<td>Same as LB broth except with 0.7% agarose; autoclave</td>
</tr>
<tr>
<td>TB Broth</td>
<td>Per litre: 5 g NaCl, 10 g Bactotryptone</td>
</tr>
<tr>
<td>2xYT</td>
<td>Per litre: 16g Bactotryptone, 10g Bacto yeast extract, 5g NaCl, pH adjusted to 7.0 with NaOH</td>
</tr>
<tr>
<td>SM Buffer</td>
<td>Per litre: 5.8g NaCl, 2.0g MgSO₄·7H₂O, 50ml 1M Tris-HCl pH 7.5, 5ml 2% gelatin; autoclave</td>
</tr>
<tr>
<td>SOC medium</td>
<td>2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5mM KCl, 10 mM each of MgCl₂ and MgSO₄; pH7.5; 20 M glucose</td>
</tr>
</tbody>
</table>

Table 2.5 Antibiotics and their working concentrations

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Stock</th>
<th>Working Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>50 mg/ml in ddH₂O</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>10 mg/ml in ddH₂O</td>
<td>50 µg/ml</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>5 mg/ml in Ethanol</td>
<td>12.5 µg/ml</td>
</tr>
</tbody>
</table>
### 2.4.2 Bacterial strains and plasmids

Bacterial strains used are listed in Table 2.6. Plasmids used are listed in Table 2.7. Libraries used are listed in Table 2.8.

#### Table 2.6 Bacterial strains used

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>Genotype</th>
<th>Relative use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y1090</strong></td>
<td>Δ(lac)U169, Δ(ion)? araD139, strA, supF, mcrA, trpC22::Tn10, [pMC9]</td>
<td>For plating λgt libraries</td>
</tr>
<tr>
<td><strong>NM422</strong></td>
<td>F', lacIΔ(lacZ)M15 proA'ΔB' /supE thi Δ(lac proAB)/Δ(hsdMS) mcrB)5 (rKmK McrBC)</td>
<td>For plating λFIX library</td>
</tr>
<tr>
<td><strong>XL1 Blue</strong></td>
<td>recA1, endA1, gyrA96, thi-1, hsdR17, supE44, relA1, lac, [F', proAB, lacIΔZΔM15, Tn10 (tet')]</td>
<td>Plasmid transformation, plating λZAP libraries</td>
</tr>
</tbody>
</table>

#### Table 2.7 Plasmids used in this work.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Features</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript</td>
<td>General cloning vector</td>
<td>Contains Δ15 region of lacZ, ampicillin resistance</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUAST</td>
<td>Containing Gal4 binding site UAS</td>
<td></td>
<td>Brand &amp; Perrimon (1994)</td>
</tr>
<tr>
<td>pΔ2-3</td>
<td>Helper plasmid encoding transposase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2.8 Genomic and cDNA libraries used in this work

<table>
<thead>
<tr>
<th>Library</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Drosophila melanogaster</em> λFIX genomic library</td>
<td>The library was constructed by ligating a size fractionated partial Sau3AI digestion of Canton S genomic DNA to partially filled XhoI sites in the vector. Insert sizes vary from 9-23kb and each end is flanked by a T3 or T7 promoter.</td>
<td>Stratagene</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em> λAZAP ovarian cDNA library</td>
<td>This library uses the λAZAP vector which allows recombinant cDNAs to be excised as pBluescript plasmids</td>
<td>A kind gift from Jan, <em>(Jongens et al., 1992)</em></td>
</tr>
</tbody>
</table>

### 2.4.3 Growth and maintenance of bacteria stocks

#### 2.4.3.1 Growth of bacteria in liquid culture

Small scale cultures were prepared by inoculating a single colony into 5ml LB-broth and grown overnight at 37°C with moderate shaking.

Large scale cultures were prepared by inoculating 100-1,000 ml LB-broth with 0.01 volume of a small scale overnight culture and growing at 37°C with moderate shaking until it reached desired $A_{600}$.

#### 2.4.3.2 Growth of bacteria on agar plates

Bacteria from a single colony were spread by an inoculating loop (flamed before used) sequentially around the LB-agar plate. Bacterial culture was spread by a glass spreader (flamed with 70% ethanol prior to use) on the plate. In both cases, plates were grown overnight at 37°C.
2.4.3.3 Long term storage of bacteria strains as glycerol stocks

0.5 ml of overnight small-scale liquid culture (2.4.3.1) was transferred to a fresh microfuge tube, 0.5 ml of LB-broth/glycerol (1:2) was added, followed by chilling in a dry ice/ethanol bath and storing at -70°C.

2.4.3.4 Long term storage of bacterial strains as stabs

A single colony was picked using straight wire and stabbed into LB-broth supplemented to 0.6% (w/v) agar. This culture was grown at overnight 37 °C with the caps slightly loose. To store the culture, the caps were tightened, the tubes were sealed with parafilm, and the stabs stored at room temperature out of direct sunlight. Stabs were revived by streaking some of the agar onto LB-agar plates and growing overnight at 37°C as described at 2.4.3.2.

2.4.4 Manipulation of bacteriophage

2.4.4.1 Preparation of plating cells

A single colony of the appropriate bacteria host was inoculated into 50 ml LB-broth (containing 0.2% maltose and 10 mM MgSO₄). Bacteria were grown overnight with moderate shaking at 30 °C (this temperature ensures that the cells will not overgrow). Cells were harvested by spinning down in a sterile conical tube (2,000 rpm, 10 minutes). The media were carefully decanted and the cell pellet was gently resuspended in ~15 ml of 10 mM MgSO₄. The cells were diluted to A₆₀₀ = 0.5 with 10 mM MgSO₄ and stored at 4°C for 2-3 days.

2.4.4.2 Determination the titre of bacteriophage

A series of phage dilutions were made in SM buffer and were used to inoculate 200 µl of A₆₀₀ = 0.5. plating cells in glass tube. After incubating at 37°C for 15 minutes, 3 ml 48°C melted LB top agar was added and the mixtures were plated on LB plates. Plates were incubated at 37°C overnight and the number of plaques was counted. Based on the dilutions, the plaque forming unit (pfu)/ml concentration was determined.
2.4.4.3 Plating of bacteriophage library

50,000 pfu with 600 μl plating cells and 30 ml LB top agar were plated on a 22 cm x 22 cm LB plate. The plate was incubated at 37°C for overnight and refrigerated at 4°C for 2 hours.

The subsequent steps for screening the library are described elsewhere. The transfer of the plaque onto the membrane is described in 2.11.3. The labelling and hybridisation are described at 2.8.7 and 2.11.4 respectively.

2.4.4.4 Excision of pBluescript phagemid from λZAP Bacteriophage

A λZAP plaque was cored and transferred to a sterile microfuge tube containing 0.5 ml of SM buffer and 20 μl of chloroform. The tube was vortexed to release the λZAP phage particles into the SM buffer (this phage stock is stable up to a year at 4°C). A 100 μl aliquot of eluted phage was added to 200 ml of XL1-Blue plating cells (2.4.4.1) with 1 μl of 1 x 10¹¹ pfu/ml helper phage (R408) also added. The mix was then incubated at 37°C for 15 minutes to allow adsorption and penetration of the phage DNA. 2 ml of 2xYT was added to this mix and incubated at 37°C for 4-6 hours with shaking. The culture was then heated to 70°C for 20 minutes and followed by centrifugation at 1,000 g for 10 minutes. The supernatant was collected as a phagemid stock with 20 μl chloroform added to prevent bacterial growth. Bacterial colonies carrying the pBluescript recombinant plasmid were prepared by adding 10 μl and 100 μl of phagemid stock to 200 ml XL1-Blue cells and 1 μl R408 helper phage with incubation at 37°C for 15 minutes. This culture was plated on to LB/ampicillin plates and allowed to incubate overnight at 37°C.

2.5 General manipulation of nucleic acids

2.5.1 Deproteinisation by Phenol/Chloroform Extraction

An equal volume of Phenol/Chloroform was added and mixed either by repeated gentle inversions (high molecular weight samples) or by vortexing. The phases were separated by centrifugation (12,000 rpm, 4°C, 10 minutes) and the aqueous phase was transferred to a fresh tube. This extraction was repeated until the interface after centrifugation was clean. A final extraction with an equal volume of Chloroform/Isomyl alcohol (25:1) was performed to ensure complete removal of phenol.
2.5.2 Precipitation of nucleic acids

Precipitation was carried out by adding 0.1 volumes of 3M sodium acetate (pH 5.2) with either 2-3 volumes of 100% ethanol or 0.6-0.8 volumes of 100% isopropanol. Typically the nucleic acid was precipitated at -20°C for at least one hour or at -70°C for 30 minutes, pelleted by centrifugation (17,000 rpm, 10 minutes), washed with 70% ethanol and dried under vacuum. The DNA was then dissolved in TE or ddH$_2$O depending on subsequent applications.

2.5.3 Estimation of the concentration of nucleic acids

Spectrophotometry was used to assess the concentration and purity of nucleic acids. Table 2.9 lists the typical concentrations of nucleic acids when reading the absorbance of the sample at X260nm. DNA was judged to be free of contaminating protein if the A260:A280 ratio was greater than or equal to 1.8. RNA was judged if this ratio was larger or equal to 2.0.

Table 2.9. Absorbency of nucleic acid solutions and inferred concentrations

<table>
<thead>
<tr>
<th>Nucleic acid</th>
<th>Absorbency ($A_{260}$)</th>
<th>Concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double stranded DNA</td>
<td>1.0</td>
<td>50</td>
</tr>
<tr>
<td>Single stranded DNA</td>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td>Single stranded RNA</td>
<td>1.0</td>
<td>40</td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>1.0</td>
<td>40</td>
</tr>
</tbody>
</table>

Approximate nucleic acid concentration was also deduced by comparing the relative fluorescence of samples to those of a known concentration on agarose gel.
Materials and Methods

2.6 DNA preparation

2.6.1 Fly genomic DNA preparation

15 to 20 anaesthetised adult flies were collected in a microfuge tube and immersed in liquid nitrogen or dry-ice/ethanol bath. 200 µl lysis buffer 1 (5% [w/v] sucrose, 80mM NaCl, 100mM Tris-HCl pH 8.5, 50mM EDTA pH 8.0,) was added. The flies were grinded thoroughly for a few moments with a plastic pestle attached to a drill (Kontab homogeniser). Then 200 µl lysis buffer 2 (lysis buffer 1 with 0.1% [w/v] SDS) were added and mixed. After incubating for 30 minutes at 70°C, 80 µl 6M potassium acetate (final concentration about 1M) was added to the homogenate and it was incubated on ice for 30 minutes. Debris and insoluble material was separated by centrifugation (17,000 rpm, 4°C, 10 minutes) and the supernatant was transferred to a fresh tube. If high quality DNA was required, the solution was deproteinised by phenol/chloroform extraction (See 2.5.1), and then precipitated with 0.6-0.8 volumes of isopropanol. The precipitate was collected by centrifugation (17,000 rpm, 15 minutes), washed with 70% ethanol and air-dried. The pellet was dissolved in 30 µl TE buffer and stored at -20°C.

2.6.2 Plasmid Preparation

This protocol was modified from the manual instruction (Qiagen).

1.5ml of overnight culture was spun down for 10 seconds at 12,000 rpm. The cell pellet was resuspended in 200µl of P1 solution (Qiagen, 40mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0, 50mM Glucose) by vortexing and incubated at room temperature for 5 minutes. Then 200µl of P2 solution (1% SDS, 200mM NaOH) was added and gently mixed and incubated on ice for 10 minutes. Then, 200µl of P3 solution (3M sodium acetate pH 4.8) was added with gentle mixing as before and incubation on ice for 10 minutes. Cell debris was pelleted by centrifugation (17,000 rpm, 4°C, 15 minutes) with the supernatant being transferred to a fresh tube. The DNA was precipitated by the addition of 0.6-0.8 volume of iso-propanol and incubation at room temperature for 10 minutes, then centrifuged in a microfuge (17,000 rpm, 4°C, 15 minutes). The supernatant was removed and the pellet washed with 70% ethanol, re-centrifuged and then dried in a speedvac desiccator. The DNA was dissolved in 30µl of TE buffer and stored at -20°C.
2.6.3 Preparation of bacteriophage lambda DNA

100μl of plating cells (2.4.4.1) of the appropriate E coli strain were infected with 100μl of resuspended bacteriophage. The infected bacterial culture was then added to 10ml LB-broth containing 10mM MgSO_4 in a 50ml plugged flask. Incubation was carried out at 37°C with vigorous shaking for 5 to 7 hours whereupon lysis of the bacteria was observed. The bacteria were completely lysed by the addition of 100μl of chloroform with shaking continued for a further 10 minutes.

The cell debris was removed by centrifugation for 10 minutes at 4,000 rpm. In every 0.8ml lysate, 1 μl of 10mg/ml DNAase I was added with incubation at 37°C for 20-30 minutes to digest the bacterial DNA. Next, 0.2ml of TES buffer (1.5% [w/v] SDS, 300mM Tris-HCl pH 9.0, 150mM EDTA, pH7.5) was added and the mixture was incubated at 70°C for 15 minutes then allowed to cool to room temperature for 10 minutes. This step was followed by the addition of 135μl of 5M Potassium-acetate, mixed, then incubated on ice for 15 minutes. The potassium-SDS precipitate was removed by centrifugation for 10 minutes at 17,000 rpm. Isopropanol at 0.7 volumes was added to the supernatant with incubation at room temperature for 10 minutes and followed by centrifugation at 17,000 rpm for 10 minutes. The supernatant was decanted off and the pellet was dried then dissolved in 100μl of TE. This solution was then extracted twice with phenol/chloroform and once with chloroform followed by precipitation with 2-3 volumes of ethanol and 0.1 volume of 3M Sodium acetate. The precipitate was washed with 70% ethanol, dried and redissolved in 50μl TE.

2.7 RNA preparation and electrophoresis

2.7.1 Preparation of total RNA

Trizol system (Gibco BRL), a solution of phenol and guanidium isothiocyanate, which is based on the method of Chomczynski and Sacchi (1987), was used to extract total RNA. 0.5ml Trizol solution was added to up to 100mg tissue, homogenised, and a further 0.5ml of Trizol was added and mixed. The homogenate was incubated at room temperature for 5 minutes. 200μl of chloroform was added to the homogenate and mixed, followed by incubation at room temperature for 3 minutes. The aqueous and organic phases were separated by centrifugation at 17,000 rpm, 15 minutes at 4°C. The aqueous phase was transferred to a fresh microfuge tube and 0.7 volumes of isopropanol was added and incubated at room temperature for 10 minutes. The RNA
was precipitated by centrifugation at 17,000 g for 15 minutes at 4°C. The supernatant was removed and the RNA pellet washed with 1ml of 70% (v/v) ethanol (in DEPC water). The pellet was air dried then dissolved in DEPC sterile water.

2.7.2 RNA electrophoresis

All solutions were prepared with DEPC water. RNA was separated on 0.7% (w/v) to 1.0% (w/v) denaturing agarose gels in a MOPS buffering system. Agarose was dissolved in 10 ml 10 x MOPS and 73 ml of water and cooled to about 55°C, then 17 ml of 37% (v/v) formaldehyde was added, the solution mixed and poured immediately into a gel tray. Gels were run in a 1 x MOPS buffer.

RNA samples were incubated at 65°C for 5 minutes in the following buffering system: RNA 5μl, formamide 12.5μl, 10 x MOPS 2.5μl, formaldehyde 4μl, then chilled immediately on ice. Prior to loading, 2.5μl RNA loading solution was added.

2.8 Manipulation of DNA

2.8.1 Restriction enzymatic digestion of DNA

DNA (dissolved in TE or ddH₂O) to be digested was placed in an eppendorf tube. 10 x restriction endonuclease buffer (provided by supplier) was added to achieve a 1 x concentration in the final reaction volume. Sterile distilled water was added to the required volume and restriction endonuclease was added (typically, 5U per 1 μg DNA). The reaction was typically carried at 37°C for two hours (plasmid and phage DNA) or overnight (genomic DNA). Digestion was stopped either by adding 1/6th 6 x electrophoresis loading buffer or by phenol/chloroform extracting (2.5.1) and followed by ethanol precipitation (2.5.2).

2.8.2 DNA de-phosphorylation

After the plasmid DNA was restriction enzyme digested, 10 x de-phosphorylation buffer (to a final 1 x concentration) and sterile distilled water were directly added to the reaction. Phosphatase (Calf Intestinal [Boehringer Mannheim] or Shrimp [USB]) was then added at the manufacturers recommended concentration. Incubation was carried out at 37°C for 60 minutes. The samples were then extracted with phenol/chloroform (2.5.1) and precipitated with ethanol (2.5.2). The samples were redissolved in TE or ddH₂O prior to ligation.
2.8.3 DNA ligation

T4 DNA ligation buffer (5x) and ddH₂O were added to restriction enzyme digested DNA to achieve a final 1x concentration, and 1 µl (1 unit) T4 DNA ligase (Gibco BRL) was added. Ligation was then allowed to proceed by incubating at 4°C for overnight.

2.8.4 Transformation of Escherichia coli

2.8.4.1 CaCl₂ Method

1 ml of overnight bacteria LB culture was inoculated into 100 ml LB medium and incubated at 37°C with moderate shaking until the A₆₀₀ reaching around 0.3. Cells were pelleted by centrifugation (4,000 rpm, 10 minutes, 4°C), then resuspended in one-half volume prechilled 0.1 M CaCl₂ and left on ice for 15 minutes. The cells were again pelleted by centrifugation, then resuspended in 1/20th volume 50 mM CaCl₂, and 15% glycerol and stored at -70°C or used immediately (Sambrook et al., 1989).

Transformation was carried out by mixing 1-10 µl DNA with 100µl of cells and incubating on ice for 30 minutes. The transformed cells were heat shocked at 42°C for 2 minutes, and LB (900µl) was added and the cells allowed growing at 37°C for 1-1.5 hours on a shaker. The culture was then spread onto antibiotic plates to select for transformants.

2.8.4.2 Electro-transformation

Fresh streaks of E. coli from a frozen glycerol stock were grown overnight at 37°C on L-broth agar plates containing appropriate antibiotics. Five or six single colonies were dispersed in 5ml L-broth and then added to 1 litre L-broth with appropriate antibiotics. Bacteria were grown at 37°C with moderate agitation until they reach A₆₀₀ of approximately 0.3. Flasks were chilled on ice for 30 minutes and cells were spun at 3,000 rpm (4°C) in 250-ml bottles. The supernatants were decanted and cells were dispersed by gentle inversion in an equal volume of cold water. Cells were washed for 3 more times as follows: One-half volume cold water, 30 ml cold 10% glycerol (v/v in de-ionised water). The final cell pellet was resuspended in 2 ml cold 10% glycerol. Cells were distributed in 40 µl aliquots into chilled microfuge tubes and then immediately flash-frozen in a dry ice/ethanol bath. Frozen electro-competent cells were stored at -70°C.
Electro-competent cell aliquots (above) were thawed on ice and appropriate quantities of DNA in water were added to 1-5 μl total volume. Mixtures were pulsed with Bio-Rad Gene Pulser apparatus and pulse controller at 2.5 kV, 25 μF and 200 Ω in chilled 0.1 cm electroporation cuvettes (Bio-Rad); immediately after pulsing, 0.5 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5mM KCl, 10 mM each MgCl₂ and MgSO₄; pH7.5; 20 M glucose) or 2 x YT (See Table 2.4) was added to the cuvette and gently mixed with the pulsed cells. Mixtures were transferred to 5 ml glass tubes, incubated at 37°C for 1 hour with moderate agitation and then plated on two L-broth agar plates. Colonies were visible after 14 hours at 37°C.

2.8.5 DNA agarose gel electrophoresis

2.8.5.1 Molecular Weight Standards

All agarose gels were run with 1 kilobase ladder (1-kb ladder, Gibco BRL) as the molecular weight (MW) standards.

2.8.5.2 Separation of DNA molecules

6 x DNA gel loading buffer and ddH₂O were added into the DNA samples to make final 1 x concentration for the loading buffer. Agarose gels were prepared by dissolving agarose in 1 x TBE buffer (in a microwave) to achieve an appropriate percentage. Ethidium Bromide (final concentration at 0.5 μg/ml) was added when the gel was cooled to approximately 65°C, followed by pouring the gel into a tray. Gels were run in 1 x TBE buffer. After electrophoresis, DNA was visualised under UV light and photographed.

2.8.6 Recovery of DNA fragments from agarose gel

Two methods were used to recover the DNA fragments from the agarose gel.

2.8.6.1 QIAquick Gel Extraction

DNA fragment was excised from the agarose gel with a clean razor blade and put into a microfuge tube containing 3 volumes (w/v) of buffer QX1 (Qiagen). The gel was dissolved at 50°C for about 10 minutes and the solution was transferred to a QIAquick spin column, which was put on a 2 ml collection tube. Followed by spinning down (60 seconds, 13,000 rpm), washing with 0.75 ml of buffer PE (Qiagen), spinning down
again and finally the spin column was placed on a fresh microfuge tube. DNA was eluted by adding 50 μl TE or ddH₂O to the column and centrifuging (60 seconds, 13,000 rpm).

2.8.6.2 Electroelution

A Model UEA Unidirectional Electroelutor (Sigma) was used to recover DNA from gel.

The DNA band of interest was cut out of the gel with a clean razor blade and briefly soaked in electroelution buffer (20 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 5 mM NaCl) to remove the electrophoresis buffer. The gel slices were paced in the horseshoe shaped slots on the UEA platform. The electroelution buffer was added as a height just barely cover the gel slice and 75 μl the high salt buffer (7.5 M NH₄OAc [for fragments <500bp use 10-13 M NH₄OAc], 0.01% [w/v] bromophenol blue) was added into the V-channel. Typically, DNA fragments (0.5-3 kb) were eluted from agarose gel for 30 minutes at 100 volts. After elution, the high salt buffer was carefully removed from the V channel into a fresh microfuge tube. The DNA fragment was precipitated by adding 0.6-1 volume of iso-propanol, followed by incubation at −70°C for at least 30 minutes, centrifugation, washing with 70% ethanol and resuspended in TE or other desired buffer.

2.8.7 Radio-labelling of DNA probes

The “Ready-To-Go” kit (Pharmacia) was used to label radio-isotope DNA probes. In this kit, the hexanucleotides, dATP, dGTP, dTTP, Klenow enzyme and buffer salts were provided as a lyophilised mix and redissolved in 20 μl of ddH₂O prior to use. The DNA (dissolved in 25 μl ddH₂O) to be labelled was denatured by boiling for 2 minutes and chilled quickly in dry-ice/ethanol bath. This was added to the reaction mix tube along with 5 μl (50 mCi) of α-³²P dCTP (Amersham) and then incubated at 37°C for 30 minutes.

Unincorporated nucleotides were removed using a Nick Column (Pharmacia) which uses the Sephadex G-50 for gel filtration allowing the larger labelled DNA molecules to pass through the column faster than the unincorporated nucleotides. The column was rinsed with 3 ml of buffer TE followed by adding the labelling reaction directly on to the top of the gel bed and 400 μl of TE was also added. The collection at
this stage contained very little labelled DNA and was discarded while a second wash of 400 µl TE was kept as it contained the bulk of the labelled DNA.

### 2.9 DNA sequencing

#### 2.9.1 Preparation of template DNA

Only double stranded DNA templates were used in this work. They were prepared as described (2.6.2).

DNA was sequenced either by manual sequencing or automated sequencing.

#### 2.9.2 Manual Sequencing

A Sequenase® version 2.0 kit (USB) which was based upon the dideoxy chain termination method (Sanger et al., 1977) was used to sequence the double stranded DNA. The α-35SdATP labelling and sequencing reaction were described in the kit support manual. The samples were run on a 6% polyacrylamide/7M urea gel in 1 x TBE buffer. After electrophoresis at a constant power setting of 40 W for 40 cm long gels or 65 W for 60 cm gels, the gel was fixed in 10% (v/v) methanol/ acetic acid for 30 minutes. Then the gel was transferred onto Whatman 3MM paper and dried under vacuum for at least 1 hour at 85°C. The dried gel was exposed to X-ray film at 4°C for 1-3 days depending on the amount of radioactive signal present.

#### 2.9.3 Automated Sequencing

The automated sequencing is also based upon the dideoxy chain termination method of Sanger et al. (1977). However, DNA bands are detected by fluorescent dyes which are incorporated into the dideoxy nucleotides. Templates were supplied for sequencing on an applied 377A machine with results provided in the form of a computer file.

The ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit was used for cycle sequencing. A sequencing reaction mix of 8µl terminator mix, 1µl of primer (3.2 pmol/µl) and 1.5-2.5 µl double-stranded DNA (0.2 µg/µl) were made to 20µl final reaction volume. The reaction mixture was overlaid with mineral oil and cycle sequencing was performed using the following conditions:
96°C  30 seconds
50°C  15 seconds
60°C  4 minutes
25 cycles

The sequenced DNA was recovered by adding the reaction mix to 2 μl of sodium acetate pH 5.2 and 50 μl ethanol after the mineral oil was carefully removed. Followed by incubation on ice for at least 10 minutes, the DNA was collected by centrifugation. The pellet was washed with 250 μl 70% (v/v) ethanol, dried and then supplied to the sequencer operator.

2.9.4 Sequence Analysis

Sequence analysis was carried out using University of Wisconsin Computer Genetics Group (GCG) software (Devereux et al., 1984) using VMS VAX and Unix operating systems. Sequences generated by the automated sequencer were edited with GeneJockey II (BioSoft) software before being transferred to GCG readable files.

2.10 The polymerase chain reaction (PCR)

2.10.1 Primers

Primers were designed by using the GCG software. Details of primers used in this work are given in the appropriate chapters.

Oligonucleotides were obtained from Oswel or Perkin Elmer and were supplied dissolved in ~1ml of sterile distilled water or ~1 ml 20% acetonitrile in water respectively.

2.10.2 Normal PCR

Polymerase Chain Reactions (PCR) were carried out using a Hybaid thermal cycler. Details of cycles used are given in the appropriate chapters. The standard reaction volume was 100 μl in sterile 0.5 ml tubes with 40-50 μl of mineral oil added to prevent evaporation. Pharmacia deoxynucleotide triphosphates (dNTPs) were used for all reactions. Standard final concentrations of dNTPs and primers were 0.1mM and 2.5 pmole/μl respectively. The standard PCR buffer used was 50 mM KCl, 20 mM Tris-HCl pH 8.3, 2 mM MgCl₂, and 1 mg/ml gelatin.
2.10.2 Reverse transcriptase (RT)-PCR

2-3 μg total RNA (see 2.7.1) was reverse transcribed by adding 1 μl AMV reverse transcriptase (Promega) and the final concentration of the RT buffer was adjusted to 1x. Usually the reaction volume was 50 μl. The reaction was carried out at 48°C for 45 minutes and stopped at 94°C for 2 minutes.

3 μl of the RT reaction mix was used as the template for PCR. The amplification procedures are the same as normal PCR.

2.11 Blotting and hybridisation

2.11.1 Southern blotting

Both alkaline and salt transfer was used in this work. For alkaline transfer, Hybond-N+(Amersham) membrane was used and the DNA agarose gel was used directly for transfer after electrophoresis. For salt transfer (using Hybond-N), the gel was washed for 30 minutes in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and 30 minutes in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001M EDTA).

Transfer buffer (0.4 M NaOH for Hybond N+ and 20 x SSC for Hybond N) was poured into a tray. The gel containing DNA was placed on two layers of Whatman filter paper which were supported by a plastic plate. Two ends of the filter paper were rested in the transfer buffer. The selected membrane with appropriate size were placed carefully over the gel (bubbles should be prevented), followed by overlaying two sheets of Whatman filter paper (cut in size), 6 cm of absorbent paper and a small weight on the top. Saran wrap was placed around the gel to prevent short-circuiting. The transfer was allowed to proceed at a minimum of 3 hours.

After transfer, the membrane was rinsed briefly in 6xSSC before drying. Hybond-N was exposed to UV light (λ254 and λ365) for 10 minutes to fix the DNA.
2.11.2 Northern blotting

The transfer of RNA to membrane is similar to the transfer of DNA from the agarose gel. Hybond-N membrane was chosen and 20 x SSC was used as the transfer buffer. Formaldehyde denaturing gel (2.7.2) was washed gently in RNAase free ddH₂O (to remove formaldehyde) for 45 minutes prior to blotting. The membrane was exposed to UV light (λ₂₅₄ and λ₃₆₅) to fix the RNA.

2.11.3 Colony and plaque lifts

Bacteriophage was plated as described in section 2.4.4.3. The plaque was transferred to the Hybond-N (Amersham) membrane by overlaying the membrane on to the agar surface for a minimum of 2 minute. The membranes were placed plaque side up for 2 minutes in a dish of denaturing solution (1.5 M NaCl, 0.5 M NaOH). The membranes were transferred in a dish of neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0) for 5 minutes followed by rinsing for 30 seconds only in washing solution (0.2 M Tris-HCl pH 7.5, 2xSSC). The DNA was fixed by exposing to UV light (λ₂₅₄ and λ₃₆₅) for 10 minutes. The membranes were then ready for pre-hybridisation and hybridisation.

2.11.4 Pre-hybridisation and hybridisation

Pre-hybridisation and hybridisation were carried out inside the hybridisation bottles in the Hybaid ovens. Membranes were pre-hybridised at 65°C for 1-2 hours in 25-50ml of pre-hybridising solution (6 x SSC, 5 x Denhardt's, 0.5% [w/v] SDS, 1 mg/ml sonicated salmon sperm DNA). The radio-labelled probe (2.8.7) was denatured in a heated block at 100°C for 2 minutes and then plunged into ice. The denatured probe was added to an appropriate volume of fresh pre-hybridisation solution after the old solution was removed. Hybridisation was allowed to proceed at 65°C for overnight.

2.11.5 Washing of hybridised membranes

Hybridisation solution was transferred into a fresh 50ml conical flask and retained for further hybridisation. Approximately 100ml washing solution 1 (2xSSC, 0.1% [w/v] SDS) was added to the hybridisation bottle and gently shaken for 15 minutes at room temperature. A second identical wash was carried out. Approximately 100ml of washing solution 2 (0.2xSSC, 0.1% [w/v] SDS) was added with gentle shaking and incubation at 65°C for 15 minutes in the Hybaid oven. This was repeated once.
2.11.6 Autoradiography

The membranes have been washed were wrapped in Saranwrap™, placed into a cassette, overlaid with X-ray film to allow it to expose at -70°C. The exposure time was dependent on the signal emanating from the membrane. The films were developed in an X-OGRAPH compact X2 automated film processor.

2.11.7 Stripping of membranes

The membranes were boiled in ddH₂O for 10 minutes to remove the old probe in order to hybridise with a new probe. The membranes were checked for absence of probe by exposing to X-ray film for overnight.

2.12 Protein manipulation

2.12.1 Preparation of protein samples:

Flies were collected (5 flies for each lane) or dissected in 1 x PBS to collect relevant tissues, and followed by homogenising in 80 μl of sample buffer. Samples were stored at -20°C and denatured by boiling for 10 minutes (Protein markers boiling for 2-3 min) prior to loading.

2.12.2 SDS-Polyacrylamide gel (PAGE) electrophoresis of proteins

Protein gel plates were washed and assembled according to the manufacture’s instruction. Then separation mix was poured (5ml 3M Tris/HCl pH8.8, 0.4ml 10% [w/v] SDS, 13.8ml 30:8 AA:Bis [30g Acrylamide + 0.8g Bis-acrylamide], 20.8ml dH₂O, 133μl 15% [w/v] AMPS and 25μl TEMED) between the plates, quickly overlaid with a small amount of overlay buffer (25ml 3M Tris-HCl pH8.8, 2ml 10% [w/v] lauryl sulphate, 173ml ddH₂O), and allowed to set for about 45 minutes at room temperature. Once set the overlay buffer was poured off, a fresh “stacker gel” (1.25ml 0.5M Tris-HCl pH6.8, 100 μl 10% SDS, 1.25 ml AA:Bis 30.8, 7.3ml ddH₂O, 75 μl 15% [w/v] AMPS, 7 μl TEMED) was poured between the plates before putting the comb at the top of the gel. The plate was then put on the tank when the gel was solid and 1 x running buffer (14.4g glycine, 3.02g Tris, 1g lauryl sulphate, ddH₂O to 1 litre) was added. Protein samples (2.12.1) was loaded and the gel was run at 50 V for overnight.
2.12.3 Western transfer of protein samples from the SDS-PAGE gel to membrane

The blotting paper was cut to the same size as the hinged plastic holder used. The blotting paper and the brillo pad were pre-soaked in transfer buffer.

A piece of brillo pad was placed into the plastic holder. One piece of blotting paper was added and the protein gel was placed on the top of the blotting paper. A piece of nitrocellulose (Hybond C super) (the same size as the protein gel) was added to the protein gel and another piece of blotting paper and the other brillo pad were put on top. The plastic holder was closed and lowered into the transfer tank. The nitrocellulose should be nearest to the positive terminal. The transfer was run at 0.3 Amps for 4 hours.

After the transfer, to check if it has worked properly, the nitrocellulose was stained with Ponseau’s stain for a few minutes, the excess stain was washed with ddH₂O. If protein bands were visible, the nitrocellulose was sealed in a bag with 15mls of milk powder buffer (m.p.b.) and left on the shaker at 37°C for 1 hour. The nitrocellulose was then put in a fresh bag which contained 15 ml of m.p.b and 50-70 μl of the first antibody. The bag was left overnight at 37°C on shaker. The nitrocellulose was then washed in 1 x milk buffer for five times at 30 minutes intervals. After washing, 50-70 μl of the secondary antibody and 15ml of m.p.b was added in the bag. The bag was then sealed and incubated at 37°C for 1 hour. The washing steps were repeated as washing off the first antibody.

The nitrocellulose was developed at room temperature in a covered chamber containing the developer (-60mg 4-chloro-l-napthol, 20ml methanol, 100ml 1 x milk buffer, 100 μl Hydrogen Peroxide). After developing, the nitrocellulose was washed in dH₂O, “pat”-dried on blotting paper before photography. The nitrocellulose can be stored between sheets of blotting paper, wrapped in foil and stored in the dark.

2.13 in situ immunohistochemical detection

2.13.1 Whole-mount ovarian antibody staining

Ovaries were dissected from the yeasted flies under the dissecting microscope in Ringer’s solution (or 1 x PBS). The anterior parts of the ovaries were torn apart to
facilitate antibody penetration. The ovaries were transferred to a microfuge tube containing 2% formaldehyde (in 1 x PBS) fixative and fixed for 30 minutes at room temperature. The fixative was carefully removed and ovaries were washed in 1ml of PTW (PBS + 1.5% [v/v] Tween-20) for 5 minutes. Then the ovaries were incubated in PBT (PTW + 1% [w/v] Bovine serum albumin [Sigma]) for 1 hour. Blocking was accomplished by incubation the ovaries in PBTG (5% [v/v] goat serum in PBT) for 2 hours at room temperature.

First antibody in PBTG at an appropriate dilution was added and incubated overnight at 4°C. The antibody solution was removed and stored at -20°C to be reused. Residual antibody was washed away with three changes of PBT with 30 minutes incubation per change. The HRP-conjugated secondary antibody was then added to the ovaries and incubated for 2 hours at room temperature or overnight at 4°C. Excess secondary antibody was removed with three PBT washes at 30 minutes intervals. DAB staining solution (Sigma) was added and the staining was allowed to proceed for 10-30 minutes before washing with several changes of PBS to stop the reaction. Stained ovaries were mounted in PBS/glycerol (1:4) to allow microscopy.

2.13.2 DAPI staining

Ovaries were dissected in PBS and fixed in 4% paraformaldehyde (w/v in 1 x PBS) for 20 minutes. This was followed by washing in 1 x PBS containing Triton-X100 (1% [v/v]) for 30 minutes. The ovaries were then washed in 3 x PBS and stained in 0.5 μg/ml DAPI (Sigma) (dissolved in 3 x PBS) in dark for 1 hour. After washing in 3 x PBS for overnight, the ovaries were mounted in PBS/glycerol (1:4) and examined under a fluorescent microscope.

2.13.3 β-Galactosidase staining

The ovaries were dissected in PBS and stained at room temperature (25°C) for 3 hours to overnight in PBS containing 0.2% X-gal (Sigma), 5 mM K₄[Fe(III)CN₆]₃, 5 mM K₃[Fe(II)CN₆], 0.3% Triton-X 100 and rinsed in PBS and mounted in PBS/glycerol (1:4) for microscopic analysis.

2.13.4 Preparation of the eggshell for dark-field microscopy

Freshly laid eggs were collected from the apple-juice plate and placed in a drop of Hoyer’s mounting (Hoyer’s mount : lactic acid=50:50) on a glass slide and covered
by a cover-slip. The slide was incubated at 65°C for overnight and was ready for dark-field microscopy.

2.14 Whole mount in situ hybridisation to ovaries

This method is modified from Tautz And Pfeifle (1989) and D. Zhao (personal communication).

2.14.1 Preparation of digoxigenin labelled DNA

10-3,000 ng of DNA dissolved in 15 µl TE (or ddH₂O) was denatured by boiling in a waterbath for 2 minutes and chilling quickly on dry ice or ice/ethanol. 2 µl hexanucleotides, 2 µl dNTP labelling mix (Boehringer Mannheim) and 1 µl (1 unit) of Klenow enzyme were added. Labelling was carried out for overnight at 37°C and stopped by adding 2 µl 0.2M EDTA (pH 8.0). The labelled DNA was precipitated by adding 2 µl 4 M LiCl and 60 µl prechilled (-20°C) ethanol, leaving at -70°C for at least 30 minutes and spinning down (17,000 rpm, 4°C, 15 minutes). The pellet was washed with 70% (v/v) ethanol, air dried and resuspended in TE or DNA hybrix. The labelled DNA was denatured by boiling for 2 minutes and chilling quickly in dry ice/ethanol bath prior to use.

