Transgenic Analysis of the Wilms' Tumour 1 gene (WT1) Expression and Regulation.

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Ph.D.
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1997
I declare

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

Adrian Walton Moore
July 1997
“First reaction, don’t drop it on your foot!”

Alan Partridge
BBC Radio 4, London, 1994
Abstract.

Wilms' tumour is a paediatric kidney neoplasia accounting for about 8% of all childhood cancers. The tumour arises by uncontrolled proliferation of the metanephric blastemal stem cells and is a clear example of a cancer caused by disrupted developmental mechanisms. Investigation of a locus, at 11p13, predisposing to Wilms' tumour led to the identification of the Wilms' tumour suppressor gene, \((WT1)\). In situ analysis of \(WT1\) mRNA expression revealed a complex developmentally regulated pattern. Mutations in the \(WT1\) gene have also been linked to gonadal dysgenesis, mesothelial defects and leukaemia. Homozygous \(Wt1\) 'knockout' mice die at E13.5 with no development of kidneys or gonads and disruption of the thoracic organs.

I used a transgenic approach to examine aspects of both \(WT1\) expression and regulation. A 5 kb region (USWT1) of the putative \(WT1\) promoter was linked to a lacZ reporter and used to generate several transgenic embryos. The expression pattern driven by this reporter construct was similar to that of the endogenous locus but very weak and susceptible to position effects. To overcome this problem a YAC spanning the human \(WT1\) locus was used to drive expression of a lacZ reporter in transgenic mouse embryos. The reporter gene expression driven by this construct mirrors that of the endogenous \(Wt1\) locus except in the sex specific Sertoli and granulosa cells. I used the YAC driven reporter gene expression in these transgenic embryos to carefully study the expression pattern of \(Wt1\). The transgene is expressed in both mesodermal and neuro-ectodermal lineages. It is expressed in mesenchymal cells produced by the proliferating coelomic epithelium; hence \(Wt1\) is a marker for this specific cell type. It is also expressed in the interdigital mesenchyme during limb morphogenesis. This newly identified site of \(Wt1\) expression was confirmed by \(Wt1\) mRNA in situ analysis and raises a potential role for \(Wt1\) in the control of apoptosis. Cells expressing the transgene are present in all the differentiating epaxial musculature of the embryo implying that \(Wt1\) may play a role in myogenesis. In the
neuro-ectodermal lineage, transgene expression implies that \( Wtl \) may play a role in neural patterning.

The YAC transgene expression studies presented in this thesis complement further YAC transgenic work we have undertaken. Transgenic animals were made with an unmodified YAC spanning the \( WTI \) locus. This transgene was able to partially rescue the \( Wtl \) homozygous null phenotype when crossed onto the \( Wtl \) knockout background.
Acknowledgements.

There are many people whom I would like to thank for helping me to get to this stage in my life. Thanks to my parents for all the support and encouragement that they have given me from an early age. Without that help I would certainly not be where I am now.

I would like to thank all of friends (near and far) for giving me so much enjoyment during the period that I have been working on my Ph.D. Thank you to Simon for ‘forcing’ me to go out all the time. Thanks to Eleanor, Gillian, Alistair, Matt, Bob, Clive, Andy, Chris, Duncan, Ana, Sarah and all the other assorted students, scumbags and divers who have helped me stray from the straight and narrow. Special thanks go out to Michelle who helped me stay sane when I wasn’t at all sure that I was going to manage it!

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Adrian
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**Abbreviations.**

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<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>Abs</td>
<td>absorbance</td>
</tr>
<tr>
<td>Ac</td>
<td>acetate</td>
</tr>
<tr>
<td>AGM</td>
<td>aorta, gonads, mesonephros</td>
</tr>
<tr>
<td>APP</td>
<td>β-amyloid precursor protein gene</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BAC</td>
<td>bacterial artificial chromosome</td>
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<tr>
<td>bFGF</td>
<td>b fibroblast growth factor</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxyl-terminal</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin dependant kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
</tr>
<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
</tr>
<tr>
<td>CSF</td>
<td>Colony Stimulating Factor gene</td>
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<tr>
<td>dATP</td>
<td>deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
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<tr>
<td>DDS</td>
<td>Denys-Drash syndrome</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<tr>
<td>DIG</td>
<td>digoxigenin</td>
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<td>EGR1</td>
<td>Early Growth Response 1 gene</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
</tr>
<tr>
<td>dH2O</td>
<td>distilled water</td>
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<tr>
<td>DMF</td>
<td>dimethylformamide</td>
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<tr>
<td>DMS</td>
<td>diffuse mesangial sclerosis</td>
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<tr>
<td>Term</td>
<td>Definition</td>
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<td>--------------</td>
<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSRCT</td>
<td>desmoplastic small round cell tumour</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>dTTP</td>
<td>deoxythymine triphosphate</td>
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<tr>
<td>dUTP</td>
<td>deoxyuracil triphosphate</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid disodium salt</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor gene</td>
</tr>
<tr>
<td>EGL</td>
<td>external granular layer</td>
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<tr>
<td>ES (cell)</td>
<td>embryonic stem (cell)</td>
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<td>ethanol</td>
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<td>FISH</td>
<td>fluorescence <em>in situ</em> Hybridization</td>
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<tr>
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<td>fluorescein isothiocyanate</td>
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<tr>
<td>G</td>
<td>guanosine</td>
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<tr>
<td>Gdnf</td>
<td>Glial cell derived neurotrophic factor</td>
</tr>
<tr>
<td>Gdnfr-α</td>
<td>Glial cell derived neurotrophic factor receptor-α gene</td>
</tr>
<tr>
<td>H&amp;E</td>
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<tr>
<td>HGU</td>
<td>Human Genetics Unit</td>
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<tr>
<td>HMG</td>
<td>high mobility group</td>
</tr>
<tr>
<td>IGF1R</td>
<td>Insulin-like growth factor 1 receptor gene</td>
</tr>
<tr>
<td>Igf2</td>
<td>Insulin-like Growth Factor 2 gene</td>
</tr>
<tr>
<td>IGL</td>
<td>inner granular layer</td>
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<tr>
<td>ILNR</td>
<td>intralobular nephrogenic rest</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthio-β-D-galactosidase</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
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<tr>
<td>Leu</td>
<td>leucine</td>
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<tr>
<td>LMP</td>
<td>low melting point</td>
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<tr>
<td>LVA</td>
<td>long vector arm</td>
</tr>
<tr>
<td>Min</td>
<td>minute(s)</td>
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<tr>
<td>MIS</td>
<td>Mullerian Inhibiting Substance</td>
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**Note:** The table includes abbreviations and their definitions commonly used in scientific literature.
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>N-terminal</td>
<td>amino-terminal</td>
</tr>
<tr>
<td>NLS</td>
<td>transgene carrying 5kb of putative $WTI$ promoter linked to a lacZ reporter with a nuclear localization sequence.</td>
</tr>
<tr>
<td>NOR</td>
<td>transgene carrying 5kb of putative $WTI$ promoter linked to a lacZ reporter</td>
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<tr>
<td>Par4</td>
<td>prostate apoptosis response 4</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDGFA</td>
<td>Platelet Derived Growth Factor A gene</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>pers. comm.</td>
<td>personal communication</td>
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<td>PEV</td>
<td>position effect variegation</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PFGE</td>
<td>pulsed field gel electrophoresis</td>
</tr>
<tr>
<td>PGD</td>
<td>partial gonadal dysgenesis</td>
</tr>
<tr>
<td>PLNR</td>
<td>perilobular nephrogenic rest</td>
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<td>PNK</td>
<td>polynucleotide kinase</td>
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<td>RB</td>
<td>Retinoblastoma gene</td>
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<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RPE</td>
<td>regulation position effect</td>
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<td>rpm</td>
<td>revolutions per minute</td>
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<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>s</td>
<td>second(s)</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Sey</td>
<td>small eye</td>
</tr>
<tr>
<td>SRY</td>
<td>sex determining region of Y gene</td>
</tr>
<tr>
<td>ssDNA</td>
<td>single stranded deoxyribonucleic acid</td>
</tr>
<tr>
<td>SVA</td>
<td>short vector arm</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
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</tr>
<tr>
<td>T</td>
<td>thymine</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TESPA</td>
<td>3-aminopropyl-triethoxy silane</td>
</tr>
<tr>
<td>TFIIIA</td>
<td>Transcription Factor IIIA gene</td>
</tr>
<tr>
<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>melting temperature</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β gene</td>
</tr>
<tr>
<td>TK</td>
<td>Thymidine kinase gene</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>Trp1</td>
<td>tyrosinase related protein1 gene</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>UBC9</td>
<td>ubiquitin-conjugating enzyme 9</td>
</tr>
<tr>
<td>ura</td>
<td>uracil</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>WAGR</td>
<td>Wilms' tumour, Aniridia, Genital abnormalities and mental Retardation</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilms' tumour 1 gene</td>
</tr>
<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
</tr>
<tr>
<td>ZF</td>
<td>zinc finger</td>
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</table>
Organization of Thesis.

This thesis describes an experimental study undertaken to investigate the developmental expression, function and regulation of the Wilms’ tumour suppressor gene $WTI$. The introductory chapter reviews the present state of knowledge regarding the developmental role and regulation of the $WTI$ gene. In this chapter I also briefly describe the reasoning which underlies the experimental approaches used in this study. In chapter two I describe the cloning of a small region of the proximal 5’ $WTI$ promoter. This region of the $WTI$ promoter is then used to drive expression of a reporter gene in cell lines and transgenic mice. Chapter three deals with the construction and molecular analysis of transgenic mice containing a YAC spanning the $WTI$ locus. Chapter four contains a detailed description of the developmental expression pattern of these YAC transgenes. In chapter five I describe work carried out in response to the data provided by studying this transgene expression. This work includes mRNA in situ hybridization analysis and study of the $WtI$ knockout mouse phenotype. Within chapters two to five I briefly discuss the significance of the results presented. Such discussion is contained within the text of the chapter rather than inserted at its end. This discussion material is reviewed in chapter six which attempts to put findings into a broader context. Chapter six also contains proposals for further studies which may be undertaken on the basis of the data described within this thesis. Chapter seven details the materials and methods used during this study.
1. Introduction.

Wilms' tumour is a childhood kidney neoplasia accounting for around 8% of all childhood cancers. It has an incidence of 1 in 10,000 children normally presenting by the seventh year (Breslow et al., 1988). The great majority of Wilms' tumours are sporadic, <1% of cases being familial. The tumour arises by uncontrolled proliferation of the metanephric blastemal stem cells involved in normal kidney development. It hence represents a clear example of a cancer being caused by disrupted developmental mechanisms, reviewed by Pritchard Jones and Hastie, 1990.

Around 10% of all sporadic Wilms' tumours are caused by a mutation or deletion in the Wilms' tumour 1 gene (WT1) (Coppes et al., 1993a). This gene gives rise to a zinc finger protein with at least 16 different isoforms (Haber et al., 1991; Hewitt and Saunders, 1996b). In addition to acting as a tumour suppressor WT1 has multiple roles in development. Loss or reduction in the expression of this gene in humans may cause developmental defects in the urogenital system (Hastie, 1992). Mice homozygous for a Wt1 null allele completely fail to develop gonads or kidneys indicating that the gene is a key player in the development of the urogenital system (Kreidberg et al., 1993). Wt1 knockout mice also have defects in the development of the organs of the thoracic cavity; they die at midgestation perhaps due to defects in heart development. The expression pattern of WT1 has been studied by mRNA in situ and immunohistochemical analysis (Pritchard Jones et al., 1990; Armstrong et al., 1992; Pelletier et al., 1991c; Mundlos et al., 1993; Rackley et al., 1993). It is expressed in a large number of sites including the developing urogenital system and the mesothelium. However a better understanding WT1 expression is still required to aid understanding the biology of the gene.
1.1 Isolation of the WTI gene.

Wilms' tumour is one of an associated congenital set of abnormalities which are present in patients with WAGR. Deletions of the chromosomal region 11p13 were identified in several cases of WAGR (Francke et al., 1979). Lewis et al. then demonstrated a homozygous deletion of an 11p13 marker in a sporadic Wilms' tumour. The smallest region of overlap between the two deletions present in this tumour defined a region of 345 kb in which the Wilms' tumour predisposition gene (named WTI) could lie. Two single copy probes isolated from a human chromosome 11p library mapped to this region and were used to isolate a partial cDNA from a pre-B cell library (WT33) (Call et al., 1990). The same gene was also concurrently, independently isolated by chromosome jumping between CpG islands (Gessler et al., 1990).

WTI is deleted or mutated in only approximately 10% of sporadic Wilms' tumours (Coppes et al., 1993a). Additional genes associated with sporadic Wilms' tumours have been located to chromosome 11p15 (designated WT2) (Koufos et al., 1989), 16q (Maw et al., 1992) and by comparative genomic hybridization to 3q, 4q 9p and 20p (Altura et al., 1996). Very few cases of inherited WTI mutation have been reported (Pelletier et al., 1991b). However linkage analysis in several large families have excluded WTI as the major familial Wilms' tumour predisposition gene (Huff et al., 1988; Grundy et al., 1988; Schwartz et al., 1991). Recently a familial predisposition gene (FWTI) was localized to 17q12-q21 (Rahman et al., 1996).

1.2 Structure of the WTI gene.

The WTI gene consists of 10 exons covering 50 kb of genomic sequence (Haber et al., 1991; Hewitt and Saunders, 1996b) (Fig. 1). Northern analysis detected a transcript of around 3.2 kb in foetal kidney, spleen, testis and ovary with an additional 2.7 kb transcript detected in foetal testis (Pritchard Jones et al., 1990).
Alternative initiation and splice sites in addition to RNA editing means that at least sixteen different WT1 isoforms are produced from the locus. Failure to produce isoforms in the correct ratio may lead to severe clinical effects (Bruening et al., 1992; Konig et al., 1993). Furthermore different isoforms may have different molecular functions. WT1 utilizes two different translation initiation sites (Fig. 9). Initiation at an AUG codon directs synthesis of protein isoforms in the range of 47-49 kDa whilst a CUG codon 204 bases 5’ of this directs synthesis of protein isoforms in the range of 54-56 kDa (Bruening and Pelletier, 1996). The protein encoded by WT1 contains an N terminal proline/glutamine rich domain (exons 1-4) and a putative leucine zipper (exon 4). The C terminal of WT1 contains four zinc fingers (exons 7-10) of the Cys2 His2 type first identified in *Xenopus* Transcription Factor IIIA (TFIIIA) (Miller et al., 1985) (Fig. 1).

The WT1 transcript shows RNA editing, U₈₃⁹ may be converted to C (Sharma et al., 1994a). It also has two alternative splice sites (Haber et al., 1991) (Fig. 1). Alternative splicing of exon 5 inserts 17 amino acids into the N terminal domain of the protein. Utilization of an alternative splice at the end of exon 9 inserts an extra three amino acids lysine, threonine and serine (KTS) between zinc fingers three and four of the protein. Throughout this thesis different WT1 isoforms utilizing alternative splice sites will be referred to as WT1 (+ or -17aa / + or-KTS). WT1 (+/+ ) refers to a WT1 protein carrying both the 17 amino acid insert and the KTS insert. WT1(-/+ ) refers to a WT1 protein lacking the 17 amino acid insert and carrying the KTS insert. WT1 (+/-) carries only the 17 amino acid insert and WT1 (-/-) lacks both inserts. In those tissues which have been analysed the ratio of different splice form remains the same 1(-/-):2.5(+/-):4(-/+):8(+/+ ) (Haber et al., 1991).

WT1 has been cloned from a number organisms: human, mouse, rat, chick, alligator, axolotl, *Xenopus* and zebrafish (Buckler et al., 1991; Sharma et al., 1992; Kent et al., 1995; Carroll and Vize, 1996; Del Rio-Tsonis et al., 1996). It is very highly conserved between mouse, rat and human with an amino acid sequence identity of over 96% rising to 100% in the zinc fingers (Buckler et al., 1991). All isolated WT1
genes show conservation of the +KTS alternative splice form implying that it has strong functional importance. On the other hand, the +17aa splice site is not conserved outside the mammalian lineage (Kent et al., 1995).
Fig 1: Schematic structure of the WT1 gene, mRNA and protein products.
After Menke 1996
1.3 Molecular function of the WT1 protein.

1.3.1 Transcription factor activity of WT1.

The WT1 protein shows nuclear localisation (Mundlos et al., 1993; Larsson et al., 1995). It has an N-terminal proline/glutamine rich domain which may act as transcription regulatory domain and four zinc fingers capable of DNA binding activity. Given this information it was originally proposed that WT1 acts as a transcription factor.

The proline/glutamine rich amino terminus of the WT1 protein is similar to transcription transregulatory domains identified in proteins such as CTF/NF-1 (proline rich) and Sp1 (glutamine rich) (Mitchell and Tjian, 1989). Domain swap experiments between WT1 and Early Growth Response 1 (EGR1) or GAL4 demonstrated the N terminal domain of WT1 to be capable of repressing transcription (Madden et al., 1991; Madden et al., 1993). Insertion of the 17aa alternative splice into the transregulatory domain may accentuate its suppressor function. In transient transfection assays the WT1(+/-) protein isoform represses around 25 times better than the WT1(-/+ ) isoform (Rupprecht et al., 1994). Moreover it has been shown that the WT1(-/+ ) protein activates transcription from a modified Platelet Derived Growth Factor A (PDGFA) promoter-reporter construct whereas isoform WT1(+/-) isoform represses it (Wang et al., 1995b).

The first of the WT1 zinc fingers (ZF1) is highly similar to those of the Sp1 transcription factor family, in particular Sp3. The final three (ZF2-4) are almost identical to those of the Early Growth Response (EGR) protein family (Call et al., 1990). The WT1 zinc fingers confer sequence specific DNA binding activity. The -KTS, but not the +KTS, isoform of the WT1 protein, binds the same GC rich sequence as EGR1 (5' GCG GGG GCG 3') (Rauscher, 3d et al., 1990). Bickmore et al. have identified non GC rich sequences to which both + and -KTS WT1 isoforms can bind, albeit with differing specificity (-KTS > +KTS) (Bickmore et al., 1992).
Additional studies have identified other potential WT1 binding sites (Wang et al., 1995b; Hofmann et al., 1993; Drummond et al., 1994; Nakagama et al., 1995).

The binding of the WT1 - KTS to the EGR1 consensus site implies that this WT1 isoform could compete with EGR1 for specific targets. To date it remains unclear what DNA sequences may represent a true in vivo target for WT1 binding. A number of potential target genes for regulation by WT1 have been hypothesised on the presence of putative WT1 binding sites in their promoters (Table 1). In transient transfection experiments, WT1 activated/repressed the activity of these promoters. The physiological relevance of these promoter regulation experiments, however, is unclear. The GC rich target sequences to which WT1 binds, are found in the promoters of thousands of genes which have CpG islands. Many of these genes are housekeeping genes and it is unlikely that WT1 regulates them all (Hastie, 1994). Furthermore the choice of cell system, expression vector and topology of the reporter construct can influence the transcriptional regulation mediated by WT1. In NIH3T3 cells WT1 -KTS represses transcription from the Egrl promoter. However, it activates transcription from the same promoter in Saos-2 and U2OS cells (Maheswaran et al., 1993). WT1(-/+), suppresses transcription from one version of the PDGFA promoter (-153/+388) yet activates transcription from a shorter one (-60/+388) (Wang et al., 1995b). Expression of WT(-/-) driven by the RSV LTR stimulates the activity of the Egrl promoter, whereas expression of the same isoform driven by the CMV (cytomegalovirus) promoter activates it (Reddy et al., 1995).

The transcription factor activity of WT1 is mediated by expression of other cellular components. Interaction with a nuclear component in NIH3T3 cells is required for transcription suppressor function (Wang et al., 1995a). p53 interacts physically and functionally with WT1. Immunoprecipitation studies demonstrate that these two proteins may be part of the same complex. In transient transfection assays WT1(-KTS), in the p53 negative Saos-2 cells, acts as a transcriptional activator. When co-transfected with p53, however, WT1(-KTS) acts as a repressor (Maheswaran et al., 1993). Cellular factors other than p53 must mediate WT1 activity given that
WT1(-/-) activates transcription from the Egr1 promoter in both the p53 negative Saos-2 and p53 positive U2OS cells (Maheswaran et al., 1993; Englert et al., 1995a). One such protein may be prostate apoptosis response 4 (Par4) which binds WT1 in the yeast two hybrid system. Transient transfection studies have demonstrated that Par4 inhibits the ability of WT1 to activate, and enhances its ability repress, transcription from target promoters (Johnstone et al., 1996).

Table 1 Putative target promoters for regulation by WT1.

<table>
<thead>
<tr>
<th>Gene promoter</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Bel2</td>
<td>(Hewitt et al., 1995b)</td>
</tr>
<tr>
<td>C-myb</td>
<td>(McCann et al., 1995)</td>
</tr>
<tr>
<td>C-myc</td>
<td>(Hewitt et al., 1995b; Wang et al., 1993b)</td>
</tr>
<tr>
<td>CSF1</td>
<td>(Harrington et al., 1993)</td>
</tr>
<tr>
<td>EGFR</td>
<td>(Englert et al., 1995a; Wang et al., 1993b)</td>
</tr>
<tr>
<td>Egr1</td>
<td>(Rauscher, 3d et al., 1990; Madden et al., 1991; Reddy et al., 1995; Gashler and Sukhatme, 1995)</td>
</tr>
<tr>
<td>G-protein α2</td>
<td>(Kinane et al., 1996)</td>
</tr>
<tr>
<td>IGF1R</td>
<td>(Werner et al., 1993; Werner et al., 1995; Werner et al., 1996)</td>
</tr>
<tr>
<td>IGF2</td>
<td>(Drummond et al., 1992; Nichols et al., 1995)</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>(Wang et al., 1993b)</td>
</tr>
<tr>
<td>Inhibin α</td>
<td>(Hsu et al., 1995)</td>
</tr>
<tr>
<td>Ki-ras</td>
<td>(Wang et al., 1993b)</td>
</tr>
<tr>
<td>Midkine</td>
<td>(Adachi et al., 1996)</td>
</tr>
<tr>
<td>nov</td>
<td>(Martinerie et al., 1996)</td>
</tr>
<tr>
<td>ODC</td>
<td>(Moshier et al., 1996)</td>
</tr>
<tr>
<td>PAX2</td>
<td>(Ryan et al., 1995)</td>
</tr>
<tr>
<td>PDGFA</td>
<td>(Wang et al., 1992; Wang et al., 1993a; Wang et al., 1995b; Gashler et al., 1992)</td>
</tr>
<tr>
<td>RARα</td>
<td>(Goodyer et al., 1995)</td>
</tr>
<tr>
<td>TGFB</td>
<td>(Dey et al., 1994)</td>
</tr>
<tr>
<td>WT1</td>
<td>(Rupprecht et al., 1994; Hewitt et al., 1996a; Malik et al., 1994)</td>
</tr>
</tbody>
</table>
1.3.2 WT1 may act as a post transcriptional regulator.