2.14.2 Pre-hybridisation treatment and hybridisation

Yeasted female flies were dissected in 1 x PBS and ovaries were taken out and fixed in 4% (w/v in 1 x PBS) paraformaldehyde (Sigma) for 20 minutes. The ovaries were washed three times at 5 minutes intervals in PTw (1 x PBS containing 0.1% Tween-20) to remove the fix. This was followed by washing for three times at 5 minutes intervals in ME (methanol : EGTA, 0.5M, pH 8; 9:1). After this step, the ovaries were able to be stored in methanol at -20°C for several months. To continue next step, the stored ovaries were washed in ME once and followed by washing for three times at 5 minutes intervals in PTw. This was followed by Proteinase K (Sigma) (100 µg/ml in PTw) treatment for 60 minutes at room temperature which was stopped by washing for 5 minutes in PTw containing 2 mg/ml glycine. After three washes in PTw to remove the residue Proteinase K, the ovaries were post-fixed in 4% paraformaldehyde and washed for 3 x 20 minutes in PTw.
Ovaries were equilibrated for 10 minutes with PTw/Hybrix (1:1) and pre-
hybridised with Hybrix (50% [v/v] formamide, 5xSSC, 50mg/ml heparin, 0.1% [v/v]
Tween-20, 100μg/ml sonicated salmon sperm DNA and 100 μg/ml tRNA) for 1 hour at
45°C. Hybridisation was carried out at 45°C for overnight by replacing digoxigenin
labelled probe dissolved in Hybrix.

2.14.3 Post-hybridisation treatment and signal detection

The probe was removed by a series of washes: first in Hybrix for 20 minutes
at 45°C, then in PTw/Hybrix for 1 hour and PTw for 20 minutes at 45°C, finally
washed five times at 5 minutes intervals in PTw at room temperature. Signals were
detected by incubation with Alkaline phosphatase (AP) conjugated anti-digoxigenin
antibody (1:1,000 in PTw) at room temperature for 1 hour. The antibody was pre-
adsorbed with post-fixed wildtype (Oregon R) ovaries at 4°C for overnight. Excess
antibody was removed with three washes of PTw at 20 minute intervals. Ovaries were
equilibrated in TLMNT buffer (100mM Tris-HCl pH 9.5, 1mM levamisole, 50mM
MgCl₂, 100mM NaCl, 0.1%(v/v) Tween-20) by three 5 minutes washes. Signal
detection solution (4.5μl NBT [75 mg/ml in 70% dimethyformamide] and 3.5μl X-
phosphate [50 mg/ml in 100% dimethyformamide in 1 ml TLMNT] was added and the
signal was developed in the dark for 15 minutes to overnight depending on the staining
intensity. The reaction was stopped by rinsing with 1 x PBS. For examination, ovaries
were mounted in a mixture of PBS and glycerol (1:4) and observed under the
microscope.

2.15 in situ hybridisation to polytene chromosomes

2.15.1 Chromosomal slides Preparation:

Both cover slips and slides were treated in 1M HCl for overnight and washed
thoroughly with tap water and distilled water. Slides were then dipped into subbing
solution (0.1% gelatine and 0.025% chromic potassium sulphate; gelatine is dissolved
at 50°C) and air-dried. Cover-slips were siliconised by dipping into 2%
Dimethyldichlorosilane and air-dried.

Salivary glands from late third instar larvae were dissected in Ringer’s solution
and fixed in a drop of 45% acetic acid for 4-5 minutes on a slide. A siliconised cover-
slip was put at the top of the glands, followed by gently tapping and streaking the slip
with the blunt-end of the forceps. An edge of the cover-slip should be held during
tapping and streaking to prevent it sliding and damaging the chromosomes. The slide was then sandwiched between layers of blotting paper and the chromosomes squashed by applying considerable thumb pressure. The chromosomes were examined for quality under the microscope. Good-quality slides were then frozen briefly in liquid nitrogen and the cover-slips were removed by a scalpel spade. Slides were dehydrated in 95% ethanol for 30 minutes and air-dried.

2.15.2 Pre-treatment of polytene chromosome slides

Slides were washed in 2 x SSPE at 65°C for 20 minutes and then at room temperature for 2 minutes. Chromosomes were acetylated by dipping the slides in freshly prepared acetic anhydride solution for 10 minutes. Slides were washed again in 2 x SSPE for 2 times at 5 minutes intervals, boiled in 2 x SSPE for 2 minutes to denature the chromosomes and put back in 2 x SSPE at room temperature for 5 minutes. Slides were re-dehydrated in 70% ethanol (2 x 5 minutes) and in 95% ethanol (5 minutes), followed by air drying.

2.15.3. Hybridisation, washing, and signal detection

16-25 µl of denatured digoxigenin-labelled DNA probe (2.14.1, dissolved in DNA-Hybrix) was added to each slide and covered by a cover-slip and sealed with nail polish. Hybridisation was carried out in a wet chamber (2 x SSPE) at 65°C for overnight. The slides were washed in 2 x SSPE at 65°C for 20 minutes; this was repeated three times. Finally the slides were washed in 2 x SSPE at room temperature for 20 minutes.

Slides were equilibrated in buffer 1 (100mM Tris-HCl pH7.5, 150mM NaCl) for 2 minutes and then incubated in buffer 2 (0.5% [w/v] blocking reagent in buffer 1) at room temperature for 60 minutes, followed by washing again in buffer 1 for 2 minutes. To detect the signals, 50 µl POD conjugated antibody, anti-digoxigenin-POD, (1:100 dilution in buffer 1) was added on the slides and the slides were incubated in a humid chamber at room temperature for 1 hour. Slides were then washed in buffer 1 for three times at 5 minutes intervals and in 1 x PBS for twice at 5 minutes intervals. 100 µl staining solution (1 set of DAB-H₂O₂ tablets [Sigma] was dissolved in 1 ml PBS) was

3 Acetic anhydride solution was prepared by adding 0.652 ml acetic anhydride in 300ml 0.1M Triethanolamine-HCL and agitated rapidly for a few seconds.
added on the slide and left at room temperature for 25 minutes. This was followed by washing the slides in 1 x PBS for three times at 2 minutes intervals. Slides were counter-stained with Giemsa solution (1:100 in water) for 15 minutes to allow the visualisation of the chromosomal bands. Slides were destained in ddH$_2$O, air-dried and mounted in aquamount (BDH) for microscopic analysis.

2.16 P-element mediated germline transformation

2.16.1 Preparation of DNA

Plasmid DNA to be injected was prepared and purified by using Qiagen plasmid kit (2.6.2) The DNA pellet was dissolved in injection buffer (5mM KCl, 0.1 mM sodium phosphate, pH 6.8) at a concentration of 400 µg/ml for the transforming plasmid and 100 µg/ml for the helper plasmid pΔ2-3.

2.16.2 Egg collection and chorion removal

Egg collection was described in section 2.3.2. Eggs were dechorionated manually by rolling the eggs gently on double-stick tape. Dechorionated embryos were transferred to a 22 x 22-mm cover-slip (attached to a 76 x 26-mm slide) which had a very thin strip of double stick tape on it. Using a dissecting needle the embryos were oriented with their posterior ends over one side of the tape. Normally, around 20 embryos could be lined on each cover-slip. When the embryos were lined, the cover-slip was placed in a petri dish with desiccant (silica gel or CuSO$_4$). As soon as the desiccation was complete (about 6-10 minutes), the embryo were covered with oil.

2.16.3 Microinjection

The embryo-laden slide was placed on the stage of the microscope with the posterior ends of the embryos towards the needle. The eggs were injected under the control of the micromanipulator at 1.5 kpsi, 0.3-0.5 seconds.

2.16.4 Post-injection treatment

After all the embryos had been injected, damaged and improperly aged embryos were removed under dissecting microscope. The cover-slip containing the injected
embryos was placed onto a tomato juice plate\textsuperscript{4} and incubated at 25°C for 2-3 days. The larvae were transferred into a fresh vial of Staffan food (about 10 larvae for each vial) and incubated at 21-25°C.

2.16.5 Examination for transformants

When the larvae developed to late pupae stage, individuals were transferred to fresh vials of food. These were crossed with flies with opposite sex when they eclosed from the pupae case. Progeny of the cross were examined for red-eyed transformants.

\textsuperscript{4} Tomato juice plate was prepared by mixing 4g of agar with 160 ml of water, brought to boil, and when the foam was reduced, 40 ml of tomato juice was added before bringing the whole mixture to boil again whereupon 5 ml of 10% Nipagin was added. The resultant mixture was allowed to cool to 60°C before being poured into petri dishes.
CHAPTER 3
Screen For Patterns Of Reporter Gene Expression In Enhancer-Trap Lines During Oogenesis
3.1 Introduction

P-element derived enhancer-trap vectors carry sufficient cis-elements such that they can transpose in the fly genome if transposase is provided. These vectors contain a reporter gene, usually the lacZ gene from *Escherichia coli*, and a marker gene for screening transformants. The expression pattern of the reporter gene is regulated by a genomic enhancer close to the P insertion site, and it is supposed to mimic the expression pattern of an endogenous gene controlled by the same enhancer (O'Kane et al., 1987; Bier et al., 1991; Wilson et al., 1991).

Enhancer-trap lines are useful cell markers. For example, novel subsets of follicle cells have been identified based on the reporter gene expression pattern during oogenesis (Spradling, 1993). This facilitates the study of cellular and genetic interactions in the follicle cells. Another use for the enhancer-trap technique is to directionally clone target genes of lines with interesting reporter gene expression patterns.

The first enhancer-trap vector was pLac92, which contains a lacZ reporter gene and a rosy marker gene (Figure 3.1A; O’Kane and Gehring, 1987). This vector has an *E. coli* plasmid fragment, which is 3’ to the inverted 3’ P-end, and is, therefore, excised before the P-element integrates into the genome. The cloning of genomic fragments flanking this P-element is time-consuming because a genomic library for each single enhancer-trap line is required. Other enhancer-trap vectors overcome this shortcoming by placing the 3’ P-end downstream of the *E. coli* plasmid fragment, and placing several restriction enzyme sites to the 5’ end of the plasmid sequence. These vectors, including plArB, plwB (Figure 3.1B,C), allow flanking genomic fragments to be recovered through a “plasmid rescue” technique, which involves restriction enzyme digestion, ligation and *E. coli* transformation (Wilson et al., 1989; Bier et al., 1989).

A distinctive enhancer-trap vector, pGawB (Figure 3.1D), uses a yeast transcription factor gene, Gal4, as the reporter. Gal4 specifically binds the upstream activation sequence (UAS) and activates expression of the gene downstream to the UAS (Brand and Perrimon, 1993). To detect Gal4 expression in the enhancer-trap lines, a transformed fly line carrying a Gal4 responsive gene, UAS-lacZ or UAS-GFP (GFP is a gene encoding green fluorescent protein), is crossed with the Gal4 lines (Figure 3.2). Thus, the tissue specific expression of Gal4 is translated in the tissue specific
expression of a second reporter, which can be visualised through histo-chemical staining (for lacZ reporter) or by fluorescent microscopy (for GFP reporter).

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**Figure 3.1** Structure of the enhancer-trap vectors.

**A.** Structure of the pLac92 plasmid (O’Kane and Gehring, 1987) plasmid.

**B.** Structure of the pIwB plasmid (Bier et al., 1989). (B, BamHI; G, BglII; H, HindIII; P, PstI; R, EcoRI; S, SacII; X, XbaI)

**C.** Structure of the plArB plasmid (Wilson et al., 1989).

**D.** Structure of the pGawB plasmid (Brand and Perrimon 1993).
Tissue-specific expression of Gal4

Figure 3.2 Detection of the Gal4 expression pattern. Enhancer-trap Gal4 lines are crossed with a fly line that carries a Gal4 responsive gene, UAS-lacZ. Expression of lacZ is specifically activated by Gal4. Thus, tissue-specific expression of Gal4 is reported by the tissue-specific expression of lacZ, which can be seen with histo-chemical staining (modified from Brand and Perrimon, 1993)
Although the detection of reporter gene expression is not straightforward, the \textit{Gal4-UAS} dual system has extended the enhancer-trap technique to a new stage. For example, if the \textit{UAS}-linked second reporter is replaced by a gene of interest, this gene can be driven in any tissue specific pattern if a suitable \textit{Gal4} line has been identified (Brand and Perrimon, 1993, 1994). Alternatively, a gene can be linked with \textit{UAS} in an anti-sense direction. Thus, tissue specific anti-sense expression can be used to silence the gene in a tissue-specific way. This targeted silencing technique has been applied to the study of the function of a myosin gene, \textit{Mhc95F}, during development (refer to Chapter 5). Furthermore, the \textit{Gal4-UAS} dual system allows cell clones to be specifically ablated or marked by different secondary markers (Hidalgo \textit{et al.}, 1995; Yeh \textit{et al.}, 1995).

The enhancer-trap technique has been used to study \textit{Drosophila} development for almost a decade, and a number of developmentally important genes have been identified. Systematic screening of reporter gene expression patterns in oogenesis has been carried out in some laboratories, and approximately 1/3 to 1/2 of enhancer trap \textit{P-lacZ} lines show staining in oogenesis (Spradling, 1993; A. González-Reyes, W.-M. Deng, H. Elliot, S. Pathirana, D. St. Johnston, M. Bownes, D. Glover, P. Deák, unpublished data). Additionally, screening of \textit{Gal4} reporter gene expression during oogenesis has also been undertaken (Brand and Perrimon, 1993; Gustafson and Boulianne, 1996; Manseau \textit{et al.}, 1997). It has been shown that \textit{Gal4} can only be detected in somatic cells, no \textit{Gal4} expression has been observed in germline cells. The reason for this is unclear. However, it facilitates the identification of patterns in the follicle cells. In order to identify interesting follicle-cell expressing genes, as well as to establish a repertoire of \textit{Gal4} lines showing specific follicle-cell expression patterns, a screen for \textit{Gal4} expression during oogenesis has been carried out (Deng \textit{et al.}, 1997) and the results are reported here.

\section*{3.2 Reporter gene expression patterns in \textit{Gal4} enhancer-trap lines$^1$}

\subsection*{3.2.1 Summary of \textit{Gal4} expression during oogenesis}

413 lines with \textit{P[GawB]} insertions on different chromosomes were crossed to the \textit{UAS-lacZ} line. Ovaries from the female progeny were dissected and histochemically

\footnote{$^1$ This work is in collaboration with Debiao Zhao, Kathleen Rothwell and Mary Bownes.}
stained with X-gal. Results of the staining are listed in Table 3.1. Approximately one third (150/413) of the lines show staining in the ovaries. Among these, 112 lines show staining in subsets of follicle cells; 4 lines are stained in all follicle cells; 22 lines are stained at patches of cells that are not always reproducible from ovary to ovary; and there are another 12 lines where staining is only visible in epithelial sheath cells. No staining has been detected in the germ-line cells, and this is consistent with previous reports (Brand and Perrimon, 1994; Gustafson and Boulianne, 1996).

The 112 lines that show staining in subsets of follicle cells have been categorised. It is noticed that a complex reporter gene expression pattern is shown in a number of lines, which exhibit staining in several groups of follicle cells. These lines are categorised into different classes according to the different groups of follicle cells that are stained. The distribution of the expression patterns is described below. Examples of some of the classes are shown in Figure 3.3.

### 3.2.2 Staining in anterior follicle cells

24.7% (37/150) of the lines stained in ovaries have been found to have staining in the anterior follicle cells. Before stage 10, anterior follicle cells refer to those located at the anterior end of the egg chambers. During and after stage 10, they include those associated with the nurse cells and those located at the anterior end of the oocyte.

Among the 37 lines stained in the anterior follicle cells, 3 lines show staining in the border cells, which delaminate from the anterior pole at about stage 9 and migrate through the nurse cell chamber to reach the nurse cell-oocyte border at about stage 10. Additionally, all 3 lines show staining in follicle cells that remain at the anterior pole after the border cells delaminate and migrate posteriorly. This may suggest a close relationship between the migrated and unmigrated anterior follicle cells. C865 is one of the 3 lines that have reporter gene expression in the border cells and the remaining anterior cells (Figure 3.3A). The staining appears at the anterior end of the egg chambers from stage 7 until stage 13, and in the border cells from stage 9 onwards. The cloning and functional analysis of this target gene will be discussed in Chapter 4 and Chapter 5 respectively.

In addition, six lines show staining in stretched cells, a thin layer of follicle cells associated with the nurse cells during stage 10. Two of them also show staining in anterior cells in egg chambers younger than stage 9. Another line is also stained in
columnar cells except at the posterior pole. C826 is one of the lines that show staining in stretched cells (Figure 3.3B). Staining is stronger in the nuclei and the ditch between nurse cells where the cytoplasm is thicker. Staining in the other parts of the cells is almost invisible, suggesting that the cells are very thin.

Furthermore, two lines show staining in the centripetal cells, which migrate along the DV axis to cover the anterior end of the oocyte at about stage 10b.

Among the 37 lines stained in the anterior follicle cells, 10 of them only show staining during stages 13 and 14.

### 3.2.3 Staining in posterior follicle cells

Posterior follicle cells are defined as the follicle cells covering the posterior end of the egg chamber. There are 32 lines that show staining in these cells. C844 is a typical line that shows a cap-like staining pattern over the posterior pole of egg chambers. The β-galactosidase staining can be detected at as early as stage-5 in the posterior cells, and it remains until later stages (Figure 3.3C). However, the majority of other lines which show staining in the posterior follicle cells have a more dynamic expression pattern. About 34.4% (11/32) of them shows staining in both the posterior and anterior follicle cells, indicating that these two groups of follicle cells co-express a number of genes. Additionally, three of the “posterior lines” show staining in the polar follicle cells at early stages. Since the posterior polar follicle cells and the neighbouring follicle cells are thought to be functionally related in sending signal(s) to the oocyte at around stage 7, the co-expression of reporter genes in these two groups of cells may be a good reflection of their related function. Furthermore, it has been found that 11 of the 32 “posterior lines” show staining only during stages 13 and 14. This observation, along with the observation that 10 of the 37 “anterior lines” only show staining during stages 13 and 14, suggests that a number of genes are required for eggshell differentiation.

### 3.2.4 Staining in columnar cells

Another major category of Gal4 lines (24.0%, 36 lines) show staining in columnar follicle cells that cover the oocyte from stage 10 to later stages. Among these, 27 lines show staining in all of the columnar cells at stage 10 and later stages, while 9 lines only show staining in the stage-14 follicle cells surrounding the egg.
3.2.5 Staining in other follicle cells

At the anterior end of each ovariole lies the germarium, where oogenesis commences. 7 lines show staining in cells located in this area. Of these, two lines show staining in terminal filaments.

As they bud from the germarium, the egg chambers are interconnected by a stack of single cells, called stalk cells. 10 lines have been found showing Gal4 expression in these cells. 133Y is one of the lines that stain at the stalk cells from very early stages (Figure 3.3D). Additionally, staining in this line is also visible in the follicle cells surrounding the anterior of the oocyte at stage 10.

At both the anterior and posterior poles of the egg chamber are located pairs of specialised follicle cells known as polar cells. They express Fascilin-III and neuralized (Ruohola et al., 1991) and are thought to be determined by and cease division in region 2b of the germarium (Margolis and Spradling, 1995). In this screening, 5 lines express Gal4 in these cells, with all of them showing expression in polar cells at both the anterior and posterior end, supporting the idea that these cells have related origins (Margolis and Spradling, 1995).

At the dorsal anterior region of the mature egg, there is a pair of chorionic appendages called dorsal appendages. 19 lines (12.7%) show staining in the follicle cells associated to these structures.
### Table 3.1 Reporter Gene Expression Patterns

In *Gal4* Enhancer-Trap Lines During Oogenesis

**Abbreviations:**
- **ant.** = anterior follicle cells;
- **bc.** = border cells;
- **f.c.** = follicle cells;
- **d.a.** = dorsal appendages;
- **ncfc** = nurse cell associated follicle cells (stretched cells);
- **st.** = stage;
- **stallk** = stalk cells;
- **oc.** = oocyte;
- **oc. as.** = follicle cells over the oocyte;
- **pc.** = polar cells;
- **post.** = posterior follicle cells.

<table>
<thead>
<tr>
<th>Stock</th>
<th>Description</th>
<th>ant</th>
<th>bc</th>
<th>post</th>
<th>ncf</th>
<th>stalk</th>
<th>oc. as.</th>
<th>pc.</th>
<th>d.a.</th>
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<tr>
<td>9Y</td>
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<td>Odd f.c. over oc.</td>
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<td>AN T. F.C., FORM A SLIGHT GRADIENT, ST.13,14</td>
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<td>Germarium f.c.; Ant. polar cells &amp; border cells</td>
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<td>Dorsal appendages and post. tip at st.14</td>
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<td>Majority of f.c. from st.9 to late</td>
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<td>Over the oocyte, st.10</td>
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<td>C538*</td>
<td>Ant. tip</td>
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<td>Majority of f.c., st.1-6, polar cells strongly stained. All f.c., st.7-11; Dorsal appendage &amp; post., st.13-14</td>
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<td>F.c., st.14; early stages(?)</td>
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<td>Ant. and post. polar cells at early stages; ant. and post. f.c., patches around oc., late stages</td>
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<td>Dorsal appendages &amp; st.14 f.c.</td>
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<td>Ant. f.c. (1/4 of st8 chamber); border cells</td>
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<td>Patches at post. pole, early stages; post. f.c., st.7-9;</td>
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<td>Polar cells, st.2-10; border cells; dark in st14 f.c.</td>
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<td>unusual pattern in early stages, then in all f.c.</td>
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<td>Basement of dorsal appendages</td>
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<tr>
<td>C621</td>
<td>Sheath + odd f.c.</td>
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<tr>
<td>C623</td>
<td>Epithelial sheath?</td>
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<tr>
<td>C628</td>
<td>Germarium(?); Patch f.c. over the oocyte, st.10; Micropyle</td>
<td>V</td>
<td></td>
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<tr>
<td>C631</td>
<td>Dorsal appendages</td>
<td>V</td>
<td></td>
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<tr>
<td>C632</td>
<td>Dorsal appendages, st.13,14</td>
<td>V</td>
<td></td>
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<tr>
<td>C633c</td>
<td>Post. pole (sheath?), st.14; early stages undetectable</td>
<td>?</td>
<td></td>
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<tr>
<td>C635</td>
<td>Over the oocyte, st.7-14</td>
<td>V</td>
<td></td>
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<tr>
<td>C646</td>
<td>F.c. over the post. half of the oocyte, st.9-10</td>
<td>V</td>
<td></td>
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<tr>
<td>C648</td>
<td>very unusual pattern, patch(?)</td>
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<tr>
<td>C648</td>
<td>Patches of f.c.</td>
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<tr>
<td>C653b</td>
<td>Random f.c.; st.6-8 Patch f.c. over the oocyte, st.9-late;</td>
<td>V</td>
<td></td>
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<tr>
<td>C661a</td>
<td>odd f.c. over oc., st9 to late</td>
<td>V</td>
<td></td>
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<tr>
<td>C665</td>
<td>odd spots</td>
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<tr>
<td>C669</td>
<td>Germarium; ant. &amp; post. f.c. in young stages</td>
<td>V</td>
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<tr>
<td>C673</td>
<td>Ant. to post. gradient from st. 10-14</td>
<td>V</td>
<td></td>
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<tr>
<td>C674</td>
<td>2 cells at ant. tip of each ovariole at tip of germarium</td>
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<tr>
<td>C696*</td>
<td>Odd spots</td>
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<tr>
<td>C702</td>
<td>All f.c., st.14</td>
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<tr>
<td>C705</td>
<td>Post. tips, st.14</td>
<td>V</td>
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<tr>
<td>C709</td>
<td>Majority of columnar cells, st.10</td>
<td>st10</td>
<td></td>
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<tr>
<td>C710</td>
<td>Strong at ant &amp; post cap, over oocyte,</td>
<td>V</td>
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<tr>
<td>C714</td>
<td>Post. f.c., st.9 onwards</td>
<td>V</td>
<td></td>
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<tr>
<td>C714b</td>
<td>Over the oc., then all f.c.</td>
<td>V</td>
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<tr>
<td>Code</td>
<td>Description</td>
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<tr>
<td>C716</td>
<td>Specific patches of post. f.c., early to late</td>
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<tr>
<td>C725c</td>
<td>Some spots post. st10</td>
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<tr>
<td>C726b</td>
<td>Two big patches of f.c. over oc. at post., st.10; small patches</td>
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<tr>
<td>C729a</td>
<td>Sheath &amp; st? f.c.</td>
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<tr>
<td>C736</td>
<td>Dorsal appendages, st14</td>
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<tr>
<td>C745</td>
<td>Ant. &amp; post. f.c., early</td>
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<tr>
<td>C751</td>
<td>All sheath cells (dark)</td>
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<tr>
<td>C752c</td>
<td>spots</td>
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<tr>
<td>C753</td>
<td>Ant. f.c. at early stages</td>
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<tr>
<td>C756</td>
<td>F.c. over oc.; then all f.c. at st.14</td>
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<tr>
<td>C760</td>
<td>Columnar cells, st.10; Post. FC, st.14</td>
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<tr>
<td>C789</td>
<td>F.c. over the oocyte, st.9; ant. &amp; post., st.10; Post. f.c., late stages</td>
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<tr>
<td>C795</td>
<td>Stalk cells; then stretched cells</td>
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<tr>
<td>C817</td>
<td>Dorsal appendages</td>
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<tr>
<td>C819</td>
<td>Dorsal appendages</td>
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<tr>
<td>C825</td>
<td>F.c. over the oocyte, st.9-11</td>
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<tr>
<td>C825a</td>
<td>Ant. and post, st.14</td>
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<tr>
<td>C825b</td>
<td>Over oocyte, st.9; stretched cells, st.10</td>
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<tr>
<td>C826</td>
<td>Band f.c. over ant. oc. &amp; stretched cells</td>
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<tr>
<td>C832</td>
<td>Post f.c. at st. 13, 14</td>
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<tr>
<td>C837</td>
<td>Patches of f.c. at st.14; more at post.</td>
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<tr>
<td>C838</td>
<td>Sheath</td>
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<tr>
<td>C840</td>
<td>Ant. tip of ovariole; stalk cells</td>
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<tr>
<td>C844</td>
<td>Post. cap over oc., from st.5 to late</td>
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<tr>
<td>C847</td>
<td>Patches of f.c.</td>
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<tr>
<td>C853</td>
<td>F.c. at st.14</td>
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<tr>
<td>C865</td>
<td>Ant. f.c and border cells</td>
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</tbody>
</table>

Notes: * Fly lines have not been kept.
3.2.6 *Gal4* lines selected for this study

Enhancer-trap *Gal4* lines provide material for cloning genes that are expressed in subsets of follicle cells and to investigate their functions that are related to these expression domains. In this study, nine *Gal4* lines (C714, C726b, C826, C832, C844, C865, 36Y, 46Y and 133Y) that exhibit reporter gene expression in different subsets of follicle cells were chosen for further characterisation of their reporter patterns and for plasmid rescue of flanking genomic DNA (Table 3.2).

**Table 3.2 *Gal4* expression patterns of the starting enhancer-trap lines**

<table>
<thead>
<tr>
<th>Stock Number</th>
<th><em>Gal4</em> expression pattern during oogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>C714</td>
<td>Posterior follicle cells, stage 9 onwards.</td>
</tr>
<tr>
<td>C726b</td>
<td>Two patches of posterior follicle cells and small random patches of columnar cells, stage 10-11.</td>
</tr>
<tr>
<td>C826</td>
<td>Band follicle cells surrounding the anterior oocyte and stretched cells, stage 10.</td>
</tr>
<tr>
<td>C832</td>
<td>Strong in stage-13 and 14 posterior follicle cells.</td>
</tr>
<tr>
<td>C844</td>
<td>Posterior follicle cells, stage 5 onwards.</td>
</tr>
<tr>
<td>C865</td>
<td>Anterior follicle cells, stages 7 to 12; border cells, stages 9 to 12.</td>
</tr>
<tr>
<td>133Y</td>
<td>Follicle cells surrounding the anterior oocyte, stage 10; stalk cells, all stages.</td>
</tr>
<tr>
<td>46Y</td>
<td>Odd dots (different from the originally reported pattern that stained in the stretched cells).</td>
</tr>
<tr>
<td>36Y</td>
<td>Stretched follicle cells, stage 10.</td>
</tr>
</tbody>
</table>

Of these nine lines, four (C714, C726b, C832, and C844) show β-galactosidase staining in follicle cells over the posterior part of the egg chamber, but the staining patterns are different from each other temporally and spatially. In line C844, the blue stain forms a cap covering the posterior end of the egg chamber from stage 5 to around stage 12 (Figure 3.3C), while in line C714, the reporter gene expression in the posterior cells starts at about stage 9 (data not shown). The staining pattern of line C726b looks like two big patches over the posterior of the oocyte but there is no expression at the posterior pole during stage 10. Staining in this line is also observed in several small patches of follicle cells at stage-10 (Figure 3.3E). In C832, the staining is strong in posterior follicle cells at stage 13 and 14 egg chambers (data not shown).
Figure 3.3 Examples of Gal4 lines directing expression of a *lacZ* gene in subsets of follicle cells during oogenesis.

A. Staining is observed in the anterior follicle cells and the border cells (arrow) in C865.

B. C826 shows staining in the nurse cell associated follicle cells and anterior columnar cells during stage 10.

C. The posterior follicle cells are stained in C844. 133Y shows staining in both the stalk cells during early stages (D1) and the anterior columnar cells at stage 10 (D2).

E. Two big patches and several small patches of columnar follicle cells are stained in C726B.
The follicle cells stained in line C865 are the anterior follicle cells from stage 7 to stage 10 and the border cells which start their migration at stage 9 (Figure 3.3A). Two lines (133Y and C826) share a common pattern of Gal4 expression in the anterior columnar follicle cells surrounding the stage-10 oocyte. They both show staining in some other follicle cells: 133Y is stained at the interfollicular stalk cells throughout oogenesis (Figure 3.3B,D); C826 is stained in the nurse cell associated follicle cells (stretched cells) at stage 10 (Figure 3.4B). Two other lines (46Y, 36Y) were originally reported to also stain the stretched follicle cells at stage 10. However, such a result for line 46Y has not been replicated. Only random blue dots can be observed in columnar cells from stage 10 onwards (data not shown). It is possible that there were originally two P[GawB] insertions in this line and they have been separated. The Gal4 expression pattern of line 36Y in the stretched cells has been confirmed.

3.3 Screen for reporter gene expression patterns in P-lacZ enhancer-trap lines

A screen for patterns of reporter gene expression during oogenesis in P-lacZ enhancer-trap lines has also been undertaken. Around 2,000 lines have been screened in collaboration with S. Pathirana, A. González-Reyes, H. Elliot, and P. Deák. These lines have placW (refer to Figure 3.1B) insertions on the third chromosome and are mostly homozygous lethal.

Around 1,000 lines which were screened in collaboration with S. Pathirana have been investigated on the pattern of β-galactosidase staining (summarised in Table 3.3). It is found that 339 lines show β-galactosidase staining in ovaries (includes staining in germaria and egg chambers). Among these, 266 (78.5%) lines show staining in the somatic cells, whilst 110 (32.4%) lines are stained in the nurse cells; these include the 37 (10.9%) lines that exhibit staining in both the somatic and germline cells, suggesting that more follicle-cell expressed genes are required for oogenesis than germline-cell expressed genes.

Table 3.3 shows the distribution of the β-galactosidase staining patterns in the 266 lines that show staining in follicle cells. It is found that 55 (20.7%) lines show staining in all follicle cells at all stages, whereas the other 211 (79.3%) lines exhibit staining in different subsets of follicle cells. It is also noticed that 38 and 36 lines are stained in follicle cells at the anterior and posterior end of the egg chambers,
respectively; 13 lines show staining in both the anterior and posterior follicle cells. The most frequent pattern observed is the staining in the columnar cells that cover the stage-10 oocyte; 101 (38.0% in 266 lines that show staining in follicle cells) lines display staining in these follicle cells. This percentage appears to be higher than in the Gal4 lines (24.0%). As has been shown in the Gal4 lines, reporter gene expression has also been detected in polar cells, border cells, stretched cells and centripetal cells. Staining has also been found in the stalk cells; however the number (2 in 266 lines) is very low. An interesting group, which includes 56 (21.1%) lines, shows β-galactosidase staining in dorsal-anterior follicle cells over the stage 10-13 oocytes. The target genes of this group may be downstream genes of the Grk-DEr signalling pathway in dorsal-ventral patterning of the follicle cells. The large number of lines belonging to this group may indicate the complexity of the patterning of the follicle cell epithelium.

Table 3.3 Estimation of reporter gene expression patterns of P-lacZ lines

<table>
<thead>
<tr>
<th>Follicle cell types that show β-galactosidase staining</th>
<th>Number of lines</th>
<th>Percentage among the 266 lines show follicle cell staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subsets of follicle cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior follicle cells</td>
<td>38</td>
<td>14.3%</td>
</tr>
<tr>
<td>Posterior follicle cells</td>
<td>36</td>
<td>13.5%</td>
</tr>
<tr>
<td>Follicle cells over the oocyte at stage 10</td>
<td>101</td>
<td>38.0%</td>
</tr>
<tr>
<td>Dorsal-anterior follicle cells</td>
<td>56</td>
<td>21.1%</td>
</tr>
<tr>
<td>Border cells</td>
<td>37</td>
<td>13.9%</td>
</tr>
<tr>
<td>Stretched cells and centripetal cells</td>
<td>21</td>
<td>7.9%</td>
</tr>
<tr>
<td>Stalk cells</td>
<td>2</td>
<td>0.8%</td>
</tr>
<tr>
<td>Polar cells</td>
<td>18</td>
<td>6.8%</td>
</tr>
<tr>
<td>All follicle cells at all stages</td>
<td>55</td>
<td>20.7%</td>
</tr>
</tbody>
</table>

Although in this study these P-lacZ lines were not selected for directional cloning of the target genes, they provide a repertoire of follicle cell markers as well as germline cell markers. When compared with the Gal4/UAS-lacZ ovaries, it is found that the β-galactosidase staining patterns in P-lacZ lines appear to be more uniformly distributed. It is very rare that staining the P-lacZ lines is present or missing in patches.
of follicle cells; they therefore are better markers than the Gal4 lines. Furthermore, since these lines are mostly homozygous lethal, they supply a source of potential mutants for genes located on the third chromosome.
CHAPTER 4

Cloning Target Genes From The Enhancer-Trap *Gal4* Lines
4.1 Introduction

Directional cloning of target genes from enhancer-trap lines can be achieved by recovering the flanking genomic DNA, then screening genomic and cDNA libraries. To verify that the cDNA obtained from library screening is the target gene, its expression pattern is compared with the reporter gene expression pattern in the starting enhancer-trap line. However, P-element insertion at different positions in a gene locus may cause the reporter gene to report different parts of the expression domains of the target gene (Wilson et al., 1989). Thus, cDNAs showing patterns which partly match the reporter gene expression pattern could also be potential target genes.

The first step towards target gene cloning from a Gal4 enhancer-trap line is "plasmid rescue", through which the genomic DNA flanking the P-element insertion site can be recovered. The "plasmid rescue" technique involves digestion of genomic DNA from the enhancer-trap line, ligation, and transformation into an E. coli host (Figure 4.1). Digestion of the genomic DNA from enhancer-trap lines creates some DNA fragments that contain both plasmid sequences, i.e. they have a DNA replication initiation site from E. coli, and host genomic DNA, which flanks the P insertion site. Following ligation and transformation into an E. coli host strain, these fragments can then multiply, whereas other DNA fragments created through enzyme digestion cannot grow in the bacterial host, since they do not have an origin of replication.

Usually, a selection of restriction endonucleases can be used to create such fragments in order to rescue flanking genomic DNA. For example, the Gal4 enhancer-trap vector pGawB contains recognition sites for the enzymes, XhoI, PstI, Sall, SacII, BstXI, and SacI, within the polylinker PL2/3. These six sites are not found further 3' of PL3 in pGawB, and therefore may be used for plasmid rescue of 3' adjacent genomic DNA (see Figure 4.1). The rescued genomic DNA can be separated from the 3' P-element sequences (the Bluescript vector) by digesting with both the enzymes used for rescue and BamHI (see Figure 4.1). Alternatively, KpnI, the only enzyme that has just one recognition site located 3' of the Bluescript sequence within pGawB, can be used to recover the 5' flanking genomic DNA (Figure 4.1).
Figure 4.1 Plasmid rescue of genomic DNA flanking P[GawB]. (PL2/3=Pst I, Sal I, Xho I, BstXI, Sac II, Sac I; PL4=Kpn I, Sma I, Sac II, Sfi I, Spe I, BamHI). Any enzyme except SacII in PL 2/3 can be chosen for the recovery of 3' flanking genomic DNA. The rescued genomic fragment can be removed from the Bluescript vector by digestion with both BamHI and the enzyme used for plasmid rescue. KpnI is the only enzyme that has a site 3' to the Bluescript sequence, but no sites 5' to it in P[GawB]. Thus KpnI is the only enzyme which can be used for the recovery of 5' flanking genomic DNA.
To verify that the rescued genomic fragment contains the target gene sequence, whole-mount RNA in situ hybridisation can be used to determine if the fragment shows an expression pattern similar to that of the reporter gene. Usually, larger genomic fragments, which can be obtained by genomic library screening, are required, because the flanking genomic fragments obtained by plasmid rescue are generally small and mostly in the 3' direction. Once larger genomic fragments have been obtained, whole-mount RNA in situ hybridisation can be used to determine if they contain target gene sequences. If the RNA in situ hybridisation pattern matches (or partly matches) the reporter gene expression pattern, cDNA libraries can then be screened to search for target cDNA clones.