WT1 may have a post transcriptional role in gene regulation. Larsson et al. discovered that the WT1(+KTS) and the WT1(-KTS) isoforms, on balance, localize to different compartments of the nucleus. WT1(-KTS) isoforms localized to areas containing transcription factors. WT1(+KTS) forms, on the other hand, localized with the speckled/coiled body domains which contain snrps and other components of the cellular splicing machinery. They also demonstrated co-immunoprecipitation of WT1 and components of the splicing machinery (Larsson et al., 1995). Other studies have indicated that WT1-mediated repression of the Insulin-like Growth Factor 2 gene (Igf2) reporter constructs is a result of post transcriptional events (Ward et al., 1995). The WT1 zinc fingers, in particular ZF1, were required for this activity. The +KTS WT1 isoform binds the Igf2 RNA much better than the -KTS (Caricasole et al., 1996). Perhaps WT1 regulates both transcription and post transcriptional processes through zinc finger binding. Such activity has been demonstrated for the TFIIB transcription factor (Pieler and Theunissen, 1993). Structural modelling has demonstrated the presence of an RNA recognition motif within the amino terminal domain of the protein in addition to RNA binding by the zinc fingers (Kennedy et al., 1996). Englert et al propose that speckled domains within the nucleus simply represent a storage site for WT1 isoforms and mutants with reduced DNA binding affinity (Englert et al., 1995b). They demonstrated that no WT1 protein isoforms co-localize with the nuclear splicing factor SC35. They also showed that truncated WT1 proteins or those with disrupted zinc fingers localize to speckles along with WT1 +KTS isoforms.

1.4 Expression of the WTI gene.

WTI expression during mouse and human embryonic development has been investigated by a number of authors using the techniques of in situ hybridisation and immunohistochemical analysis (Pritchard Jones et al., 1990; Armstrong et al., 1992; Pelletier et al., 1991c; Mundlos et al., 1993; Rackley et al., 1993). The WTI gene shows a complex and developmentally regulated expression pattern. It is first expressed in the embryonic intermediate mesoderm, then subsequently in the organs
of the urogenital system which derive from this structure. The \textit{WT1} gene is also expressed in the mesothelium, an epithelial tissue which surrounds the internal organs of the body. It shows additional sites of expression within the developing nervous system (Armstrong et al., 1992).

Expression of \textit{WT1} is evolutionarily conserved. \textit{WT1} expression in rat, chick and alligator embryos parallels the pattern seen in mouse and human (Sharma et al., 1992; Kent et al., 1995). In \textit{Xenopus} \textit{xWT1} expression is observed in the dorsal portion of the splanchnic lateral plate in tailbud embryos, the glomus of early tadpoles and in the adult mesonephros (Carroll and Vize, 1996).

\textbf{1.4.1 \textit{WT1} expression during kidney formation.}

\textit{WT1} is expressed during the development of the foetal kidneys. Three different kidneys are sequentially formed during embryogenesis. These are the pronephros, mesonephros and metanephros. The following account deals with the formation of the murine kidneys, but those of humans follow a very similar developmental pathway. For a review of kidney development see Saxen 1987, ‘Gray’s Anatomy’ (Bannister et al., 1995) and Lechner and Dressler 1997. A schematic representation of the development of the mouse nephric system is shown in Fig. 2.

The pronephros, mesonephros and metanephros all arise from the nephrogenic mesenchyme of the intermediate mesoderm. From E8.5, a wave nephrogenic differentiation proceeds caudally from the region of about the fifth somite. The pronephric (Wolffian) duct invaginates into the nephrogenic mesenchyme simultaneous with forming the pronephros. As the duct extends in a rostro-caudal direction along the embryo (becoming the mesonephric duct) a series of epithelial tubules form in the nephrogenic mesenchyme which lay adjacent to it. At the level of the pronephros, these tubules remain as rudimentary clusters of cells. However those of the mesonephros form more complex structures. The first 6-8 tubules of the mesonephros (the cranial tubules) develop by budding directly from the mesonephric duct. At more caudal levels the tubules form within the mesonephric mesenchyme.
They lie close to the duct but are still separated from it by a basement membrane. The two different populations of mesonephric tubules have different modes of formation. The cranial tubules form by extension of the mesonephric duct. The caudal ones form by a mesenchyme to epithelial cell type transition within the nephrogenic mesenchyme (Sainio et al., 1997).

*Wt1* is expressed in the nephrogenic mesenchyme prior to tubule formation but it is not expressed in the epithelial mesonephric duct. The state of gene expression in the pronephric and cranial mesonephric tubules is not clear. However it is expressed in the glomeruli of the caudal mesonephric tubules after they have formed (Armstrong et al., 1992; Pritchard Jones et al., 1990; Rackley et al., 1993). Expression in the mesonephric tubules is in structures analogous to those of the metanephros. Expression of the gene has been studied in more detail in the process of metanephric nephrogenesis (Fig. 3).

At around E10.5 the ureteric bud evaginates from the mesonephric duct just above the level of the cloaca. This bud, as with the rest of the mesonephric duct, does not express *Wt1*. The bud invades the mesenchymal metanephric blastema, a spatially distinct population of cells lying at the caudal end of the nephrogenic mesenchyme. Cells of the metanephric mesenchyme express *Wt1* at low levels. Invasion of the ureteric bud induces cells of the metanephric blastema to condense at its tip. An upregulation of the level of *Wt1* expression occurs in those blastemal cells condensing around the ureter tip. Interestingly the spectrum of nephric structures found within a Wilms' tumour implies that it is at this stage of development where loss of *WT1* function can lead to tumourigenesis (Pritchard Jones and Fleming, 1991; Hastie, 1994). Following invasion, the ureteric bud proceeds to divide forming many branches within the metanephric blastema. The blastema is induced to condense at the tip of each branch. Continued branching of the ureter is responsible for the radial wave of nephrogenesis which occurs throughout the developing kidney.
The condensed mesenchyme undergoes a burst of proliferation and then differentiates into an epithelial cyst known as the renal vesicle. The renal vesicle continues to express \( Wt1 \). It now undergoes a series of morphological changes. It first forms a comma shaped body; cells furthest from the collecting duct (the proximal part of the vesicle) become elongated and a slit forms within the vesicle. Next a second slit forms at the opposite distal pole, resulting in the comma shaped body becoming S-shaped. \( Wt1 \) expression becomes localized during the transition from comma to S-shaped body in the proximal part of the body. The distal part of the S-shaped body extends and joins the collecting duct which then undergoes extension to form the proximal and distal convoluted tubules and the loop of Henle. The \( Wt1 \) expressing cells at the proximal part of the S-shaped body become flattened and are destined to form the Bowman’s capsule. The inner cells of the Bowman’s capsule undergo differentiation into the glomerular podocyte cells. \( Wt1 \) expression is restricted to the podocytes where it remains expressed postnatally and continues in adult life.

Angiogenic cells, possibly derived from the lateral mesenchyme, invade the proximal slit to form the capillaries and mesangial cells of the glomerulus. The podocytes overlay the capillaries of the glomerular tuft.

Cells of the metanephric mesenchyme which are not induced or do not undergo nephrogenesis form either the supporting stroma of the kidney or undergo apoptosis (Koseki et al., 1992). \( Wt1 \) is not expressed in the kidney stroma and hence is lost from the metanephric blastemal cells as they undergo this transition.

Within the nephrogenic cord a wave of regression follows that of differentiation. This removes the pronephros and subsequently the mesonephros. By around E13.5 only the developing metanephros remains.
Fig. 2 Schematic representation of the nephric system of the developing mouse embryo.

Adapted from Lechner and Dressler 1997. A composite is depicted so that the pronephros (P) and mesonephros (cranial (Cr-M)and caudal tubules(Ca-M)) are seen together, although by the time the ureteric bud (UB) has formed the presumptive pronephros has begun degenerating. The mesonephric or Wolffian duct (W) is first observed about the level of the fifth somite in the pronephric region and develops caudally toward the cloaca (Cl). The metanephric mesencyme (MM, not drawn to scale) is found at the caudal end of the intermediate mesoderm near the level of the developing hind limb (HL). (FL) forelimb.
Fig. 3 Schematic representation of the different stages of nephrogenesis showing the localization of WT1 expression.

After Hastie 1994. Light shading low levels of WT1 mRNA. Heavy shading high levels of WT1 mRNA.
1.4.2 *WT1* expression during the development of the gonads.

In addition to the pronephros, mesonephros and metanephros the gonads are also formed from the intermediate mesoderm. They develop in close proximity to the mesonephros and also express *WtI*. For a review of human gonad development see 'Gray’s Anatomy' 1995 (Bannister et al., 1995). A schematic representation of *WT1* expression during gonad development is shown in Fig. 4.

In the mouse, gonad development begins at around E11.5 (Kaufman, 1992). At this stage all of the coelomic epithelium surrounding the internal organs of the body including the developing kidneys and gonads is expressing the *WtI* gene (Armstrong et al., 1992; Rackley et al., 1993; Pritchard Jones et al., 1990). Formation of the gonads begins as a thickening of the coelomic epithelium on ventromedial aspect of the mesonephric ridge. This thickening is known as the primary sex cords. The gonad rapidly grows out from the urogenital ridge into the coelomic cavity forming the genital ridge which continues to express *WtI*. The genital ridge runs the full length of mesonephros. It is longer at this stage than the final gonad which is formed.

Concurrent with the formation of the genital ridge, a second duct, the paramesonephric (Mullerian) duct, appears in the mesonephric ridge. It invaginates into the lateral mesonephric ridge and runs parallel to the mesonephric duct. Similar to the mesonephric duct the paramesonephric duct does not express *WtI*.

At around E13 the male and female gonads begin to follow different developmental pathways (Kaufman, 1992). In the male gonad (testis), 'secondary' sex cords, also known as seminiferous cords, form within the gonadal mesenchyme. These cords enclose the germ cells which have migrated into the gonad primordium from near the allantois. The walls of the seminiferous cords consist of epithelial Sertoli cells. Formation of Sertoli cells is required to switch the developing gonad to a male specific developmental pathway rather than a default female one (Ramkissoon and Goodfellow, 1996). Expression of *WT1* is lost from the mesenchymal cells of the testis but is retained in the Sertoli cells and the coelomic epithelium surrounding it.
(Armstrong et al., 1992; Pritchard Jones et al., 1990). As the seminiferous cords form within the gonad a second set of tubules, the rete testis, differentiate within the gonadal mesenchyme. These also express Wt1 (Mundlos et al., 1993). By the stage of gonadal sex determination the mesonephric tubules have regressed. In male embryos the most cranial tubules do not regress. The rete testis provides a bridge between the seminiferous cords and the remaining cranial mesonephric tubules which differentiate into the efferent ducts of the testis.

The non-WT1 expressing mesenchymal cells of the testis differentiate into Leydig cells. These cells produce the sex hormone testosterone. The Sertoli cells produce anti-Mullerian hormone (AMH) also known as Mullerian inhibiting substance (MIS). Under the influence of MIS the paramesonephric duct degenerates. Under the influence of testosterone the mesonephric duct differentiates into the vas deferens and becomes convoluted at the level of the gonad to form the epididymis (Fig. 5).

In the female gonad (ovary) no sex cords form. The gonadal mesenchyme maintains a grainy appearance. Individual germ cells within the gonad become surrounded by follicular/granulosa cells. During gonad maturation, Wt1 expression is lost from the mesenchyme but continues to be expressed in the granulosa cells and the mesothelium overlaying the ovary (Mundlos et al., 1993; Hsu et al., 1995). The rete ovarii form within the developing ovary but do not connect to other structures. The follicular cells of the ovary produce the steroid hormone oestrogen which causes the regression of the entire mesonephric duct and the rete ovarii. Under the influence of this hormone the paramesonephric ducts differentiates into the oviducts and the cranial ends of these ducts open to form the ostia. The caudal ends of the oviducts fuse at the cloaca and extend to produce the uterus and contribute to the vagina (Fig. 5). The myometrial and the endometrial stroma of the uterus express Wt1 (Pelletier et al., 1991c; Zhou et al., 1993). This expression remains into adult life staying constant during normal hormonal cycling. In pregnant rats expression of WTI was highly upregulated in endometrium undergoing decidualization in response to embryo implantation (Zhou et al., 1993).
Expression of \textit{WTI} in Sertoli and granulosa cells continues into adult life. Expression of the gene within these cells is related to the state of maturation of their associated germ cells. In the adult rat ovary \textit{WTI} is expressed and in the granulosa cells of primordial, primary and secondary follicles. \textit{WTI} levels decrease with follicular development (Hsu et al., 1995). \textit{WTI} expression in Sertoli cells has been studied in the Mexican axolotl \textit{Amblystoma mexicanum}. The testis of the axolotl contain zones containing germ cells at different stages of maturation. \textit{WTI} expression was only found in Sertoli cells associated with early spermatogonia (Del Rio-Tsonis et al., 1996).
Fig. 4 Schematic representation of the development of the gonads and associated ducts as seen in transverse section showing localization of \( WTI \) expression.

Adapted from ‘Grays Anatomy’ 1995 eds. Bannister et. al. Light green low levels of \( WTI \) mRNA. Dark green high levels of \( WTI \) mRNA.
Fig. 5 Schematic representation of secondary sexual determination

After Gilbert 1991. (A) The indifferent gonad differentiates on the medial side of the mesonephric ridge. The Mullerian (paramesonephric) duct differentiates alongside the Wolffian (mesonephric) duct and both join the cloaca. (B) In the male the testis produces testosterone and MIS which induces the retention and differentiation of the mesonephric duct into the epididymis and the regression of the paramesonephric duct. (C) In the female the mesonephric duct and tubules degenerate. The paramesonephric duct forms the oviducts and uterus under the influence of oestrogen.
1.4.3 Expression of WTI in the mesothelium and underlying mesenchyme.

In addition to expression in the mesothelium surrounding the developing urogenital system, WTI is expressed in the mesothelial coat of all organs in the coelomic cavity (Armstrong et al., 1992; Rackley et al., 1993). It is expressed in the pericardium, on the surface of the diaphragm, gut mesenteries and on the inner surface of the coelomic cavity.

In the wall of the thoracic cavity WTI is not just expressed on the inner surface but is found in deeper layer of cells adjacent to differentiating muscle in the intercostal region (Kent et al., 1995) and within the developing musculature of the body wall (Armstrong et al., 1992). In stage 13-14 alligator whole mount in situ studies, WTI expression was seen in the developing body wall muscle and also in a novel site in the presumptive wrist and armpit regions of the forelimb (Kent et al., 1995). WTI is also expressed at high levels in the spleen which develops from the surface of the splanchnopleric mesenchyme (Armstrong et al., 1992). Further expression of the gene has been detected by RT-PCR in the extra-embryonic mesoderm of the placenta (Jinno et al., 1994).

1.4.4 WTI expression in haematopoietic cells.

WTI expression has been detected in foetal yolk sac by RT-PCR (Fraizer et al., 1995). The yolk sac contains the blood islands which are a site of embryonic haematopoiesis. WTI is likely to be expressed during formation of some haematopoietic cell types (King Underwood et al., 1996) and the original WTI cDNA was isolated from a pre B-cell library (Call et al., 1990). WTI expression has also been detected in adult thymus (in situ and northern analysis) (Park et al., 1993b) and at low levels in bone marrow and peripheral blood (RT-PCR) (Inoue et al., 1994; Fraizer et al., 1995; King Underwood et al., 1996).
1.4.5 Expression of WTI in ectodermally derived tissues.

WTI is expressed in ectodermally derived tissues of the nervous system. In the developing mouse embryo Wti is expressed in the neural tube from E10.5. This expression becomes localized to the ventral horns of the cord by E15.5. Similar domains have also been detected in human and rat (Armstrong et al., 1992; Sharma et al., 1992; Rackley et al., 1993). WTI is also expressed in the eye (RT-PCR on E12.5 mouse embryo) and the developing and adult brain. It is detected by in situ hybridisation analysis in the roof of the fourth ventricle of the brain at E15 in mouse (Armstrong et al., 1992) and the area postrema of the adult rat brain (Sharma et al., 1992).

1.5 Involvement of WTI (mutations) in disrupted development.

The first insights into the biological role of WTI came from the clinical study of humans. Approximately 8% of all Wilms’ tumours are associated with other developmental abnormalities. The WAGR (Wilms’ tumour, Aniridia, Genital abnormalities, mental Retardation) syndrome (Miller et al., 1964) is cause by deletion of a region of 11p13 containing the WTI gene. The more severe Denys-Drash syndrome (DDS) (Denys et al., 1967; Drash et al., 1970) is due to the effects of a WTI dominant negative allele.

1.5.1 WAGR syndrome.

WAGR patients have a constitutional heterozygous deletion of chr. 11p13 (Francke et al., 1979). Virtually all cases are sporadic with only very few cases of an inherited 11p13 deletion reported (Fantes et al., 1992; Henry et al. 1993). WAGR patients have a 100% incidence of aniridia - congenital absence of the iris. Aniridia is due the loss of one copy of the PAX6 gene also lying at 11p13 (Ton et al., 1991). Around 30% of WAGR patients present Wilms’ tumours (Coppes and Williams, 1994). There is a marked excess of male WAGR patients (almost 2:1). Over 50% of these develop
genital abnormalities ranging from cryptorchidism (undescended testes) and hypospadias (misplaced urethral opening on the penis) to more severe forms of sexual ambiguity. Loss of one copy of the \textit{WT1} gene (haploinsufficiency) is likely to be responsible for these genital abnormalities (van Heyningen et al., 1990).

Monoallelic \textit{WT1} expression has been detected in the brains of two human foetuses by RT-PCR (Jinno et al., 1994). However loss of \textit{WT1} activity does not appear to be responsible for the mental retardation seen in WAGR patients. Retardation in patients is independent of the parental origin of a deleted chromosome. Loss of a gene or genes other than \textit{WT1} lying in 11p13 may be responsible for WAGR associated mental retardation. Retardation appears to be more prevalent in patients with larger deletions in the 11p13 region (Huff, 1994).

1.5.2 Denys-Drash syndrome.

Denys-Drash patients are characterized a triad of partial gonadal dysgenesis (PGD), diffuse mesangial sclerosis (DMS) and Wilms' tumour (Denys et al., 1967; Drash et al., 1970). Both Wilms' tumour (90%) and the DMS (100%) are highly penetrant (Coppes and Williams, 1994). The DMS seen in DDS patients is potentially lethal unless these patients are offered a renal transplant. They suffer from hypertension caused by collapse of the glomerular capillary. This in turn is caused by the production of fibrotic material by frequently hypercellular mesangial cells (Jadresic et al., 1990). Genital abnormalities seen in DDS patients are more severe than those in WAGR. As in WAGR they are more frequent in XY individuals, who often have pseudohermaphroditism (look phenotypically female). Internal abnormalities range from streak gonad, in which the gonadal ridge does not fully form, to the development of ovo-testes.

The spectra of \textit{WT1} mutations associated with DDS are different to those associated with sporadic Wilms' tumours. A representative sample of reported \textit{WT1} mutations is shown in Fig. 6. Nearly all mutations described in sporadic tumours are nonsense or frame shift mutations which are localized anywhere throughout the gene (Coppes et al., 1993a). The majority of DDS patients however show missense mutations in \textit{WT1}
zinc fingers 2 and 3. These mutations specifically affect residues which either chelate a zinc ion or have been shown to interact with DNA in the co-crystal structure of EGR1 (Pavletich and Pabo, 1991). 60% of these mutations affect either arginine$^{394}$ or aspartate$^{396}$ of ZF3. By analogy to EGR1, R$^{394}$ probably binds a guanidine base in the target DNA molecule and D$^{396}$ interacts with the arginine stabilizing this interaction. Fig. 7 illustrates the position of residues in the WT1 protein predicted to be altered by a representative sample of mutations detected in DDS.

Haploinsufficiency at the $WTI$ locus may be responsible for the genital defects associated with WAGR syndrome. However the more severe dominant genital and renal phenotype associated DDS implies that a different molecular mechanism is at work. A neomorphic dominant effect of DDS associated $WTI$ mutations is unlikely given that a number truncate the protein at the level of the zinc fingers and hence abolish any DNA binding (Baird et al., 1992; Schneider et al., 1993; Little et al., 1993). In addition several mutations in the zinc fingers of WT1 associated with DDS have been demonstrated to destroy WT1 binding to the EGR1 consensus site (Pelletier et al., 1991a). A more likely mechanism of action is a dominant negative one (Hastie, 1992). The WT1 protein can self associate and the self-association domain maps to the N-terminus of the molecule (Moffett et al., 1995). In fact oligomers of full length protein form less stable complexes than oligomers between full length and truncated proteins, a mechanism which may serve to amplify the dominant negative effects of DDS associated $WTI$ mutations. In two cases a separate class of mutation causes DDS; mutation in the splice donor site at the end of exon 9 prevents the inclusion of the +KTS alternative splice (Bruening et al., 1992; Konig et al., 1993). This illustrates the importance of the correct ratio of splice forms for the cellular function of WT1.

The differences between WAGR and DDS and are not always distinct. For example one report (Baird et al., 1992) describes a case with a 11p12-13 deletion with DMS normally characteristic of DDS. In another case the same deletion of 11p13 was inherited by two cousins the XX individual developed aniridia whilst the XY case
also had PGD (Henry et al., 1993). It seem likely that the balance between developing WAGR like and DDS phenotypes is due to a combination of the type of \textit{WTI} mutation and genetic background with higher susceptibility in XY cases.
Fig. 6 Spectrum of intragenic \textit{WTI} mutations detected in unilateral, bilateral, familial and WAGR-WT, and in individuals with DDS.