In this study, nine Gal4 lines (C714, C726b, C826, C832, C844, C865, 36Y, 46Y and 133Y) that show reporter gene expression in different subsets of follicle cells were chosen for plasmid rescue of flanking genomic DNA (refer to Chapter 3). Although the study of one or two target genes in depth is sufficient for this thesis, data obtained in each step used in the cloning of these nine lines will be beneficial for future work. Flanking genomic DNA from nine selected lines has been recovered. Four lines (C726b, C826, C844 and C865) that showed reporter gene expression in four different subsets of stage-10 follicle cells were then chosen and used to clone larger flanking genomic fragments by genomic library screening. Of these, potential target cDNAs from three lines (C726b, C826 and C865) have been isolated. Two of them (C726b and C865) have been confirmed and further study of their function during oogenesis has been carried out. Results of cloning are reported in this chapter.

4.2 Plasmid rescue

Genomic DNA from the nine chosen Gal4 lines was prepared according to the method described in 2.2.1. The samples were then digested separately with XhoI, PstI, and SalI to recover the 3' adjacent genomic DNA. KpnI was used to rescue the 5' flanking genomic DNA. After ligation and transformation, 3' adjacent genomic DNA from the selected nine lines was recovered using at least one of the three enzymes (Table 4.1, Figure 4.2). In contrast, the 5' adjacent genomic DNA was only recovered in line C865. Positive clones appeared when 5' rescue was performed in two other lines, however, one of the rescued plasmid is smaller than the vector, whilst the other one appeared to have no inserts. The reason for this is unknown.
Table 4.1 Plasmid rescue results

<table>
<thead>
<tr>
<th>Name of stock</th>
<th>3' rescue</th>
<th>5' rescue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PstI</td>
<td>SalI</td>
</tr>
<tr>
<td>C714</td>
<td>0.7 kb</td>
<td></td>
</tr>
<tr>
<td>C726b</td>
<td>2.0 kb</td>
<td></td>
</tr>
<tr>
<td>C826</td>
<td>0.45 kb</td>
<td>1.1 kb</td>
</tr>
<tr>
<td>C832</td>
<td>0.5 kb</td>
<td></td>
</tr>
<tr>
<td>C844</td>
<td>7 kb</td>
<td></td>
</tr>
<tr>
<td>C865</td>
<td>7.5 kb</td>
<td>2.0 kb</td>
</tr>
<tr>
<td>133Y</td>
<td>0.4 kb</td>
<td>1.0 kb</td>
</tr>
<tr>
<td>46Y</td>
<td>0.7 kb</td>
<td></td>
</tr>
<tr>
<td>36Y</td>
<td>0.7 kb</td>
<td></td>
</tr>
</tbody>
</table>

1. The sizes shown in the table are the recovered genomic DNA fragment sizes.
2. The rescued plasmid is smaller than pGawB; its origin is unidentified.
3. No insert is found in this rescued plasmid.

Figure 4.2 Plasmid rescue of 3' flanking genomic DNA in GAL4 lines.

A. Lane 1, 1-kb ladder; lane 2, Bluescript KS(-), BamHI digestion.
   Lane 3-8, XhoI+BamHI digestion: lane 3, 4, pXB (C726B); lane 5, 6, pXC (C826); lane 7, pXD (C832); lane 8, pXE (C844);
   lane 9, pPF (C865), PstI+BamHI digestion;
   lane 10-11, pPA (C714), PstI+BamHI digestion;
   lane 12, pPD (C832), PstI+BamHI digestion.

B. Using 32P labeled Bluescript to probe the Southern blot made from A, a ~3-kb band was detected in each rescued plasmid.
4.2.1 Line C865

Both 3' and 5' flanking genomic DNA fragments have been rescued in line C865. In the 3' rescue, two fragments were obtained using the enzymes PstI and XhoI. The PstI rescued plasmid, pPF, contains a large insert at 7.5-kb. Using XhoI and BamHI to double digest pPF, a 2-kb band was obtained, which is the same size as the insert in XhoI rescued plasmid, pXF (Figure 4.3A). They should, therefore, be homologous fragments. Additionally, a 5' rescued plasmid containing a 7.1-kb insert has been obtained (data not shown). The cloning and analysing of the target gene of this line will be discussed in section 4.3 and Chapter 5.

**Figure 4.3** Plasmid rescue of the 3' adjacent genomic fragment in C865.
A. Lane 1, 1-kb ladder; lane 2, pPF, PstI+ BamHI digestion; lane 3, pPF, XhoI+ BamHI digestion; lane 4, pXF, XhoI+ BamHI digestion.
B. Lane 1, 1-kb ladder; lane 2, pXF, XhoI+ EcoRI double digestion; lane 3, pPF, XhoI+ EcoRI digestion; lane 4, pPF, XhoI digestion.
C. A diagram of the restriction map of the rescued plasmid, pPF (linearised); based on gel A and B.
4.2.2 Line C726b

In line C726b, only XhoI digested 3' adjacent DNA has been rescued. The rescued plasmid, pXB, contains 2.0-kb of genomic sequence (Figure 4.2A, lane 3,4). No signal has been detected when using it as a probe to hybridise whole-mount ovaries. Target gene cloning carried out in this line has revealed it to be a gene encoding zinc-finger proteins. These results will be presented in both section 4.4 and Chapter 6.

4.2.3 Line C826

In line C826, the rescued plasmids, pXC and pSC, from XhoI and SalI digested genomic DNA, contain a 1.1-kb and a 0.45-kb insert respectively (Figure 4.2A, lane 5,6 and data not shown). The larger one, pXC, gives rise to a 0.45-kb fragment after SalI-XhoI digestion (data not shown). This indicates that these two fragments may be homologous to each other. Further efforts have been made to clone its target gene, which will be discussed in section 4.5.

4.2.4 Line C844

In line C844, 7-kb of genomic DNA was rescued after digestion with XhoI (Figure 4.2A, lane 8), but no signal was detected when this was used to probe whole-mount ovaries. Larger genomic fragments from three positive clones have been obtained after screening a λFix D. melanogaster genomic DNA library. It is unlikely that these larger fragments contain the target gene sequence, since a whole-mount ovarian in situ assay using the λFix clones as probes showed staining only in the nurse cells (data not shown). This may indicate that the target gene is located at a chromosomal position which is far from the P[GawB] insertion. Chromosomal walking would therefore be required in order to clone the target gene. No further efforts have been made with this line.

4.2.5 Other lines

0.7-kb of 3' flanking genomic DNA from line C714 has been rescued after PstI digestion (Figure 4.2). In line C832, 0.5-kb of 3' adjacent DNA has been rescued after PstI digestion (Figure 4.2). 3' fragments from lines, 133Y, 46Y, and 36Y, have also been rescued (Table 2.1). However, no target gene sequence seems to be contained in any of these rescued fragments as determined by ovarian RNA in situ analysis (data not shown). No further efforts have been made to clone the target genes from these lines.
4.3 Target gene cloning from C865

4.3.1 Genomic library screening

The plasmid rescued DNA fragments from C865 contain about 14.6-kb of the genomic DNA surrounding the P[GawB] insertion site (section 4.2.1). Preliminary whole-mount ovarian RNA in situ hybridisation showed no apparent staining pattern in the follicle cells when recovered flanking DNA was used as the probe. In order to obtain larger genomic fragments that may contain the target gene sequence, a D. melanogaster λFix genomic library (Stratagene) was screened with 32P-dATP labelled 7.5-kb insert from the 3' rescue, pPF.

Five positive clones, namely λF1a, λF1b, λF2a, λF7a, and λF8a, were isolated after secondary screening. These have been confirmed to be true positives by using the 7.5-kb pPF insert to probe Southern blots of restricted phage clone DNA. Physical mapping with SstI, EcoRI and XhoI shows that these phage clones cover about 30-kb genomic DNA, which extends about 22-kb to the 3' end of the P[GawB] insertion, and about 8-kb to the 5' end (Figure 4.4A).

![Figure 4.4](image)

**Figure 4.4** Restriction map of the genomic region surrounding P[GawB] insertion site in C865. (X=Xho I, R=EcoR I, S=Sst I)
4.3.2 Do the larger genomic fragments contain the target gene?

In order to determine if these larger genomic fragments contain the target gene, inserts from λF7a and λF2a, which cover the entire 28-kb genomic sequence were labelled with Digoxigenin-11-dUTP for use in in situ hybridisations to whole-mount ovaries.

Signals were detected in the anterior follicle cells at stages 7-8 (see Figure 4.5B). The border cells are also stained from stage 9 to stage 11. However, this is obscured because staining is also seen in nurse cells. The staining pattern in the anterior and border cells is very similar to the β-galactosidase staining pattern in the starting enhancer-trap line, C865 (Figure 4.5A). This, therefore, indicates that the target gene sequence is likely to be present in these larger genomic fragments. Signals have also been detected in other subsets of follicle cells and nurse cells (data not shown). There are two possible explanations for this complex expression pattern. Firstly, more than one gene may be included in these genomic fragments, each of which contributes to a different part of the follicle cell expression pattern. Secondly, Gal4 may only report a part of the complex expression pattern of the target gene, as is the case in some other enhancer-trap lines (Wilson et al., 1989; Clinton and Bownes, personal communication).

4.3.3 Isolation of cDNAs corresponding to the genomic fragments

In order to obtain the target gene, a D. melanogaster ovarian λZAP cDNA library (a gift from YN Jan, San Francisco) has been screened using the genomic inserts from λF7a and λF2a as probes. After the secondary screening, two positive λZAP phage clones were isolated. These were then excised to Bluescript (SK-) clones following the protocol provided by Stratagene (this technique is also called “plasmid rescue”, refer to section 2.4.4.4). These two Bluescript clones are named cSA41 and cSA42. cSA42 contains a 2.2-kb cDNA insert, whereas cSA41 contains a 1.3-kb insert and lacks one EcoRI site. These two cDNAs do not cross hybridise (data not shown), suggesting they have different origins.
Figure 4.5 Whole-mount RNA in situ hybridisation analysis to verify target gene cloning of the enhancer-trap Gal4 line C865.

A. The reporter gene of C865 is expressed in the anterior follicle cells during stage 7 of oogenesis.

B. Using digoxigenin labelled larger genomic fragments as probes for whole-mount RNA in situ hybridisations, signals are detected in the anterior follicle cells (arrow). This is similar to the reporter gene expression pattern in C865.

C. The Mhc95F cDNA probe also detects signals in the anterior follicle cells, indicating that the Mhc95F is the target gene in C865.

D. Although a part of the yolkless cDNA is coligated with Mhc95F cDNA in cDNA clone cSA42, yolkless is not the target gene. Its RNA is found in the oocyte from stages 2-10, and no expression is found in the follicle cells.
4.3.4 Partial sequencing of the cDNA clones

Both ends of the two cDNA clones have been sequenced using primers T3 and T7. The sequence data was then used to search for homologous genes in EMBL and GENBANK. No homologous sequences have been found to cSA41. It is therefore thought to represent a novel gene. The sequence of one end of cSA42 using the T3 primer shows that it is from the *yolkless* (*yl*) gene. However, the sequence at the other end using the T7 primer shows homology to *Myosin heavy chain at 95F* (*Mhc95F*), which encodes a class VI unconventional myosin. Obviously, cSA42 contains two co-ligated heterogeneous cDNAs. After analysing the sequence data of *yl* and *Mhc95F* obtained from the database, the enzymes *Pst*I and *Msl*II have been chosen to map the cSA42 insert. Physical mapping of the cSA42 insert shows that about 300bp is from *yl*, whereas the rest, about 1.9-kb, is from *Mhc95F* (Figure 4.6A). These two heterogeneous cDNA fragments were separated after digestion with *Pst*I and *Msl*II (see Figure 4.6A).

**Figure 4.6 A.** cDNA clone cSA42 is co-ligated and contains two cDNAs, *yolkless* (*yl*) and *Mhc95F*. It contains around 300-bp of sequence from the *yl* cDNA and around 1.9-kb from the *Mhc95F* cDNA. The co-ligation point of these two cDNAs is close to two *Msl*II sites (*yl* 6,000-bp and *Mhc95F* 1,700-bp) which are shown on the map, while other *Msl*II sites in this cDNA clone are not shown here.

**B.** The restriction map of EM3, a full-length *Mhc95F* cDNA.
4.3.5 Whole-mount *in situ* hybridisation to ovaries with the cDNA probes

The sequence data from *Mhc95F*, *yl* and the novel cDNA, cSA41, are unrelated. To determine which one is the target gene, analysis of their expression patterns during oogenesis was required.

Using Digoxigenin labelled cSA41 to probe the ovary, a signal was only detected in nurse cells (data not shown). Thus, cSA41 is not the target gene of C865.

*yl* transcripts are detected in the oocyte from stage 2 to 10 when using the 300-bp *yl* fragment in cSA42 as the probe (Figure 4.5D). However, no signal is detected in the follicle cells, and it is also not the target gene.

The expression pattern of *Mhc95F* matches that of the larger genomic fragments. From stage 7 to stage 11, it is expressed in anterior follicle cells and border cells. This also matches the original expression pattern of the reporter gene in C865 (Figure 4.5A). In addition, *Mhc95F* expression also occurs in other parts of the egg chambers (for the expression pattern of *Mhc95F*, refer to Figure 5.2, Chapter 5). The staining starts to appear in the germarium. At about stage 9, most of the follicle cells that are undergoing migration to cover the oocyte are stained heavily. During stages 10b to 12, the signal is also detected in the dorsal anterior follicle cells over the oocyte. Nurse cells are also heavily stained at about stage 10. Taken together, these data suggest that the reporter gene in the starting enhancer-trap line C865, only reports part of the expression pattern of *Mhc95F*. A *yl* fragment appears to have been co-ligated with it when the library was constructed.

4.3.6 Mapping the full-length *Mhc95F* cDNA to the genomic fragments

Analysis of the expression pattern suggests that *Mhc95F* is the target gene of the enhancer-trap line C865. This has been further established through mapping of the 4.4-kb full-length *Mhc95F* cDNA (Figure 4.6B; kindly provided by K. Miller) on the larger cloned genomic fragments after Southern hybridisation analysis. It has been shown that the 5' end of *Mhc95F* lies within 2 kb of the 3' end of P[GawB] (Figure 4.7). The first exon, which is likely to be smaller than 500-bp, is followed by a large intron of about 7-kb, and then the rest of the coding sequence.
Figure 4.7 Mapping of the $Mhc95F$ cDNA to cloned genomic fragments.

A. $Mhc95F$ cDNA was labelled and used to probe the Southern membrane which contained restriction enzyme digested DNA from $\lambda$Fix clones (A1). Lane 2,5,8,11,14, SstI digestion; lane 3,6,9,12,15, SstI+EcoRI double digestion; lane 4,7,10,13,16, EcoRI digestion. Hybridisation bands are observed in all five clones (A2).

B. Mapping of the 5' end of the $Mhc95F$ cDNA to the 3' flanking genomic DNA. The 3' rescued plasmid, pPF, and the 5' rescued plasmid, pKF, were digested by restriction enzymes and Southern blotted. Using 1.5-kb of the 5' end of the $Mhc95F$ cDNA fragment as a probe, a 2-kb $XhoI$- BamHI band (lane 3) and a $XhoI$- EcoRI band (lane 2) showed positive hybridisation; no signals were detected in pKF (lane 4 and 5). This indicates that the 5' end of the $Mhc95F$ cDNA is located 3' of P[GawB] and is within 2-kb of the P[GawB] insertion in C865.

C. Mapping of the $Mhc95F$ cDNA onto the restriction map (X=$XhoI$, S=SstI, R= EcoRI).
Target Gene Cloning

A1

\[ \lambda F_{1a}, \lambda F_{1b}, \lambda F_{2a}, \ldots, \lambda F_{8a} \]

Lanes:
1. 1kb ladder
2. pPF (XhoI + EcoRI)
3. pPF (XhoI + BamHI + EcoRI)
4. pKF (EcoRI)
5. pKF (EcoRI + HindIII + KpnI)
6. 1kb ladder

A2

\[ \lambda F_{1a}, \lambda F_{1b}, \lambda F_{2a}, \ldots, \lambda F_{8a} \]

B

1 2 3 4 5 6

C

5' rescue: KF

3' rescue: PF

PGawB

\[ \lambda F_{7a}, \lambda F_{1a}, \lambda F_{8a}, \lambda F_{2a} \]

1kb
4.4 Target gene cloning from C726b

The reporter gene Gal4 in enhancer-trap Gal4 line C726b is expressed in the two patches of posterior follicle cells during oogenesis (Figure 4.9A). A 2-kb genomic fragment adjacent to the 3' end of the P[GawB] insertion site has been recovered in the plasmid, pXB (see section 4.2.2).

4.4.1 Genomic library screening

Using the 2-kb XhoI-BamHI genomic sequence in pXB as a probe, the λFix genomic library was screened for larger genomic fragments. Three positive clones, namely λB1, λB2 and λB6, were isolated after the secondary screen. It has been shown that they are related to each other by physical mapping with SstI, EcoRI, and XhoI (see Figure 4.8A). These genomic fragments cover about 24-kb of the chromosome, extending about 15-kb to the 3' end of the P[GawB] insertion and about 9-kb to the 5' end (Figure 4.8C).

4.4.2 Looking at the expression pattern

Using the mixed λB6 and λB2 clones as a probe, signal can be detected in two patches of follicle cells over the mid-dorsal region of the oocyte during stages 10-11, (Figure 4.9B). This is similar to the Gal4 expression pattern in the starting line C726b (Figure 4.9A); both of them have two big patches of columnar follicle cells which are stained during stages 10-11. This observation suggests that the target gene sequence is likely to be present in these two lambda clones.

4.4.3 cDNA library screening and the analysis of cDNA clones

Using λB6 and λB2 inserts as probes to screen a λZAP ovarian cDNA library, two positive clones were isolated. The two clones, named cB1 and cB2, contain inserts of 2.0-kb and 1.4-kb respectively. The two cDNA fragments are not homologous, as shown by cross hybridisation analysis (data not shown). Partial sequencing of both ends of these two cDNAs, and database searching, has shown that they are both novel sequences.
**Figure 4.8** Restriction Mapping of genomic DNA surrounding the P[GawB] insertion site in C726b. A. DNA made from λFix clones (shown at the top of the gel picture) was digested with restriction enzymes (listed in the text box next to the gel picture) and Southern blotted. B. The southern blot was probed with $^{32}$P labelled 3' flanking genomic DNA, pXB, positive bands were present in clones λB1, λB2 and λB6. C. Shows a diagram of the restriction map of the genomic DNA surrounding the P[GawB] insertion site in C726b. The cDNA clones are also shown in this map after Southern analysis (data not shown).
Using Southern hybridisation, cB2 has been mapped to a 1.4-kb EcoRI fragment, within 2 kb of the 5’ end of the P[GawB] insertion site. cB1 has been mapped to a 2.6-kb XhoI fragment on λB2, and is about 10-kb downstream of the P[GawB] insertion site in C726b (Figure 4.8).

4.4.4 Expression patterns of the cDNAs during oogenesis

When cB1 was used as a probe for in situ hybridisation, signal was detected in the nurse cells (data not shown), which differs from the expression pattern of Gal4 in the starting line C726b, indicating that it is not the target gene.

The expression pattern of cB2 in the follicle cell epithelium is the same as that of the larger genomic fragments. Two groups of lateral-dorsal-anterior follicle cells are stained during stages 10b and 11 (Figure 4.9C). Thus, cB2 is a representative cDNA of the target gene of the enhancer-trap line C726b. The position of these two groups of stained follicle cells appears to shift slightly from the posterior to the anterior as the egg chambers are developed. In addition, signals are detected by both the cDNA and genomic probes in all follicle cells during stage 6 and the follicle cells over the oocyte from stage 7 to 9. This will be discussed in Chapter 6.

4.4.6 Sequence analysis of cB2

Sequence analysis shows that the target cDNA of C726b, cB2, is 1392-bp long (Figure 4.10). This cDNA clone does not appear to have a long open reading frame. A FASTA search in the GENBANK shows no homologous sequence to other recorded data. However, cB2 has been mapped to a well characterised locus called the Broad-Complex (BR-C). The mapping of cB2 into the BR-C locus and the functional analysis of the BR-C during oogenesis will be discussed in Chapter 6.
Figure 4.9 Whole-mount RNA in situ hybridisation analysis to verify target gene cloning of the enhancer-trap Gal4 line C726b. A. Gal4 reporter gene is expressed in two big patches and several random patches of columnar follicle cells during stage 10. B. Using probes made from the λFix clones which contain larger genomic fragments flanking the P[GawB] insertion site, signals are detected in two groups of lateral-dorsal columnar follicle cells at stage 10 (the lateral view shows one group). This expression pattern is similar, although not completely the same as the reporter gene expression pattern in C726b (A). C, Using the cDNA cB2 as a probe, signals are also detected in these lateral-dorsal follicle cells during stage 10.
Figure 4.10 Nucleic acid sequence of the cDNA clone, cB2.

1  gaattcgacg  cgagaccttg  aaccttggaact  cagtttacga  gttgaagaat
51  tgggcttttg  ttttggcttg  aatggctatc  tctctctccc  tctgccgcgtg
101  ctaatttcgct  ctgacacctc  ctctgggtgc  tctgctcgtt  ccatgtgctc
151  ctcctccaaac  cgttaagctgc  ttacaattag  cgtaataaggc  gaattaacac
201  ccgttaaatca  gatgccccac  agggccactg  gaataggtac  cgatgtacgt
251  gtgtgtgtttt  gagtgtgcgg  cagtgcaaat  ggttaatgtaa  atgaaatgcc
301  ccgtttgtgta  gttggcgagatg  gattttggttt  gttgggtgac  tgggtgtgtggt
351  cccctctgcce  tttggtgtctt  agcatttgcct  tggccgaca  cagacacaca
401  cacgcgcaca  cagatgcaact  ctgcaattgg  ccgttaatca  gatgccccac
451  ttgccaccgc  caacctcttt  tctctctccg  ctatggcttag  tttttgcaca
501  gaacttttgc  ttttaccact  ctttctgtttg  ctatggttgg  gccgttaaat
551  tttacatttg  gccgccccac  agggccactg  gttgtgctgg  cagtgcaaat
601  ggggcatttcg  cagtgctggtc  gatagcccat  gtagaatgtg  gatgtggggg
651  ccacggcgtc  tgcgtctagtt  cggccacaag  tggaaaggga  atgtggtcag
701  aggtgggaaa  gtggatgtggt  actgaccctg  tgggaatggt  tggtaagctgt
751  ccteacacaa  ttcgtgatacg  ctcctctctt  tgggaatggt  gatgtggggtt
801  aatagaaaga  gaagaagcgg  ccagataagc  taagacacga  cagagagac
851  ggttggtttgg  ggggcggacg  gaaaaacgac  tcggcaaatc  gtcaaatgtg
901  cctgaaaata  gaaccacaca  caattggcat  acctgagac  acctgagac
951  gcacaaagggc  gatgggcagag  gagaacacaa  gcaaaagtcc  ggacacacac
1001  aatagaaaga  gaagaagcgg  ccagataagc  taagacacga  cagagagac
1051  tgggggggggg  gggggggggg  cgaggacggg  ccacagacggg  ccacagacggg
1101  gtgcagcaga  tatggcacac  caaatctgatc  cggccgatac  accatcagac
1151  gcacacatac  atatgtgatg  atgtatactat  acacatcata  tttcgccatt
1201  cgcaaatatat  gtacaattta  gtttccaatc  gcacacatac  ctaacctggg
1251  cttgctctattc  taatggcagtt  ccgccccgct  ctttctccct  tttataagct
1301  acagcgcagt  cagcccaata  atgtgttttt  tccaacaaaa  cacacacaca
1351  ctcgattttg  gggccgagccg  aaaaaacgac  tgcaacgaaat  tc
Figure 4.12 Whole-mount RNA *in situ* hybridisation assay to verify target gene cloning of the enhancer-trap *Gal4* line C826. A. The reporter gene is expressed in the nurse cell associated follicle cells (NCFC) during stage 10. B. The λFix clones (λC4 and λC7) contain sequences from the target gene, which shows the similar expression pattern in the NCFC during stage 10. C. Strong signals are detected in the nurse cells when using the cDNA probe to hybridise the whole-mount ovary. However, the staining is not clear in the NCFC.
4.5 Cloning the target gene of C826

The reporter gene Gal4, of enhancer-trap line C826 is expressed in the stretched follicle cells and the centripetal cells during stage 10 of oogenesis (Figure 4.4E, and 4.13A). The rescued plasmid, pXC, contains a 1.1-kb genomic fragment which flanks the P[GawB] insertion site.

4.5.1 Genomic library screening

Two positive clones, namely λC4 and λC7, have been isolated by using the pXC insert as a probe to screen a λFix genomic library. It seems that these two genomic clones cover about 20 kb of genomic fragment, as shown by mapping with SstI, XhoI and EcoRI (Figure 4.12).

![Restriction map of C826 group λFix clones. It is unclear at which end of the 3’ rescue the P[GawB] insertion site is located (X=XhoI, S=SstI, R=EcoRI).](image)

Figure 4.11: Restriction map of C826 group λFix clones. It is unclear at which end of the 3' rescue the P[GawB] insertion site is located (X=XhoI, S=SstI, R=EcoRI).

4.5.2 Looking at the expression pattern

In order to determine whether the cloned genomic fragments contain the target gene sequence, λC4 and λC7 were mixed and labelled with Digoxigenin-11-dUTP and used as probes for in situ hybridisations on whole-mount ovaries. Signal was detected the nurse cell associated follicle cells during stage 10. This is similar to the expression pattern of the reporter gene in C826 (Figure 4.12A), suggesting that the target gene sequence is included in the larger genomic fragments. In addition to the staining in follicle cells, the signal is also detected in nurse cells.
4.5.3 cDNA isolation and mapping

Four separate positive cDNA clones have been identified after screening a λZAP ovarian cDNA library and Southern hybridisation using mixed λC4 and λC7 inserts as a probe. These five clones, namely cC2a, cC3, cC6, cC7 and cC8, can be divided into four groups (Figure 4.13). cC3 and cC8 gave rise to the first group, which contains a 0.9-kb insert. Group two, which has a sole member, cC6, contains a 1.7-kb fragment. The third cDNA, cC2a, contains two EcoRI fragments of 0.9-kb and 1.6-kb respectively. The 1.6-kb fragment does not hybridise to the genomic DNA probe, and could, therefore, have been co-ligated with the 0.9-kb fragment when the library was constructed. The fourth cDNA clone, cC7, appears to have lost an EcoRI site. It was shown on the gel as a single band which was larger than the 2.96-kb Bluescript fragment (arrows in Figure 4.13). The cC8 insert was labelled to study cross hybridisation between the other cDNA fragments, and it has been shown that the 0.9-kb EcoRI fragments in cC2a, cC3 and cC8 are homologous. The 0.9-kb EcoRI fragment is also homologous to the 1.7-kb insert in cC6 and the cC7 fragment (data not shown). So these cDNA fragments are derived from the same gene.

4.5.4 Expression pattern of cC8 during oogenesis

In order to determine whether the cloned cDNAs are from the target gene in C826, cC8 was Digoxigenin labelled to study its expression pattern during oogenesis. Since strong staining is seen in the nurse cells, it is unclear if the nurse cell associated follicle cells are stained. Using cC8 as the probe, the signal detected in nurse cells seems to be higher when compared to signals produced by the λC4/λC7 probe (Figure 4.12C). As the expression pattern of cC8 does not completely match that of the genomic fragment, it is still unclear whether the target gene of C826 has been cloned.

Partial sequencing of both ends of the cC8 cDNA fragment and GENBANK searching, has shown that the cloned cDNA is from a novel gene (data not shown). Since this cDNA does not seem to be expressed in an interesting pattern in follicle cells, no further efforts have been made to characterise its function.
Figure 4.13 Southern analysis of cDNA clones screened by the probe λC4+ λC7. All cDNA clones were digested with EcoRI. It was found that cC2, cC4, cC6, cC7 and cC8 are real positive clones and can be divided into four groups (for details see text).

4.6 Discussion

It was shown in this chapter that genomic fragments 3' flanking the P[GawB] insertion have been recovered in nine selected Gal4 enhancer-trap lines. Of these, larger genomic fragments surrounding the P[GawB] insertion site have been obtained by screening a genomic library in four chosen lines. These lines show reporter gene
expression in different subsets of follicle cells around stage 10. This includes the anterior and border cells (C865), the nurse cell associated follicle cells (C826), the posterior follicle cells (C844), and patches of the columnar cells (C726b). Furthermore, cDNAs corresponding to these larger genomic fragments have been cloned in three of these four lines (C865, C826 and C726b). Finally, target genes of two lines have been identified (C865 and C726b).

In order to study follicle cell behaviour and function during oogenesis, it is more important to analyse the function of several representative genes in detail, rather than adding more and more genes to the list that show expression in the follicle cells. On the other hand, time does not allow the cloning and analysis of the function of the target genes in all nine lines. Thus, efforts have been put into functional characterisation of two target genes, the Mhc95F (Chapter 5) and the Broad-Complex (Chapter 6). Cloning of the target genes in seven other lines had to be stopped at a certain stage. Nevertheless, the flanking DNA and larger genomic DNA fragments provide useful tools for future cloning if time allows. For example, although it is unknown whether or not the target cDNA sequence has been cloned in C826, it is known that the larger genomic fragments, λC4 and λC7 include at least a part of the target gene, which shows expression in the nurse cell associated follicle cells during stage 10. Thus, λC4 and λC7 provide a good starting point in identification of this target gene. It will be helpful to DIGoxigenin label λC4 and λC7, for use as probes for in situ hybridisations on whole-mount ovaries. This could localise the target gene on either λC4 or λC7, since they appear to overlap only at a small region. The lambda clone that shows expression in the nurse cell associated follicle cells can then be used to screen a cDNA library for cloning of the target gene.

3' flanking genomic fragments have been recovered in all of the nine Gal4 enhancer-trap lines. Among these, three lines have been rescued by two enzymes, while the other six lines have been rescued by one enzyme. Thus, there are twelve 3' rescued plasmids altogether. Of these, only two have inserts larger than 2-kb. In contrast, six of them have inserts smaller than 1-kb, indicating that smaller fragments may be easier to be rescued. This could explain why the 5' adjacent DNA fragment was only recovered in one line by plasmid rescue, since the 5' rescued plasmid will contain the entire 11-kb pGawB sequences, and is much larger than the 3' rescued plasmid, which only contains the 2.9-kb Bluescript sequences from pGawB. However, the sole successful 5' rescue pKF has a large insert of 7.1-kb, so the entire rescued plasmid is around 18-kb, suggesting that plasmid rescue of large fragments is not impossible. Additionally, the
fact that KpnI is the only enzyme that can be employed for 5' rescue also prevents the 5' adjacent genomic DNA from being efficiently rescued in every enhancer-trap line.

In this work, whole-mount in situ hybridisation to RNA was used to determine if the target gene sequence is contained in the plasmid rescued genomic fragments and larger genomic fragments obtained from genomic library screening. Using this straightforward method, it was shown that the target gene sequences of C865, C726b and C826 are present in the larger genomic fragments. This method also led to the identification of the target genes in C865 and C726b. Nevertheless, in certain circumstances, some subtle expression patterns were missed. For example, the first exon of the C865 target gene Mhc95F is actually present in the 3' rescued genomic fragment. However, the expression pattern of this exon in the follicle cells was missed in the preliminary in situ hybridisation assay when using the rescued 7.5-kb genomic fragment as a probe. Two possibilities could be used to explain this. Firstly, when compared to the entire 7.5-kb rescued genomic fragment, the first exon of the Mhc95F gene is very small. Secondly, strong staining was visible in the nurse cells, and this obscured staining in the follicle cells. This observation also suggests that one must be careful when using the in situ hybridisation technique to verify if a cDNA clone is a potential target gene of an enhancer-trap line, particularly of the Gal4 lines where germline cells never show reporter gene expression.

The reporter gene expression pattern in the anterior follicle cells and the border cells in C865 partly matches the complex expression pattern of the target gene Mhc95F, indicating that different cis-elements may be required for the regulation of expression in different follicle cell groups. The rescued genomic fragments may contain these cis-elements. If so, they may be useful for identification of cis-elements that are required for establishing a specific expression pattern in the anterior follicle cells and the border cells. These cis-elements can then be used to explore trans-elements that are involved in the establishment of the expression pattern in these cells.

It is noticed that the patches of follicle cells showing Gal4 expression in C726b are more posterior than the follicle cells stained by in situ hybridisation using the cB2 probe. Since cB2 is expressed in the follicle cell epithelium in a very dynamic pattern, it is necessary to switch on and off gene expression quickly, and remove the transcripts rapidly. The reporter gene product β-galactosidase, however, may not be cleared as quickly as the transcripts; it may therefore interfere with the genuineness of the report of the endogenous gene expression pattern.
CHAPTER 5

Functional Characterisation of

Myosin heavy chain at 95F

(Mhc95F)
5.1 Introduction: myosins and the Drosophila members

Myosins (myosin heavy chains) are a large family of structurally diverse mechanoenzymes that, upon interaction with actin filaments, convert energy from ATP into mechanical force. They contain a conserved N-terminal head (motor) domain of around 80-kDa, a neck (regulatory) domain for light chain binding and a class-specific tail domain. Previously, the myosins had been divided into two groups, myosin-Is and myosin-IIs, based upon whether they form monomers or dimers (Cheney and Mooseker, 1992). Myosin-IIs are double-headed and filament-forming myosins involved in cytoplasmic contractile phenomena in muscle and non-muscle cells. They have been intensively studied because of their role in muscle contraction. Myosin-Is, on the other hand, were firstly identified as monomeric and membrane-associated myosins from Acanthamoeba. More recently, studies on these molecular motors have uncovered the existence of a large, evolutionary conserved superfamily. In addition to the conventional myosin-IIs, more than ten additional classes of unconventional myosins have been identified (for review see Mooseker and Cheney, 1995; Titus, 1997).

The head domains are conserved among all myosins. However, there are still numerous critical class-specific features which are important for their functional diversity. Based upon the primary amino acid sequence divergence within the head domain, the myosin superfamily is divided into eleven evolutionary conserved classes, termed myosin-I to -XI (see Figure 5.1A). The neck domains of all known myosin heavy chains consist of a variable number of putative light-chain binding motifs, termed the IQ motifs. The sizes of the neck domains vary from 1-6 IQ repeats in different myosins. The IQ motif is an amino sequence of approximately 23 basic and hydrophobic amino acids whose most conserved residues usually conform to the consensus IQxxxRGxxxRK (Cheney and Mooseker 1992). The structures of the tail domains, on the other hand, are conserved within each class but distinct from other classes. One feature shared by the tail domains of myosins from several different classes, including myosin-VI, is the presence of regions predicted to form segments of coiled-coil α-helix, suggesting that these myosins form dimers. Another property attributed to the tail domains of several myosins is the ability to interact with membranes.
Figure 5.1 A, An unrooted phylogenetic tree of the myosin superfamily showing the division of the myosins into 11 classes. Each class is indicated by a Roman numeral and illustrated by a hypothetical model based on sequence predictions (adapted from Mooseker and Cheney, 1995). B, A schematic diagram of myosin 95F. It contains ATP and actin binding motifs (open boxes) in the head.
5.1.1 Conventional myosin-II

Of the myosin superfamily, conventional myosin-IIIs were the first discovered and have been most extensively studied. All members of this class contain an $\alpha$-helical tail that enables them to form double-headed dimers and assemble into bipolar anti-parallel filaments. They bind filamentous actin to produce chemomechanical force. Their function is regulated and modified by a pair of myosin light chains.

In *Drosophila*, all muscle myosin isoforms appear to be alternatively spliced from the transcripts encoded by a single-copy gene at the chromosomal locus 36B. Two classes of flightless mutations have been mapped to this locus (Bernstein *et al.*, 1983; Rozek & Davidson, 1986). In addition, non-muscle (or cytoplasmic) myosin-II is encoded by the *zipper* gene (Kiehart *et al.*, 1989; Young *et al.*, 1993). Homozygous *zipper* mutant embryos exhibit defects in cytokinesis during dorsal closure (Young *et al.*, 1993). The proposal that non-muscle myosin-II is required for cytokinesis in dividing cells is supported by the fact that a mutation which blocks cytokinesis is mapped to *spaghetti-squash* (*sqh*), a gene encoding the non-muscle myosin regulatory light chain (RLC) (Karess *et al.*, 1991; Edwards *et al.*, 1995). Beyond its function in cytokinesis, non-muscle myosin-II plays an essential role in cell shape change and cell sheet movement, as shown by analysis of homozygous *zipper* mutant embryos, which have aberrant cell shapes during dorsal closure (Young *et al.*, 1993). Furthermore, by using strong and weak alleles of *sqh*, non-muscle myosin-II is found to have multiple essential roles in imaginal disc and egg chamber morphogenesis. When the RLC is transiently depleted in larvae the resulting adults show malformed wings and legs, demonstrating that non-muscle myosin-II is required for proper development of eye and leg imaginal discs (Edwards & Kiehart, 1996). During oogenesis, non-muscle myosin-II is shown to be involved in morphogenesis of the interfollicular stalks, follicle cell migrations (including border cell migration, centripetal migration and dorsal anterior cell migration), as well as in dumping of nurse cell cytoplasm into the oocyte (Edwards & Kiehart, 1996; Wheatley *et al.*, 1995; also refer to Chapter 1).

5.1.2 Unconventional myosins

5.1.2.1 Myosin-I

The single-headed, short-tailed myosin-IIs constitute the most extensively studied unconventional myosin class. Four subgroups have been further divided by primary
sequence comparison. Subclass one (amoeboid-type myosin-Is) consists of more than a
dozen members, primarily from amoeboid organisms *Acanthamoeba* and *Dictyostelium.*
It is suggested that amoeboid myosin-Is participate in a wide range of actin-based motile
phenomena including pseudopod and lamellapodial extension, phagocytosis and
chemotaxis (Cheney and Mooseker, 1992). Subclass two, on the other hand, constitutes
several vertebrate myosin-Is. The *Drosophila* myosin-IB (MIB) and myosin-IA (MIA)
belong to subclasses three and four, respectively. During embryogenesis, the presence
of both proteins correlates with the formation of a brush border within the alimentary
canal. It has been shown that MIA and MIB have distinct but overlapping subcellular
distribution in larval and adult gut. Additionally, MIB is found in egg chambers at both
the basolateral and apical surfaces of the somatic follicle cells during oogenesis. Since
no mutations have been reported in these two myosin genes, their function during
development remains unclear (Morgan *et al.*, 1995; Morgan, 1995).

5.1.2.2 Class III and IV myosins

*Drosophila* NinaC myosins are the sole members of the class III myosin family
that have been identified (Montell and Rubin, 1988). The *ninaC* gene encodes two
unconventional myosins, p174 and p132, which result from alternative RNA splicing.
These myosins are notable for the fact that they both contain a putative kinase
domain which is to the N-terminal side of the head domain. Three aspects of the null
phenotype of *ninaC* have been identified. These include phototransduction, proper
rhabdomere targeting, and the maintenance of retinal structure. The kinase domain
seems only to be involved in phototransduction, while the head domain seems to be
involved in all three functions (Porter *et al.*, 1992; Porter and Montell, 1993).

Class IV only contains the high-molecular weight “myosin-I” from
*Acanthamoeba.* Although termed myosin-I based on its presumed single headedness, it
is distinct from the class I myosins in its head and tail structures.

5.1.2.3 Myosin-V

Class V myosins are the second best characterised unconventional myosins after
myosin-Is. In the yeast *Saccaromyces cerevisiae*, two myosin-Vs have been identified.
The *MYO2* myosin functions as a molecular motor to transport secretory vesicles along
actin cables to the site of bud development. The second yeast myosin-V gene, *MYO4,*
has been found to be among the five genes that are required for mother-specific
expression of *HO*, a gene only transcribed in mother cells in *S. cerevisiae* (Jansen *et al.*, 1996). *MYO4* is specifically accumulated in buds, which relates to its role in switching
of the mating type. The *Drosophila* myosin-V has been cloned in our group (Maclver, McCormick & Bownes, submitted). During oogenesis, its transcripts are selectively transferred into the oocyte from nurse cells, and become localised at the anterior part of the oocyte before stage 10. This transfer and localisation of the transcripts is disrupted in a number of mutants that affect the localisation of some well-known maternal determinants. It is hypothesised that *Drosophila* myosin-V may play a similar role to its yeast homologue in selective transportation of maternal gene products. The mouse myosin-V is also known encoded by the *dilute* gene and a second *Myo V* gene in the mouse has been cloned in our lab which is closely related to the new *Drosophila* myosin-V (Maclver, Wilkie, Loke and Bownes, personal communication).

5.1.2.4 Class VII-XI myosins

The first member of myosin-VIIIs, *Drosophila* myosin 35BC, was isolated using a PCR-based screen. The molecular characterisation of this class is still in a nascent state. It has been found that the myosin-VII genes are targets for shaker-1, a recessive locus causing deafness in mice, and human Usher syndrome type 1B, an inherited disease characterised by congenital deafness (Weil et al., 1996). The precise function of this class of myosins is still unknown. Genetic analysis of the *Drosophila* 35BC myosin may provide critical insights.

Class VIII and class XI are plant myosins which were discovered in *Arabidopsis*; class IX are myosins that have RHO gap domains; Class X myosins are thought to have a PH domain (Mooseker and Cheney, 1995).

5.1.2.5 *Drosophila* myosin 95F and Class VI myosins

The *Drosophila* unconventional myosin 95F is encoded by *Myosin heavy chain at 95F* (gene and mRNA: *Mhc95F*; protein: myosin 95F), which is a single copy gene located at polytene chromosomal position 95F. It was first detected as a 140kD actin binding protein by Miller et al. (1991) with their 3C7 monoclonal antibody. The gene was then cloned by using the antibody to screen an ovarian cDNA expression library (Kellerman and Miller, 1992). *Mhc95F* transcripts are differentially spliced within the tail domain at a site immediately C-terminal to the coiled-coil domain, and it is suggested that this probably leads to the production of four protein isoforms (Kellerman and Miller, 1992).
Myosin 95F is the founding member of class VI myosins. Some vertebrate homologues have been identified, including the pig myosin-VI (Hasson and Mooseker, 1994). The head domains of *Drosophila* Myosin 95F and pig myosin-VI are 56% identical, and share some notable structural features when compared with other myosin classes. For example, they both have a ~50aa insert just N-terminal to the neck domain, which is distinct from the neck-head junction of any other known myosin. This domain is thought to be a novel light-chain-binding site. In addition to the head domains, similarities between these two class VI myosins are also found in the neck and tail domains. The neck domains of both myosins contain a single IQ motif, which is probably the binding site for the calmodulin light chain. The tail domains, on the other hand, consist of a coiled-coil domain followed by a globular domain, which is very similar in both the pig and fly myosins (64% identical). It is significant that -COOH tails of these two myosin-VIs are more similar than their head domains (Figure 5.1B; for reviews see Mooseker and Cheney, 1995; Miller and Kiehart, 1995).

Expression of *Mhc95F* during development is complex. Transcripts and protein are present throughout the life cycle, with variable levels found in different tissues and development stages. During embryogenesis, this myosin is associated with particles throughout the cytoplasm from the blastoderm onwards. High levels of protein are expressed in the CNS and PNS and in the ventral lip of the stomadeum. In addition, high levels of protein are also present in the posterior spiracles and in the dorsal-lateral epidermal cells undergoing dorsal closure (Kellerman and Miller, 1992; Kellerman *et al.*, 1992). Expression of Myosin 95F in cells undergoing dorsal closure is similar to that of the non-muscle myosin-II. This may suggest a similar role played by both myosin-II and myosin-VI in epidermal cell sheet movement.

Time lapse computational optical sectioning microscopy has shown that Myosin 95F is involved in intracellular transport of cytoplasmic particles, which are distributed throughout the cytoplasm surrounding the interphase nuclei (Mermall *et al.*, 1994, Mermall & Miller, 1995). During mitosis, these particles move to the invaginating actin-rich "pseudo"-cleavage furrows that form between adjacent mitotic spindles (Mermall & Miller, 1995). Disruption of this particle movement by injecting a Myosin 95F specific antibody results in abnormal furrow formation and, as a result, improper segregation of adjacent spindles. Effects of antibody inhibition on the function of Myosin 95F are quite similar to those caused by the disruption of the actin-based cytoskeleton (Mermall & Miller, 1995). These studies demonstrate that Myosin 95F is involved in the cell-cycle-dependent movement of organelles, and in the assembly of a cleavage furrow-like array.
Although antibody injection allowed part of the *Mhc95F* function in early embryogenesis to be studied, it was not sufficient to identify its role throughout development. Other strategies are, therefore, required for complete functional characterisation. Since *Mhc95F* has been characterised as the target gene of a *Gal4* enhancer-trap line (see Chapter 4), P-element mediated mutagenesis can be applied to create mutations. In addition, the *Gal4-UAS* dual system can be used in order to activate or silence *Mhc95F* expression in a tissue-specific way. In this chapter, the expressional and functional analysis of *Mhc95F* during oogenesis and other developmental stages will be discussed.

### 5.2 Expressional analysis of *Mhc95F* during oogenesis

#### 5.2.1 *Mhc95F* is expressed in migrating follicle cells during oogenesis

The distribution pattern of the *Mhc95F* transcripts during oogenesis was studied by whole-mount *in situ* hybridisation. It is shown in Figure 5.2A that the transcripts are present in the anterior follicle cells prior to their delamination. At stage 9-10, expression is also detected in border cells during and after their migration through the nurse cell cluster (Figure 5.2C). Expression in these two groups of follicle cells is similar to that of the reporter gene in the starting *Gal4* line, C865. Additionally, its expression is also found in other follicle cells that undergo morphogenetic movement during the middle and late stages of oogenesis. These include the follicle cells that retract from the nurse cell cluster to cover the oocyte at stage 9 (Figure 5.2B), the centripetal follicle cells at stage 10b (Figure 5.2D), and the dorsal-anterior follicle cells during stages 10b to 12 (Figure 5.2E,F). The expression domain in dorsal-anterior follicle cells shifts rapidly. Initially it appears as two curved lines over the stage-10b oocyte (Figure 5.2E); this changes to a two-circle pattern at stage 12 (Figure 5.2F). The two to three rows of dorsal-anterior follicle cells stained during stage 10b are presumably at the leading edge of the follicle cell epithelium that undergo dorsal-anterior migration (Figure 5.2E). In summary, all types of follicle cell migration during stages 9-12 exhibit *Mhc95F* expression. This observation, along with the report that strong expression of myosin 95F is detected in the three of the four rows of dorsal-lateral epidermal cells which were at the leading edge of the epithelial sheet involved in dorsal closure (Kellerman and
Miller, 1992), indicates that myosin 95F may be widely involved in morphogenetic movements of epithelial sheets.

In addition to the migrating follicle cells, \textit{Mhc95F} expression is also detected in a small group of posterior follicle cells at stage 10b (Figure 5.2C,D). The function related to expression in these follicle cells is unclear. Strong expression of \textit{Mhc95F} is also present in the germarium region 2 (Figure 5.2A) and the nurse cells during stages 9 to 13 (Figure 5.2B-F). This may reflect a role played by myosin 95F in nurse cell cytoplasm dumping, similar to non-muscle myosin-II.

5.2.2 \textit{Mhc95F} expression in the dorsal-anterior follicle cells is downstream of the Grk-DER signalling pathway

It is shown in Figure 5.2E,F that \textit{Mhc95F} is expressed in the dorsal-anterior follicle cells, the fate of which induced by the Grk-DER signalling pathway (refer to Chapter 1). To determine if dorsal-anterior expression is induced by Grk-DER signalling, \textit{Mhc95F} expression was examined in the \textit{fs(1)K10}\textsuperscript{o} egg chambers, in which the Grk signal is mis-localised to form an anterior ring in the oocyte during stage 8-10. It was found that the \textit{Mhc95F} transcripts are present in a ring of anterior columnar cells during stage 10B in these egg chambers (Figure 5.3B), indicating that \textit{Mhc95F} expression in the dorsal-anterior follicle cells is indeed induced by the Grk-DER signalling pathway. In contrast, its expression in the anterior follicle cells at stage 9 is not affected in the \textit{fs(1)K10}\textsuperscript{o} mutant (Figure 5.3A).
**Figure 5.2** The expression pattern of *Mhc95F* during oogenesis. **A.** *Mhc95F* is expressed in the germarium (arrowhead). Expression is also observed in the anterior follicle cells during stages 7-8 (arrow). **B.** During stage 9, the follicle cells that retract from the nurse cell cluster to cover the oocyte appear to be stained. **C.** *Mhc95F* expression is also found in the border cells (arrow) and the posterior follicle cells (arrowheads in C and D) during stage 10. **D.** The centripetally migrating follicle cells (brackets) are stained. In addition, expression of *Mhc95F* is observed in the dorsal anterior follicle cells. During stages 10b-11, 2-3 rows of lateral-dorsal-anterior follicle cells are stained (E, arrow). During stages 12-13, more dorsal-anterior follicle cells are stained (F, arrow).

**Figure 5.3** Expression of Mhc95F in the dorsal-anterior follicle cells is dependent on the Grk/DER signalling pathway. **A.** Early expression of *Mhc95F* in the anterior follicle cells (arrow) is not disrupted in the *fs(1)K10* mutant egg chamber. However, the localised expression in the dorsal-anterior region is expanded (arrows) in the *fs(1)K10* mutant during late stages (B).
5.3 Screen for mutations in the *Mhc95F* gene

5.3.1 Looking for existing insertional mutations in *Mhc95F*

Expressional analysis has suggested that *Mhc95F* may be involved in follicle cell migration. This, however, had to be confirmed by examination of the "mutant" phenotype. A relatively simple approach which has been used to obtain mutations in a gene locus is to search for existing mutations that have been mapped in the same chromosomal region. To apply this approach for screening mutations in the *Mhc95F* gene, 13 *P-lacZ* insertional mutants were chosen which fail to complement a deficiency stock, *Df(3R)crbS87-5*, which covers 95F7-96A17-18. These *P-lacZ* lines, which were screened from about 2,400 third chromosomal homozygous lethal mutants, were divided into nine complementation groups (P. Deák, personal communication, Table 5.1).

To determine if any of the nine complementation groups had a *P-lacZ* insertion in the *Mhc95F* gene, Southern hybridisation analysis was applied. Genomic DNA from the 13 representative lines was digested by *EcoRI* (*EcoRI* digested genomic DNA was applied to all of the following Southern blots in Section 5.3 and 5.4) and blotted. The probes used for Southern hybridisation in this experiment and the following experiments are shown in Figure 5.4A. These probes include the 4.4-kb full-length *Mhc95F* cDNA probe (hereafter called the cDNA probe), a 2.0-kb and a 3.9-kb probe made from genomic fragments which are close to the 3' end of the *P* insertion site in the starting line C865 (hereafter called XF2.0 and PF3.9 respectively), and a 1.7-kb probe made from the 5' flanking genomic fragment (hereafter called KF1.7 probe). In the wildtype, the cDNA probe yielded two hybridisation bands, one is around 14-kb, which contains the major part (the 3' part) of the *Mhc95F* cDNA, the other is around 8-kb, which includes the first exon of the *Mhc95F* gene (Figure 5.4B). Since this 22-kb genomic region detected by the cDNA probe covers the whole coding region of *Mhc95F*, as well as the 5' and 3' regulatory sequence, it is predicted that a *P* element insertion in this gene will be localised in this 22-kb region. The insertion can be shown as a band shift on the Southern blots when compared to the wildtype. Indeed, in the *Gal4* line C865, the 8-kb *EcoRI* fragment detected by the cDNA probe was enlarged to around 14-kb, due to the insertion of *P[GawB]* in the 8-kb *EcoRI* fragment. For this reason, homozygous C865 showed only one 14-kb hybridisation band on the Southern membrane (Figure 5.4B). The PF3.9 probe, on the other hand, yielded the 8-kb band in
the wildtype and a 14-kb band in C865 (Figure 5.4C). As shown in Figure 5.4D, probe KF1.7 produced a 4.7-kb hybridisation band in C865 and an 8-kb band in the wildtype.

**Figure 5.4** Genomic structure of the $Mhc95F$ genomic region in the wildtype (A1) and in the starting line C865 (A2). The probes used in this work are shown by plain lines. *EcoRI* sites are marked with open triangles.

B, C, D. Southern hybridisation analysis of the $Mhc95F$ genomic region in the wildtype (OrR) and the starting line C865. B, C and D are the same membrane which has been hybridised with different probes.

B. The $Mhc95F$ cDNA probe detects two bands (8-kb and 14-kb) in the wildtype and one band (14kb) in C865.

C. Probe XF2.0 detects an 8kb band in the wildtype and a 14kb band in C865.

D. KF1.7 detects an 8kb band in the wildtype and a 4.7-kb band in C865.
Table 5.1: Complementation groups covered by Df(3R)crbS87-5

<table>
<thead>
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<th>Group</th>
<th>Representative lines</th>
<th>Chromosomal position</th>
<th>Other information</th>
</tr>
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<td>95F10-15</td>
<td>Lethal phase</td>
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<td>95F and 90D, two insertions</td>
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<td>95F</td>
<td>PA-A</td>
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<tr>
<td></td>
<td>258/7</td>
<td>85A and 95F, two insertions</td>
<td>Embryo</td>
</tr>
<tr>
<td></td>
<td>509/11</td>
<td>95F</td>
<td>Embryo</td>
</tr>
<tr>
<td></td>
<td>581/4</td>
<td>95F</td>
<td>Embryo</td>
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<td>95F</td>
<td>A</td>
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<td>96A8-14, DTS-3 allele</td>
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<td>A</td>
</tr>
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<td>417/6</td>
<td>96A3-16</td>
<td>Embryo</td>
</tr>
<tr>
<td>8</td>
<td>677/12</td>
<td>second site at 95F-96A</td>
<td>P</td>
</tr>
<tr>
<td>9</td>
<td>87/2</td>
<td>68F, second site at 95F-96A</td>
<td>P-pA</td>
</tr>
</tbody>
</table>

1. Based on P. Deák (personal communication).
2. L1= 1st larval instar; L2= 2nd larval instar; P= pupae; pA= pharate adult; A= adult.

Both the cDNA and XF2.0 probes were used to screen the lines listed in Table 5.1. However, none of them showed a shift of the hybridisation-band (Figure 5.5 and data not shown), which indicates that none of the P-lacZ lines examined have insertion in the 22-kb Mhc95F genomic region. Thus, it is unlikely that the Mhc95F gene is mutated in any of these lines. However, this experiment does not rule out the possibility that the Mhc95F gene locus is larger than the 22kb region that the probe recognised. Although the coding region is included, there may be regulatory sequence outside of this 22-kb region. Insertions in the outside region are not detected by these two probes.
Figure 5.5 Screen of P-lacZ insertional mutations in the Mhc95F gene. A, B and C are the same membrane which was hybridised with different probes. A. The Mhc95F cDNA probe. B. Probe XF2.0. C. Probe KF1.7. D. The cDNA probe. Similar hybridisation bands to the wildtype are found in most of the P-lacZ lines, suggesting that no P-elements are inserted in the detected 22-kb region of the Mhc95F gene in these lines. Larger bands appeared in lines 104/9, 1426/3 and 87/2 (D) were probably due to the poor quality of DNA made in these lines (E). Southern analysis was repeated in these lines and has shown similar hybridisation bands to the wildtype chromosome.
5.3.2 Genetic scheme for screening deletion mutations in the \textit{Mhc95F} gene

In addition to searching for existing mutations, we also attempted to create new mutations in the \textit{Mhc95F} gene using the P-element mediated mutagenesis technique. Since the \textit{P[GawB]} insertion site is close to the first exon of the \textit{Mhc95F} gene in the starting enhancer-trap line C865 (Figure 5.4A and refer to Chapter 4), it is possible to generate deletion or insertional mutations in the \textit{Mhc95F} locus by mobilising \textit{P[GawB]} in the C865 genome.

Figure 5.6 shows the crossing scheme used in order to generate imprecise excisions by mobilising the \textit{P[GawB]} (\textit{P[w\textsuperscript{+}, Gal4]}) in the C865 genome. The line C865 was crossed with a transposase-providing line that carried \textit{P[\Delta2-3]}, a truncated P-element which is not itself mobile, but can provide transposase to mobilise other P-elements in the genome. (Cooley \textit{et al.}, 1988; Robertson \textit{et al.}, 1988). Among the F\textit{1} progeny, virgin female or male flies were selected that had both \textit{w\textsuperscript{+}} (red-eye) and \textit{Drop} (\textit{Dr}), which marks the \textit{P[\Delta2-3]} chromosome. These flies were then crossed with the balancer strain, \textit{w; TM3, Sb/TM6B, Tb}, to balance the third chromosome. Excision of \textit{P[w\textsuperscript{+}, Gal4]} should have occurred in some of the germline cells in the F\textit{1} flies, which would lead to some of the F\textit{2} progeny being \textit{w\textsuperscript{−}} (white-eye) over either TM3 or TM6B. These w\textsuperscript{−} F\textit{2} progeny were then established as strains by crossing each single fly with the third chromosomal balancer, \textit{w; TM3, Sb/TM6B, Tb}. By the F\textit{3} generation, flies were ready for screening for deletions in the \textit{Mhc95F} gene by Southern hybridisation analysis.

It was also possible to examine homozygous viability in the F\textit{4} generation. If none of the F\textit{4} progeny in an established fly line appeared to be either \textit{Sb\textsuperscript{−}} or \textit{Tb\textsuperscript{−}}, a lethal event might have occurred on chromosome 3. If this was not the case, the fly lines examined would be homozygous viable, which presents two possibilities. Firstly, the excision of \textit{P[w\textsuperscript{+}, Gal4]} might not lead to a mutation or, secondly, any mutations present might be zygotically non-lethal. These crosses, therefore, needed to be checked further for fertility and other possible phenotypes.
5.3.3 Screening deletions in the *Mhc95F* locus by Southern hybridisation

187 white-eyed (w') fly lines were set up. Of these, 105 lines were examined by Southern analysis. The probes used in this experiment were XF2.0, containing the 2.0-kb 3' flanking genomic fragment to the *P[w', Gal4]* insertion in C865, and KF1.7, including the 1.7-kb 5' flanking genomic sequence (see Figure 5.4). The XF2.0 probe yielded an 8-kb *EcoRI* hybridisation band in the wildtype and the balancer third chromosomes, whilst a 14-kb fragment was detected in the third chromosome of C865 (hereafter called C865 chromosome; see Figure 5.4C). If excision occurred precisely in the line examined, the 14-kb band should be undetectable. Conversely, if excision was imprecise, a new hybridisation band should have been detected. The KF1.7 probe yielded the 8-kb band in the wildtype and balancer chromosome, and a 4.7-kb band in
the C865 chromosome (see Figure 5.4D). In the primary screen, the XF2.0 probe was used, whilst the KF1.7 probe allowed further characterisation of the excision events.

Based on Southern analysis, the excision events were placed into one of three groups (Table 5.2). In the first group, excision occurred precisely, as was shown by an 8-kb band detected by XF2.0(Figure 5.7), similar to the one found in the wildtype (Figure 5.4). Of the 105 lines examined, it was found that 88 (83.8%) showed precise excision of $P[w^+, \text{Gal4}]$.

<table>
<thead>
<tr>
<th>Classes</th>
<th>Numbers (percentage)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precise excision</td>
<td>88 (83.8%)</td>
</tr>
<tr>
<td>Truncation in the P-element</td>
<td>12 (11.4%)</td>
</tr>
<tr>
<td>Truncation in flanking genomic</td>
<td>2 (1.8%)</td>
</tr>
<tr>
<td>Not determined *</td>
<td>3 (2.9%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>105 (100%)</td>
</tr>
</tbody>
</table>

(*Not determined: 3 lines showed unidentified hybridisation bands.)

12 of the 105 lines (11.4%) were placed in group two. Lines in this group yielded an XF2.0 detected band at around 12- to 14-kb, in addition to the 8-kb balancer band (Figure 5.7A). It seems likely that a truncated $P[w^+, \text{Gal4}]$ remained at the original position, and the flanking genomic DNA was not deleted. For example, a novel 12-kb band was detected by the XF2.0 probe in line mmw1; the KF1.7 probe, however, detected a 4.7-kb band, which was the same size as that on the C865 chromosome (Figure 5.7B, lane 2), indicating that the 5' part of the $P[w^+, \text{Gal4}]$ sequence remained in the original position. The 12-kb band was possibly generated by a 2-kb deletion in the 5' part of $P[w^+, \text{Gal4}]$, presumably in the white ($w$) transgene, since it showed a white-eye phenotype. This 12-kb band was observed in other three lines, namely mmw13, mmw48 and mfw109 (Figure 5.7, 5.8 and data not shown). The viability of these lines appeared normal when homozygous on the third chromosome.
Functional Study of \textit{Mhc95F}

\section{A.}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{A.png}
\caption{A novel \textasciitilde14-kb band detected by the XF2.0 probe was also detected by the KF1.7 probe in all three cases when the KF1.7 probe was applied (Figure 5.7, lane 4.5; Figure 5.8, lane 9). Because the KF1.7 probe detected a 4.7-kb band on the C865 chromosome (Figure 5.4D), the novel 14-kb band may indicate that a deletion event occurred and removed about 5-kb of the \textit{P[w\textsuperscript{+}, Gal4]} sequence, which should include the only \textit{EcoRI} site in this plasmid (Figure 5.4A). It was found that seven lines showed this \textasciitilde14-kb band, namely, \textit{mfw11}, \textit{mfw91}, \textit{mfw112}, \textit{mmw3}, \textit{mmw4}, \textit{mmw17} and \textit{mmw46} (Figure 5.7, 5.8 and data not shown). The data shown by the analysis of this group suggest that there may exist some \textit{cis}-elements which are sensitive to P-element excision within the \textit{P[w\textsuperscript{+}, Gal4]} sequence. \textit{mmw10} is similar to this group and}\end{figure}
produces a novel 13-kb band which was detected by both the KF1.7 and XF2.0 probes (figure 5.7, lane 11).

In the third group, imprecise excision did occur and resulted in deletion of the flanking genomic region. Two of the 105 lines, mmwl14 and mfw98, were found to belong to this group. The line mmwl14 showed a novel 7-kb band detected by XF2.0 (Figure 5.7, lane 14), indicating that about 1-kb of adjacent genomic DNA sequence was deleted, whilst mfw98 showed a novel 6-kb fragment by PF3.9 (a 3' probe detects the same 8-kb band in the wildtype as the XF2.0 probe; see Figure 5.4A), indicating that 2-kb of sequence was deleted (Figure 5.8B, lane 6).

The imprecise excision of the P-element in the genomic region could have occurred in both directions. If the deleted sequence was in the 3' direction, it would possibly truncate the Mhc95F gene, the first exon of which is located within 2-kb of the 3' end of P[w+, Gal4] in C865. However, if excision was in the 5' direction, it might not affect the Mhc95F gene. In deletion line mfw98, it appears that excision was in the 5' direction. Using the KF1.7 probe, only the 8-kb band from the balancer chromosome was detected, suggesting that the 2-kb deletion region covered the common sequences of the 5' flanking genomic DNA and the probe KF1.7 (Figure 5.8A, lane 6). It was found that line mfw98 exhibits male homozygous lethality. Complementation analysis showed that mfw98 complemented the deficiency stock, Df(3R)crbS87-5. Furthermore, it was found by RT-PCR that Mhc95F transcription was not affected in homozygous mfw98 females (data not shown). Taken together, these data suggest that the Mhc95F gene is not mutated in mfw98.

The case of mmwl14 was different, because it only had a 1-kb deletion, and it was found that the 7-kb hybridisation band remained when the KF1.7 probe was applied. The deletion in this line will be discussed in the next section.

The viability of mmwl14 and mfw98 flies homozygous for the third chromosome appeared to be reduced. However, they were complementary to each other. mmwl14 showed homozygous lethality; the homozygous mutant was developmentally arrested at third instar larvae, and metamorphosis never occurred. These larvae survived for about 30 days at 25°C, as compared to the 2-day life span for the third instar larvae in the wildtype. mfw98 showed male homozygous lethality; the females developed normally and were fertile.
Figure 5.8 Southern hybridisation to screen for deletions and insertions in the Mhc95F gene. Genomic DNA is digested by EcoRI. Probes used in this work are: A, KF1.7; B, PF3.9 and D. The cDNA probe. C is the gel of probes A and B, while E is the gel of probe D.

Using PF3.9 as a probe, white-eyed fly line mfw98 showed a 6-kb extra band (lane 7 in B) when compared with the wildtype (refer to Figure 5.4). No extra bands were found when probe KF1.7 was used (lane 7 in A). The cDNA probe detected two similar hybridisation bands (lane 6 in D) to the wildtype (lane 2 in D) and the balancer chromosomes (lane 3 in D).

Using KF1.7 as a probe, Red-eyed fly line mfr53 showed a 4.7-kb band (lane 1 in A), which is identical to the C865 chromosome (refer to Figure 5.4). When PF3.9 was used as a probe, mfr53 showed no extra hybridisation bands (lane 1 in B). The cDNA probe detected two bands (lane 13, in D) which are identical to the wildtype (lane 2 in D) and the balancer chromosomes (lane 3 in D).
The XP2.0 and KF1.7 probes, derived from the flanking genomic region, could not detect large deletions. In order to see if any of the white-eye fly lines showed larger deletions, the cDNA probe was used (Figure 5.4A). This probe detected a 14-kb fragment which included the coding region of the Mhc95F gene. However, it appeared that none of the white-eyed fly lines affected the 14-kb hybridisation band (Figure 5.8D), indicating that no large deletions had occurred.

5.3.4 Characterisation of the deletion line, mmmw14

In order to determine if Mhc95F transcription was truncated in mmmw14, an RT-PCR assay was applied. The primers used in this experiment are shown in Figure 5.10A. The primer pair, p1 and p1027, was used to amplify the first 1027bp sequence of the Mhc95F transcripts in both the wildtype and mmmw14 third instar larvae. A 1-kb band appeared in the wildtype, while no signal was detected in homozygous mmmw14, indicating that a deletion event did occur and the 5' end of the Mhc95F gene was removed (Figure 5.9). However, since it has been reported that several transcripts with different lengths exist in the wildtype (Kellerman and Miller, 1992), there was a possibility that the deletion of the first exon might have no effect on Mhc95F transcription. In order to address this question, primers p517 and p1027 were used to amplify the Mhc95F transcripts at the coding region, which was reported to start at the 341-bp position (Kellerman and Miller, 1992). It was found that a 0.5-kb band was produced from both the control wildtype and homozygous mmmw14 third instar larvae. Taken together, these data indicate that although the 5' end, including the first exon, was truncated in mmmw14, the Mhc95F gene is still transcribed. There could be several transcription initiation sites present, which provide the Mhc95F transcripts with variable lengths.

Phenotypic analysis showed that mmmw14 homozygotes were developmentally arrested at the third instar larvae. In order to determine if the lethality was due to the deletion in the 5' end of the Mhc95F gene, complementation analysis was used. It was found that mmmw14 did complement the deficiency strains, Df(3R)crbS87-5 and Df(3R)crb87-4, which were thought to have no Mhc95F gene as determined by quantitative Southern analysis (Kellerman and Miller, 1992). This observation, along with the fact that the Mhc95F is still transcribed in mmmw14 homozygous larvae, demonstrates that the lethality is unlikely to be due to truncation of the Mhc95F locus.
Figure 5.9 RT-PCR to detect transcription of *Mhc95F* in *mmw14* homozygous third instar larvae (A). *p1027* was used as the reverse transcription primer in lanes 2-5, while *at*, *atal* (*atl*) 3' primer (Dr. A. Jarman, personal communication) was used as the reverse transcription primer in lanes 6 and 7, which are used as positive controls. Amplification primer pairs are listed in the text box beside the gel picture. It was shown in lane 3 that no products were present in the *mmw14* homozygous third instar larvae when primer pair *p1* and *p1027* was used, while the control wildtype (*Oregon R*) larvae had a 1-kb product, indicating a deletion event which removes the 5' end of the *Mhc95F* cDNA had occurred. This deletion does not seem to disrupt transcription of the *Mhc95F* gene, since a band is observed when using the primer pair *p517* and *p1027* to amplify the reverse transcriptional products (lane 5).

B. A schematic diagram shows the location of the *Mhc95F* primers used in this experiment.

\[ \text{p1: GAGTTCGACTCGACTCTCATCCAC} \]
\[ \text{p517: ATCACGATGACAACTGCTGAC} \]
\[ \text{p1027: TAGTGCGATATGTAACCACCCGACC} \]
5.3.5 A screen for insertional mutations by mobilising the P element

An alternative scheme used in creating mutations in the Mhc95F locus was to screen for insertional mutants by mobilising P[w+, Gal4] in the C865 genome. This is based on the fact that the P-element tends to transpose to a nearby position, with respect to the original site. The scheme was identical, during the first two steps, to that of the screen for imprecise excision mutations; it differed in the third step, in which the w+ (red-eye) rather than the w- (white-eye) F2 progeny over the TM3 or TM613 balancers were selected and established as fly lines by crossing with the third chromosomal balancers. These lines were then ready for use with Southern analysis to determine if the P-element was transposed into the Mhc95F coding region, and were also ready for an examination of homozygous lethality on chromosome 3 in the F4 generation.

![Southern blot example](image)

**Figure 5.10** A Southern blot shows an example the screen of red-eyed fly lines whose genomic DNA was digested with EcoRI. The probe used in this experiment was XF2.0 (see Figure 5.4). The majority of lines still have a P[GawB] insertion at the original position, and this is shown as a 14-kb hybridisation band, as well as a 8-kb band which is from the balancer chromosome. One line shows excision of the P-element; it only has a 8-kb hybridisation, as seen in the wildtype (refer to Figure 5.4).

290 red-eye (w+) lines were established, and 222 of these were scored by Southern analysis using the 3' flanking DNA probe XF2.0. Surprisingly, 97.5% of these lines (212/222) had P[w+, Gal4] insertion in the original position, whilst only 10 lines showed excision of the P[w+, Gal4]. Figure 5.10 shows an example of these
screens. It was found that none of the lines showed local jumping into the 3' part of the *Mhc95F* gene when they were tested with the cDNA probe (data not shown).

### 5.3.6 Analysis of line *mfr53*

94 Red-eyed lines were screened for homozygous lethality on the third chromosome. Of these, it was found that 5 lines showed homozygous lethality. Complementation analysis was then undertaken by crossing with *Df(3R)crbS87-5* to determine if lethality in these lines was due to a mutation covered by the deficiency. It was found that one of them, *mfr53*, failed to complement this deficiency, indicating that a mutation in chromosomal 95F region may cause the homozygous lethality, whilst the lethality in the remaining 4 lines were presumably due to mutations elsewhere on the third chromosome. Homozygous *mfr53* on the third chromosome was found to be lethal at the embryonic stage.

In order to determine if the *mfr53* genome was truncated at the *Mhc95F* locus, different probes were used to detect the genomic structure within this gene in the *mfr53/TM3* flies. Surprisingly, it was observed that the KF1.7 probe, yielded an 8-kb product (Figure 5.8A, lane 1; page 138), apparently from the balancer chromosome, and a 4.7-kb product identical to that from the C865 chromosome. This 4.7-kb fragment included the 1.7-kb flanking genomic DNA and the *Gal4* transgene in *P[w*, *Gal4]*, indicating that *P[w*, *Gal4]* remained at the original position (see Figure 5.4). However, the 5' flanking genomic DNA probe, PF3.9, yielded only an 8-kb band (Figure 5.8B, lane 1; page 138), which is thought to be from the balancer chromosome. No 14-kb product, which was observed in the C865 chromosome, was present, suggesting that a deletion event may have occurred and resulted in the truncation of *P[w*, *Gal4]* at its 3' part. In order to determine if the coding region of the *Mhc95F* gene was truncated in the *mfr53/TM3* flies, the cDNA probe (see Figure 5.4) was used. Two products were seen at 8- and 14-kb, respectively, identical to those detected on the wildtype and balancer chromosomes (Figure 5.8D, lane 13; page 138). This observation may suggest that the *Mhc95F* gene is not truncated in the *mfr53* line. However, there exists another possibility; a large deletion may have occurred, resulting in the removal of a large area of the 5' flanking DNA, which includes the sequences common to both the PF4.0 and cDNA probes. To check this, a probe that detects genomic sequence 3' to the *Mhc95F* coding region is required, and this remains to be identified. This possibility could also be verified if a *mfr53* allele shows chromosome rearrangement in the *Mhc95F* coding region. Since *mfr53* was mutated at the chromosomal 95F region, complementation
analysis was undertaken to determine if any of the \textit{P-lacZ} deletion mutants (refer to 5.3.1) in the same chromosomal region was an \textit{mfr53} allele. It was found that \textit{mfr53} failed to complement lethality of the 581/4 group lines; thus they belong to the same complementation group. However, all five members of the 581/4 complementation group (581/4, 509/11, 104/9, 1384/4, 258/7) failed to show a \textit{P-lacZ} insertion in the \textit{Mhc95F} coding region by Southern analysis (see Figure 5.5). This observation proposes that the \textit{Mhc95F} gene is unlikely to be mutated in line \textit{mfr53}.

\textbf{5.4 Targeted silencing and activation of \textit{Mhc95F} expression during development}

In addition to genetic analysis, the \textit{Gal4-UAS} technique was also used in order to characterise the function of \textit{Mhc95F} during development. This technique has been widely used to express a gene (or a modified gene) in a spatial- and temporal-specific manner, thus allowing the function of the gene to be studied. Furthermore, if the \textit{Gal4-UAS} dual system is combined with the antisense RNA technique, a novel "targeted silencing" technique can be introduced. The assumption made in this approach is that tissue specific \textit{Gal4} induces expression of a UAS linked antisense gene, and this in turn suppresses translation of the target gene in cells in which the antisense gene is expressed (see Figure 5.11). Here, the application of these two techniques to the study of \textit{Mhc95F} function is reported.

\textbf{5.4.1 Cloning of the \textit{Mhc95F} cDNA into the pUAST plasmid}

Two strategies were used in order to clone the full-length \textit{Mhc95F} cDNA into pUAST, a vector containing UAS for Gal4 binding and cis-elements for P-element integration into the \textit{Drosophila} genome. In the first scheme, the plasmid EM5, which had the full-length \textit{Mhc95F} cDNA inserted into a pNB40 vector (refer to Figure 5.12, kindly provided by K. Miller), was digested with \textit{EcoRI} and a 4.7-kb DNA fragment was recovered from the electrophoresis gel. This 4.7-kb fragment included the 4.4-kb full-length \textit{Mhc95F} cDNA sequence and \textasciitilde{}0.3-kb pNB40 sequence, which was linked to the 5’end of the cDNA. This fragment was then ligated to the \textit{EcoRI} digested and CIP dephosphorised pUAST vector, and the ligations were used to transform the \textit{E. coli} host cell XL1-Blue. Two positive clones that had the 4.7-kb DNA insert, as shown by \textit{EcoRI} digestion, were obtained. Physical mapping of these two clones by \textit{Bgl II} digestion showed that the cDNA had inserted downstream of UAS in an antisense
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Figure 5.11 Targeted silencing technique. A selection of enhancer-trap Gal4 lines are crossed with a fly line that carries a UAS linked antisense Mhc95F gene, UAS-Mhc95Fas. Expression of Mhc95F antisense RNA is activated by Gal4 in a tissue-specific manner. If the antisense Mhc95F transgene is expressed in the same cells as the endogenous Mhc95F gene, binding of antisense Mhc95F RNA with endogenous Mhc95F mRNA will block translation of the endogenous Mhc95F gene.

The −0.3-kb pNB40 vector sequence attached to the full-length Mhc95F cDNA was not expected to affect the antisense RNA expression. However, it might interfere with the translation of ectopic Mhc95F mRNA. An alternative approach was therefore chosen to clone the Mhc95F cDNA, detached from the leading pNB40 sequence, into the pUAST in a sense direction (see Figure 5.12). In this approach, the pNB40 was completely digested by EcoRI and partially digested by Hind III. The complete digestion
Figure 5.12 Cloning of the *Mhc95F* cDNA into pUAST in a sense direction. For details see text.