After Williamson 1996. Each deletion and insertion depicted with a solid symbol creates a frameshift.
Fig. 7 Position of the residues predicted to be altered by a representative sample of WT1 mutations detected in DDS. After Williamson 1996.
1.5.3 The *Wt1* knockout mouse.

The phenotype of human patients with a mutation in *WT1* gives an indication of the possible biological roles of the gene. However for further information direct experimental analysis is required. To that end Kreidberg *et al.* disrupted the *Wt1* gene in mice. Deletion of the *Wt1* gene by demonstrated it to be necessary for both normal kidney and gonad development (Kreidberg *et al.*, 1993).

Mice heterozygous for a *Wt1* null allele are phenotypically normal and do not develop Wilms' tumours. This is also the case for *Sey*<sup>Dey</sup> mice which contain a large deletion in the *Wt1-Pax6* region similar to that which occurs in WAGR patients (Glaser *et al.*, 1990).

Mice homozygous for the *Wt1* null allele however die *in utero* between E13 and 15. Development of both the mesonephros and metanephros was severely affected in the *Wt1* null embryos. Cranial tubules but no caudal tubules form in the mesonephros (Sainio *et al.*, 1997). At the level of the metanephros the ureteric bud does not branch from the mesonephric duct. The metanephric blastema forms but undergoes apoptosis and regresses. Whilst spinal cord is a strong inducer of nephrogenesis in cultures of wild type metanephric blastemal explants, it was unable to promote nephrogenic induction in mutant metanephric blastemal explants (Kreidberg *et al.*, 1993). These data imply that *Wt1* is playing two roles in metanephric development. It is required for both the branching of the ureteric bud and for the cell autonomous differentiation of the metanephric blastema during nephrogenesis.

The gonads of homozygous null *Wt1* mice also fail develop. A reduced thickening of the coelomic epithelium overlying the presumptive gondal ridge occurs at E11. Further development was aborted. Germ cells were still present in the region of the presumptive gonad at E12 so *Wt1* activity is not required for their migration.

*Wt1* homozygous null mutants show defects in heart development and occasional pericardial bleeding; mutants older than E12 have massive edema. It is these defects
in the circulatory system which probably lead to foetal death, however this is by no means clear. In addition to a malformed heart, mutants have small, malformed lungs which often herniate through an incomplete diaphragm (Kreidberg et al., 1993). Wt1 is not expressed in the heart or the lungs but is expressed in the mesothelium surrounding them. Defects in the development of this tissue may therefore seriously affect the organ which it surrounds. Cases of diaphragmatic hernia in DDS patients also provides evidence that WTI activity in the developing diaphragm is of functional consequence in humans (Devriendt et al., 1995). Wt1 is also expressed in the mesenchymal cell of the urogenital ridge and cloaca which give rise to the uterus (Armstrong et al., 1992). This structure does not develop in the Wt1 homozygous null embryos (Kreidberg et al., 1993). Similarly the uterus is also malformed in some human Wilms’ tumour survivors (Nicholson et al., 1996).

1.5.4 Involvement of WTI in Wilms’ tumourigenesis.

Mutations in the WTI gene have been found in approximately 10% of sporadic Wilms’ tumours. The tumour is a malignant embryonal tumour of the kidney. The majority of Wilms’ tumours have a ‘triphasic’ histology comprising of blastemal, epithelial and stromal elements (Beckwith et al., 1990). Although this tumour develops postnatally it contains immature blastema and epithelial structures which are normally present during embryonic kidney organogenesis. Wilms’ tumour is a clear example of a cancer caused by loss of developmental control (Pritchard Jones and Hastie, 1990). Tumours may contain renal vesicles, comma, S-shaped and ‘glomeruloid’ bodies, but the formation of more mature structures such as glomeruli and proximal and distal tubules does not occur. In addition Wilms’ tumours may contain foci of heterotrophic elements such as striated and smooth muscle, chondrocytes, and adipocytes. These elements are believed to derive from the aberrant differentiation of blastemal stem cells. The blastema cells must therefore have a degree of pluripotency to form these tissues types which is restricted during normal kidney development.
Wilms tumours are found in association with kidney lesions known as nephrogenic rests. Nephrogenic rests are normally found in approximately 1% of infant post-mortems. This level increases to approximately 40% in patients with unilateral and 100% with bilateral Wilms' tumours (Beckwith et al., 1990). Nephrogenic rests may represent premalignant lesions which serve to immortalize stem cells within the kidney. Nephrogenic rests can be categorized into two types. Intralobular nephrogenic rests (ILNR) consist of islands of stromal and epithelial components and appear to be generated from a defect in early nephrogenesis. INLRs are associated with tumours of 'triphasic type' which often have mutations at the WTI locus. Two cases have been reported where specific mutations in the WTI gene are present in a Wilms' tumour and its associated rest but not in the normal surrounding kidney tissue (Park et al., 1993a). Perilobular nephrogenic rests (PLNRs) on the other hand consist of mainly epithelial structures and appear to have arisen late in the process of nephrogenesis. They are associated with tumours which are not triphasic but consist of more uniform epithelial tubular and glomeruloid structures. PLNRs are more associated with Wilms' tumours caused by disruptions of loci in 11p15 and Beckwith-Wiedemann syndrome (BWS).

Several growth factor gene represent potential targets for transcriptional regulation by WT1, e.g. Platelet Derived Growth Factor A (PGDFA), Epidermal Growth Factor Receptor (EGFR), Colony Stimulating Factor (CSF), Transforming growth factor β (TGF-β), Insulin-like growth factor 1 receptor (IGF1R) and Insulin-like growth factor 2 (IGF2) (see Table 1). Deregulation of WT1 mediated regulation of these growth factor genes could potentially lead to increased cell proliferation and neoplasia. IGF2 is a very strong candidate for interaction with WT1 during tumourigenesis. It is a potent mitogen for mesenchymal cells and is required for correct kidney development in vitro (Gansler et al., 1989). IGF2 is expressed at high levels in undifferentiated metanephric blastema. IGF2 levels decline during nephrogenesis as those of WT1 increase. Furthermore IGF2 is overexpressed in 95% of Wilms' tumours and its expression may persist in epithelial structures where it is normally down regulated (Yun et al., 1993).
Widespread programmed cell death occurs during normal nephrogenesis (Koseki et al., 1992). Growth factors such as EGF and bFGF (fibroblast growth factor) can prevent cell death when added to cultured metanephric blastema (Weller et al., 1991; Perantoni et al., 1995). The relationship between WT1 activity and the process of apoptosis is not clear. The protein has been shown to either inhibit or induce apoptosis. Interaction between WT1 and p53 stabilizes the p53 protein and inhibits p53 mediated cell death (Maheswaran et al., 1995). In contrast expression of high levels of WT1 in p53 negative Saos-2 cells induces apoptosis which may be caused by the transcriptional repression of the endogenous \(EGFR\) gene (Englert et al., 1995a). \(PAX2\) represents another target for potential regulation by WT1 (Ryan et al., 1995), it downregulates expression of p53 (Stuart et al., 1995) and is often overexpressed in Wilms’ tumours (Eccles et al., 1995).

WT1 may also interact with the cell cycle machinery. WT1(+/-) and (+/+ can block cell cycle progression. This block may be relieved by expression of endogenous cyclinE/CDK2 and cyclinD1/CDK4 (Kudoh et al., 1995). WT1 has been shown to physically interact with human ubiquitin-conjugating enzyme 9 (UBC9). In yeast UBC9 has been shown to be involved in cell cycle progression and is required for the proteolytic degradation of S and M phase cyclins (Wang et al., 1996).

An alternative mechanism by which tumours may arise is by disruption of the ratio between WT1 splice variants. Simms et al. (Simms et al., 1995) reported that 7 out of 10 Wilms’ tumours have an increase in the ratio between WT1(-17aa) and WT1(+17aa). Expression of the WT1(-/-) isoform in adenovirus transformed baby rat kidney (BRK) cells increased the tumourigenic potential of those cells whilst WT1(-/+ strongly suppressed their tumourigenicity (Menke et al., 1996).

Heterozygous \(Sey^{Dey}\) and \(Wt\) knockout mice fail to develop Wilms’ tumours (Kreidberg et al., 1993). A Wilms’ tumour has been observed, however, in a single mouse chimeric for wild type ES cells and cells in which the endogenous \(Wt\) gene has been has been truncated in the zinc fingers similar to some DDS mutations.
A potential experimental model for Wilms' tumour exists in rats. Kidney neoplasia in rats can be induced by N-nitroso-N'-methylurea chemical mutagenesis. At least 40% of rat nephroblastomas induced in this way have mutations in the \textit{WTI} gene (Sharma et al., 1994b).

1.5.5 Involvement of \textit{WTI} in tumours other than Wilms' tumours.

Disruption of the \textit{WTI} gene plays a role in the development of a significant proportion of Wilms' tumours. Given the expression pattern of \textit{WTI} in a range of different tissues it seems likely that the gene may also be involved in the development of a range of tumour types. In rare cases, tumours histologically identical to Wilms' tumours arise outside the metanephric kidney and in the adult. These extra-renal nephroblastomas are mainly found in the retroperitoneal space and gonads. Aterman argues these tumours may be formed by misplaced metanephric tissue. However these tumours could arise from mesonephric or the gonadal mesenchyme which has failed to regress (Aterman, 1989).

\textit{WTI} may also play a role in the development of gondal tumours. Pelletier \textit{et al.} have described one case where bilateral juvenile granulosa cell tumours co-developed with Wilms' tumour in a DDS patient (Pelletier \textit{et al.}, 1991a). However no \textit{WTI} mutation have been described in Frasier's syndrome (nephrotic syndrome and gonadoblastoma) (Poulat \textit{et al.}, 1993), ovarian tumours (Bruening \textit{et al.}, 1993), sex cord-stroma tumours (Coppes \textit{et al.}, 1993b) or other urogenital tumours (Quek \textit{et al.}, 1993).

The mesothelium is another tissue in which the \textit{WTI} gene is expressed. Park \textit{et al.} examined 32 cases of malignant mesothelioma (Park \textit{et al.}, 1993b). This study identified one mutation in a mesothelial lesion, which was not malignant, and likely to represent a mesothelial developmental defect. Similarly Langerak \textit{et. al.} identified no \textit{WTI} disruption in a panel of 12 malignant mesothelioma cell lines (Langerak \textit{et al.}, 1995).
WTI is strongly implicated to be involved in the genesis of leukaemias. WTI mutations have been found in 4/36 of cases of sporadic acute adult myeloid leukaemia (King Underwood et al., 1996). All of these resulted in a truncated protein and 3/4 were present in a heterozygous state implying that a dominant or dominant negative mechanism may be operating. In another study of 33 cases of childhood acute lymphatic leukaemia and 15 cases of childhood acute myeloid leukaemia no mutations in the WTI gene were found (Algar et al., 1997). In fact all of these leukaemic cell lines which were tested expressed WTI. Other studies have reported high levels of WTI expression in leukaemia cell lines and patient leukaemias (Miwa et al., 1992; Miyagi et al., 1993). In leukaemia patients elevated expression of the gene is correlated with both poor prognosis and treatment outcomes (Inoue et al., 1994). In leukaemia cell lines, induced to differentiate, down regulation of the WTI gene is specifically associated with differentiation along myeloid and erythroid pathways (Sekiya et al., 1994; Phelan et al., 1994). However blocking the expression of WTI with antisense oligonucleotides, inhibited proliferation and induced apoptosis in 2 out of 3 myeloid leukaemia lines (Algar et al., 1996). WTI may be playing multiple roles in the process of haematopoiesis. It may act to repress genes in immature haematopoietic cells which are required to allow cells to proceed along specific differentiation pathways. In support of this hypothesis, WTI is expressed in immature leukaemic cells with high rates of proliferation and in immature normal haematopoietic cells (King Underwood et al., 1996; Inoue et al., 1994). However the identification of WTI mutations in adult acute myeloid leukaemias implies that the gene may play a separate later role in myeloid differentiation.