By *EcoRI* allowed the 3' end of the *Mhc95F* cDNA to be disconnected from the vector pNB40. The *HindIII* digestion allowed the 5' end of the cDNA to be separated from the vector. However, the presence of two *HindIII* sites within the *Mhc95F* coding sequence meant that the 4.4-kb complete cDNA fragment could only be maintained by partial
digestion. After recovery from an agarose gel, the 4.4-kb cDNA fragment was ligated to an EcoRI-HindIII double digested pBluescript(SK+) vector. The ligations were then used to transform the E. coli host, XL1-Blue. Next, the 4.4-kb cDNA fragment was recovered from the DNA made from the positive clones by Xho I-Xba I double digestion, and was ligated to a XhoI-XbaI double digested pUAST vector. Positive clones from this transformation, named p[w⁺, UAS-95Fs] ("s" means sense), should therefore contain the full-length Mhc95F cDNA in sense direction. This was confirmed by physical mapping by Bgl II digestion and sequence analysis (data not shown).

5.4.2 Germline transformation of the p[w⁺, UAS-95Fs] and p[w⁺, UAS-95Fas] constructs

DNA from the constructs (p[w⁺, UAS-95Fs] and p[w⁺, UAS-95Fas]) was co-injected with the helper plasmid pΔ2-3 into the w' blastoderm prior to pole cell formation. Two rounds of injection were undertaken in order to obtain transformants. In the first round of injection, about 100 embryos were injected with either p[w⁺, UAS-95Fs] or p[w⁺, UAS-95Fas]. 20 larvae were hatched from the p[w⁺, UAS-95Fs] injected embryos, which gave rise to 9 adults. However, no red-eyed progeny were observed from the crossing of the 9 adults to w flies. (Table 5.1). From the 100 embryos injected with the p[w⁺, UAS-95Fas] construct, 17 larvae hatched, and 11 adults survived. These fly lines were set up separately by crossing with w flies. Red-eyed transformants, named Am8, appeared in the progeny of one of these crosses.

<table>
<thead>
<tr>
<th>Table 5.3 P-element mediated germline transformation</th>
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<tbody>
<tr>
<td>Constructs</td>
</tr>
<tr>
<td>1st round</td>
</tr>
<tr>
<td>p[w⁺, UAS-95Fs]</td>
</tr>
<tr>
<td>p[w⁺, UAS-95Fas]</td>
</tr>
<tr>
<td>2nd round</td>
</tr>
<tr>
<td>p[w⁺, UAS-95Fs]</td>
</tr>
<tr>
<td>p[w⁺, UAS-95Fas]</td>
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</table>

In the second round of injection, 330 w' embryos were injected with the p[w⁺, UAS-95Fs] construct. Of these, 112 larvae hatched and 74 adults survived, with five
transforms, namely Tmh98, Tmh118, Tmh100, Tmh121, and Tmh139, obtained. No transformants were obtained from the second round of injection with p[w⁺, UAS-95Fas], although 55 larvae hatched from the 200 injected embryos.

5.4.3 Chromosomal locations of the transformed fly lines

To determine if the transformed P-element was located on the X-chromosome, male transformants were crossed with w female virgins, and the eye phenotype of F1 progeny was examined. If all female F1 progeny and no male F1 progeny appeared to be red-eye, then the insertion site was determined to be on the X-chromosome. In order to find out on which autosome the transformed P-element was located, transformants were crossed with the second and third chromosomal balancers over a w background. The F1 progeny that had both the dominant balancer marker and the red-eye (w⁺) phenotype were then crossed with w flies. The segregation ratio was used to determine which chromosome the P-element was located on.

It was found that the five sense lines had p[w⁺, UAS-95Fs] insertion sites dispersed over all three of the major chromosomes. The insertions in Tmh98 and Tmh118 were on the X-chromosome, while the insertion in Tmh139 was on the second chromosome, and the insertion in Tmh100 and Tmh122 were both on the third chromosome. The antisense Mhc95F line, Am8, had at least two insertion sites of p[w⁺, UAS-95Fas] on the second and the third chromosomes. No developmental defects were observed in any of these lines when homozygous for the chromosome on which p[w⁺, UAS-95Fas] is inserted.

5.4.4 Targeted expression of Mhc95F

In order to obtain targeted expression of Mhc95F during oogenesis, several Gal4 lines that show reporter gene expression in different subsets of follicle cells were crossed with the five UAS-95Fs lines. The Gal4 lines chosen to cross with the transformed sense lines were C324, C532, C535, C606, C714, C826, C844 and 133Y (for their reporter gene expression patterns, see Table 3.1, Chapter 3). However, no apparent defects were observed in the F1 progeny from all of these crosses. They survived until adulthood and the females produced normal eggs.
Figure 5.13 RT-PCR to analyse expression of the $Mhc95F$ transgene in Gal4-C714/UAS-Mhc95Fs flies (A). The reverse transcription primer used in this experiment was p1027, and the template was total RNA made from Gal4-C714/UAS-Mhc95Fs flies. Using the primer pair, p1027 and KS primer, a 1.1-kb product was observed (lane 2). Since the KS primer is specific to the $UAS-Mhc95F$ transgene, the 1.1-kb product is thought to have been amplified from $Mhc95F$ transcripts driven by the Gal4 driver. Lane 3 shows a 1kb product amplified from the total RNA using the primer pair p1027 and p1. Since the concentration of the KS primer used in this experiment was five times lower than that of p1 and p1027, the 1.1-kb band in lane 2 appears to be weaker than the 1kb band in lane 3. No products are found in lane 4 when using the primer pair p1027 and pUASL, which is specific to the $UAS-Mhc95F$ transgene but is not transcribed, indicating that the 1.1-kb product in lane 1 is not due to contamination of the genomic DNA.

B and C show the location of the primers used in this experiment in the endogenous $Mhc95F$ gene and the $UAS-Mhc95F$ transgene.

Primer UASL:GCAACTACTGAAATCTGCC;
Primer KS:TCGAGGTCGACGGTATC.
Several possibilities could explain why no defects were detected in the progeny of these crosses. Firstly, the \textit{UAS-Mhc95F} transgene might not have been transcribed; no gene product would then be available. Secondly, the \textit{UAS-Mhc95F} transgene might have been transcribed but not translated; no protein products would therefore be produced. Thirdly, targeted \textit{Mhc95F} expression might not be able to disturb normal development and induce a phenotype. In order to choose between these possibilities, an RT-PCR assay was undertaken in the \textit{Gal4-C714/UAS-95Fs} flies. The 3' primer was p1027, from the 1027-1005bp region of the \textit{Mhc95F} coding sequence, whereas the 5' primer was the KS primer, which had been linked with the \textit{Mhc95F} cDNA since it was inserted into pBluescript (KS+) to attain the \textit{XhoI} and \textit{XbaI} sites. Using this primer pair, a band around 1-kb was detected in the C714/UAS-95Fs lane. This DNA band was obviously amplified from the RNA template produced by the \textit{UAS-Mhc95F} transgene whose expression was driven by Gal4. The endogenous transcripts, on the other hand, were not amplified because only one primer (p1027) was from the \textit{Mhc95F} coding region. From this experiment it was shown that the \textit{UAS-Mhc95F} transgene was transcribed in the \textit{Gal4/UAS-95Fs} flies.

Theoretically, the other two possibilities could be distinguished between by using antibody that recognises the Myosin 95F protein to investigate extracts from the \textit{Gal4/UAS-95Fs} flies by western blots. Since Myosin 95F is found in nearly all tissues throughout development (Kellerman and Miller, 1992), a quantitative Western blot analysis would be required. However, time has not permitted such experiments to be done.

\subsection*{5.4.5 Targeted expression of antisense \textit{Mhc95F} RNA causes lethality in different developmental stages}

When the \textit{UAS-95Fas} lines, Am8, was crossed with a number of different \textit{Gal4} lines, disturbance of development was detected in many cases (Table 5.6). The cross of \textit{Gal4-C22c} with Am8 resulted in lethality of the F1 progeny during late embryogenesis. It is known that C22c has a high-level of ubiquitous Gal4 expression during embryogenesis (R. Kilby, personal communication), and the control C22c/UAS-lacZ flies showed normal development; the lethal phenotype of C22c/Am8 was therefore proposed to be caused by antisense \textit{Mhc95F} RNA expression. It was also found that progeny of crosses of Am8 with a number of other \textit{Gal4} lines, including C606, C648 and F173, died during the first larval instar; C612b/AM8 and C673/Am8 showed lethality during the second larval instar. Moreover, antisense \textit{Mhc95F} expression driven
by Gal4 in lines C524b, C577, C601a and C844 resulted in lethality during the third larval instar. Lethal phenotypes were also observed during the white prepupal stage when antisense Mhc95F expression was driven by Gal4 in the five lines, C320a, C789, C714, C726b and C760. All control crosses of the UAS-lacZ line with the above Gal4 lines showed normal development in F1 progeny. Taken together, these results indicate that lethality in Gal4/Am8 during different developmental stages resulted from the developmental onset of Mhc95F antisense RNA expression, which was dependent on the temporal and spatial specific expression of Gal4.

**Table 5.4. Targeted silencing of Mhc95F**

<table>
<thead>
<tr>
<th>Gal4 lines</th>
<th>Chromosomal locations</th>
<th>Lethal stages of Gal4/Am8 flies</th>
</tr>
</thead>
<tbody>
<tr>
<td>C22c</td>
<td>2</td>
<td>late embryo</td>
</tr>
<tr>
<td>C606</td>
<td>1st-instar larvae</td>
<td></td>
</tr>
<tr>
<td>C648</td>
<td>1st-2nd instar larvae</td>
<td></td>
</tr>
<tr>
<td>F173</td>
<td>3</td>
<td>1st-instar larvae</td>
</tr>
<tr>
<td>C612b</td>
<td>2nd-instar larvae</td>
<td></td>
</tr>
<tr>
<td>C673</td>
<td>2nd-instar larvae</td>
<td></td>
</tr>
<tr>
<td>C524b</td>
<td>before 3rd-instar larvae(stage unidentified)</td>
<td></td>
</tr>
<tr>
<td>C577</td>
<td>before 3rd-instar larvae(stage unidentified)</td>
<td></td>
</tr>
<tr>
<td>C601a</td>
<td>before 3rd-instar larvae(stage unidentified)</td>
<td></td>
</tr>
<tr>
<td>C844</td>
<td>before 3rd-instar larvae(stage unidentified)</td>
<td></td>
</tr>
<tr>
<td>C320a</td>
<td>white pupae</td>
<td></td>
</tr>
<tr>
<td>C789</td>
<td>X</td>
<td>white pupae</td>
</tr>
<tr>
<td>C714</td>
<td>X</td>
<td>white pupae</td>
</tr>
<tr>
<td>C726b</td>
<td>X</td>
<td>white pupae</td>
</tr>
<tr>
<td>C760</td>
<td>X</td>
<td>white pupae</td>
</tr>
<tr>
<td>C865</td>
<td>3</td>
<td>Pharate adults, flies fail to eclosion</td>
</tr>
<tr>
<td>C532</td>
<td></td>
<td>malformed legs and wings; oogenesis stopped at stages 9, 10; centripetal migration blocked</td>
</tr>
<tr>
<td>C535</td>
<td>X</td>
<td>malformed legs and wings</td>
</tr>
</tbody>
</table>
Detailed analysis was undertaken in C726b/Am8 flies; it was found that the cuticle in third instar larvae was abnormal. In the wild-type larvae, dark denticles could only be detected on the ventral side, and the dorsal denticles were normally transparent (Figure 5.14A,C,D). However, dark denticles were detected on both the ventral and dorsal sides of each segment in C726b/Am8 larvae (Figure 5.14B,E,F). In some cases, the whole segment was surrounded by a circle of dark denticles. In order to address if this phenotype could have been due to antisense Mhc95F expression, C726b was crossed with UAS-GFP line. Green fluorescence was detected in the cuticle of C726b/UAS-GFP larvae (data not shown), suggesting that the cuticle phenotype was due to antisense Mhc95F expression. These observations may suggest that the Mhc95F function is required for cuticle development.

It was interesting to find out what the phenotype of C865/Am8 flies was, because expression of antisense Mhc95F RNA in these flies is driven by a Gal4 transgene which is regulated by the Mhc95F enhancer. It was found that they developed quite normally until late metamorphosis. This is probably due to the fact that the Gal4 reporter in C865 only represents part of the endogenous Mhc95F expression (See Chapter 4, Figure 4.4), which must be insufficient to induce an early development arrest. The C865/Am8 adults failed to eclose from the pupal case, indicating that Mhc95F plays a role in this developmental process (data not shown).

5.4.6 Targeted expression of antisense Mhc95F RNA causes defects in leg eversion

The cross of Am8 with C532 gave a striking phenotype in F1 adults. Most of the F1 progeny showed an unexpanded wing phenotype (Figure 5.15B), possibly due to developmental arrest in newly hatched flies, which have unexpanded wings in a short period before they are fully expanded in the wildtype. Furthermore, the C532/Am8 flies showed malformed legs (Figure 5.15E) when compared with wildtype legs (Figure 5.15F). Legs were bent, shortened and thickened. This phenotype was similar to that of the sqh mutant flies (Edwards and Kiehart, 1996), and this may indicate a similar function of myosin-II and myosin-VI in the cell shape changes that drive leg disc eversion. The C532/Am8 flies were less active when compared to the wildtype flies, and survived only for three to four days before they died.
Figure 5.14 Analysis of the denticle phenotype in Gal4-C726b/Am8 larvae/white prepupae (B, E and F) and the wildtype (A, C and D). In the wild-type larvae/white prepupae, dorsal denticles are normally transparent (A and C); dark denticles can only be detected on the ventral side (D). In C726b/Am8 larvae/white prepupae, dark denticles are detected on both the dorsal (B and E) and ventral (F) sides.
**Figure 5.15** Targeted silencing of *Mhc95F* expression causes adult flies with unexpanded wings and malformed legs. 

A. Shows a wild-type fly with its wings fully expanded. B (dorsal view) and C (lateral view) show a C532/Am8 fly with unexpanded wings and malformed legs. D. The cross of C532 with Amj122, which has a different chromosomal location of *P[w+;UAS-95Fas]* to that in Am8, produces progeny with similar but less severe unexpanded wing phenotype. E. Shows a close-up look at the malformed legs (arrow) of the C532/Am8 flies. F. Shows a wildtype leg.
Additionally, the cross of a different Gal4 line C535 with Am8 also resulted in offspring with malformed legs and unexpanded wings, as seen in the C532/Am8 flies, indicating that this phenotype was reproducible, using different Gal4 drivers.

5.4.7 Targeted expression of antisense Mhc95F RNA arrests oogenesis

When female C532/Am8 flies were dissected to study ovaries (Figure 5.16A shows the Gal4 expression pattern in a stage 9 egg chamber), it was found that oogenesis stopped at stages 9 and 10 and egg chambers degenerated after this stage. No defects were detected earlier than stage 9 (Figure 5.1 B,D).

In egg chambers just prior to degeneration, centripetal follicle cell migration appears to have been disrupted. In the wildtype egg chamber, the anterior most columnar cells migrate along the nurse cell-oocyte border at stage 10b when the retraction of follicle cells from the nurse cell chamber to the oocyte is completed. In contrast, in C532/Am8 egg chambers, the centripetal migration takes place at a more posterior position rather than the nurse cell-oocyte border, resulting in separation of the oocyte into two regions (Figure 5.16E,F). Additionally, the follicle cells at the leading edge of the migrating cell sheet do not appear to be the anterior most columnar cells. Moreover, the centripetal migration seems to occur at stage 9 when follicle cell retraction from the nurse cells to the oocyte has not been completed. (Figure 5.16E,F). Taken together, it is likely that myosin 95F is involved in the regulation of centripetal migration. Additionally, it was noticed that migration of the follicle cells from the nurse cell chamber to cover the oocyte does not seem to have been affected.

As shown by DAPI staining, the nurse cell nuclei appeared abnormal in stages 9-10 C532/Am8 egg chambers (Figure 5.16B). It is shown in Figure 5.16A that in C532 the Gal4 reporter is expressed in most of the follicle cells during stages 9 and 10, no germline expression has been detected. Thus, the degeneration of the nurse cell nuclei was likely a secondary effect. It is proposed that antisense Mhc95F expression in the follicle cells may interfere with the follicle cell behaviour, and in turn disturb nurse cell differentiation.
Figure 5.16 Targeted silencing of \textit{Mhc95F} expression arrests oogenesis. A, The Gal4 driver C532 used in this experiment shows reporter gene expression in the majority of follicle cells during stages 9-10. B, DAPI staining shows that nurse cells in C532/Am8 egg chambers degenerate (arrow) at around stage 9. C, DAPI staining of the wildtype egg chambers. D, The C532/Am8 egg chambers degenerate during stages 9-10. E and F show that centripetal migration of the follicle cells in stage-10 egg chambers appear to be at an incorrect position (arrows; for the wildtype position \textit{Mhc95F} expression refer to Figure 16D).
5.4.8 Verification of antisense Mhc95F RNA expression in C532/Am8

In order to verify that the phenotype observed in C532/AM8 was indeed due to antisense Mhc95F RNA expression, an RT-PCR assay was undertaken. The reverse transcription primer used in this experiment was p1, while the amplification primers were p1 and p1027 (refer to Figure 5.9). A 1-kb band was obtained when total RNA from C532/Am8 flies was used as the template (Figure 5.17), whereas no product was observed when using total RNA from the wildtype flies as the template. This result suggests that antisense Mhc95F RNA is expressed in C532/AM8 flies, and supports that the presence of antisense Mhc95F RNA is the reason for the unexpanded wing and malformed leg phenotype.

![Figure 5.17](image)

**Figure 5.17** RT-PCR to detect Mhc95F antisense RNA expression in Ga4-C532/Am8 flies. The reverse transcription primer used in this experiment was p1, which could only reverse transcribe the Mhc95F antisense RNA. The amplification primer pairs and the templates are listed in the text box next to the gel picture.

Lane 2 and 3 show positive RT-PCR products, indicating that the antisense Mhc95F transgene is transcribed in Ga4-C532/Am8 flies. However, residual expression of the antisense Mhc95F transgene may exist in Am8 flies, as shown by a weak band in lane 4. No products were yielded in wildtype control flies (lane 5).

Since Am8 has at least two P[w\(^+\), UAS-95Fas] insertions on the second and the third chromosomes, they were then separated, named Am8-2 and Am8-3 respectively, and crossed with C532 flies. It was found that crosses of C532 with Am8-2, which has
the insertion on the second chromosome, but not Am8-3, resulted in the malformed leg and unexpanded wing phenotype. This suggests that insertions in different chromosomal positions may affect the levels of antisense RNA expression. However, there was another possibility that disturbance of development in Gal4/Am8 flies was due to activation of a nearby gene which is close to the $P[w^+, UAS-95Fas]$ insertion in Am8-2. In order to rule out this possibility, a cross scheme was carried out to mobilise the $P[w^+, UAS-95Fas]$ into a new chromosomal position. If a similar phenotype was observed after the $P[w^+, UAS-95Fas]$ moved to a new position, this possibility could then be ruled out.

**Figure 5.18** Cross scheme to mobilise $P[w^+, UAS-95Fas]$ from the second chromosome, $P[w^+, UAS-95Fas]*$ may have jumped from the original position.

As shown in Figure 5.18, Am8 was crossed with a transposase-providing strain. In F1 generation, flies with the genotype $w^+$; $P[w^+, UAS-95Fas]$; $P[(\Delta 2-3), Sb]$ were selected, and crossed with $w^+$; $Pin/Cyo$ flies. In F2 generation, flies with both $w^+$ and $Cyo^-$ genotype were selected and established as fly lines by crossing each fly with a third chromosomal balancer in a $w^+$ background. In F3 generation, the segregation ratio
of \( P[w^+, UAS-95Fas] \) with \( Cyo^+ \) was calculated to assess if the \( P[w^+, UAS-95Fas] \) was mobilised from the second to a new chromosome.

92 lines were established and assessed for chromosomal locations of the \( P[w^+, UAS-95Fas] \) element. Of these, 47 lines had \( P[w^+, UAS-95Fas] \) insertion remaining on the second chromosome, while 14 other lines showed insertion both in the second and another chromosome. 28 lines showed insertions into a new chromosome, while no insertions were found in the second chromosome. These 28 lines were then crossed with the \( Gal4 \) line C532 to detect the wing phenotype. It was found that progeny from one of these crosses, C532/Amj122, showed a similar, although less severe, unexpanded wing phenotype (Figure 5.15D). Since Amj122 had a different position of the \( P[w^+, UAS-95Fas] \) insertion to Am8-2, the similar phenotype observed in C532/Amj122 and C532/Am8-2 suggests that the \( Mhc95F \) function, as determined by targeted antisense RNA expression, is reliable. It also shows that only lines happen to be inserted into the chromosome, such that they are expressed at a high level, generate a phenotype. This has also been observed in antisense experiment using transgenic plants (Edwards et al., 1995; Zrenner et al., 1996).

### 5.5 Discussion

#### 5.5.1 \( Mhc95F \) expression during oogenesis

The expression pattern of \( Mhc95F \) during oogenesis is fascinating and provides some clues for predicting its function. Significantly, the \( Mhc95F \) is expressed in follicle cells undergoing dramatic cell shape change and cell movement. These include border cells, follicle cells migrating to cover the oocyte, centripetal cells, and dorsal-appendage producing cells. The only major type of follicle cell migration not included in this list is migration to envelop germline cysts in the germarium region 2. However, since \( Mhc95F \) expression has also been detected in the germarium (see Figure 5.2A), it is quite possible that the stained cells are the cyst enveloping follicle cells. If this is the case, all major follicle cell migrations appear to be coincident with \( Mhc95F \) expression. Since \( Mhc95F \) expression is also observed in cells at the leading edge of the epithelium that undergoes dorsal closure during embryogenesis (Kellerman and Miller, 1992), it is reasonable to hypothesise that \( Mhc95F \) is widely involved in cell motility and morphogenesis during development.
Functional Study of Mhc95F

Since myosins are actin-binding proteins, myosin 95F is likely to be involved in the regulation of cell shape change and cell migration by modifying the actin cytoskeleton. Another myosin, non-muscle myosin-II, is also expressed in most of the migrating follicle cells during oogenesis (Young et al., 1993). The requirement for non-muscle myosin-II in follicle cell movement has been shown by analysing the mutant phenotype of the regulatory light chain (RLC) gene, sqh (Edwards and Kiehart, 1996). Coincident expression of non-muscle myosin-II and myosin-VI (Myosin 95F) in the migrating follicle cells proposes that these two myosins are likely to be functionally related. Since the requirement for myosin-II in follicle cell migration was only shown by the study of the sqh mutant phenotype, it is possible that this phenotype reflects a requirement of both myosin-II and myosin 95F for follicle cell migration, because myosin 95F also contains the light-chain binding IQ motif.

5.5.2 Genetic analysis of Mhc95F function

Although efforts have been made in searching for existing lethal mutations and in generating novel lethal mutations in the Mhc95F gene, no strong evidence has been produced to link the lethal phenotypes to a mutation in the Mhc95F locus. As no mutation has been identified which results in truncation of the coding region of the Mhc95F gene, it is not known what the null phenotype of Mhc95F is. In order to determine this, it is necessary to generate mutations that have the entire, or most of, the coding region deleted. Since an enhancer-trap line has been identified to have a P-element insertion near the Mhc95F gene, it is possible to generate large deletions using P-element mediated male recombination technique (Preston et al., 1996A, 1996B).

The mfr53 line appeared to be a good candidate for a Mhc95F mutant, since its lethality has been mapped to the same deficiency as the Mhc95F gene has. It is interesting to note that mfr53 may be a deletion mutant, despite the fact that it was originally screened as a potential local-hopping mutant. The deletion probably includes the 3-kb pBluescript sequence within the P[w+, Gal4], because the w transgene must remain to give rise to the red-eye phenotype. However, it is also possible that a new P[w+, Gal4] insertion may be responsible for this phenotype. If it is true that a deletion event occurred and resulted in the removal of a large genomic fragment, including the Mhc95F coding region, it will of course help to characterise the function of this myosin in more detail. However, failure to find any band-shift of the five P-lacZ insertion alleles of mfr53 by Southern analysis suggests that Mhc95F is unlikely to be mutated in mfr53 (Figure 5.5).
The 5' end of the \textit{Mhc95F} gene, including both transcribed and untranscribed sequences does not appear to be essential for the expression and function of this gene. This was shown by analysis of a deletion mutant, \textit{mmw14}, which removes around 1-kb genomic sequence at the 5' end, including the first exon of \textit{Mhc95F}. Although homozygous lethality was observed in this mutant, it does not seem to be caused by this 1-kb deletion. It is surprising that after the first exon has been deleted, the \textit{Mhc95F} is still transcribed in \textit{mmw14}. The transcripts produced by this line appear to be functional, as shown by its fully complementation of a deficiency, \textit{Df(3R)crb587-5}, which is thought to have removed the \textit{Mhc95F} gene (Kellerman and Miller, 1992).

\subsection*{5.5.3 Targeted expression of \textit{Mhc95F}}

It has been shown by RT-PCR analysis that the \textit{Mhc95F} transgene is transcribed in \textit{Gal4/UAS-Mhc95F} flies. Since normal development is not disturbed in these flies, it is unclear whether or not the ectopic transcripts are translated. To determine this, a quantitative Western analysis may be helpful. Another possibility could be that the level of the \textit{Mhc95F} transgene expression is not high enough to interfere with normal development. This can be tested either by crossing of the \textit{UAS-Mhc95F} transgenic lines with different \textit{Gal4} lines or by making a line with multiple copies of the \textit{UAS-Mhc95F} transgene. This is unlikely to be the case, because the cross of a number of different \textit{Gal4} lines, which should have different levels of \textit{Gal4} expression, with all five \textit{UAS-Mhc95F} transgenic lines showed no defects in any of the F1 progeny. It is also possible that ectopic \textit{Mhc95F} expression may not disturb normal development. If this is the case, it may mean that myosin 95F must co-operate with another gene product in order to be functional, and if that gene is not expressed no defect will occur.

It may be possible to design a UAS linked dominant negative \textit{Mhc95F} gene and transform it into the fly genome. By crossing the transformant with tissue-specific \textit{Gal4} lines, a dominant negative phenotype may be observed. To use this approach, a more detailed biochemical characterisation of Myosin-VI is required. It is known that the coiled-coil domain at the 3' tail region is required for dimerisation of this myosin (Cheney and Mooseker, 1995). If this domain is expressed in specific cells, dimerisation of myosin 95F may then be disturbed, which could possibly result in a dominant negative phenotype.
5.5.4 The targeted silencing technique

Targeted silencing, which combines the Gal4-UAS system and the antisense technique, has been successfully used for the study of Mhc95F function during development. This technique provides an alternative way to generate mosaics. Under the control of tissue-specific Gal4, antisense RNA is only expressed in cells in which Gal4 is expressed, thus, silencing of gene expression only occurs in these cells. This technique is useful in studying the function of a gene that plays multiple essential roles during development. By crossing the UAS-antisense gene strain with a selection of tissue specific Gal4 lines, the function of the target gene can be identified in different developmental processes. This technique also provides a means for studying the function of a gene for which mutants are not available.

Usually, the antisense transgene in flies is under the control of a heat shock (hs) inducible promoter (Ruohola-Baker, et al., 1993; Hsu et al., 1996), which allows the antisense gene to be switched on and off at a chosen developmental stage. However, heat shock allows the antisense gene to be expressed in every tissue at the time when it is applied, which differs from the tissue-specific expression of the antisense gene induced by the Gal4-UAS dual system. Theoretically, it is possible to mimic heat inducible antisense RNA expression by crossing an hs-Gal4 line with the UAS-antisense gene line. However, the hs-Gal4/UAS combination cannot be used for the study of oogenesis, as two separate hs-Gal4 lines show degeneration of egg chambers after standard heat shock (39°C, 15 min) without crossing with any UAS lines (data not shown).

The chromosomal position of a transgene insertion may influence the level of its expression, despite the fact that the transgene has its own regulatory sequences. The $P[w^+, UAS-95Fas]$ in Am8-2 is presumably expressed at a high level. Antisense RNA technique has been widely used for the study of plant genes. It is known that insertion of an antisense transgene into different chromosomal sites generally results in different levels of transgenic expression, and the expression level appears to be crucial for the function of the antisense transgene (Edwards et al., 1995; Zrenner et al., 1996).

5.5.5 Mhc95F function during development

Using different tissue-specific Gal4 lines crossed with the UAS-antisense-Mhc95F strain, Am8, lethality was observed during late embryogenesis, early larval
development and metamorphosis, suggesting that multiple roles are played by myosin 95F during development.

The malformed leg and unexpanded wing phenotype of the C532/Am8 flies demonstrates that myosin 95F is likely to be involved in regulating cell shape changes and cell movement in imaginal disc eversion during metamorphosis. This phenotype appears to be similar to mutants of the non-muscle myosin-II RLC gene, sqh (Edwards and Kiehart, 1996). The phenotypic similarity of C532/Am8 antisense flies and the sqh mutants is also found during oogenesis. sqh mutants show disruption of cyst formation, border cell migration, centripetal migration and dorsal-appendage producing cell migration (Edwards and Kiehart, 1996). It has been found that centripetal migration is disrupted in C532/Am8 egg chambers. Although direct evidence linking myosin 95F with border cell migration and dorsal-appendage producing cell migration has not yet obtained, the presence of Mhc95F transcripts in the migrating cells suggests a functional involvement of Mhc95F in these two types of follicle cell migration. However, it is noticed that the migration of follicle cells from the nurse cell chamber to cover the oocyte seems to be unaffected in both C532/Am8 and sqh mutant egg chambers (Figure 5.15E,F; Edwards and Kiehart, 1996). This may indicate that this type of follicle cell migration requires different molecular motors. Nevertheless, this observation further suggests a functional relationship exists between myosin 95F and non-muscle myosin-II.
CHAPTER 6

Functional Analysis of the Broad-Complex

During Oogenesis
6.1 Introduction

The Broad-Complex (BR-C) has been previously identified as a key gene required for *Drosophila* metamorphosis, a developmental process which is triggered by fluctuations in the titre of steroid moulting hormone, 20-hydroxyecdysone (ecdysone) at the end of larval development. A sharp rise in the concentration of ecdysone induces expression of a hierarchy of ecdysone responsive genes. The BR-C is among the "early ecdysone responsive genes" which are directly activated by the ecdysone receptor. The "early genes" then coordinate to activate transcription of the tissue-specific "late genes". As one of the "early genes", the BR-C plays a critical role in both coordinating the ecdysone response among tissues and regulating expression of effector genes (Karim and Thummel, 1992; Karim et al., 1993).

![Figure 6.1](image)

**Figure 6.1** The Broad-Complex locus and protein isoforms (adapted from Bayer et al., 1997).

**A.** BR-C transcripts synthesised from either of two ecdysone-inducible promoters, P_{distal} and P_{prox}, encode four different pairs of C_{2}H_{2} zinc-finger domains (Z1, Z2, Z3 and Z4) that are linked by alternative splicing to the common "core" exons. There are six exons in the core region. Open boxes indicate open reading frames; filled boxes indicate untranslated regions of BR-C transcripts.

**B.** The first 113 aa of the 431 aa core region shared by all BR-C proteins encode the conserved BTB domain, which is thought to mediate protein-protein interactions. Each BR-C protein isoform is distinguished by its unique linker and zinc-finger containing domains. Alternative splicing of the linker region generates three Z1 isoforms.
The *BR-C* encodes a family of C$_2$H$_2$ zinc-finger proteins (Z1, Z2, Z3, and Z4) which share a common amino terminus (the *BR-C* “core”) domain but differ in zinc-finger domains (DiBello *et al.*, 1991; Bayer *et al.*, 1996). The core contains a highly conserved amino-terminal motif, called BTB or POZ domain, which appears to be involved in protein-protein interaction and is widely distributed among metazoans (DiBello *et al.*, 1991; Bardwell and Treisman, 1994; Zollman *et al.*, 1994). The core is alternatively spliced to link with one of the four zinc-finger domains, generating four classes of proteins, the Z1, Z2, Z3 and Z4 isoforms (Figure 6.1). Additionally, three variants of the Z1 isoforms have been identified. They differ in the linker region between the core motif and the zinc-finger domain.

Expression of the *BR-C* isoforms does not seem to be tissue-specific. Most of the tissues examined express all *BR-C* isoforms. However, the relative level of each isoform changes at the different stages of metamorphosis (Bayer *et al.*, 1996, 1997).

The *BR-C* is located at the chromosomal region 2B5. Genetically, the *BR-C* locus has three fully complementing functions: *br* (*broad*), *rbp* (reduced bristle number on palpus) and 2Bc, as well as a non-complementing *npr* (nonpupariating) class (Figure 6.2) (Belyaeva *et al.*, 1980). Additionally, a number of *BR-C* alleles have been categorised to the 2Bab group. These alleles do not complement *br* or *rbp* mutations, but do complement 2Bc mutations (Belyaeva *et al.*, 1980). Kiss *et al.* (1988) have classified *br* and *rbp* to one complementation group, however, this categorisation has not been used by most other researchers (Emery *et al.*, 1995; Bayer *et al.*, 1996, 1997). The *npr* class is thought to be the null mutation, because alleles in this class fail to complement mutations in each of the three-complementing groups. They are also phenotypically indistinguishable from deletions of the locus.

```
    npr  
    |   |   |   |
  br  rbp 2Bc  
  |   |   |   |
2Bab
```

Figure 6.2 Complementation maps of *Broad-Complex* (based on Balyaeva *et al.*, 1980). The *BR-C* locus has three fully complementing functions: *br*, *rbp* and 2Bc, as well as a non-complementing *npr* class. Additionally, a number of *BR-C* alleles were categorised to the 2Bab group. These alleles do not complement *br* or *rbp*, but do complement 2Bc mutations.
It has been shown by genetic analysis that the BR-C is essential for the morphogenesis of imaginal discs. br* function is primarily required in the elongation and eversion of appendages by imaginal discs as well as tanning and hardening of the larval cuticle. rbp* function, on the other hand, is essential for muscle and bristle development. Additionally, both rbp* and 2Bc* functions are vital for histolysis of the larval tissues and gut morphogenesis. In the fusion of discs to form a continuous adult epidermis, 2Bc* function was shown to be essential (Kiss et al., 1988). All three functions are also required for the reorganisation of the CNS (Kiss et al., 1988; Emery et al., 1994). Moreover, rbp* function seems to be necessary for chorion gene amplification during oogenesis (Orr et al., 1989; Huang and Orr, 1992).

Some genetic studies suggest a one-to-one link between the specific complementing genetic functions and protein isoforms. However, other data suggests that the relationships between the complementing groups and protein isoforms are more complicated. For example, in the br28 mutant, Z3 transcripts and protein levels are reduced and all Z1 isoforms are truncated. Clearer data on these relationships were provided by Bayer et al. (1997) who showed that lethality associated with each of the complementing group was rescued using heat-inducible transgenes. It was found that br* function is only provided by the Z2 isoform. Despite this, there may be functional redundancy or regulatory dependency associated with rbp* and 2Bc* functions. It was found that Z1 transgenes provide full rbp* function, while Z4 provides partial function. The 2Bc lethality is fully rescued by Z3 protein expression, and partially rescued by Z2 protein expression.

In this chapter, I will report that the target cDNA, cB2, of the enhancer-trap line Ga4-C726b, is mapped to the BR-C locus. During oogenesis, the BR-C is expressed in the follicle cells in a dynamic pattern, which is highlighted in two groups of dorsal-anterior follicle cells at stage 10b. This dorsal-anterior expression pattern is specified by the Grk-DER and Dpp signalling pathways along the two major axes, and is associated with its function in dorsal appendage formation. The involvement of BR-C function in dorsal appendage morphogenesis was shown by mutational analysis of BR-C partial "loss-of-function" mutants, and was supported by ectopic expression of BR-C transgenes during oogenesis. It is proposed that the BR-C may provide a link between pattern formation and cell behaviour in morphogenesis.
6.2 Mapping of the cDNA clone cB2 to the BR-C locus

6.2.1 The cB2 is mapped on the chromosomal 2B5 region

The target cDNA, cB2, of the enhancer-trap line Ga14-C726b was digoxigenin labelled to hybridise to the polytene chromosome squashes. A single hybridisation band at the distal end of the X chromosome was observed. This band, located at an ecdysone-induced puff, was identified to be the 2B5 chromosomal region (Figure 6.3A).

![Figure 6.3](image)

**Figure 6.3** Mapping of cB2 into the Broad-Complex locus.

A. Polytene chromosome in situ hybridisation shows that cB2 has a single hybridisation band at chromosomal position 2B5.

B. Deficiency mapping of cB2 to the Broad-Complex locus. Southern hybridisation shows that no band is present in a double deficiency line which removes the BR-C locus, while a 1.4-kb band is present in all control lines (B1). B2. Using a control probe from the third chromosome to detect the same membrane, a hybridisation band is found in the double deficiency line, indicating the DNA used in this experiment is reliable.
6.2.2 Deficiency mapping of the cB2 to the Broad-Complex locus

The 2B5 chromosome is a genetically and molecularly well-characterised region (Belyaeva, et al., 1980; Dibello, et al., 1991; Lindsley and Zimm, 1992). The Broad-Complex (BR-C), which occupies about 100-kb of chromosomal DNA in this region (Figure 6.4; Chao and Guild, 1986; Dibello, et al., 1991), appears to be a good candidate target gene for cB2. In order to determine this, the DNA from the third instar larvae of a double deficiency line, Df(1)S391Dp(1;Y)ySz280, which removes the entire BR-C gene, was digested by EcoRI and Southern blotted onto a nylon membrane. This membrane was incubated with the 32P-labelled CB2 probe. No signal was detected in

![Diagram of the 2B chromosome and the Broad-Complex exons](image)

Figure 6.4 Diagram of the 2B chromosome and the Broad-Complex exons (adapted from Chao and Guild (1986); C. Bayer, personal communication). Only EcoRI (R) sites are shown in part of the map (160kb-190kb). The map is oriented left to right, telomere to centromere. A coordinate scale in kb has been set with zero as the most telomeric sequence. The P[GawB] insertion site (arrowhead) and cB2 cDNA (open box) are shown on the map.
he double deficiency line, whereas a 1.4-kb band appeared in all control lines (Figure 6.3B). Subsequently, when a third chromosomal probe was used to hybridise the same membrane, a hybridisation band was detected in the DNA of the double deficiency line, suggesting that the DNA used in this experiment was intact. These data clearly demonstrate that cB2 is part of the \textit{BR-C} locus.