Fusion of the N terminal domain of the Ewing's sarcoma gene EWS and the C terminal end of WTI leads a chimeric transcript implicated in the genesis of desmoplastic small round-cell tumour (Gerald et al., 1995; Ladanyi and Gerald, 1994). These cancers are found closely associated in the main with the mesothelial membranes of the abdominal cavity, three cases have been reported in the pleura and one in the brain (Tison et al., 1996).
1.6 Possible developmental roles of WTI.

WTI seems to have several roles during development. The gene is expressed in the developing nephron, mesothelium and secondary sex cords (Pritchard Jones et al., 1990; Armstrong et al., 1992; Rackley et al., 1993; Mundlos et al., 1993). The phenotypes of the Wt1 knockout mouse and WAGR and DDS patients demonstrate that proper WTI function is required for the normal development of all these tissues. The developing nephron, mesothelium and secondary sex cords all undergo a mesenchymal to epithelial cell type transition during development. On the basis of WTI expression in these tissues it was proposed that the gene may play a role in mediating this transition (Pritchard Jones et al., 1990).

In fact WTI may play several alternative roles at different stages of development, this is most clearly demonstrated in metanephric nephrogenesis reviewed by Hastie 1994 (Fig. 3). The expression pattern of WTI is very dynamic during the process of nephrogenesis. It is expressed in very low levels in the uninduced metanephric blastemal stem cells. The level of expression is upregulated in the cells condensing at the ureter tip. WTI is then localized during nephrogenesis to the podocyte layer of the glomerulus where it remains being expressed during adult life. Examination of kidneys from the knockout mouse, WAGR, DDS and sporadic Wilms' tumour patients indicates that the gene may have different roles at these different stages of nephrogenesis.

In a Wt1 homozygous null knockout mouse only cranial mesonephric tubules are formed and no branching of the ureteric bud occurs (Kreidberg et al., 1993). A number of other genes involved in different stages of kidney development have recently been identified, for a review see Lechner and Dressler 1997. The Ret-k gene is expressed in the ureteric bud as it emerges, and later in the growing tips of its branches. Ret-k knockout mice also show a reduction in the number of mesonephric tubules and failure of ureteric bud formation. Ret is co expressed with Glial cell derived neurotrophic factor receptor-α (Gdnfr-α) and in tandem, these molecules act
as a receptor for the TGF-β related molecule Glial cell derived neurotrophic factor Gdnf to induce the branching of the ureteric bud. In Gdnf knockout animals, the ureteric bud also does not branch from the mesonephric duct and the metanephric blastema subsequently undergoes apoptosis. Gdnf is expressed in the metanephric mesenchyme simultaneously with Wt1. Potentially Wt1 expression may precede and permit Gdnf expression. To date however, the state of Gdnf expression in Wt1 knockout mice has not been investigated.

In addition to being required for branching of the ureteric bud Wt1 is required cell autonomously to mediate the response of the metanephric mesenchyme to signals inducing nephrogenesis (Kreidberg et al., 1993). Wnt molecules are prime candidates for this signal. Wnt11 is expressed at the growing ureter tips. Other signals may be required within the blastema to promote nephrogenesis. Wnt4 is switched on in the condensing mesenchyme around the tips of the ureteric buds and required for nephrogenesis (Stark et al., 1994). Bmp7, a member of the TGFβ family, is expressed in the ureteric bud and is upregulated in the condensing mesenchyme. It is also involved in the control of nephrogenesis (Dudley et al., 1995; Luo et al., 1995; Vukicevic et al., 1996). Perhaps Wt1 activity within the metanephric blastema is required for the subsequent upregulation of Wnt4 and Bmp7 expression in this tissue.

Wt1 is upregulated in those cells of the metanephric blastema which undergo a mesenchymal to epithelial cell type transition during nephrogenesis and may play a role in this process (Pritchard Jones et al., 1990). It may do this by regulating other genes involved in this transition e.g. Pax2 and Pax8. Pax2 is a putative target gene for control by Wt1. It is overexpressed in many Wilms' tumours and has an expression pattern complementary to that of Wt1 (Eccles et al., 1995). Pax2 is essential for the mesenchyme to epithelial transition during metanephric development. In homozygous Pax2 null mice, no epithelial tissues are formed within the intermediate mesoderm (Torres et al., 1995). Anti Pax2 oligonucleotides prevent any epithelium formation in metanephric blastemal explant cultures (Charite et al., 1994).
WT1 may not only be involved in the onset and control of nephrogenesis but it may play a role in the maintenance of the nephron. In the adult nephron, \textit{WT1} continues to be expressed in the podocytes. The podocytes can synthesise a complete glomerular basement membrane and may play a role in its degradation since they show endocytic activity (Mundel and Kriz, 1995). The DMS seen in DDS patients demonstrates the requirement for \textit{WTI} in the proper functioning of the podocyte cells in the adult nephron.

1.7 Regulation of the \textit{WTI} gene.

Expression of the \textit{WTI} gene is tightly controlled during embryonic development. Abberant expression of the gene can have severe clinical effects. Study of the transcription regulation of the gene is therefore essential. Firstly mutations of the regulatory elements of the gene may be of clinical importance. Secondly analysis of the regulatory elements will lead to the identification of factors interacting with the \textit{WTI} promoter and help unravel the regulatory cascade of which the \textit{WTI} gene is part.

To date however \textit{WTI} transcriptional regulation has proven to be very difficult to unravel. The putative \textit{WTI}/\textit{WtI} promoter region is highly conserved between mice and humans (~73% identical) (Gessler and Bruns, 1993) (Fig. 8). There are long stretches of complete homology between the two promoters. The promoters are ~70% GC rich and lack TATA or CCAAT boxes (Gessler and Bruns, 1993; Hofmann et al., 1993). The overall structure of the promoter is more similar to housekeeping and other ubiquitous tumour suppressor genes e.g. \textit{Retinoblastoma} (\textit{RB}) and \textit{P53} than tissue specific or developmentally regulated genes. In keeping with the similarity to ubiquitously expressed genes a putative promoter lying in the 5' region of the \textit{WTI} gene displays promiscuous activity when tested in a large number of cell lines (Table 2). In a 24 kb stretch of the 5' promoter a 9 bp (CTC)$^3$ repeat is responsible for around 80% of promoter activity (Cohen et al., 1997) (Fig. 8). The Sp1 transcription factor binds to this repeat in extracts of cell lines and murine tissues. Versions of the \textit{WTI} promoter in which this repeat is mutated have a greatly
reduced ability to be bound by Sp1 and to drive transcription. Sp1 regulates the promoter of other TATA less genes, e.g. Tgfβ, Egfr and c-Ha-ras, by binding to GC boxes within the promoter (Blake et al., 1990). Interestingly, although the WTI promoter contains several GC boxes, it is through the CTC repeat that Sp1 mediated transcriptional activation is modulated.

Table 2 Cells lines in which WTI promoter activity has been demonstrated.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Reference</th>
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<tr>
<td>293</td>
<td>human embryonic kidney</td>
<td>(Nornes et al., 1990; Fraizer et al., 1994; Rupprecht et al., 1994; Campbell et al., 1994; Wu et al., 1995)</td>
</tr>
<tr>
<td>JMN</td>
<td>human mesothelioma</td>
<td>(Nornes et al., 1990)</td>
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<tr>
<td>HeLa</td>
<td>human cervical carcinoma</td>
<td>(Nornes et al., 1990; Wu et al., 1995)</td>
</tr>
<tr>
<td>Hep3B</td>
<td>human hepatocellular carcinoma</td>
<td>(Fraizer et al., 1994; Wu et al., 1995)</td>
</tr>
<tr>
<td>G401</td>
<td>human rhabdoid tumour of the kidney</td>
<td>(Fraizer et al., 1994)</td>
</tr>
<tr>
<td>K652</td>
<td>human chronic myelogenous leukemia in blast crisis</td>
<td>(Fraizer et al., 1994; Wu et al., 1995)</td>
</tr>
<tr>
<td>HEL</td>
<td>human erythroleukemia</td>
<td>(Wu et al., 1995)</td>
</tr>
<tr>
<td>CEM</td>
<td>human acute lymphoblastic T cell leukaemia</td>
<td>(Wu et al., 1995)</td>
</tr>
<tr>
<td>TM3</td>
<td>mouse leydig</td>
<td>(Campbell et al., 1994)</td>
</tr>
<tr>
<td>COS7</td>
<td>African green monkey kidney</td>
<td>(Hofmann et al., 1993)</td>
</tr>
</tbody>
</table>

The WTI promoter contains several WT1/EGR1 binding sites raising the possibility that WT1 may bind and regulate its own promoter. Footprinting studies identified seven putative regions of WT1 binding with the 5' region (Fig. 8). Transient transfection assays have demonstrated that high levels of the WT1 protein repress the activity of the WTI promoter by 50% (Rupprecht et al., 1994). One of the WT1 footprints identified in the WTI promoter overlaps with the Sp1 binding CTC repeat. This raises the possibility that that competition between Sp1 and WT1 for the CTC repeat is involved in the regulation of the WTI gene. WT1 has been shown to negatively regulate expression of the Egfr promoter and this repression is mediated by binding a CTC repeat region in this promoter (Englert et al., 1995a).
In transient transfection experiments the WTI promoter appears to be constitutively active. However, Cohen et al. have reported differential DNaseI hypersensitivity in the endogenous WTI locus between WTI expressing and non expressing cells (Cohen et al., 1997). Therefore there must be regulatory elements which are involved in ‘opening up’ the WTI promoter in specific cell types. A number of such potential tissue specific regulatory elements have been identified in cell culture experiments. Lying immediately 3’ to the WTI gene is a haematopoietic enhancer. This enhancer can activate gene expression in erythroid cell lines through the binding of GATA1 (Wu et al., 1995). Lying within intron 3 of the WTI gene is a repressor element containing an alu repeat. This repressor is capable of repressing transcription from the WTI locus in all cell lines in which it has been tested except those of kidney origin (Hewitt et al., 1995a). Around 250 bp 5’ of the CTC repeats in both the mouse and human promoters is an element (CR) which contains two paired box binding sites (Fig. 8). High levels of PAX8 and PAX2, but not PAX5, transfected into cell lines, upregulate reporter gene expression via this CR element. PAX8 binding to the CR element has been demonstrated in transfected cells; PAX2 binding to the element has not been detected (Dehbi et al., 1996; Dehbi and Pelletier, 1996).