### 6.2.3 Further mapping of the cB2 within the \textit{BR-C} locus

In order to further map cB2 within the \textit{BR-C} locus, the physical map of cloned genomic fragments which contain the cB2 sequence, was compared with the published physical map of the 2B chromosome (DiBello \textit{et al.}, 1991; Figure 6.5). It was found that these cloned genomic fragments have similar physical map to the 170-180 kb chromosomal region. Therefore, two lambda phage clones, \(\lambda 192\) and \(\lambda 193\), which are known to be mapped to this chromosomal area (kindly provided by C. Bayer; DiBello \textit{et al.}, 1991) were used to localise the cB2. Using the \(^{32}\text{P}-\text{CB2}\) probe to hybridise the Southern membrane, a 1.4-kb hybridisation band was observed in the lane which was loaded with the \textit{EcoRI} digested \(\lambda 193\) DNA (Figure 6.5). This 1.4-kb \textit{EcoRI} fragment is mapped to the 173-175-kb chromosomal region, which is about 2-kb away from the 5' end of \textit{BR-Core} coding region, and is about 6-kb from the 3' end of exon 4, which includes the \textit{BR-C} proximal promoter (P167) (see Figure 6.4).

**Figure 6.5** Mapping of cB2 to \(\lambda 193\). Using \(^{32}\text{P}\)-labelled cB2 as a probe, a 1.4-kb \textit{EcoRI} band is detected in both \(\lambda 193\) and \(\lambda B6\); the latter is used as a positive control.
The fact that the cB2 is part of the BR-C gene has been confirmed through expressional analysis. Whole-mount RNA in situ hybridisation has shown that both the cB2 and BR-C have exactly the same expression pattern during oogenesis. The expression pattern of the BR-C will be discussed in the next section.

6.3 The BR-C expression pattern during oogenesis

6.3.1 The BR-C RNA distribution pattern during oogenesis

Digoxigenin labelled DNA probes from the BR-C core domain and the four distinctive zinc-finger isoforms, Z1, Z2, Z3, and Z4, (kindly provided by C. Bayer) were used to detect BR-C expression during oogenesis. Z1 is the only zinc-finger isoform is expressed at detectable levels by whole mount in situ hybridisation during oogenesis. Its expression pattern mirrors that of the core domain (hereafter called the BR-C expression pattern). Expression of the BR-C is first detected in all follicle cells at stage 6 of oogenesis (Figure 6.6A). At stage 10a, it is expressed in all columnar cells but absent in the dorsal-anterior most region, facing the oocyte nucleus (Figure 6.6B, C). The expression is stronger in the follicle cells close to the anterior-dorsal region of the oocyte, and becomes gradually weaker posteriorly and ventrally, diminishing at the posterior pole (Figure 6.6B). At stages 10b and 11, BR-C expression disappears entirely in the ventral and posterior follicle cells, and remains in just two groups of dorsal-anterior follicle cells which lie symmetrically at either side of the dorsal midline (hereafter called the BR-C late expression pattern) (Figure 6.6D, E). These follicle cells, which are thought to be the progenitor cells of the dorsal appendages, include about 55-65 cells in each group and are 2 cells away from the dorsal midline and 2-3 cells from the centripetal cells (Figure 6.6C, E).
Figure 6.6 The wildtype BR-C expression pattern during oogenesis.
A. Expression of the BR-C is first detected in all follicle cells surrounding the stage-6 egg chamber.
B and C. A stage-10a egg chamber shows BR-C expression in all columnar cells except the dorsal-anterior region. Stronger expression is detected in follicle cells close to the dorsal-anterior gap. Its expression is also missing at the posterior pole (B).
D and E. During stage 10b, the BR-C is expressed in two groups of lateral-dorsal-anterior follicle cells, which are about 2 cells away (arrowheads) from the dorsal midline and 2-3 cells away (curved line) from the centripetal cells.
6.3.2 The BR-C Protein distribution pattern during oogenesis

In order to determine whether the BR-C protein is distributed in a similar pattern to the RNA expression pattern during oogenesis, antibodies which recognise the BR-core, Z1 or Z3 domains respectively (Emery et al., 1994) were used to stain the whole-mount ovaries. Both the Z1 and BR-core antibodies exhibited similar staining patterns, while the Z3 antibody showed no staining during oogenesis. These observations are consistent with the result shown by RNA in situ hybridisation, that Z1 is the only zinc-finger isoform whose expression can be detected during oogenesis.

The distribution pattern of the BR-C protein appears to be similar to that of its RNA during stages 6-8 of oogenesis, when all follicle cells have been stained (Figure 6.7A1). The protein is also detected in all columnar cells except the dorsal anterior cells at stage 10 (Figure 6.7A1-A3), similar to the pattern of RNA distribution (Figure 6.6B,C). However, the follicle cells at the posterior pole appear to be stained at this stage (Figure 6.7A1), which differs from the RNA distribution pattern (Figure 6.6B). In contrast, the late distribution pattern of the BR-C protein appears to be quite different to that of its RNA. Although a very strong signal is observed in two groups of the lateral-dorsal follicle cells at stages 11 and 12, the posterior and ventral follicle cells are still stained (Figure 6.5B). The signal in the posterior and ventral region disappears until around late stage 13, leaving only the dorsal appendage associated follicle cells stained (Figure 6.5D). The differences between the distribution patterns of the protein and RNA are presumably due to the fact that the half-life of the protein is much longer than that of the RNA. By the time late BR-C transcription occurs at the lateral-dorsal-anterior follicle cells, the protein translated from the early BR-C transcripts remains at the posterior and ventral side, whilst the RNA has been cleared. Thus, the early and late protein distribution patterns overlap to form a gradient-like pattern at stages 11-12. The same reasoning could also be used to explain why the protein, but not the RNA, is detected in the follicle cells at the posterior pole during stage 10.

Another feature of the BR-C protein distribution is that it only appear in the nuclei of the follicle cells (Figure 6.7A1-D). This is consistent with the fact that the BR-C encodes transcription factors.
Figure 6.7 The distribution pattern of the BR-C protein during oogenesis. A1. Using BR-core antibody to stain the ovaries, signals are initially detected in all follicle cells at around stage 6 (arrowhead). Staining is also observed in all follicle cells at stage 7. During stage 10, columnar follicle cells over the oocyte are stained. However, staining is not seen at the dorsal anterior region. A2 shows a close look at the dorsal region. The dorsal gap is marked by a double headed arrow, while the anterior gap is labelled by two curved lines. A3 shows a ventral view of the same egg chamber. It appears that all ventral columnar cells are stained. B. During stage 12, two groups of lateral-dorsal-anterior follicle cells are heavily stained, while the posterior and the ventral follicle cells are weakly stained. The dorsal gap between the two groups of the lateral-dorsal cells still exists (arrow). Expression in the posterior follicle cells becomes weaker and weaker (C), until it disappears at about stage 13 (D). Expression is only detectable in the dorsal appendage associated follicle cells. The arrow shows a growing dorsal appendage (D).
6.4 Functional analysis of the BR-C during oogenesis

6.4.1 The BR-C is required for dorsal appendage morphogenesis

Both RNA in situ hybridisation and antibody staining showed that the BR-C is expressed in the dorsal appendage producing follicle cells. Does this expression pattern reflect a function in dorsal appendage formation? In order to address this question, viable combinations of the BR-C mutants are required.

A47 is a mutant generated by mobilising the P[Gal4] in the starting enhancer-trap line, C726b. Complementation analysis showed that A47 fails to complement npr, br and rbp, but complements 2BC (Table 6.1; Figure 6.8). Thus, A47 is mapped to the 2Bab complementing group. A47 was used to cross with a weak BR-C allele, br'. The female progeny, br'/A47, survived until adulthood and produced eggs with reduced dorsal appendages (Figure 6.9A), when compared to the long appendages in the wildtype egg (Figure 6.9B). Normal embryos developed in these eggs and hatched as first instar larvae. Since progeny from the cross of the BR-C null allele, npr', with br' can also survive until adulthood (Kiss et al., 1988), the eggs produced by br'/npr' females were also examined. These eggs exhibited a similar phenotype to the eggs produced by the br'/A47 mothers, and had reduced dorsal appendages (data not shown). These observations, along with the report that another BR-C mutant, br'12/br'12, produces "appendageless" eggs (Huang and Orr, 1992), indicate that BR-C function is required for dorsal appendage morphogenesis during oogenesis.

<table>
<thead>
<tr>
<th>BR-C mutants*</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>npr1'</td>
<td>(-) lethal</td>
</tr>
<tr>
<td>br'</td>
<td>(-) viable with broad wing in adults (Figure 6.8)</td>
</tr>
<tr>
<td>br2'</td>
<td>(-) lethal</td>
</tr>
<tr>
<td>rbp'</td>
<td>(-) lethal</td>
</tr>
<tr>
<td>2Bc'</td>
<td>(+) viable</td>
</tr>
</tbody>
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* the BR-C mutants are described in Chapter 2. A47/FM6 were mated to BR-C/y6719.1 males to generate trans-heterozygotes and more than 25 female progeny were tested.

(+, complementation; -, non-complementation.)
Figure 6.8 $A47$ fails to complement $br^t$. A. Shows a normal wildtype wing. B. The $A47/br^t$ wing exhibits the broad wing phenotype, which is shorter than the wildtype. C. The $npr^6/br^t$ wing exhibits a more severe phenotype than the $A47/br^t$ wing.

Figure 6.9 The $BR-C$ is required for dorsal appendage formation. A. The wildtype eggshell has a pair of dorsal appendages secreted at the dorsal-anterior region of the egg. B. A $br^t/A47$ stage-14 egg shows reduced dorsal appendages.
6.4.2 $rbp^+$ function is required for dorsal appendage formation

It was observed that eggs produced by $br'/A47$, $br'/npr^6$ and $br^{de12}/br^{de12}$ mothers have reduced dorsal appendages, suggesting that the $br$ functional domain seems likely to be required for dorsal appendage formation. However, this is contradictory to the observation that $Z1$, which provides the full $rbp$ function (Bayer et al., 1997), is the only zinc-finger isoform that has detectable levels of expression in oogenesis by RNA in situ hybridisation. In order to verify whether the $br$ functional domain is involved in dorsal appendage formation, eggs produced by $br'/br'$ and $br^6/br'$ females were examined, and found to have normal dorsal appendages. This observation, along with the fact that the $br^{de12}$ is actually an $rbp$ allele (Huang and Orr, 1992), indicates that the $br$ functional domain is not involved in dorsal appendage formation.

How can the phenotype of $A47/br'$ and $npr^6/br'$ eggshells be explained if $br$ is not the functional domain required for dorsal appendage formation? This could be understood if the $br'$ mutant not only affected $br$ function, but also affected $rbp$ function. In order to test this possibility, the eggshell phenotype in the $br'/rbp'$ mutant was examined. It was shown that eggs produced by the $br'/rbp'$ mothers have reduced dorsal appendages, similar to those produced by the $br'/A47$ females. This, therefore, indicates that the $br'$ is in fact a weak $2Bab$ allele, which fails to complement either $rbp$ or $br$ function. Moreover, this also suggests that $rbp$ is the functional domain involved in dorsal appendage formation during oogenesis, consistent with the observation that $Z1$, which provides full $rbp^+$ function, is the only zinc-finger isoform detected in oogenesis.

6.4.3 Ectopic $BR-C$ expression induces ectopic dorsal appendage material

Is $BR-C$ function sufficient to direct the formation of the dorsal appendages? To address this, the heat-inducible $Z1$ transgenic flies ($hsp70/Z1$) were used to examine the effect of ectopic $BR-C$ expression during oogenesis. Following standard heat shock ($37^\circ C$, 30min) and incubation at $25^\circ C$ for 7 to 24 hours, ovaries of the $hsp70/Z1$ females were dissected. It was observed that extra dorsal-appendage material was produced in the dorsal-anterior region of the eggshells (Figure 6.10B,C). In most cases, dorsal appendage material appeared in the dorsal-gap between the two appendages, and the basements for the dorsal appendages in these eggshells are larger than those in the wildtype eggshell (Figure 6.10A). The dorsal appendages could not elongate properly,
which resulted in a stack of dorsal appendage material in the dorsal-anterior eggshell (Figure 6.10B,C). These observations indicate that the ectopic Z1 can induce formation of the ectopic dorsal appendage material. Nevertheless, the ectopic dorsal appendage material is restricted in the dorsal anterior eggshell, suggesting that the fate of the follicle cells is pre-determined along the two major axes prior to the requirement for BR-C function.

Z1 seems to be the sole BR-C zinc-finger isoform functioning during oogenesis. Thus, oogenesis provides an ideal model to investigate functional diversity of the four different zinc-finger isoforms. In order to test if Z2, Z3 and Z4 exhibit a similar phenotype when ectopically expressed during oogenesis, hsp70/Z2, hsp70/Z3 and hsp70/Z4 flies were heat-shocked and the eggshell phenotype was examined. It was found that ectopic dorsal appendage material is produced in the dorsal-anterior region of the eggshells produced by all three transgenic flies (Figure 6.10D,E,F). This phenotype is similar to that exhibited by eggs produced by the hsp70/Z1 flies after heat-shock, indicating that all of the four zinc-finger isoforms may be functional in dorsal appendage formation during oogenesis.

Additionally, ectopic BR-C expression appears to induce pre-mature production of the chorion. Figure 6.10G1 and G2 show that the chorion is already visible in stage 12 egg chambers; and this appears to have blocked the dumping of cytoplasm from the nurse cells to the oocyte (Figure 6.10G1,G2). This observation is consistent with the report that a mutation in the BR-C locus cause pre-mature arrest of chorion gene amplification (Huang and Orr, 1992).
Figure 6.10 Ectopic expression of different BR-C zinc-finger isoforms during oogenesis. A-F, dark field microscopy; G1, DCl microscopy; G2, DAPI staining.

A. Shows a wildtype egg after heat-shock treatment. B, C. Two distinctive hsp70-Z1 transgenic lines exhibit a similar phenotype. Dorsal appendage materials are present in the dorsal most region, resulting in fused, thickened and shortened dorsal appendages. D. hsp70-Z2 shows a similar, but less severe eggshell phenotype. Fused, thickened and shortened appendages are also found in eggshells produced by the Z3 and Z4 transgenic lines (E, F). G1 and G2 show that transgenic Z1 also causes pre-maturation of chorion synthesis, and this blocks dumping of the nurse cell cytoplasm into the oocyte during stage 11-12 of oogenesis.
6.5 The *BR-C* is downstream of the Grk-DER signalling pathway

6.5.1 The *BR-C* expression is regulated by Grk-DER signalling

Since the *BR-C* is expressed in an asymmetrical pattern along the DV axis, it is interesting to find out whether its expression in dorsal-lateral follicle cells is a consequence of Grk-DER signalling. This has been studied by investigating *BR-C* expression in mutants that affect this signalling pathway. *fs(1)K10* is required for the localisation of *grk* RNA to the dorsal-anterior region of the oocyte at stage 8 (Cheung *et al.*, 1992). In *fs(1)K10′* mutant, *grk* RNA is mis-localised to form a ring in the anterior oocyte (Roth and Schüpbach, 1994; also see Figure 1.3B). This induces a *BR-C* late expression pattern which is expanded to the ventral follicle cells surrounding the oocyte. However, the lack of dorsal *BR-C* expression in the follicle cells still exists in this mutant (Figure 6.11C). Since the *BR-C* is required for dorsal appendage formation, it is suggested that ventrally expanded expression of *BR-C* is the reason for the ventral expansion of the dorsal appendages in the *fs(1)K10′* mutant (Figure 6.11D). In strong *grk* mutants, in which the Grk signal is absent, the localised *BR-C* expression in the lateral-dorsal-anterior follicle cells cannot be detected, and no dorsal appendages are generated in these mutants (data not shown). Nevertheless, the early *BR-C* expression in all follicle cells at stage 6 does not seem to be affected in the strong *grk* mutant. Taken together, these data indicate that *BR-C* late expression is downstream of Grk-DER signalling in DV polarity formation, and an intermediate in dorsal appendage morphogenesis.

6.5.2 The *BR-C* responds to Grk-DER signalling in a dose-dependent manner

Why is *BR-C* expression missing in the dorsal-most follicle cells in which the highest dose of Grk signal is supposed to have been received (Figure 6.12A,B)? If the *BR-C* responds to Grk-DER signalling in a dose dependent manner, increases or decreases of the signal should shift the *BR-C* late expression pattern along the DV axis. In order to test this, *BR-C* expression was examined in the transgenic flies with four extra copies of *grk* gene in the genome (Neuman-Silberberg and Schüpbach, 1994). It was found that the dorsal gap between the two groups of *BR-C* expressing cells is enlarged to about 8 cells wide (Figure 6.11E), in comparison with the 4-cell-wide dorsal gap in the wildtype (Figure 6.11A). Consequently, the dorsal gap between the two dorsal appendages is widened in the mature egg (Figure 6.11F). The expansion of the
Figure 6.11. Expression of the \textit{BR-C} is dependent on the Grk-DER signalling pathway. A,C,E,G,I, Whole-mount in situ hybridisation of a \textit{BR-C} probe to ovaries. B,D,F,H,J, Corresponding dorsal appendage phenotype. The \textit{BR-C} late expression pattern in wildtype ovaries includes two groups of cells separated by a 4-cell-wide dorsal gap (A). The wildtype egg has two long dorsal appendages (B). C. In the \textit{fs(1)K10}\textsuperscript{f} mutant, \textit{BR-C} expression is expanded to the ventral side and fused ventrally, but the dorsal gap remains (arrow), which results in ventrally fused dorsal appendages (D); notice that the dorsal gap exists. In transgenic flies with 4 extra copies of the \textit{grk} gene, the dorsal gap between the patches of \textit{BR-C} late expression is expanded to about 8 cells wide (E), which leads to the expansion of the gap between the two dorsal appendages (F). G. In \textit{top}\textsuperscript{D7}, \textit{BR-C} expression fuses at the dorsal-most region, only one group of cells is stained, and results in dorsally fused dorsal appendages (H). I. Ectopic \textit{pnt} expression down regulates \textit{BR-C} expression in dorsal appendage progenitor cells when \textit{pntP2} is expressed in most follicle cells at stage 9 (\textit{UAS-pntP2} is driven by a \textit{Ga14} driver \textit{C532}, which shows expression in most follicle cells at stage 9; see Figure 6.12C). \textit{C532/UAS-pntP2} results in a reduced number of cells expressing the \textit{BR-C} (I), which in turn leads to thinner and shorter dorsal appendages (J).
Functional Analysis of the Broad-Complex
dorsal gap is due to the shift of the BR-C expressing cells ventrally, since the number of cells in each group is approximately the same as in the wildtype. In contrast, decreases of Grk-DER signalling in a top^Q^ mutant (Schüpbach, 1987) result in BR-C expression in the dorsal most follicle cells (Figure 6.11G). The two groups of BR-C expressing cells shift dorsally and fuse to form one group along the dorsal midline (Figure 6.11G). This leads to the formation of one dorsal appendage in the dorsal most region (Figure 6.11H). Taken together, these data indicate that the absence of BR-C expression in the dorsal most region is due to the high dose of Grk-DER signalling. They suggest that if the signal is higher than a certain level, it represses rather than activates BR-C expression. In the fs(1)K10^I^ egg chambers, the Grk signal is stronger in the dorsal corner than in the rest of the anterior ring (Roth and Schüpbach, 1994; also see Figure 1.3B), and this could explain why BR-C expression is still absent in the dorsal most follicle cells when it expands ventrally (Figure 6.11C). In order to further test the dose-dependency on the Grk-DER signalling pathway, BR-C expression and the eggshell phenotype were examined in egg chambers that have increased levels of a constitutively activated Draf, a serine/threonine kinase downstream of Grk and DER in the signalling pathway (Brand and Perrimon, 1994). It was found that when Draf is highly expressed in most of the follicle cells at stages 9 and 10 (a Gal4 driver C532, crossed with a UAS linked gain-of-function Draf gene, UAS-Draf^G^, Brand and Perrimon, 1994; Deng et al., 1997), about 90% of the mature eggs have no dorsal appendages, and the BR-C late expression pattern could not be detected in the majority of the stage 10b-11 egg chambers (data not shown).

6.5.3 Ectopic expression of Pnt decreases the number of BR-C expressing cells

What is the link between the high dose Grk-DER signalling and the repression of BR-C expression in the dorsal most follicle cells. An ideal candidate is pointed (pnt), an ETS domain transcription factor, found downstream of the Ras signalling pathway in a number of developmental processes (Klämbt, 1993; Brunner et al., 1994). During stages 9-10a of oogenesis, both isoforms of pnt (pnt^P1^ and pnt^P2^) are expressed in the dorsal-anterior most columnar cells in response to the Grk-DER signalling pathway (Morimoto et al., 1996). Ectopic pnt expression in all anterior columnar cells resulted in eggs with reduced dorsal appendages (Morimoto et al., 1996). In order to determine if pnt is the repressor of BR-C expression, BR-C expression was examined in egg chambers with ectopic pnt^P2^ in most of the follicle cells at stage 9-10 (both UAS-pnt^P1^ and UAS-pnt^P2^ were crossed with Gal4-C532, but only C532/UAS-pnt^P2^ survived until adulthood). The reporter gene expression pattern of Gal4-C532 is shown in Figure
6.12C, where it has been crossed to a *UAS-lacZ* line. There are about 10-15 follicle cells expressing *BR-C* at both sides of the dorsal middle line at stage 10b (Figure 6.11I), 4-5 times less than in wildtype. The dorsal appendages produced in these flies are thinner and shorter than the wildtype (Figure 6.11J). These observations indicate that *pnt* is a mediator of the high dose Grk-DER signalling in the dorsal-anterior most follicle cells, acting to repress the expression of *BR-C*. The remaining *BR-C* expression in a small number of follicle cells is probably due to the fact that the *Gal4* reporter gene is not expressed uniformly in *Gal4-C532*, as shown in Figure 6.12C that some follicle cells fail to show β-galactosidase staining while their neighbouring cells are stained.
6.6 BR-C expression along the AP axis is regulated by Dpp signalling

An hypothesis for why BR-C expression is also missing in the anterior most columnar cells in the dorsal side is that there is probably another repressor produced in these cells. A good candidate is a downstream gene of the Dpp signalling pathway (Padgett et al., 1987), which is required for anterior eggshell patterning (Twombly et al., 1996). If Dpp signalling acts as a repressor of BR-C expression in oogenesis, a decrease in Dpp levels should result in expression of the BR-C in the anterior most columnar cells, where it is absent in the wildtype eggshell. In order to determine if this is the case, a temperature sensitive mutant, dpp^{kr56}/dpp^{ts}, was used to examine the BR-C expression pattern. The mutants were raised at 18°C and then shifted to restrictive conditions after the flies eclosed. As predicted, the anterior most columnar cells express the BR-C when the level of Dpp is decreased in stage-10b mutant egg chambers (Figure 6.13A). Since the anterior frontier of the BR-C late expression pattern in the wildtype is about 2-3 cells from the Dpp source in the centripetal cells, Dpp could act as a morphogen here. Conversely, when Dpp is ectopically expressed in most of the follicle cells at stage 9 (Gal4-C532 crossed with a UAS-dpp) (Figure 6.12C), BR-C expression is found over the middle part of the oocyte at a more posterior position in the dorsal side when compared to the wildtype egg chambers (Figure 6.13C). As a result, the dorsal appendages are also generated at a more posterior site (Figure 6.13D). Additionally, the ectopic expression of Dpp also leads to the disruption of the bilaterally symmetrical expression pattern of BR-C. It was often observed that three or more groups of the follicle cells expressing the BR-C, which results in the formation of three or more malformed dorsal appendages (Figure 6.13D). Taken together, these results indicate that late expression of the BR-C is specified by Dpp along the AP axis. It is probably via an indirect route, because ectopic expression of Dpp in most of the follicle cells does not eliminate BR-C expression.
Figure 6.12. (Left panel) The Grk localisation pattern in Oregon R and the reporter gene expression pattern in Gal4-C532. A,B. Anti-Grk antibody staining. A. A dorsal view focused in the apical region of the oocyte shows that the high doses of the Grk signal are received by the dorsal-anterior most follicle cells facing the oocyte nucleus. B, A lateral view focused in the oocyte to show that the Grk protein is localised at the dorsal-anterior corner in a stage-9 oocyte. C, β-galactosidase staining in Gal4-C532/UAS-lacZ shows that expression of the reporter gene is detected in most follicle cells at stage-9; however, staining is not uniformly distributed, and some follicle cells are not stained.

Figure 6.13. (Right panel) Expression of the BR-C along the AP axis is regulated by Dpp. A, In dpp<sup>1rs56/dpp<sup>87> stage-10b egg chambers, BR-C expression is detected in the anterior most columnar follicle cells, which leads to the dorsal appendages secreted at a more anterior position (B) when compared to the wildtype (see Figure 6.11B). C. BR-C late expression is detected at a more posterior position in Gal4-C532/UAS-dpp egg chambers when dpp is ectopically expressed in most columnar cells, as compared to the wildtype (see Figure 6.6D,E). D. Gal4-C532/UAS-dpp produces eggs with more posteriorly located and malformed dorsal appendages; sometimes, more than two chorionic appendages have been produced in one egg.
Functional Analysis of the Broad-Complex
6.7 DISCUSSION

6.7.1 The \textit{BR-C} expression pattern and eggshell patterning

The data presented in this chapter suggests that the follicle cell and eggshell pattern, which is partly dependent upon the late expression pattern of the \textit{BR-C}, requires signals from both the germline and somatic follicle cells. The patterning along the DV axis is specified by Grk-DER signalling from the germline in a dose dependent way, as the dorsal-anterior columnar cells respond differently, according to how much signal they receive. The Dpp signalling pathway is required for the patterning of the eggshell along the AP axis. These two signalling pathways, one from the oocyte and the other from the follicle cells, coordinately specify patches of follicle cells to express the \textit{BR-C} in a unique position in respect to both major axes. This, subsequently, directs the differentiation of dorsal appendages in the correct position on the eggshell. Hence, the interaction of these two signalling pathways can divide the dorsal-anterior columnar follicle cells into at least three different subgroups: \textit{BR-C} late expression marks the dorsal appendage forming cells in the lateral-dorsal-anterior region; follicle cells anterior to them form the operculum and other anterior structures; follicle cells dorsal to them form the gap between the two appendages (Figure 6.14).

\textit{BR-C} expression in the lateral-dorsal cells is induced by a medium concentration of Grk-DER signalling. Higher concentrations of Grk signal seem to induce another transcriptional factor Pnt, which is likely to be a repressor of \textit{BR-C} in the dorsal midline cells. It has been reported that the ectopic expression of \textit{pntP1} but, not \textit{pntP2}, results in reduced appendage material (Monmoto \textit{et al.}, 1996). This is different from the observations shown in this Chapter. The reason for this is probably that the \textit{Gal4} driver used in this experiment has higher level of \textit{Gal4} expression in the columnar cells. This may signify that \textit{pntP1} and \textit{pntP2} are functionally redundant but work at different concentrations.

Is the \textit{BR-C} under the control of ecdysone pathway during oogenesis, as it is during metamorphosis? The answer could be yes, because expression of the \textit{BR-C} observed in all follicle cells at stage 6 apparently is not induced by the Grk-DER signalling pathway, and expression of the ecdysone receptor (EcR) is detected in all follicle cells during the same stage (data not shown). This coincidence might imply that the earliest \textit{BR-C} expression is mediated by the ecdysone pathway. However, the \textit{BR-C} late expression pattern in dorsal-lateral follicle cells is unlikely to be induced by
ecdysone, since the mis-localisation of Grk signal in the ventral-anterior oocyte is sufficient to induce the ectopic expression of BR-C in the ventral follicle cells in egg chambers mutant for fs(1)K10.

Figure 6.14 A model for specification of BR-C expression in the lateral-dorsal-anterior follicle cells. The anterior-dorsally localised Grk signal from the oocyte induces the follicle cells to express different genes in a dose-dependent way. The dorsal most cells, which receive the highest Grk signal, express pnt, which appears to be a repressor of BR-C expression. Thus, the BR-C, which also responds to the grk signalling pathway, is expressed in lateral-dorsal follicle cells. Along the AP axis, the Dpp morphogen from the centripetal cells activates an unidentified repressor to inactivate the BR-C in the anterior most columnar cells. The interaction of these two signalling pathways specifies localised expression of the BR-C in the lateral-dorsal-anterior follicle cells and divides the columnar cells into different subgroups.
6.7.2 The relationship between dorsal/ventral patterning in the eggshell and embryo

The DV polarities of both the embryo and eggshell are initiated by the dorsal-anteriorly localised Grk. The downstream genes, Ras1, Draf and Mek, which are involved in transmitting the signal, are involved in the formation of both these DV polarities. However, the defects caused by mutation or mis-expression of these signal-transmitting genes are more severe in the eggshell patterning than in the embryonic patterning when compared with the grk and top/DER mutants. This led to the hypothesis that another signalling pathway may be involved in the transmission of the DV signal (Schnorr and Berg, 1996).

In addition, the existence of some mutants which only affect the embryonic DV pattern formation indicates that there is a branch of the DV patterning pathway used only for eggshell polarity. It is suggested that the BR-C, which only affects the eggshell pattern when it is mutated during oogenesis, is an effector gene for this branch. Ectopic expression of the BR-C isoforms during oogenesis only transforms the fate of the dorsal most follicle cells, which are located between the two groups of appendage producing cells, indicating that BR-C function is only required after the dorsal-ventral polarity is established in the follicle cell epithelium. Another transcription factor, Pnt, which is expressed in the dorsal-midline follicle cells could also be part of this branch, because no embryonic defects have been reported when it is mis-expressed in oogenesis. BR-C and Pnt may antagonise each other in the dorsal-anterior follicle cells. A increase of the Pnt level results in the reduction of the dorsal appendages, whilst an increase in BR-C levels induces the production of extra dorsal appendage material.

Additionally, a ventrally expressed transcription factor, CF2, has been shown involved in both eggshell and embryonic DV pattern formation (Hsu et al., 1996). Therefore, there should be a transcriptional repressor of CF2 in the dorsal follicle cells downstream of the Grk-DER signalling pathway. This transcriptional repressor must be involved in the establishment of DV polarities of both the embryo and eggshell, and remains to be identified.

6.7.3 Dpp is involved in anterior/posterior patterning in the eggshell

The dorsal-anteriorly located appendages also mark the AP polarity of the eggshell. Their position is induced by the Grk-DER signalling pathway, thus the AP
position of the appendages in the eggshell is originally established by the Grk signal. It is suggested that this position is further refined by Dpp signalling, which is initiated in the nearby centripetal cells. Dpp signalling is directly involved in the specification of the anterior-most columnar cells and anterior eggshell structure, the operculum (Twombly, 1996). However, the regulation of the AP position of BR-C late expression and the subsequent AP position of the dorsal appendages seems to be indirectly controlled by Dpp signalling. Cells have to choose between the columnar cell fate and the appendage producing cell fate. When Dpp levels decrease, the number of anterior columnar cells decreases, and this leads to the BR-C being expressed in a more anterior position when compared with the wildtype. Conversely, the ectopic expression of Dpp in most of the follicle cells at stage 9 leads to the expansion of anterior-follicle cell fate. Consequently, BR-C expressing cells are pushed towards the posterior. However, the posterior follicle cells seem to be predetermined, probably by the Grk-DER signalling pathway during the early stages of oogenesis when the AP axis is initiated (González-Reyes et al., 1995; Roth, et al., 1995), and this may define the posterior frontier of the appendage-producing cells, which are marked by BR-C expression. Therefore, BR-C expressing cells are squeezed to form a curved line over the oocyte at a more posterior position than in the wildtype egg chambers.

The Dpp signalling pathway is widely used during development, including dorsal epidermal determination during embryogenesis, as well as wing, leg and eye-antennal disc patterning. A number of genes downstream of Dpp have been identified in other developmental processes. It has been reported that the Dpp receptor, saxophone is also involved in eggshell patterning (Twombly et al., 1996). A preliminary search for the target genes has been undertaken in the follicle cells by examining the expression pattern of some known Dpp downstream genes used during wing morphogenesis, including spalt, spalt-related and omb (Lecuit et al., 1996; de Celis et al., 1996). However, they may not be the targets of Dpp in follicle cell patterning, since no β-galactosidase expression is detected in oogenesis in enhancer-trap lines containing P-lacZ insertion in these genes (data not shown). It seems likely there are other genes downstream of Dpp in follicle cell patterning. These remain to be identified.

6.7.4 The BR-C complementing groups and zinc-finger isoforms

It has been shown by genetic analysis that rbp+ function is required for dorsal appendage formation, and it was observed that Z1 is the sole zinc-finger isoform expressed in the appendage-producing cells. These observations are compatible with the report that Z1 provides rbp+ function (Bayer et al., 1997). It was also found that rbp+
function is partially provided by Z4, but is not provided by Z2 and Z3, (Bayer et al., 1997). However, heat-shock induced expression of all four zinc-finger isoforms (Z1, Z2, Z3 and Z4) leads to a similar phenotype that extra dorsal appendage material is produced in the dorsal-gap, indicating that the functional diversity of the four zinc-finger isoforms may not be dramatic. They may substitute for each other functionally in dorsal appendage formation.

The homozygous viable mutant, br', is the first mutant identified in the BR-C locus. It exhibits a broad wing phenotype and fails to complement other mutants which are categorised in the br complementing group. However, complementation analysis shown in this chapter suggests that the br' mutant also partially loses rbp function. Therefore, it is in fact a 2Bab allele. It is known the 2Bab mutations cause reduction of both Z1 and Z2 expression. Thus in the br'/A47 and br'/npr6 females, both Z1 and Z2 are reduced. The reduction of Z1 levels results in the reduction of dorsal appendages, while no effect produced by the reduction of Z2 levels. This is why no defects were observed in eggs produced by br'/br5 and br'/br6 females.

6.7.5 The BR-C is involved in morphogenesis

Genetic analysis of the partial “loss-of-function” BR-C mutants indicates that dorsal appendage morphogenesis requires the BR-C function. This has also been supported by the examination of BR-C expression in different mutants that affect DV patterning. Furthermore, ectopic expression of the BR-C transgene induces extra dorsal appendage material at the dorsal-gap, and the dorsal appendages fail to elongate properly.

The BR-C is found to be involved in morphogenetic movement during metamorphosis. A few target genes of the BR-C, including Sgs4, Ddc and Fbp2, have been identified in larval and pupal tissues (Bayer et al., 1997). Other target genes could include actin-binding proteins, because the morphogenetic movements require cell shape changes, which result from the rearrangement of the actin cytoskeleton within the cells. The non-muscle myosin-II gene, zipper, is a good candidate gene, since it has been shown to be involved in morphogenetic movements associated with dorsal appendage formation (Edwards and Kiehart, 1996). Strikingly, zipper has been identified as the Enhancer of broad (E(br)) locus during imaginal disc morphogenesis. E(br)/+, in br' flies, interferes with appendage elongation during metamorphosis (Gotwals and Fristrom, 1991; Fristrom and Fristrom, 1993). These observations may suggest a
conserved regulatory pathway mediated by the Broad-Complex involved in morphogenetic movements throughout development.

6.7.6 The BR-C and the evolution of eggshell morphology

The discovery of the requirement for BR-C function in dorsal appendage formation and the role BR-C links pattern formation and morphogenesis, indicating that the BR-C may provide a useful tool to study eggshell morphogenetic evolution in Drosophilinae. Sturtevant (1921) reported that variable numbers of dorsal filaments are present in different Drosophilinae species (Table 6.2). For example, D. melanogaster has two appendages, while D. hydei has four, whilst other species have 3, 8, or 10 appendages, suggesting that the number and size of dorsal appendages has evolved rapidly. In this paper, we show that the size and number of dorsal appendages is modified when the size and number of groups of BR-C expressing cells is changed (Figure 6.11J, Figure 6.13D). Therefore, the modification of the regulation of BR-C expression in the dorsal-anterior follicle cells may be the driving force for this morphogenetic evolution. By studying the mechanisms regulating the BR-C expression in a small number of representative Drosophilinae species, some aspects of morphogenetic evolution may be understood at a molecular level.

Table 6.2 Eggshell morphology in Drosophilinae

<table>
<thead>
<tr>
<th>Species</th>
<th>Classification</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymomyza:</td>
<td>Usually eight filaments</td>
<td>C. procnemis</td>
</tr>
<tr>
<td>Eight or more filaments</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ten filaments</td>
<td>C. amaena</td>
</tr>
<tr>
<td>Scaiptomyza</td>
<td>Two filaments</td>
<td>S. graminum</td>
</tr>
<tr>
<td>With a ridge above; filaments short</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Four filaments</td>
<td>S. adusta</td>
</tr>
<tr>
<td>Drosophila</td>
<td>Three filaments</td>
<td>D. quinaria</td>
</tr>
<tr>
<td>No ridge on upper side</td>
<td></td>
<td>D. transversa</td>
</tr>
<tr>
<td></td>
<td>Two filaments</td>
<td>D. melanogaster</td>
</tr>
<tr>
<td></td>
<td>Four filaments</td>
<td>D. funebris</td>
</tr>
</tbody>
</table>
CHAPTER 7

Final Discussion
7.1 Enhancer-trap lines and target genes

The enhancer-trap technique has been used in this work to identify genes expressed in subsets of follicle cells during oogenesis. The target genes of two enhancer-trap *Gal4* lines have been cloned. One is an unconventional myosin gene *Mhc95F*, which is expressed in migrating follicle cells during oogenesis. The reporter gene *Gal4* in the starting enhancer-trap line C865 only shows expression in anterior follicle and border cells, which is part of the complex expression pattern of *Mhc95F* during oogenesis. This observation is consistent with the report that the *lacZ* reporter gene does not always entirely match the expression pattern of the target gene (Wilson et al., 1991). Indeed, since most eukaryotic genes have a large chromosomal region for regulatory cis-elements to co-ordinate expression spatially and temporally during development, the position in which the P-element is inserted in a gene locus appears to be very important for reporter gene expression. In the case of C865, the reporter gene *Gal4* is likely to be controlled by cis-elements specific for border cell and anterior cell-expression of the *Mhc95F* gene.

The target cDNA cB2 for C726b contains novel sequences. However, deficiency analysis showed that it is from a well-known locus, the *Broad-Complex* (*BR-C*), which encodes a family of zinc-finger transcription factors sharing a common core domain. The cDNA cB2 shows the same expression pattern as that of the core and Z1 zinc-finger domains during oogenesis, indicating that cB2 is likely to be co-transcribed with these domains. Since the *BR-C* is reportedly post-trancriptionally spliced during development (Bayer et al. 1996), and cB2 does not seem to have a large open reading frame, it may be possible that cB2 is an intronic fragment that has been transcribed but is not present in mature *BR-C* mRNA.

The *BR-C* is also expressed in a dynamic pattern in follicle cells, including two groups of lateral-dorsal follicle cells which secrete the dorsal appendages. The β-galactosidase staining pattern in the starting enhancer-trap line C726b is visualised as two big patches and several random small patches of columnar follicle cells. Although both the reporter gene and target gene show staining in columnar cells, the two patterns do not completely match. It was found that a number of *Gal4* lines seem to exhibit a non-uniformly distributed reporter gene expression pattern (Chapter 3). For example, a number of lines were found to show reporter gene expression that vary from ovary to ovary. Even in line C532 when the reporter gene is expressed in the majority of follicle cells at stage 9 (Figure 5.16A and Figure 6.12C), staining is not observed in random patches of follicle cells. This is unlikely to be the case with an endogenous gene.
Therefore, it seems impractical to obtain a complete match of the expression patterns between the target gene and the reporter gene of a \textit{Gal4} lines which have random patches of follicle cells either stained or missing for \textit{Gal4} expression. The reason for the presence of these random patches of follicle cells is unidentified, but is presumably due to the dual amplification system used in the \textit{Gal4-UAS} technique, since the random staining pattern is very rare in \textit{P-lacZ} enhancer-trap lines (refer to section 3.3).

### 7.2 Expression in the follicle cells

The follicle cells form an epithelial sheet which covers germline cells during oogenesis. They are highly adapted to support the development of the oocyte/egg. Morphologically, they can be divided into different groups. However, more detailed knowledge of follicle cell differentiation comes from the analysis of molecular markers (Spradling, 1993). It is interesting to note that both the \textit{BR-C} and \textit{Mhc95F} are expressed in a dynamic pattern in follicle cells, and can be used to identify different follicle cell groups. For example, the \textit{BR-C} late expression domain in the lateral-dorsal-anterior follicle cells marks the dorsal appendage progenitor cells. Thus, \textit{BR-C} expression can be a useful marker for the exploration of dorsal-ventral polarity formation during oogenesis, as well as for the study of signal transduction pathways involved in follicle cell patterning.

During stages 9-12, the follicle cells undergo a series of morphogenetic movements, which are associated with their function in synthesising different parts of the eggshell. It is interesting to note that the \textit{Drosophila} myosin VI, myosin 95F, is expressed in all of these migrating follicle cells. It has been reported that another myosin, non-muscle myosin-II, is also expressed and functionally involved in these follicle cells (Edwards and Kiehart, 1996). They can therefore be used as models for the study of cell movement and cytoskeleton function.

Interestingly, both target genes, \textit{Mhc95F} and the \textit{BR-C}, appear to be expressed in the dorsal-anterior follicle cells, and both of them seem to be downstream of the Grk-DER signalling pathway. Both genes may be functionally related in dorsal-appendage morphogenesis. During stage 10B, \textit{Mhc95F} is expressed in two to three rows of the lateral-dorsal follicle cells, which are presumably the leading follicle cells that provide a driving force for the dorsal-anterior migration of the appendage producing cells. Thus, myosin 95F is probably directly involved in follicle cell migration. The \textit{BR-C}, in contrast, is expressed in the appendage producing cells, and appears to be required for the formation of dorsal appendage materials. It is unclear if \textit{Mhc95F} is a direct
downstream target gene of the *BR-C* zinc-finger transcription factor. If this is the case, the establishment of the *Mhc95F* expression pattern in the dorsal-anterior follicle cells may require the co-operation of another (or other) transcription factor(s) with the *BR-C*, because the *BR-C* and *Mhc95F* expression domains in the dorsal anterior region do not overlap completely.

### 7.3 Functional analysis

Genetic analysis has been used to investigate the function of the *BR-C* during oogenesis. It has been shown that reduced dorsal appendages are produced in partial "loss-of-function" *BR-C* mutants, suggesting that *BR-C* late expression in the lateral-dorsal-anterior follicle cells is related to its function in dorsal appendage formation. It has been further demonstrated that *rbp* is the functional domain, and Z1 is the zinc-finger isoform related to dorsal appendage formation.

To determine *BR-C* function in relation to its early expression in all follicle cells at stage 6 and most of the columnar cells at stage 10A, strong *rbp* alleles may be required. If lethality of *rbp* strong alleles during metamorphosis is rescued by introducing the heat-inducible *BR-C* transgenes (Bayer *et al.*, 1997), the "loss-of-function" phenotype could then be determined during oogenesis. Huang and Orr (1992) have proposed that the *BR-C* is required for chorion gene amplification prior to choriogenesis by analysing a *rbp* allele, *brJ2Ib,hJe2*, which causes a premature arrest of chorion gene amplification during later oogenesis. This function is likely to be related to the *BR-C* early expression pattern, since chorion gene amplification occurs in most of the follicle cells. Furthermore, it was found that chorion is synthesised prematurely in stage-12 egg chambers in which heat-induced *BR-C* is expressed, which supports the hypothesis that *BR-C* early expression in all columnar cells is related to its function in switching on chorion gene amplification.

Efforts made to search for mutant phenotypes of *Mhc95F* have been unfruitful, although a deletion mutation which removes the 5' end, including the first exon, was created during our work. A saturation screen for mutations in 95F chromosome has also been undertaken in K. Miller's lab. Six complementation groups have been identified, but it is unclear which one affects the *Mhc95F* gene (Hicks and Miller, personal communication). Nevertheless, using an antisense transgene to silence expression of *Mhc95F* in a tissue-specific manner led us to suggest that this myosin may be involved in a number of developmental processes. These include leg and wing imaginal disc eversion during late morphogenesis, a function similar to that of non-muscle myosin-II
It is interesting that some \textit{BR-C} mutants appear to show a similar phenotype. Thus the \textit{BR-C}, myosin 95F and non-muscle myosin-II may functionally related in several morphogenetic activities. It is possible also that to some extent that they substitute for each other, and this is possibly why the mutants do not always have the expected phenotypes.

Using antisense-\textit{Mhc95F} RNA expression, it has been found that \textit{Mhc95F} is likely to be required for centripetal migration, which is consistent with its expression in these follicle cells. However, the function of \textit{Mhc95F} in relation to its expression in border cells, and dorsal anterior follicle cells has not been ascertained using the antisense expression assay. To address this, \textit{Gal4} lines with strong \textit{Gal4} expression in these subsets of follicle cells can be crossed with the UAS-antisense-\textit{Mhc95F} line. To overcome the early lethal phases, the FLP-FRT technique may be applied; this would involve inserting a copy of \textit{white} gene, which is flanked by FLP target sites, between the antisense \textit{Mhc95F} gene and the UAS. The antisense gene would not then be expressed until a heat-inducible source of FLP was introduced. Recombination between the two FRT sites will excise the \textit{white} gene. Thus the antisense-\textit{Mhc95F} gene can be activated by tissue-specific \textit{Gal4} (Figure 7.1). The early lethal phase can be passed if FLP is introduced during later stages of development.

\textbf{Figure 7.1} Schematic drawing of a modified technique of targeted antisense RNA expression. A copy of the \textit{white} gene was inserted between the UAS and the antisense gene. Flp recombinase target sites (FRTs) flank the \textit{white} gene and, following transformation, \textit{white} can be excised by introduction of \textit{flp} recombinase in a genetic cross. Thus, the antisense gene can be activated by \textit{Gal4} in a tissue-specific manner.


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Two signalling pathways specify localised expression of the Broad-Complex in Drosophila eggshell patterning and morphogenesis

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SUMMARY

The Drosophila eggshell, which has a pair of chorionic appendages (dorsal appendages) located asymmetrically along both the anterior/posterior and dorsal/ventral axes, provides a good model to study signal instructed morphogenesis. We show that the Broad-Complex, a gene encoding zinc-finger transcription factors, is essential for the morphogenesis of dorsal appendages and is expressed in a bilaterally symmetrical pattern in the lateral-dorsal-anterior follicle cells during late oogenesis. This is induced and specified along the dorsoventral axis by an epidermal growth factor receptor signalling pathway, which includes a localised transforming growth factor-alpha like molecule, Gurken, in the oocyte and the Drosophila EGF receptor homologue, Torpedo, in the surrounding somatic follicle cells. Furthermore, the precisely localised expression of BR-C along the AP axis requires a separate signalling pathway, initiated by a transforming growth factor-beta homologue, Decapentaplegic, in nearby follicle cells. These two signalling pathways, one from the oocyte and the other from the follicle cells, co-ordinately specify patches of follicle cells to express the Broad-Complex in a unique position in respect to both major axes, which in turn directs the differentiation of the dorsal appendages in the correct position on the eggshell.

Key word: Drosophila, eggshell, follicle cells, Broad-Complex, Gurken, Torpedo, Decapentaplegic, signalling pathway, patterning, morphogenesis, oogenesis, transcription factor

INTRODUCTION

Pattern formation and morphogenesis are two key areas of developmental biology. The Drosophila follicle cells, which produce a polarised eggshell, provide a good model to study the relationship between pattern formation and morphogenesis. During oogenesis, the somatically derived follicle cells interact with the germline cells, and this is critical in the establishment of both the anterior/posterior (AP) and dorsal/ventral (DV) axes (Schupbach, 1987; Gonzalez-Reyes et al., 1995; Roth et al., 1995). The cell-cell interactions also cause the follicle cells to be divided into several subgroups, which have different morphology, function, and position along the two major axes. Additionally, during stages 9-12 of oogenesis (staging of oogenesis is based on Spradling, 1993), a series of follicle cell migrations occur. A group of 6-10 follicle cells at the anterior tip of egg chambers migrate through the nurse cell cluster to the oocyte border during stage 9. At the same time, the majority of the follicle cells move towards the posterior of the egg chamber to form a columnar epithelium covering the oocyte, while the remaining follicle cells stretch to cover the nurse cell cluster. During stage 10b, the anterior columnar cells migrate centripetally between the oocyte/nurse cell border to cover the anterior end of the oocyte. Finally, two groups of columnar cells from the dorsal-anterior region migrate anteriorly to produce a pair of dorsal appendages (filaments). These are bilaterally located in a dorsal-anterior position in the mature egg, marking both the DV and AP polarity of the eggshell (reviewed by Spradling, 1993).

The DV polarity and dorsal appendages are induced by a signalling pathway initiated in the stage-8 oocyte. Gurken (Grk), a transforming growth factor-alpha (TGF-α) homologue, is the germline signal and its transcript is localised in the dorsal-anterior region of the oocyte during stages 8 to 10 of oogenesis (Neuman-Silberberg and Schupbach, 1993). The Grk signal is thought to be received by Torpedo (Top), the Drosophila epidermal growth factor receptor homologue (EGF-R/DER), in the adjacent follicle cells (Price et al., 1989). The binding of Grk and Top/DER activates a receptor tyrosine kinase signalling pathway in these follicle cells, and induces them to adopt a dorsal fate (Neuman-Silberberg and Schupbach, 1993; Roth and Schupbach, 1994). These follicle cells are further divided into two different cell types: the dorsal midline cells and the appendage-producing lateral dorsal follicles (Spradling, 1993).

Many genes have been found to be involved in the Grk-DER signalling pathway in the differentiation of follicle cell fate along the DV axis. cappuccino, spire, fs(1)K10, cornichon, orb and squid are required for the anterior-dorsal localisation of grk transcripts in the oocyte (Manseau and Schupbach, 1989; Neuman-Silberberg and Schupbach, 1993; Christenson and McKearin, 1994; Roth and Schupbach, 1993; Roth et al., 1995). Drosophila homologues of Ras1, Raf and MEK have been shown to transmit the signal in the follicle cells and are required for dorsal follicle cell fate determination in oogenesis.
et al., 1996). CF2, a zinc-finger transcription factor, has been expressed in subsets of follicle cells along the DV axis and are written), an ETS domain transcription factor, is a downstream effector of the Grk-DER signalling pathway have been identified. Pointed (Pnt), an ETS domain transcription factor, has been found to be expressed in the ventral follicle cells and is thought to be a suppresser of Grk-DER signalling on the ventral side (Hsu et al., 1996). Both CF2 and Pnt are transcription factors expressed in subsets of follicle cells along the DV axis and are thought to define the cell fate in these cells. However, the transcription factor directly involved in dorsal appendage formation has not been identified.

The patterning of the eggshell along the AP axis requires a transforming growth factor-beta (TGF-beta) family member, Decapentaplegic (Dpp) (Padgett et al., 1987; Twombly et al., 1996). Dpp is a morphogen and can produce a long range signal to organise the AP axis in wing imaginal discs (Lecuit et al., 1996). It is expressed in both the central and nurse cell associated follicle cells at stage 10, and is required for the formation of the anterior eggshell. Decreases or increases in the level of Dpp expression during oogenesis shift the AP position of the dorsal appendages (Twombly et al., 1996).

Since both the Dpp and Grk-DER signalling pathways are required for the patterning of the eggshell, how do they cooperate? Is there an effector gene regulated by both signalling pathways? Here we show that the Broad-Complex (BR-C) is downstream of both these signalling pathways and is an intermediate in eggshell patterning. The BR-C encodes a family of C2H2 zinc-finger proteins (Z1, Z2, Z3, and Z4) which share a common amino terminus (the BR-C 'core') domain but differ in zinc-finger DNA binding domains (DiBello et al., 1991; Bayer et al., 1996). The core contains a highly conserved amino-terminal motif, called the BTB or POZ domain, which appears to be involved in protein-protein interaction and is widely distributed among metazoans (DiBello et al., 1991; Bardwell and Treisman, 1994; Zollman et al., 1994). The core is alternatively spliced to link with one of the four zinc-finger domains, generating four classes of proteins, the Z1, Z2, Z3 and Z4 isoforms (DiBello et al., 1991; Bayer et al., 1996).

Genetically, the BR-C locus has three fully complementing functions: br (broad), rbp (reduced bristle number on palpus) and 2Bc, as well as a non-complementing npr (nonpupariating) class (Belyaeva et al., 1980). Additionally, a number of BR-C alleles have been categorised to the 2Bah complementation group. These alleles do not complement br or rbp, but do complement 2Bc mutations (Belyaeva et al., 1980). The npr class is thought to be the null mutation, because alleles in this class fail to complement mutations in each of the three-complementing groups. They are also phenotypically indistinguishable from deletions of the locus.

During metamorphosis, the BR-C belongs to the 'early genes' that respond to the steroid hormone, 20-hydroxysteroid (ecdysone), and co-ordinate the ecdysone response among tissues by regulating the expression of effector genes (Emery et al., 1994; Talbot et al., 1993). It is required for a number of morphogenetic activities during metamorphosis, including wing and leg imaginal disc elongation and eversion (Kiss et al., 1988; Emery et al., 1994).

In the studies presented here we report that the transcription factor BR-C is expressed in the lateral-dorsal follicle cells and is required for dorsal appendage formation. Its expression is induced by the Grk-DER signalling pathway in a dose-dependent manner, and further regulated by the Dpp signalling pathway along the AP axis. The study of the generation and regulation of the bilaterally symmetrical expression pattern of a morphogenetic gene, namely the BR-C, may help us to understand the general mechanisms underlying pattern formation and morphogenesis.

**MATERIALS AND METHODS**

**Drosophila strains**

The following *Drosophila melanogaster* strains were used: *Oregon R*, *ts1(1)K10* (Wieschaus et al., 1978); *top(1)* (Schüpbach, 1987); *dpp*<sup>66</sup>, *dpp*<sup>67</sup> (Twombly, 1996); *Ga4-552* (Deng et al., 1997); *br<sup>l</sup>, *npr<sup>l</sup>* (Kiss et al., 1988); *A47* (Deng and Bow, unpublished data); *UAS-lacZ*, *UAS-Drk<sup>66</sup>* (Brand and Perrimon, 1993, 1994); *UAS-dpp* (Stachling-Hampton and Hoffmann, 1994); *UAS-pntP1*, *UAS-pntP2* (Klaes et al., 1994). All stocks were raised on standard corn meal food at 18°C.

**RNA in situ hybridisation**

The hybridisation probes used were a 0.5-kb BR-C core domain cDNA fragment from plasmid BSCDS5; and Z1, Z2, Z3 and Z4 zinc finger domain fragments from plasmids containing PCR products for each of them respectively (kindly provided by C. Bayer). The probes were labelled with digoxigenin as described by the supplier (Boehringer Mannheim Biochemicals). The temperature sensitive flies incubated at 18°C were shifted to 29°C after eclosion and were fed with yeast for 2 days before dissection. Other flies were incubated at 25°C and yeasted for 2 days before dissection.

The staining and detection procedure have been described (Tautz and Pfeifle, 1989) and modified as follows. Ovaries were dissected in PBS and fixed in 4% paraformaldehyde (Sigma) in PBS for 20 minutes, then washed in PTw (PBS containing 0.1% Tween-20) for 3x5 minutes to remove the fix. After washing in methanol/EGTA (0.5 M, pH 8) (9:1) for 3x5 minutes, the ovaries can be stored in methanol at ~20°C for several months. The stored ovaries were washed in methanol/EGTA once followed by washing in PTw 3 times. They were then incubated in protease K (100 mg/ml) (Sigma) for 60 minutes at room temperature and followed by washing for 5 minutes in PTw containing 2 mg/ml glycine to stop the protease K reaction. The postfixation, hybridisation, washing and detection procedures are similar to those of Tautz and Pfeifle (1989). Anti-DIG-AP-conjugate was preabsorbed with postfixed wild-type (*Oregon R*) ovaries at 4°C for overnight. For examination, ovaries were mounted in a mixture of PBS/glycerol (1:4).

**Detection of Ga4 expression**

The female progeny of the cross of *C532-Ga4* with *UAS-lacZ* were dissected in PBS and stained at room temperature (25°C) overnight in PBS containing 0.2% X-gal (from Sigma), 5 mM K<sub>4</sub>Fe(II)(III)CN<sub>6</sub>, 5 mM K<sub>4</sub>Fe(II)CN<sub>6</sub>, 0.3% Triton X-100, rinsed in PBS and mounted in PBS 80% glycerol for microscopic analysis.

**Ectopic expression analysis**

*C532-Ga4* flies were crossed with *UAS-dpp*, *UAS-pntP1*, *UAS-pntP2*, *UAS-Drk<sup>66</sup>* respectively. The ovaries of the female progeny of these flies were raised on standard corn meal food at 18°C.
crosses were dissected in PBS to score the eggshell phenotype and to examine the BR-C expression after fixation and treatment as described in the section on 'RNA in situ hybridisation'.

RESULTS

The BR-C is expressed in the cells which secrete the dorsal appendages

We have searched for target genes from P[Gal4] enhancer-trap lines which show reporter gene expression in subsets of follicle cells (Deng et al., 1997). The BR-C was identified as the target gene in one of these lines, which shows reporter gene expression in two patches of columnar cells at stage 10 of oogenesis (Deng and Bownes, unpublished data).

The four BR-C isoforms share a 5' common core domain but differ in zinc-finger domains (Z1, Z2, Z3, and Z4) (DiBello et al., 1991, Bayer et al., 1996). Using digoxigenin-labelled probes from the core domain and different zinc-finger domains to detect BR-C expression, we find that Z1 is the only zinc-finger isoform which is expressed at levels detectable by whole-mount in situ hybridisation during oogenesis. Its expression pattern mirrors that of the core domain (hereafter called BR-C expression pattern). Expression of the BR-C is first detected in all follicle cells at stage 6 of oogenesis (Fig. 1A). At stage 10a, it is expressed in all columnar cells but absent in the dorsal-anterior most region, facing the oocyte nucleus and the localised Grk signal (Fig. 1B,1C). The expression is stronger in the follicle cells close to the anterior-dorsal region of the oocyte, and becomes gradually weaker posteriorly and ventrally, diminishing at the posterior pole (Fig. 1B). At stages 10b and 11, BR-C expression disappears entirely in the ventral and posterior follicle cells, and remains in just two groups of dorsal-anterior follicle cells which lie symmetrically at either side of the dorsal midline (hereafter called the BR-C late expression pattern) (Fig. 1D,1E). These follicle cells, which are thought to be the progenitor cells of the dorsal appendages, include about 55-65 cells in each group and are 2 cells away from the dorsal midline and 2-3 cells from the centripetal cells (Fig. 1D,1E).

The BR-C is required for dorsal appendage morphogenesis

We asked if the late expression of BR-C in the lateral-dorsal follicle cells reflected a function in dorsal appendage formation. In order to answer this, a newly generated BR-C mutant, A47, which has been mapped to the 2Bab complementation group (Deng and Bownes, unpublished data), was crossed with a weak mutant, br1. The female progeny, br1/A47, survived until adulthood and produced eggs with reduced dorsal appendages (Fig. 2B), when compared to the long appendages in the wild-type egg (Fig. 2A). Normal embryos developed in these eggs and hatched as first instar larvae. Since progeny from the cross of the BR-C null allele, npr1, with br1 can also survive until adulthood (Kiss et al., 1988), the eggs produced by br1/npr1 females were also examined. These eggs exhibited a similar phenotype to the eggs produced by the br1/A47 mothers, and had reduced dorsal appendages (data not shown). These observations, along with the report that another BR-C mutant, br1012/pd1012, produces 'appendageless' eggs (Huang et al., 1992), indicate that BR-C function is required for dorsal appendage morphogenesis during oogenesis.

The BR-C is downstream of the Grk-DER signalling pathway

Since the BR-C is expressed in an asymmetrical pattern along the DV axis, we asked whether its expression in the lateral-dorsal follicle cells is a consequence of Grk-DER signalling, which directs DV pattern formation during oogenesis. This has been investigated by analysing the BR-C expression pattern in mutants that affect this signalling pathway, fsl1/IK10 is required for the localisation of grk RNA to the dorsal-anterior corner of the oocyte at stage 8 (Cheung et al., 1992). In the fsl1/IK10 mutant egg chambers, grk RNA is mis-localised to form a ring in the anterior oocyte (Roth and Schüpbach, 1994; and data not shown). We observed that this induces a BR-C late expression pattern which is expanded to the ventral follicle cells surrounding the oocyte. However, the lack of dorsal BR-C expression in the follicle cells still exists in this mutant (Fig. 3C). Since the BR-C is required for dorsal appendage formation, it can be deduced that the ventrally expanded domain of BR-C expression is the reason for the expansion of the dorsal appendages to the ventral region of the fsl1/IK10 egg (Fig. 3D). Conversely, in strong grk- mutants, in which the Grk signal is absent, we cannot detect the localised BR-C expression pattern in the lateral-dorsal-anterior follicle cells (data not shown), and no dorsal appendages are generated in these mutants. Nevertheless, early expression of the BR-C in all follicle cells during stage 6 seems to be unaffected in the strong grk- mutant (data not shown). Taken together, these data indicate that the BR-C late expression is downstream of the Grk-DER signalling pathway in DV polarity formation during oogenesis.

The BR-C responds to the Grk-DER signalling pathway in a dose-dependent manner

We next investigated why BR-C expression is absent in the dorsal most follicle cells in which the highest dose of Grk signal is supposed to be received (the fact that these cells receive a higher level of Grk signal is shown in Fig. 4A,B). If the BR-C responds to the Grk-DER signalling pathway in a dose-dependent manner, increases or decreases in the signal should shift the BR-C late expression domain along the DV axis. In order to test this, the BR-C expression pattern was examined in transgenic flies with four extra copies of grk gene in the genome (Neuman-Silberberg and Schüpbach, 1994). It was found that the dorsal gap between the two groups of BR-C expressing cells is enlarged to about 8 cells wide (Fig. 3E), in comparison with the 4-cell-wide gap in the wild type (Fig. 3A). Consequently, the dorsal gap between the two dorsal appendages is widened in the mature egg (Fig. 3F). The expansion of the dorsal gap is apparently due to the shift of the BR-C expressing cells ventrally, since the number of cells in each group is approximately the same as in the wild type (data not shown). In contrast, a decrease in Grk-DER signalling in a top01 mutant (Schüpbach, 1987) results in BR-C expression in the dorsal most follicle cells (Fig. 3G). The two groups of BR-C expressing cells shift dorsally and fuse to form one group along the dorsal midline (Fig. 3G). This fusion leads to the fusion of the dorsal appendages in the dorsal most region (Fig. 3H). Taken together, these data indicate that the absence of BR-
expression in the dorsal most region is due to the high dose of Grk-DER signalling. They suggest that if the signal is higher than a certain level, it represses rather than activates BR-C expression. In the $f_5(1)K10^f$ egg chambers, the Grk signal is stronger in the dorsal corner than in the rest of the anterior ring (Roth and Schüpbach, 1994; and data not shown), and this could explain why BR-C expression is still absent in the dorsal most follicle cells when it expands ventrally (Fig. 3C). In order to further test this dose-dependency on the Grk-DER signalling pathway, BR-C expression and the eggshell phenotype were examined in egg chambers that have increased levels of a constitutively activated Draf, a serine/threonine kinase downstream of Grk and DER in the signalling pathway (Brand and Perrimon, 1994). We found that when Draf is highly expressed in most of the follicle cells at stages 9 and 10 (a $Gal4$ driver $C532$, crossed with a UAS linked gain-of-function Draf gene, $UAS-Draf^{rf}$; Brand and Perrimon, 1994; Deng et al., 1997), about 90% of the mature eggs have no dorsal appendages, and the BR-C late expression pattern could not be detected in the majority of the stage 10b-11 egg chambers (data not shown).

**Ectopic expression of Pnt decreases the number of BR-C expressing cells**

We next investigated what the link is between the high dose Grk-DER signalling and the repression of BR-C expression in the dorsal most follicle cells. An ideal candidate is *pointed* (pnt), an ETS domain transcription factor, which acts downstream of the Ras signalling pathway in a number of developmental processes (Klarcembi, 1993; Brunner et al., 1994). During stages 9-10a of oogenesis, both isoforms of pnt (pntP1 and pntP2) are expressed in the dorsal-anterior most columnar cells in response to the Grk-DER signalling pathway (Morimoto et al., 1996). Ectopic pnt expression in all anterior
Fig. 3. Expression of the BR-C is dependent on the Grk-DER signalling pathway. (A,C,E,G,I) Whole-mount in situ hybridisation of a BR-C probe to ovaries. (B,D,F,H,J) Corresponding dorsal appendage phenotype. The BR-C late expression pattern in wild-type ovaries includes two groups of cells separated by a 4-cell-wide dorsal gap (A). The wild-type egg has two long dorsal appendages (B). (C) In the fs(1)K101 mutant, BR-C expression is expanded and fused ventrally, but the dorsal gap remains (arrow), which results in ventrally fused dorsal appendages (D); notice that the dorsal gap exists. In transgenic flies with 4 extra copies of the grk gene, the dorsal gap between the patches of BR-C late expression is expanded to about 8 cells (E), which leads to the expansion of the gap between the two dorsal appendages (F). (G) In topQY1, BR-C expression fuses dorsally, only one group of cells is stained, and results in dorsally fused dorsal appendages (H). (I) Ectopic pnt expression down regulates BR-C expression in dorsal appendage progenitor cells when pntP2 is expressed in most follicle cells at stage 9 (UAS-pntP2 is driven by a Ga4 driver C532, which shows expression in most follicle cells from stage 9). C532/UAS-pntP2 results in a reduced number of cells expressing the BR-C (I), which in turn leads to thinner and shorter dorsal appendages (J). Magnifications in all panels are the same. Anterior is to the left.

Fig. 4. The Grk localisation pattern in Oregon R flies and the reporter gene expression pattern in Gal4-C532. (A,B) Anti-Grk antibody staining. (A) Dorsal view focused in the apical region of the oocyte shows that the high doses of the Grk signal are received by the dorsal-anterior most follicle cells facing the oocyte nucleus. (B) A lateral view focused in the oocyte to show that the Grk protein is localised at the dorsal-anterior corner in a stage-9 oocyte. (C) Staining for β-galactosidase in Gal4-C532/UAS-lacZ shows that expression of the reporter gene is detected in most follicle cells at stage-9; however, staining is not uniformly distributed, and some follicle cells are not stained.
Fig. 5. Expression of the BR-C along the AP axis is regulated by Dpp. (A) In dpphr50/dppe87 stage-10b egg chambers, BR-C expression is detected in the anterior-most columnar follicle cells, which leads to the dorsal appendages being secreted at a more anterior position (B) than in the wild type (see Fig. 3A). (C) BR-C late expression is detected at a more posterior position in Gal4-C532/UAS-dpp egg chambers when dpp is ectopically expressed in most columnar cells, compared to the wild type (Fig. 1D,E). (D) Gal4-C532/UAS-dpp produces eggs with more posteriorly located and malformed dorsal appendages, sometimes, more than two chorionic appendages have been produced in one egg. Magnifications in all panels are the same. Anterior is to the left.

Fig. 6. A model for specification of BR-C expression in the lateral-dorsal-anterior follicle cells, and patterning of the eggshell. The anterior-dorsally localised Grk signal from the oocyte induces the follicle cells to express different genes in a dose-dependent way. The dorsal most cells, which receive the highest Grk signal, express pnt, which appears to be a repressor of BR-C expression. Thus, the BR-C, which also responds to the grk signalling pathway, is expressed in lateral-dorsal follicle cells. Along the AP axis, the Dpp morphogen from the centripetal cells activates an unidentified repressor to inactivate the BR-C in the anterior most columnar cells. The interaction of these two signalling pathways specifies localised expression of the BR-C in the lateral-dorsal-anterior follicle cells and divides the columnar cells into different subgroups.

columnar cells resulted in eggs with reduced dorsal appendages (Morimoto et al., 1996). In order to determine if pnt is the repressor of BR-C expression, we examined BR-C expression in egg chambers with ectopic pntP2 in most of the follicle cells at stage 9-10 (Fig.4) (both UAS-pntP1 and UAS-pntP2 were crossed with Gal4-C532, but only C532/UAS-pntP2 survived until adulthood). The reporter gene expression pattern of Gal4-C532, crossed to a UAS-lacZ line, is shown in Fig. 4C. There are about 10-15 follicle cells expressing BR-C at both sides of the dorsal midline at stage 10b (Fig. 3J), 4-5 times less than in the wild type. The dorsal appendages produced by these flies are thinner and shorter than those produced by the wild type (Fig. 3J). These observations indicate that pnt is a mediator of high dose Grk-DER signalling in the dorsal-anterior-most follicle cells, acting to repress the expression of BR-C. The remaining BR-C expression in a small number of follicle cells is probably due to the fact that the Gal4 reporter gene is not expressed uniformly in Gal4-C532. As shown in Fig 4C some follicle cells fail to show β-galactosidase staining while neighbouring cells are stained.

BR-C expression along the AP axis is regulated by Dpp signalling

An hypothesis for why BR-C expression is also missing in the anterior most columnar cells is that there is probably another repressor produced in these cells. A good candidate is a downstream gene of the Dpp signalling pathway (Padgett et al., 1987), which is required for anterior eggshell patterning...
expression pattern. The mutants were raised at 18°C and then shifted to restrictive conditions after the flies eclosed. As predicted, the anterior most columnar cells express the BR-C when the level of DPP expression is decreased in stage-10b mutant egg chambers (Fig. 5A). Since the anterior frontier of the BR-C late expression pattern in the wild type is about 2-3 cells distant from the Dpp source in the centripetal cells, Dpp could act as a morphogen here. Conversely, when Dpp is ectopically expressed in most of the follicle cells at stage 9 (Gal4-C53.2 crossed with a UAS-dpp) (Fig. 4C), BR-C expression is found over the middle part of the oocyte at a more posterior position than in the wild-type egg chambers (Fig. 4C). As a result, the dorsal appendages are also generated at a more posterior site (Fig. 5D). Additionally, ectopic expression of Dpp also leads to the disruption of the bilaterally symmetrical expression pattern of BR-C. We often observed that three or more groups of the follicle cells expressed the BR-C, which results in the formation of three or more malformed dorsal appendages (Fig. 5D). Taken together, these results indicate that expression of the BR-C is specified by Dpp along the AP axis. It is probably via an indirect route, because ectopic expression of Dpp in all follicle cells does not eliminate BR-C expression.

DISCUSSION

Our data suggest that the follicle cell and eggshell pattern, which is partly dependent upon the late expression pattern of the BR-C, requires signals from both the germline and the somatic follicle cells. The patterning along the DV axis is specified by Grk-DER signalling from the germline in a dose dependent way, as the dorsal-anterior columnar cells respond differently, according to how much signal they receive. Dpp signalling is required for the patterning of the eggshell along the AP axis. These two signalling pathways, one from the oocyte and the other from the follicle cells, co-ordinately specify patches of follicle cells to express the BR-C in a unique position in respect to both major axes. This, subsequently, directs the differentiation of the dorsal appendages in the correct position on the eggshell. Hence, the interaction of these two signalling pathways can divide the dorsal-anterior columnar follicle cells into at least three separate subgroups: BR-C late expression marks the dorsal appendage forming cells in the lateral-dorsal-anterior region; follicle cells anterior to them form the operculum and other anterior structures; follicle cells dorsal to them form the gap between the two appendages (Fig. 6).

In this paper we show that the generation of bilateral morphological structures, is directed by a symmetrical expression of a transcription factor which directs the subsequent morphogenesis of these cells. The localised spatial pattern is established by a TGF-α signalling pathway in a dose dependent manner and is further regulated by a TGF-β signalling pathway from different sources. The mechanisms described here, using the eggshell as a model, could be used to explain the generation of other bilaterally symmetrical structures.

The regulation of the BR-C expression pattern

BR-C expression in the lateral-dorsal follicle cells is induced by a medium concentration of Grk-DER signalling. Higher concentrations of Grk signal seems to induce another transcription factor Pnt, which is likely to be a repressor of BR-C in the dorsal midline cells. It has been reported that the ectopic expression of pntP1 but, not pntP2, results in reduced appendage materials (Morimoto et al., 1996). This is different from our observations. The reason for this is probably that the Gal4 driver we used has higher level of Gal4 expression in columnar cells. This may signify that pntP1 and pntP2 are functionally redundant, but work at different concentrations.

Is the BR-C under the control of ecysdose during oogenesis, as it is during metamorphosis? The answer could be yes, because expression of the BR-C observed in all follicle cells at stage 6 is not induced by the Grk-DER signalling pathway, and expression of the ecysdose receptor (EcR) is detected in all follicle cells during the same stage (Deng, Mauchline and Bowes, unpublished data). This coincidence might imply that the earliest BR-C expression pattern is mediated by ecysdose. However, the BR-C late expression pattern in the dorsal-lateral follicle cells is unlikely to be induced by ecysdose, since the mis-localisation of Grk signal in the ventral-anterior oocyte is sufficient to induce the ectopic expression of BR-C in the ventral follicle cells in egg chambers mutant for fs(1)K10.

The relationship between dorsal/ventral patterning in the eggshell and embryo

The DV polarities of both the embryo and eggshell are initiated by the dorsal-anteriorly localised Grk. The downstream genes, Ras1, Draf and Mek, which are involved in transmitting the signal, are involved in the formation of both these DV polarities. However, the defects caused by mutation or mis-expression of these signal-transmitting genes are more severe in the eggshell patterning than in the embryonic patterning when compared with the grk and top/DER mutants. This led to the hypothesis that another signalling pathway may be involved in the transmission of the DV signal (Schnorr and Berg, 1996).

In addition, the existence of some mutants which only affect embryonic DV pattern formation indicates that there is a branch of the DV patterning pathway used only for eggshell polarity. We suggest that the BR-C, which only affects the eggshell pattern when it is mutated during oogenesis, is an effector gene for this branch. Another transcription factor, Pnt, which is expressed in the dorsal-midline follicle cells could also be part of this branch, because no embryonic defects have been reported when it is mis-expressed in oogenesis. Additionally, a ventrally expressed transcription factor, CF2, has been shown to be involved in both the eggshell and embryonic DV pattern formation (Hsu et al., 1996). Therefore, there should be a transcriptional repressor of CF2 in the dorsal follicle cells downstream of the Grk-DER signalling pathway. This transcriptional repressor must be involved in the establishment of DV polarities of both the embryo and eggshell, and remains to be identified.
Dpp is involved in anterior/posterior patterning in the eggshell

The dorsal-anteriorly located appendages also mark the AP polarity of the eggshell. Their position is induced by the Grk-DER signalling pathway, thus the AP position of the appendages in the eggshell is originally established by the Grk signal. Our data suggest that this position is further refined by Dpp signalling, which is initiated in the nearby centripetal cells. Dpp signalling is directly involved in the specification of the anterior-most columnar cells and anterior eggshell structure, the operculum (Twombly, 1996). However, the regulation of the AP position of BR-C late expression and the subsequent AP position of the dorsal appendages seems to be indirectly controlled by Dpp signalling. Cells have to choose between the columnar cell fate and appendage producing fate. When Dpp levels decrease, the number of anterior columnar cells decreases, and this leads to the BR-C being expressed in a more anterior position when compared with the wild type. Conversely, the ectopic expression of Dpp in most of the follicle cells at stage 9 leads to the expansion of the anterior follicle cell fate. Consequently, BR-C expressing cells are pushed towards the posterior. However, the posterior follicle cells seem to be predetermined, probably by the Grk-DER signalling pathway during the early stages of oogenesis when the AP axis is initiated (Gonzlez-Reyes et al., 1995; Roth, et al., 1995), and this may define the posterior frontier of the appendage-producing cells, which are marked by BR-C expression. Therefore, BR-C expressing cells are squeezed to form a curved line over the oocyte at a more posterior position than in the wild-type egg chambers.