The WTI gene may be post transcriptionally regulated. The WITI gene lies around 800 bp 5’ of the WTI gene; it is transcribed in a centromeric direction, as opposed to the telomeric direction of WTI (Gessler and Bruns, 1993) (Fig. 9). The WITI gene does not appear to encode a protein and may exert its activity as an RNA. WTI and WITI are transcriptionally co-regulated. They are expressed in the same spatio-temporal pattern during development and at an invariable ratio of 5-10WTI:1WITI transcripts (Yeger et al., 1992). Uncoupling of WTI WITI regulation occurs only in a few Wilms’ tumours where deregulation of WTI expression is likely (Huang et al., 1990). Eccles et al. 1994 and Campbell et al. 1994 both identified a number of cDNAs, running antisense to WTI, spanning part of WTI intron 1, the upstream sequences between WTI and WITI and part of the WITI gene. Malik et al. 1995 showed that DNA sequences within the first intron of WTI can act as a promoter producing an antisense transcript of exon 1. The putative WITI promoter as with the
putative WTI promoter has no TATA or CCAAT boxes but several consensus Sp1, WT1 and PAX2 binding sites. Unlike the WTI promoter, that of WIT1 is not CG rich and is positively activated by WT1 protein. Stable transfection with a construct encoding the message from the putative WIT1 promoter significantly reduced (to 50%) the level of WT1 protein in cell lines with high endogenous WTI activity. WTI gene expression therefore may be regulated by antisense transcripts. This method of regulation has been proposed for other genes than WTI e.g. N and c-myc, and c-Ha-ras. Similarly to WTI/WIT1, N-myc and its antisense transcript N-cym are transcriptionally co regulated (Armstrong and Krystal, 1992). To date a mouse homologue of WIT1 has not been isolated. The mouse as well as human however exhibits bi-directional transcription through the first exon of WTI implying that antisense control may also occur at the Wti locus (Campbell et al., 1994).

Analysis of putative WTI promoter activity in cell lines gives some indication as to the localization of regions required for promoter activity and the factors which bind to them. For a true in vivo analysis of WTI promoter function transgenic analysis is required. Cohen et al. created four transgenic mouse lines containing a WTI promoter-lacZ reporter construct (Cohen et al., 1997). The promoter region they used ran from 1.9 kb upstream of the main transcription initiation site to 0.2 kb downstream (see Fig. 9). These transgenic mice showed no reporter gene expression. Hewitt et al. isolated a further 6 mice containing a 2.5 kb WTI promoter-lacZ reporter constructs and 15 with shorter versions of this construct (Hewitt et al., 1996). None of these mice expressed the reporter gene in transgenic embryos. It is difficult to explain why the same region of the WTI promoter activates transcription in cell lines but is inactive in transgenic animals. As the putative WTI promoter region contains CpG islands WTI may potentially be regulated by tissue specific DNA methylation. If this is the case the lack of methylation in transfected DNA may lead promiscuous promoter activity in cell lines. A larger 42 kb cosmid containing the WTI gene was also introduced into transgenic lines by Hewitt et al. Transcription is activated from the WTI locus of this transgene but it is unclear whether it has any tissue specific activity (Hewitt, J. et al., 1996).
Fig. 8 Sequence of the DNA 5’ of WT1.
uppercase - human, lowercase - mouse. The (CTC)³ repeat and CR element are highlighted along with a putative Sry binding site. Arrows represent transcription start sites and the two alternative translation initiation sites are indicated (TLSS1, TLSS2). Lines above the sequence represent WT1 footprints.
Fig. 9 Schematic representation of WT1 and WIT1 transcription start sites.
1.8 Analysis of gene regulation - methodology.

In the study reported in this thesis we wished to analyse the mechanism of \( WT1 \) gene regulation and also to create a good marker for \( WT1 \) expression during development. In this section I discuss why the transgenic method and in particular the YAC transgenic method was chosen for this study. We made transgenic lines using three different reporter constructs. The first contains a region of the 5' \( WT1 \) promoter linked to a lacZ reporter gene. The region of \( WT1 \) promoter used in this construct is slightly bigger than that used by Cohen et al. and Hewitt et al. in recent reports (Fig. 9). The second and third constructs were both made by integrating a lacZ reporter gene into exon 1 of the \( WT1 \) gene locus on a YAC. By using these YAC constructs we hoped that the inserted transgenic locus would carry all \( WT1 \) regulatory sequences in the correct chromatin environment. LacZ expression from this locus therefore, would mirror exactly that of the endogenous \( Wti1 \) gene. We could then use such a transgene exactly mimicking the expression pattern of \( Wti1 \) as a marker to examine the expression of the gene on both wildtype and mutant genetic backgrounds.

Cell lines are useful for biochemical studies of promoter activity such as identification of DNaseI hypersensitive sites and protein footprints. These sites may indicate regions of the promoter which may be bound by transcription factor molecules. The relative importance of different elements within a promoter in controlling gene expression from a locus can be assessed by introducing mutations into the putative promoter. The ability of these 'mutant' promoters to activate transcription of a reporter gene 'hooked up' to the promoter can then be assayed by introducing these constructs into cell lines. The tissue specific activity of the promoter can be tested, to some degree, by assaying activity in a range of cell types. Although such techniques are relatively quick and easy to carry out, putative enhancers revealed by such analysis may often not be utilized in vivo.

To assess the in vivo developmental control, mediated by elements within a gene promoter, a transgenic assay is required. A short transgene containing enhancers
lying immediately proximal to a gene is often enough to drive proper tissue specific activity of a reporter gene in a transgenic animal, e.g. Msx1 (MacKenzie et al., 1997). On the other hand the activity of many promoters is often weak and the expression pattern subject to ‘position effects’, e.g. genes of the β globin cluster (Martin et al., 1996). Position effects are caused by the chromatin at the site of transgene integration affecting the expression of that transgene. Elements lying at the site of integration can act as enhancers driving expression of the transgene in incorrect cell types, this is know as regulation position effects (RPE) (Milot et al., 1996). Alternatively, transgenes can take up an ‘inactive’ chromatin configuration. This may be caused by spreading of inactive chromatin present at the site of transgene integration, similar to the effect of position effect variegation (PEV) first described in Drosophila melanogaster (Martin and Whitelaw, 1996).

Enhancer elements may lie a great distance from the region of gene initiation. Transgenic constructs carrying only a short region of a gene may therefore not contain all elements required to drive transgene expression in the correct cell types. For example, lying 6-22 kb 5' of the human ε globin gene are five DNaseI hypersensitive sites. These sites contain multiple binding sites for tissue specific transcription factors. Mutations in humans in this region disrupt correct expression of the genes of the β globin cluster and cause haemoglobinopathies (Townes and Behringer, 1990). This region of hypersensitive sites plays a very important role in the control of the genes of the β globin cluster and has been termed the β globin locus control region (LCR). Inclusion of the LCR on a transgenic construct which also carries the genes of the β globin cluster increases the level of tissue specific transcription form each of these genes. It further prevents transgenes from being subject to both RPEs and PEV. In many cases the spatial arrangement of elements within a locus may also be essential for its proper expression. For example the correct order of the genes within the globin cluster and their relationship with the LCR is essential for correct gene switching during development (Hanscombe et al., 1991).
In order to attempt to recreate the endogenous locus and include potential long range regulatory elements in a transgenic animal it is often advantageous to use a very large transgenic construct. Yeast artificial chromosomes have a number of advantages for use in transgenic experiments. They are capable of maintaining DNA inserts in excess of 1 Mb in size and have been successfully utilised for the generation of transgenic animals. For example, YAC constructs encoding \textit{PAX6} (420 kb) (Schedl et al., 1996b), \textit{β-amyloid precursor protein (APP)} (650 kb) and human chromosome region 21p22.2 (430-1100 kb) have all been successfully used to generate transgenic animals. An added advantage for YAC constructs is that they can be quickly and easily altered, for example to introduce reporter genes or promoter mutations, by homologous recombination in yeast. For a review of the uses of YACs in transgenic studies consult Peterson \textit{et al.,} 1997.

To get a faithful study of gene expression, however, the endogenous locus is obviously the best promoter to study. An endogenous gene locus can be targeted and manipulated in ES (embryonic stem) cells. These cells can then be used to generate transgenic lines. Deletion individually of each of the 5 DNaseI hypersensitive sites contained with the β globin LCR has been undertaken at the endogenous locus. These deletions have no effect on expression or mildly reduce expression of linked globin genes. Using YAC transgenic analysis however deletion of individual hypersensitive site significantly reduced the level of transcription from linked β globin genes (Martin et al., 1996). The discrepancies between these different experiments indicate that YAC transgenic constructs may still, to a degree, be influenced by position effects in the same way as much smaller constructs. On balance however using YAC transgenes is often advantageous over targeting the endogenous locus. As carrying out homologous recombination and manipulation of sequences in ES cell is a very much more difficult and time consuming process than in YACs.
2. Analysis of WT1 regulation by a 5kb promoter sequence.

The putative WT1 promoter in the region between WT1 and WIT1 and spanning the WT1 transcription start sites has promiscuous promoter activity in transfected cell lines (Table 2). In order to analyze the ability of this putative WT1 promoter to regulate gene expression in vivo, we isolated 5kb of proximal WT1 promoter. This 5kb promoter sequence (hereafter referred to as USWT1) consisted of around 3 kb 5' of the WT1 transcription initiation site, WT1 exon 1 and 1 kb of intron 1. USWT1 spans sequences identified as a minimal promiscuous WT1 promoter (Hofmann et al., 1993; Fraizer et al., 1994). It includes WT1, Sp1 and PAX binding sites proposed to have a role in the regulation of the WT1 gene (Dehbi et al., 1996; Dehbi and Pelletier, 1996; Cohen et al., 1997; Rupprecht et al., 1994) (Figs. 8 and 9).

2.1 Cloning of USWT1, a 5kb WT1 promoter sequence.

The bacteriophage construct λWT4, contains around 15 kb of human genomic sequence upstream of WT1, WT1 exon 1 and about the first kilobase of WT1 intron 1. A 5 kb BamHI/SalI fragment (USWT1) was isolated from λWT4 and cloned into pUC18 to give plasmid pUSWT1. One of two different lacZ cassettes was subsequently cloned into the SacII site of pUSWT1 by blunt end ligation. Inserting a 3.7 kb XbaI/BamHI lacZ fragment, isolated from RB8Z (Charite et al., 1994), into pUSWT1 gave rise to pNLS. The XbaI/BamHI lacZ fragment inserted into pNLS contains, in addition to the lacZ gene, a nuclear localization sequence. A second plasmid pNOR was constructed by ligating the 3.7 kb HindIII/BamHI lacZ fragment isolated from pCH110 (Pharmacia) into the pUSWT1 SacII site. The lacZ cassette in pNOR unlike that of pNLS carries no nuclear localization signal. In both pNLS and pNOR the lacZ cassette inserted is under the control of its own translation initiation site.
Fig. 10: Cloning of a 5 kb potential W77 promoter (pUSWT1) from the human W77 locus and subsequent insertion of a lacZ reporter (pNLS/pNOR).
2.2 Transient transfection of cell lines.