The Dpp signalling pathway is widely used during development, including dorsal epidermal determination during embryogenesis, as well as wing, leg and eye-antennal disc patterning. A number of genes downstream of Dpp have been identified in other developmental processes. It has been reported that the Dpp receptors, saxophone (sax) and thick veins (tkv) are also involved in eggshell patterning (Twombly et al., 1996). However, in these sax and tkv mutants the dorsal-ventral positioning of the appendages, rather than the anterior-posterior positions was affected by the dpp mutants. A preliminary search for the target genes in the follicle cells has been undertaken by examining the expression pattern of some known Dpp downstream genes used during wing morphogenesis, including split, split-related and omb (Lecuit et al., 1996; de Celis et al., 1996). However, they may not be targets of Dpp in follicle cell patterning, since no β-galactosidase expression is detected in oogenesis in enhancer-trap lines containing P(lac-Z) insertions in these genes (Deng and Bownes, unpublished data). It seems likely there are other genes downstream of Dpp in follicle cell patterning, which remain to be identified.

The BR-C is involved in morphogenesis

Genetic analysis of the partial 'loss-of-function' BR-C mutants indicates that dorsal appendage morphogenesis requires a functional BR-C locus. This has been supported by the examination of BR-C expression in different mutants that affect DV patterning. Additionally, the BR-C is found to be involved in morphogenetic movements during metamorphosis. A few target genes of the BR-C, including Sgs4, Ddc and Fhp2, have been identified in larval and pupal tissues (Bayer et al., 1997).

Other target genes could include actin-binding proteins, because the morphogenetic movements require cell shape changes, which result from the rearrangement of the actin cytoskeleton within the cells. The non-muscle myosin-II gene, zipper, is a good candidate gene, since it has been shown to be involved in morphogenetic movements associated with dorsal appendage formation (Edwards and Kiehart, 1996). Strikingly, zipper has been identified as the Enhancer of broad (E(br)) locus during imaginal disc morphogenesis. E(br)I+, in br flies, interferes with appendage elongation during metamorphosis (Gotwals and Fristrom, 1991; Fristrom and Fristrom, 1993). These observations may suggest a conserved regulatory pathway mediated by the BR-C involved in morphogenetic movements throughout development.

The discovery of the requirement for BR-C function in dorsal appendage formation and the role BR-C plays in linking pattern formation and morphogenesis indicates that the BR-C may provide a useful tool to study eggshell morphogenetic evolution in Drosophilinae. Bridges (1928) reported that variable numbers of dorsal filaments are present in different Drosophilinae species. For example, D. melanogaster has two appendages, while D. hydei has four, and other species have 3, 8, or 10 appendages, suggesting that the number and size of dorsal appendages has evolved rapidly. In this paper, we show that the size and number of dorsal appendages is modified when the size and number of groups of BR-C expressing cells is changed (Figs 3J, 5D). Therefore, the modification of the regulation of BR-C expression in the dorsal-anterior follicle cells may be the driving force for this morphogenetic evolution. By studying the mechanisms regulating BR-C expression in a small number of representative Drosophilinae species, some aspects of morphogenetic evolution may be understood at a molecular level.

We thank C. Bayer and J. Fristrom for sending us BR-C cDNAs; T. Schüpbach for anti-Grk antibody; C. Bayer, J. Fristrom, T. Schüpbach, A. Gonzalez-Reyes, D. St Johnstone, V. Twombly, M. Singer, F. M. Hoffmann, K. Matthews, A. Jarman, K. Kaisar and D. Glover for fly stocks; D. Zhao and other colleagues in M. Bownes lab for helpful discussions; T. Schlitt for help in complementation analysis; S.A. Krauss and M. Heck for dark-field microscopy; A. Jarman and two anonymous reviewers for critical reading of the manuscript. W.-M. D. is a Darwin Trust Ph.D. student. This research was supported by the Wellcome Trust.

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(Submitted 4 September 1997)
Analysis of \( P[\text{gal}^4] \) insertion lines of \textit{Drosophila melanogaster} as a route to identifying genes important in the follicle cells during oogenesis

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We report the analysis of a number of lines of \textit{Drosophila melanogaster} containing insertions of the yeast \( \text{gal}^4 \) gene. By crossing a \text{UAS-lacZ} fusion gene as a reporter into these lines, we analysed the expression patterns of \( \beta \)-galactosidase during oogenesis. Since there is no expression of \( \text{GAL}^4 \) in the germ-line in these experiments, this is an ideal system for the analysis of expression patterns in sub-sets of follicle cells. These lines provide ideal markers for sets of follicle cells, e.g. anterior or posterior polar cells for studying genetic interactions in oogenesis; however, they can also be used in the same way as conventional enhancer traps to clone nearby genes with similar expression patterns. The advantages of this dual \( \text{gal}^4 \)/\text{UAS} system over conventional enhancer trapping includes the possibility of \( \text{GAL}^4 \) directed misexpression and antisense expression studies to establish the function of the genes we identified during follicle cell determination and differentiation. These studies could lead to the isolation of homologous genes crucial in mammalian oogenesis. Understanding how the somatic cells and germ cells interact to promote growth and maturation of the mammalian follicle and oocyte could well be crucial for improving the fertility of eggs used for in-vitro fertilization programmes, and could provide methods for assessing the quality of eggs.

\textbf{Key words:} \textit{Drosophila} melanogaster/follicles/gene insertion/oogenesis

Introduction

The egg is uniquely able to give rise to all the cell types of an organism. To do this it is a complex cell, assembled in the mother's ovary. Progress in human and animal reproductive technologies such as in-vitro fertilization make it imperative that we understand how the egg is assembled. Only then can we discover what can go wrong in its development and, perhaps in the long term, how to correct defects. Research at the molecular level on mammalian oogenesis lags behind some other species; this is because they are small, experimentally difficult and, of course, there are ethical considerations. Using our knowledge of other systems and the recent substantive advances in molecular developmental biology it should be possible to gain entry into understanding some key aspects of mammalian oogenesis.

Oogenesis in \textit{Drosophila}

The ovaries of \textit{Drosophila} are arranged as ovarioles, each containing egg chambers of increasing developmental age, passing from anterior to posterior. The oocytes arise from stem cells located in the germarium and, in order to generate each egg chamber, the germ cell divides four times to generate a 16-cell cluster of linked cells; these become surrounded by somatic follicle cells. The germ cell lying at the posterior of the chamber is determined to become the germ cell whilst the others become nurse cells. They provide materials to the developing oocyte. The nurse cells become polyploid and are connected to each other and to the oocyte. They not only supply the building blocks of the egg, such as ribosomes, tRNAs, histones, etc., but also supply information to direct the subsequent embryogenesis. They degenerate late in oogenesis. The follicle cells which surround first the egg cluster, then the oocyte, supply yolk and make the vitelline membranes and chorion which protect the egg. They too then degenerate. The egg which is produced is able to develop to the blastoderm stage using the stored maternal mRNAs. During oogenesis it has already acquired an anterior–posterior and dorsal–ventral polarity which is apparent in the shape and morphology of the egg. The embryo that develops from it has its anterior–posterior and dorsal–ventral polarity determined by information stored in the egg by the mother.

Once the egg is laid the embryo is divided into a segmented embryo and this is set in motion by determinants located at the anterior and posterior poles. These determinants are present as localized mRNAs. The anterior determinant is encoded by the \text{bicoid} gene; it is localized at the anterior of the egg. Once it is translated after fertilization a gradient of Bicoid protein forms which diffuses from anterior to posterior. The protein is a transcription factor which activates zygotic genes needed to establish the anterior of the embryo and turn on other genes needed to divide it into segments.

At the posterior is a complex of determinants needed to set aside the germ cells of the next generation and to determine the posterior regions of the embryo. \textit{Nanos} is the key gene here and it acts to prevent the translation of a protein needed to determine the anterior of the embryo and allow posterior development. The Nanos protein is part of a complex called...
the polar granules. These are distinct structures containing many maternally-derived mRNAs and proteins. Some of these components, such as the product of the germ-cell-less gene and a cyclin-B mRNA, are important in germ cell development.

So how is all this set up during oogenesis? One of the most crucial parts of the system is the cytoskeleton as it controls the polarized transport of the stored determinants. A polarized microtubule network forms in the germarium and extends through all the nurse cells and the oocyte. It is set up with the plus ends in the nurse cells and the minus ends at the posterior of the oocyte. Just before the major growth of the oocyte, this network is completely repolarized within the oocyte so that the minus ends are located anterior to the oocyte.

Many events occur during oogenesis to ensure that the mature egg is ready for development. This requires not only the information and materials passed to the oocyte by the nurse cells, follicle cells and other somatic cells, but also constant interaction with the surrounding layer of follicle cells. It is the cell–cell signalling between these cells which gives the oocyte its polarity.

The first step is to determine the oocyte itself within the 16-cell germ cell cluster. This depends upon the genes Bic-D and orb. As this happens, several mRNAs become transported to and localized within the oocyte. So at this point, in the posterior of the germarium, mRNAs are already being moved to the pro-oocyte and they determine that it becomes the oocyte. The microtubule network is crucial for this transfer mechanism and oocyte determination.

As the oocyte is determined at the posterior of the cyst, it lies next to a specific group of posterior follicle cells. The product of the grk gene signals to these follicle cells. It encodes a TGFα-like molecule and this signal is received in the follicle cells by the EGF receptor homologue of Drosophila (topl DER). This sends a signal to the nucleus of these follicle cells via a kinase signalling pathway. The follicle cells then signal back to the oocyte. Several genes are needed for this to occur properly, including notch and Delta in the follicle cells and protein kinase A in the germ cells. As a result of this, the microtubule array is reoriented as can be seen with kinesin which defines the plus-end-directed microtubule motor. Stored mRNAs then tend to be localized at the anterior of the oocyte when this reorganization of the network occurs. Eventually bicoid mRNA remains at the anterior of the oocyte.

To set up dorsal–ventral polarity requires a second signal. As the microtubules reorganize, the oocyte nucleus is carried to the anterior of the oocyte and it comes to lie at one end at the periphery of the oocyte. The grk product signals again to the overlying follicle cells; the signal is received by the EGF receptor homologue and the information is transmitted to the nucleus, defining the anterior–dorsal follicle cells. Later these follicle cells migrate and produce specialized parts of the egg membranes. The follicle cells become further divided into subpopulations and later the ventral follicle cells signal to determine the dorsal–ventral axes of the embryo.

The follicle cells at the anterior of the egg chamber migrate to lie at the anterior of the oocyte and, along with the posterior follicle cells, are important in sending signals which determine the terminal regions at the extreme poles of the egg. Thus the whole process of producing the highly organized egg occurs rapidly during oogenesis by constant interplay between the oocyte and follicle cells.

The localization of mRNAs depends not only upon the cytoskeleton but also upon the 3' UTRs of the mRNAs themselves. These are not only required for the correct (anterior or posterior) localization of the mRNA but also for translational repression of these stored RNAs, since they need to be stored untranslated until after fertilization.

A number of protein factors have been found which bind 3' UTRs, such as the products of exuperantia, swallow and staufen for the localization of bicoid mRNA. Posterior localization requires more genes. Staufen and vasa proteins are needed and they localize oskar, nanos, cyclin-B, germ-cell-less mRNAs. These products also localize along the microtubule network.

The dorsal–ventral axis, as mentioned earlier, depends upon a signal from grk which becomes associated with the oocyte nucleus when it moves to the oocyte anterior as the cytoskeleton repolarizes. Several mutants disrupt this localization such as fs(1)K10, squid, cappuccino and spire. K10 is a DNA binding protein and squid is involved in RNA processing and both are found in the oocyte nucleus at this time. However, the oocyte nucleus itself is transcriptionally inactive. It is the position of the oocyte nucleus, by virtue of grk localising to it, that determines the position of the dorsal–ventral axis.

The above summary has drawn from the contents of many papers; for key reviews see Lasko's book (1994) and a review by Micklem (1995).

Mammalian oogenesis

Our aim here is to describe briefly mouse oogenesis and to look for clues that may help us to understand the molecular mechanisms important in other mammals. Mammalian oogenesis and early embryonic development looks rather different to that in Drosophila where everything is compressed in time. Furthermore, historically the methods of study and questions asked have been very different in these systems. The approach of looking at homologous genes in such different species has already been demonstrated to be extremely valuable at later developmental stages and will be discussed later.

One big difference is the division of mouse embryonic development into two phases, the short phase where a blastocyst develops prior to implantation and the subsequent phase when just one group of cells in the blastocyst develops to make the embryo and differentiates into all the appropriate tissues and organs. These stages are very difficult to study and rather few mutants have been analysed. Indeed there are very few maternal effect mutants; the zygotic genome takes over at the 2-cell stage in mouse rather than at the blastoderm formation as in flies. So some of the events occurring in oogenesis in Drosophila are probably delayed in the mouse and occur in early embryogenesis. Nevertheless, maternal mRNAs are important.

The implantation event seems to be crucial for nutrition rather than instruction for embryonic development, so we need...
to look at oogenesis and the blastocyst to discover the instructions for development.

The primordial germ cells divide in the genital ridges and come to lie in the peripheral regions of the ovary where they become surrounded by follicle cells, and thus they form a follicle. The follicles gradually become separated from one another and remain quiescent until sexual maturation. Hormonal cues then stimulate them to grow and develop in small groups. The oocyte dramatically increases in size and the follicle cells continue to divide; eventually the oocyte occupies the antrum, which is fluid-filled.

For this process to occur, interactions between the growing oocyte and surrounding follicle (granulosa cells) are required. The oocyte influences the development of the follicle and the follicle cells influence the oocyte. During this phase there is an accumulation of materials needed for embryogenesis, including a huge store of polyA+ mRNAs, reaching very high proportions (~20%) of the total RNA. We know that this includes histones, ribosomes, etc., but rather little is known about the nature of the stored mRNAs and how they are translationally repressed. Although the zygotic genome takes over at the 2-cell stage in mouse, this takes 24 h, whereas in Drosophila the zygotic genome takes over at the blastoderm stage, in only 3 h. However, the rapid activation of the zygotic genome does not mean that the stored mRNAs no longer have any function.

Little is known about the recruitment, translation and function of the maternally stored mRNAs or about the oocyte–nurse cell interactions required during follicle development at the molecular level. Certainly new species of protein are translated from the maternal mRNA at the 1- and 2-cell stages.

The first differences in determination are seen as the embryo segregates into inner cell mass and trophectoderm cells. Quite what sets up this initial difference is still the subject of debate; there seems to be a difference gradually established between cells on the inside and cells on the outside but cell fate takes some time to be really fixed. Even less is known about how cells are later determined and how the embryonic axes are determined and developed. There may be some polarized pre-existing differences in the newly ovulated egg which generate these differences in the 2-cell types first established. There is also a major difference between the mouse and flies in terms of the genomic imprinting phenomenon observed in mammals. This section also covers information from many papers; for reviews see Eppig (1991), Gosden (1995), Gosden and Bownes (1995) and Wilkins (1993).

**Evolutionary conservation of genes used in later development**

There are several methods of obtaining mouse mutants, but one of the most fruitful approaches has been to use Drosophila homologues to isolate the mouse homologues. This has been extremely successful with the homeotic gene complexes of Drosophila, essential for the establishment of segment identity. What has been significant is that not only do the genes exist in mammals, but also their genomic organization and expression patterns in development are remarkably conserved.

These are the *Hox* gene clusters in the mouse. In both mouse and *Drosophila* the chromosomal order of the genes in the cluster is reflected in their anterior–posterior expression pattern along the embryonic axis. The genes probably even perform similar functions. This is reviewed well in Wilkins (1993) and extensively by Edwards and Beard (1997). It is hoped that some of the genes already shown to be important in *Drosophila* oogenesis, such as *nanos*, and some of the new genes we are isolating, using novel methods, in *Drosophila* (described below) will also be crucial in mammalian oogenesis and early embryogenesis. The genes can be isolated in mammals using the *Drosophila* genes as a starting point to look for homologues, as has often been successful at later developmental stages.

**Why look at follicle cell gene expression in Drosophila?**

The follicle cells have crucial roles in oogenesis and are far from homogeneous. They are very dynamic and often involved in morphogenetic movements, first surrounding the whole egg chamber and then reorganizing to leave a very thin layer of cells over the nurse cells and a columnar layer around the oocyte. Cells from the anterior of the egg chamber migrate through the nurse cells to the anterior border of the oocyte where they will produce the micropyle, and dorsal–ventral differences appear in the shape of the egg chamber and follicle cells. Still later, choric appendages are formed dorsally from materials secreted by the follicle cells (for reviews see King, 1970; Spradling, 1993).

Although some of the functions of follicle cells are well known, such as supplying yolk proteins, chorion proteins and vitelline membrane proteins, and their role in determining embryonic dorsal–ventral polarity and terminal embryonic regions is reasonably well established, rather little is known about the earlier interactions between follicle cells and oocyte. Recently this has begun to be addressed (González-Reyes and St Johnston, 1994; González-Reyes et al., 1995; Roth et al., 1995). The posterior localization of the oocyte in the egg chamber provides a signal to the posterior follicle cells setting them upon a posterior developmental pathway. Later these same posterior follicle cells signal back to polarize the cytoskeleton along the anterior–posterior axis of the oocyte. This is used for the RNA and protein localization machinery which has been extensively studied (e.g. Pokrywka and Stephenson, 1991; St Johnston and Nüsslein-Volhard, 1992; Cooley and Therkauf, 1994; Lane and Kalderon, 1994; Therkauf, 1994). It now seems that the molecules and genes generating these signals are the same as those which had already been shown to determine the dorsal–ventral axis a little later in oogenesis, that is a *grk* signal is received in the posterior follicle cells. A gene called *cornichon* (*cni*) affects both anterior–posterior and dorsal–ventral patterning and causes mislocalization of both *bicoid* and *oskar* RNAs. The phenotypes observed are identical to *grk* and *top*, thus it seems to represent a new component in the *grk*/*top* signalling pathway in the germine (Roth et al., 1995). The links that have not yet been identified are between receipt of these signals in a subset of follicle cells...
to specific groups of follicle cells sending new signals back to the oocyte or egg depending upon the stage of development.

Although there are clear gaps in our understanding we do have some pieces of the puzzle of what happens in the dorsal anterior follicle cells when they receive the grk signal. It has been shown that the Raf serine/threonine kinase is also involved in the EGF signalling pathway in the follicle cells, acting downstream of the grkstop signal. Expressing Raf ectopically in follicle cells dorsalizes the eggshell and embryo, whereas reducing it ventralizes the eggshell (Brand and Perrimon, 1994). Raf is thus required in follicle cells. It had previously been suggested that Raf was involved in transducing signals from the torso and sevenless pathways for the determination of terminal regions of the embryo and the determination of the R7 photoreceptor in the developing eye, acting downstream of Ras. Presumably then in the germ cell–follicle cell signalling, there is a pathway that begins with a signal grk (germline) → EGF receptor (follicle cells) → Ras → Raf. It is likely that Dso I (which encodes the MAP kinase activator MEK) and MAPK are then intermediates before a signal is received in the dorsal follicle cell nuclei to modify their gene expression. Clearly this pathway is not fully understood in the follicle cells and there may be other components to be found. One such component is encoded by the rhomboid (rho) gene. Mutants cause ventralization of the eggshell and embryo. The product is localized on the apical surface of the anterior dorsal follicle cells, in the region receiving the grk signal. The relationship between rho and the grkstop, Raf etc. signalling pathway is not entirely clear (Ruohola-Baker et al., 1993). Figure 1 shows a summary of what is happening during signalling between the oocyte and follicle cells in relation to dorsal ventral polarity. The same signalling cascade is thus used twice in oogenesis resulting in very different consequences for the groups of follicle cells that receive the signal on each occasion. The changes in gene expression initiated are essential for the differentiation of the oocyte and embryo.

Our aim is to find genes expressed in subsets of follicle cells which are involved in their differentiation and their interactions with the oocyte. This seems especially relevant to mammalian oogenesis as some components of the system are already known to be involved, such as transforming growth factor (TGF)-α and TGF-β, but their exact functions are unclear.

For many years developmental problems in Drosophila have been approached genetically by inducing mutations that affect the process of interest and investigating their phenotypes and interactions with other genes, followed by a molecular analysis of the genes. Oogenesis, however, is an immensely complex process and many of the genes essential for the differentiation of an oocyte have been used at earlier developmental stages. This means that mutations in many of the genes crucial for oogenesis die at earlier developmental stages, so the role of these genes remains unclear. More recently this has been addressed by using 'enhancer trapping'.

The technique involving P-element-mediated enhancer detection (O‘Kane and Gehring, 1987) has great potential for finding genes expressed specifically in oogenesis since the pattern of reporter gene expression details the spatial and temporal regulation of the gene normally controlled by that enhancer (Bellin et al., 1989; Grossniklaus et al., 1989). Basically a bacterial gene encoding β-galactosidase is placed in a transposable P-element and moved around the genome using a transposase provided from another insertion which cannot itself transpose. By using genetic crosses, this transposase is present for just one generation, and then the resulting transpositions are stable. To determine whether the P-element now lies near to an enhancer of interest, the adults from all of the lines are dissected and male and female carcasses, ovaries and testes are stained for β-galactosidase, and those giving ovary specific staining are selected. The DNA flanking the inserts which give interesting ovary specific patterns of expression is then cloned. We have used this approach in our laboratory to clone several genes expressed in the ovary.

More recently a 2-step technique has been developed which has special advantages for investigating the roles of follicle cells in oogenesis, and it is this method which we describe herein.

Results and discussion

The gal4/UAS enhancer trap system and its advantages

A modified version of enhancer trapping uses the yeast gal4 gene as the first reporter gene, which is driven by a minimal Jsp 70 promoter, including only the TATA box and the transcription initiation site of Jsp 70. Therefore, the expression of GAL4 is dependent upon a genomic enhancer close to the P[gal4] insertion site. The gal4 gene encodes a specific transpositional activator which binds to a UAS (upstream activation sequence) target sequence. To visualize the expression pattern the lines expressing GAL4 are crossed to a line carrying a secondary reporter gene UAS-lacZ and as a result β-galactosidase is produced in those cells expressing GAL4 (Brand and Perrimon, 1993). The principles of this method are shown in Figure 2. Although the initial screening for lines of flies expressing relevant expression patterns is slower as each line must be crossed to the P[UAS-lacZ] flies to visualize the expression pattern, there are numerous advantages. For reasons which are not understood, no GAL4 expression occurs in the germ-line. With conventional enhancer traps, many genes show high levels of expression in the germ-line-derived nurse-cell, and as a result obscure subtle expression patterns in follicle cells. This system allows us very clearly to observe even single follicle cells expressing the reporter gene. As with conventional enhancer traps we can clone flanking DNA to search for the nearby gene expressed in this spatial and temporal pattern in oogenesis and we can create mutations by excision and mobilization of the inserted P[gal4] elements. In addition we can create transgenic flies carrying the gene we clone under UAS control. This enables us to ectopically express it in the wrong follicle cells or at the wrong time in development and observe the results of misexpression of the genes. We can also make constructs designed to express antisense RNA under UAS control, and by crossing with appropriate P[gal4] lines we can 'turn off' expression of the gene of interest in subsets of follicle cells, since the GAL4...
Genetics of oogenesis in *D. melanogaster*
on the dorsal side

<table>
<thead>
<tr>
<th>Original signal</th>
<th>Receptor</th>
<th>Signal transduction</th>
<th>Transcription factors</th>
<th>Dorsal fate</th>
</tr>
</thead>
<tbody>
<tr>
<td>grk, cni, brn</td>
<td>top</td>
<td>Gap1, ras, raf, D-mek, MAPK</td>
<td>1. Repressor</td>
<td><em>rho</em></td>
</tr>
<tr>
<td>Distribution</td>
<td></td>
<td></td>
<td>2. Activator</td>
<td>Ventral fate</td>
</tr>
<tr>
<td>K10, sqd, orb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>capu, spir</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Generation of ventral signal</th>
<th>Ventral fate</th>
</tr>
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<tbody>
<tr>
<td><em>pip, ndl</em></td>
<td></td>
</tr>
<tr>
<td><em>wind, gd</em></td>
<td></td>
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</tbody>
</table>

**Figure 1.** Signal transduction in the secretion of dorsal–ventral polarity of the *Drosophila* egg and embryo. Two signal transduction pathways are required in the determination of the dorsal–ventral axes of both the oocyte and early embryo, one on the dorsal side and the other on the ventral side of egg chamber, respectively. Production of the active *grk* signal requires *cni* and *brn*, but its distribution depends on K10, *sqd*, *orb*, *capu*, and *spir*. Once *grk* activates *Top* in the dorsal–anterior follicle cells, a *ras* signal pathway is activated, leading to the phosphorylation of unidentified transcription factors (activators or repressors) which determine the dorsal fate of these follicle cells. On the other hand, *ndl*, *wind* and *pip*, expressed in the ventral follicle cells somehow cooperatively produce an unknown ventral signal which, as a cue for the embryonic polarity, activates a series of serine and threonine proteinase activation steps, leading to the localized proteolytic processing of *spz*. The binding of *Ti* receptor by the *spz* product in turn activates *tub* and *pll*, which release *dl* from its complex with *cact* protein by phosphorylation, leading to the nuclear localization of the *dl* protein. The gradient of the nuclear *dl* morphogen finally determines the dorsal–ventral axis of embryo, by either activation or repression of downstream zygotic genes, such as *twi, dpp* etc. Abbreviations: *grk*: gurken; *cni*: cornichon; *brn*: brainiac; *K10*: fs(1)K10; *sqd*: squid; *capu*: cappuccino; *spir*: sipre; *pip*: pipe; *ndl*: nudle; *snk*: snake; *ea*: easter; *sp*: spatzie; *Ti*: Toll; *tub*: tube; *pll*: pelle; *dl*: dorsal; *cact*: cactus; *twi*: twist; *sna*: snail; *dpp*: decapentaplegic; *wind*: windbeutel; *gd*: gastrulation defective; *zen*: zerknuellr; *tld*: toloid; *srw*: shrew; *sog*: short gastrulation; *put*: punt; *tkv*: thick vein; *sax*: saxophone; *tag*: twisted gastrulation; *Gap1*: gurken, *ras*: ras, *raf*: D-mek and MAPK: members of ras signal transduction pathway.

**Table 1a.** Summary of staining in oogenesis

<table>
<thead>
<tr>
<th>No. of lines</th>
<th>% of 413 lines checked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining in ovaries</td>
<td>150</td>
</tr>
<tr>
<td>Staining in subsets of follicle cells</td>
<td>112</td>
</tr>
<tr>
<td>No staining in ovaries</td>
<td>263</td>
</tr>
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**Table 1b.** Patterns of staining of oogenesis

<table>
<thead>
<tr>
<th>Class</th>
<th>Cells which stain</th>
<th>No. of lines</th>
<th>% of 150 lines stained in ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Anterior follicle cells</td>
<td>37</td>
<td>24.7</td>
</tr>
<tr>
<td>2</td>
<td>Posterior follicle cells</td>
<td>32</td>
<td>21.3</td>
</tr>
<tr>
<td>3</td>
<td>Follicle cells over the oocyte</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>4</td>
<td>Germarium</td>
<td>7</td>
<td>4.7</td>
</tr>
<tr>
<td>5</td>
<td>Interconnecting stalk cells</td>
<td>10</td>
<td>6.7</td>
</tr>
<tr>
<td>6</td>
<td>Polar follicle cells</td>
<td>5</td>
<td>3.3</td>
</tr>
<tr>
<td>7</td>
<td>All follicle cells</td>
<td>22</td>
<td>14.7</td>
</tr>
<tr>
<td>8</td>
<td>Patches of cells, not always reproducible from ovary to ovary</td>
<td>19</td>
<td>12.7</td>
</tr>
<tr>
<td>9</td>
<td>Chorionic appendages</td>
<td>12</td>
<td>8.0</td>
</tr>
</tbody>
</table>

**Figure 2.** GAL4 expression detection in ovaries. The *P[gat4]* element which contains a reporter gene *gat4*, a marker gene *white* and sequence from the *E.coli* plasmid, pBlueScript, has been inserted into the fly genome. The expression of the *gat4* gene is regulated by the enhancer of a gene, *X*, which is very close to *P* insertion. The cross of the *P[gat4]* line with a transgenic fly line *UAS-lacZ* triggers the expression of a secondary reporter *lacZ* in the same cells that express GAL4. After dissection of the female progeny and staining for β-galactosidase, expression can be detected as a blue stain.

**UAS-DNA system can actually amplify gene expression, the antisense is able to block the native gene expression. This targeted silencing technique is especially valuable when the mutations are lethal at an earlier developmental stage, as we can select a *P[gat4]* line which only switches on the follicle cell expression pattern, thus allowing flies to survive the earlier
lethal stages and enabling us to analyse the phenotypic effect of disrupting expression in oogenesis. Using this technique to repress expression of an unconventional myosin during oogenesis, we were able to identify its function in follicle cell migration (W.-M. Deng and M. Bownes, unpublished data). We can also use the $P[gal^{4}]$ lines as markers to define where in the genetic pathway the genes we are studying can be fitted, by asking if expression patterns are altered in given mutant backgrounds.

**Expression patterns in oogenesis**

We observed expression in 413 lines with $P[gal^{4}]$ insertions on different chromosomes and the results are shown in Table Ia. Approximately one-third of the lines showed expression in the ovary and 22% were stained in subsets of follicle cells. Table Ib shows the distribution of expression patterns. Examples of some of these classes are shown diagrammatically in Figure 3 and some examples of ovaries stained for β-galactosidase are shown in Figure 4.

As can be seen, the follicle cells show highly dynamic and detailed expression patterns. Some genes are expressed in anterior follicle cells, others in posterior follicle cells, some in both (Figure 4a). The developmental stage at which this occurs is variable. In the follicle cells overlying the oocyte, subsets of expression can be seen as a narrow cap at the posterior (Figure 4a), a broad cap at the posterior, or all the follicle cells occupying the posterior half of the oocyte (Figure 4e). Some subsets occupy a band around the anterior region of the oocyte. Follicle cells which are stretched over the nurse cells can also be uniquely stained (Figure 4b).

We have divided the expression patterns into 10 classes which are detailed below.

We observed that 24.7% (37/150) of the lines stained in ovaries show staining in the anterior follicle cells. Anterior follicle cells include those that lie at the anterior end of the egg chambers during the early stages (stages 2–7) and the follicle cells that form the anterior structures of the mature egg. The latter includes the border cells, the centripetal migrating follicle cells (CMFC), and the nurse cell-associated follicle cells (NCFC). Border cells migrate from the anterior pole to the egg chamber between the nurse cells and lie at the anterior of the oocyte during stages 6–10. They are required to direct the production of the micropyle (the sperm entry point) (Montell et al., 1992). We have seen three $P[gal^{4}]$ lines which show staining in the border cells, and all of them still show staining at the anterior pole when border cells are undergoing migration; this may suggest a close relationship between the border cells and the cells at the anterior of the egg chamber during later stages. There are two lines which show staining in CMFC, which migrate centripetally to cover the anterior end of the oocyte during stage 10b. They are required to form the operculum (needed for the embryo to hatch.
from the egg) and the ventral collar (a structure surrounding the operculum). NCFC are the follicle cells that remain associated with the nurse cells, when most of the follicle cells migrate posteriorly at stage 10. Their function is not understood. We have observed six lines staining in these cells. There is one line which stains in all follicle cells except those at the very posterior end at stage 10. There are 10 lines which show staining in anterior follicle cells at stage 13 and 14 only, suggesting that a group of genes is required for the late stages of egg differentiation.

The follicle cells which lie at the posterior end of the egg chamber have been shown to receive the grk signal from the oocyte during early stages (stages 2–7) and send a signal back to re-organize the anterior and posterior polarity of the oocyte. The functions of the posterior follicle cells at later stages have not yet been identified. We found that 21.3% of the lines show staining in the posterior follicle cells. Most of them form a cap-like pattern to cover the posterior end of the oocyte at about stage 10. Interestingly, a large proportion (11/32) of these lines stain in both anterior and posterior follicle cells, suggesting that these two groups of cells share the expression of many genes. We observed 11 lines expressed in posterior follicle cells at stages 13 and 14 only.

Another major category of P[gat] lines (24.0%) showed staining in the follicle cells over the oocyte during the middle and later stages; these follicle cells form most of the egg shell of the mature egg. Among these, 27 lines show staining in the follicle cells over the oocyte from stages 10 to later stages, while nine lines only showed staining in the stage 14 follicle cells.

At the anterior end of each ovariole lies the germarium, where oogenesis commences. We observed that 4.7% of the lines showed staining in cells located in this area. They are likely to be follicle cell precursors since we never observed staining in germ line cells at any stage of oogenesis.

When the egg chambers are budding from the germarium, they are interconnected by several specialized stalk cells. It has been suggested that the Notch and Delta signalling pathway may have a function in the specification of these follicle cells (Ruohola et al., 1991). We observed that 6.7% (10/150) of the lines showed staining in these follicle cells.

A pair of specialized follicle cells located at both the anterior and posterior poles are called polar cells, they express Fascillin-III and neuralized (Ruohola et al., 1991) and are thought to be determined and cease division in region 2b of the germarium (Margolis and Spradling, 1995). Notch and Delta signalling is also required for their determination. We observed five lines expressing the reporter gene in these cells, and all of them show expression in both the anterior and posterior polar cells, supporting the idea that these cells have related origins.

There are only four lines among the 150 lines which showed

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**Figure 5.** Cloning the target gene of a P[gat] enhancer trap line. **Step 1:** Rescue of the flanking genomic fragment: the genomic DNA of the enhancer line is digested with a restriction enzyme 'X' which has a recognition site in polylinker 'PL3'. If there is also a site of 'X' in the genomic sequence close to the P insertion site, the flanking genomic DNA can be recovered to a plasmid containing vector pBluescript, after ligation and transformation to Escherichia coli. **Step 2:** Larger genomic fragments are isolated by screening the genomic library with the larger genomic fragments as probes. **Step 3:** Target cDNAs are isolated by screening the ovarian cDNA library with the larger genomic fragments as probes. **Step 4:** Whole mount ovarian in-situ hybridization is used to compare the expression pattern of the cloned cDNA with the reporter gene expression pattern in the starting P[gat] enhancer line.

**Figure 6.** Example of correlation between β-galactosidase staining and expression pattern of a flanking gene. (a) β-Galactosidase staining in the anterior follicle cells at stage 8/9 of oogenesis. The procedure in Figure 5 was followed and (b) shows the in-situ hybridization of the cDNA corresponding to a gene flanking the P[gat] insert to RNA in ovaries. It shows that the gene is expressed in the anterior follicle cell at stage 8/9 of oogenesis. This gene encodes an unconventional myosin and is expressed in the anterior follicle cells just prior to their migration through the nurse cells to lie at the anterior of the oocyte.
staining in all the follicle cells. This may suggest that the target genes of these lines are 'house-keeping' genes which are constitutively expressed in development. A further 22 lines show staining in patches of follicle cells, but are not always reproducible from ovary to ovary; these have not been further analysed.

At the dorsal anterior region of the mature egg, there is a pair of chorionic appendages, also known as dorsal appendages. Dorsal appendages are secreted by two groups of dorsal anterior lateral follicle cells in response to the grk signal from the oocyte at about stage 10. We observed that 12.7% of the lines showed staining in the follicle cells surrounding these structures.

Outside the egg chambers there is a layer of somatic cells, called the epithelial sheath cells, which surrounds the ovarioles. Their function has not been investigated in detail. We observed 12 lines showing expression in these cells.

Cloning nearby genes with related expression patterns

The method of cloning the flanking DNA and isolating a gene of interest, with a potential role in the follicle cells during oogenesis, is described in Figure 5. The cloning of flanking genomic DNA can be achieved by using a 'plasmid rescue' technique, which includes cutting the genomic DNA from the P[gal4] line with an appropriate enzyme, followed by religation and transformation into E.coli (Figure 5, Step 1). Larger flanking genomic fragments can be isolated by using this rescued DNA fragment as a probe to screen a fly genomic library. Several cDNA clones may then be isolated by screening an ovarian cDNA library with the larger genomic fragments as probes. Whole mount RNA in-situ hybridization to the ovaries with the cloned cDNA as a probe helps to identify whether these cDNA clones are from the target gene, by comparing their expression patterns with the reporter gene expression pattern. Since there are often numerous enhancer sites located near any given gene and the P[gal4] may well only be influenced by a subset of them, one would expect to find near to the insert a gene expressed in at least the cells observed to contain β-galactosidase, but probably extending to further cells and developmental stages.

An example is shown in Figure 6, which shows the relationship between the β-galactosidase staining and an in-situ hybridization to RNA in the ovary (Figure 6b). This gene encodes an unconventional myosin VI which is expressed in the anterior follicle cells prior to their migration through the nurse cells (W.-M. Deng and M. Bownes, unpublished data; Kellerman and Miller, 1992).

Conclusions

This approach has given us a method of identifying genes used in subsets of follicle cells in oogenesis and the flexibility of the system will allow us to investigate the function of these genes in the differentiation of the oocytes.

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