To investigate whether the pNOR and pNLS constructs were able to drive lacZ expression, a series of cell line transfection experiments was undertaken. A range of different cell lines was used in an attempt to estimate any potential tissue specificity residing in the USWT1 promoter fragment. The results of this investigation are summarized in table 3. Transfection of cells was carried out as described in materials and methods using pNOR, pNLS or pCMV. pCMV contains the lacZ gene driven by the highly active ubiquitous CMV promoter (MacGregor and Caskey, 1989). Efficiency of transfection is highly divergent between different cell lines; hence using pCMV as a positive control gives an indication of the transfection efficiency of each line. The level of β-galactosidase activity of each promoter (CMV, NOR and NLS) was assessed by estimating the percentage of cells per transfected plate which stain blue following incubation with X gal. Such a technique is crude, but is believed to be a reliable method of estimating β-galactosidase activity (MacGregor et al., 1989).

The 5kb of putative WTI promoter (USWT1) in pNOR and pNLS has promoter activity. It is highly active in mouse mesothelioma (AC29) cells and also to a lesser extent in human granulosa cell tumour (COV343) cell lines. Both of these cell lines express WTI endogenously. pNOR and pNLS show equivalent activity in each cell line. β-galactosidase expression in cells transfected with pNLS is localized to the nucleus (Fig. 11). Cell lines which do not express endogenous WTI (10T1/2 and C2 cells) do not show pNOR or pNLS promoter activity. However both the 10T1/2 and C2 cells were only transfected once and showed a low level of transfection efficiency. No definite conclusion can be drawn from the data collected in these lines.
Fig. 11 β-galactosidase expression in AC29 cells transfected with pNOR, pNLS or pCMV.

β-galactosidase transcribed from pCMV and pNOR is present throughout the cell or mainly in the cytoplasm. β-galactosidase transcribed from pNLS is located mainly in the cell nucleus.
Surprisingly the USWT1 promoter sequence does not drive β-galactosidase expression in mouse mesonephric cells (M15) and Sertoli cells (PyT45) indicating that it is not a promiscuous promoter. These cell lines are both easily transfecatable and express endogenous WTL in high levels. The inability of USWT1 sequences to drive reporter gene expression in M15, PyT45, C2 and 10T1/2 cells is in contradiction to findings of other investigators. Several groups have demonstrated that the region of WTL promoter spanning the transcription start site acts as a promiscuous promoter (Table 2). In particular both Frazier et al. 1994 and Cohen et al. 1997 have demonstrated that constructs very similar to USWT1 are active in 293 foetal kidney cells, K562 leukaemia cells, JMN mesothelioma cells and HeLa cervical carcinoma cells. It is difficult to explain the difference between the findings of this study and those of other groups. Potentially with a more careful and sensitive analysis we could identify a low level of USWT1 promoter activity in those cell lines in which we have so far failed to do so. However the purpose of this experiment was in the main to determine if the pNOR and pNL5 constructs were able to drive reporter gene expression. This is the case.
<table>
<thead>
<tr>
<th>cell line name</th>
<th>cell type &amp; reference</th>
<th>WT1 activity</th>
<th>CMV</th>
<th>NLS and NOR</th>
<th>No. of experimenta l repeats.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC29</td>
<td>mouse mesothelioma (Langerak et al., 1995)</td>
<td>++</td>
<td>10-30</td>
<td>1-2</td>
<td>2</td>
</tr>
<tr>
<td>COV434</td>
<td>human granulosa cell tumour (van den Berg Bakker et al., 1993)</td>
<td>+</td>
<td>3-10</td>
<td>0.1</td>
<td>6</td>
</tr>
<tr>
<td>M15</td>
<td>polyoma large T immortalized mouse mesonephric (Larsson et al., 1995)</td>
<td>++</td>
<td>10-30</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>PyT45</td>
<td>polyoma large T immortalized Sertoli cells from adult mouse testicular tumours (Rassoulzadegan et al., 1993)</td>
<td>++</td>
<td>10</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>10T1/2</td>
<td>mouse fibroblastic (Larsson et al., 1995)</td>
<td>-</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>C2</td>
<td>mouse myoblastic (Larsson et al., 1995)</td>
<td>-</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
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</table>

A schematic indication of the level of endogenous WT1 activity is given in the column headed (WT1 expression) along with the relevant references for cell type and WT1 activity, (++ = high, + = medium, - = none). Promoter activity of pCMV, pNOR and pNLS is given as an estimate of the percentage of cells per transfected plate which stain blue following incubation with X-Gal.
2.3 Establishment of transgenic lines containing NOR and NLS transgenes.

Transient transfection of cell lines demonstrated that USWT1 has a promoter activity. Potentially this activity was not promiscuous in contradiction to the findings of other groups. In order to investigate the true tissue specificity of this promoter in vivo, transgenic lines were made using pNOR and pNLS as transgenes.

pNOR and pNLS were digested with BamHI and SphI. This digestion released an 8 kb fragment containing the USWT1 sequence with integrated lacZ cassette. The 8 kb fragment was then used to microinject oocytes isolated from superovulated (C57BL/6 x CBA)F1 mice. 167 of the oocytes injected survived overnight culture to the 2 cell stage. These were transferred into pseudopregnant host mothers. 54 mice were born of which 3 were transgenic, NOR21 (male), NLS52 (female) and NLS53 (female). Founder NLS53 did not transmit the transgene (around 50 offspring were analyzed). NOR21 and NLS52 transmitted the transgene founding lines NORp21 and NLSp52 respectively. A genomic Southern blot of offspring from founders NOR21 and NLS52 indicates that the transgenes have inserted intact (Fig. 12). Embryos isolated from NORp21 and NLSp52 showed developmentally regulated β-galactosidase expression. There was little similarity however, in the expression pattern seen in these two lines. For a detailed description of the expression pattern of NORp21 and NLSp53 refer to section 2.6.
Genomic DNA of offspring from breeding lines NLSp52 (lanes 1-11) and NORp21 (lanes 12-22) was digested with EcoRI and XhoI and Southern blotted. Control lanes are EcoRI/XhoI digested plasmid (pNLS or pNOR) DNA. The blot was probed with TRPLZ (see section 7.14). TPLZ highlights the lacZ EcoRI fragment in NLSp52 (2.5 kb) and NORp21 (5.5 kb). In lanes pNLS and pNOR another band of around 700bp indicates the EcoRI/XhoI fragment spanning the 3’ end of lacZ and the 3’end of WTI exon 1. TPLZ also hybridizes to the murine TrpI gene giving a strong band at 2.6kb and weak bands at 5.5 and 5 kb. A further band in lanes pNLS and pNOR, slightly bigger than the TrpI 2.6 kb band, represents hybridization to pUC18 plasmid sequences (2.66kb). The NLS transgene is present intact in lanes 3, 4, 5, 8 and 10. The NOR transgene is intact in lane 19 but has undergone a rearrangement in lane 15. This was the only example of rearrangement recorded in all offspring analyzed.
<table>
<thead>
<tr>
<th>MW (kb)</th>
<th>PNLS</th>
<th>PNOR</th>
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<tr>
<td>23.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9.4</td>
<td></td>
<td></td>
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<tr>
<td>6.6</td>
<td></td>
<td></td>
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<tr>
<td>2.3</td>
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The diagram shows a gel electrophoresis with bands at 23.1, 9.4, 6.6, and 2.3 kb.
2.4 Isolation of ‘transient’ transgenic embryos.

The developmental expression pattern of the transgene was very different between NORp21 and NLSp52. To allow quicker analysis of several different transgenic embryos a ‘transient’ approach was used. Mouse oocytes were microinjected, the resulting embryos isolated at E12.5 and examined for transgene expression. E12.5 was chosen on the basis of the reported Wt1 expression pattern. It is a stage where the gene is expressed in a number of different developing structures. Analysis of NORp21 and NLSp52 embryos had further highlighted transgene expression in the interdigital areas of the developing handplate which would not be seen earlier than E12.5. Finally E12.5 is the most developed stage at which there is full penetration of the X-gal stain allowing embryos to be stained whole. With microinjection help from Dr. Andreas Schedl and Ms Lesley McInnes 8 transgene expressing embryos (T1-8) were isolated, stained and vibrotome sectioned. These embryos were examined for any conserved expression domains. The conservation of any such domains between a number of the embryos may indicate that elements responsible this expression are present on USWT1.

2.5 Comparison of expression between NORp21, NLSp52 and T1-8.

Comparison of the expression pattern between each of the NOR or NLS transgenic embryos allows any potential tissue specific activity of USWT1 to be assessed. There is a large degree of variation between the β-galactosidase expression pattern in each of the embryos. This variation indicates that the USWT1 promoter is weak and susceptible to position effects. (For a detailed description of the expression pattern in each of these embryos refer to section 2.6). Between the 10 embryos analysed it was possible to identify domains of transgene expression which are similar to the endogenous expression of Wt1 and/or reoccur in more than one embryo. These domains are summarized in table 4. Fig. 13 illustrates the β-galactosidase expression pattern in two of these transient transgenic embryos.
A main site of expression for \textit{WtI} is within the developing urogenital system in particular in the mesonephros, metanephros and gonads (Pritchard Jones et al., 1990; Armstrong et al., 1992; Rackley et al., 1993; Pelletier et al., 1991c). No expression of the transgenes could be detected in any of these tissues which was distinguishable from background endogenous $\beta$-galactosidase activity. \textit{WtI} is expressed in the pericardium, diaphragm and mesenteries of the gut. 4/10 of the embryos examined showed transgene expression in some of these tissues and 3/10 had expression on the surface of the umbilical hernia which is an extension of gut mesentery. 2/10 of embryos show NOR and NLS promoter activity in the body wall muscle, another previously reported domain of gene expression. Whilst all of the embryos examined showed expression in the brain, the domains of expression appeared to differ between each. However expression in the ventral part of the spinal chord was seen in 3/10 of the lines and appears to be a similar region to that in which \textit{WtI} is active.

A number of other sites of transgene expression, not similar to domains of \textit{WtI} activity, are common between several of these transgenic embryos. In particular 6/10 of the embryos show varying degrees of expression in limb mesenchyme In the main this appears to be in differentiating limb musculature which is perhaps analogous to transgene expression in differentiating body wall muscle. 3/10 of the embryos show transgene expression in intercostal mesenchyme which again could potentially be in developing muscle. Another domain of transgene expression in 5/10 of these transgenic embryos is the hand plate in particular the interdigital regions and/or the marginal zones. These regions of loose mesenchyme undergo apoptosis raising the possibility that the transgenes may be marking domains of apoptosis in these embryos (Konig et al., 1993).

\textit{NORp21}, T8 and to some degree T4 have a general expression pattern of $\beta$-galactosidase similar to that of \textit{WtI}. Hence the 5kb of \textit{WtI} promoter sequence (USWT1) present in pNOR and pNLS has tissue specific promoter activity. It is able to drive reporter gene expression in a tissue specific manner similar to endogenous
expression. It is not however, able to drive reporter gene expression within the developing urogenital system. USWT1 does drive reporter gene activity in a potentially novel site for \textit{Wt1} activity. Half the embryos isolated in this study show expression of lacZ within the interdigital regions of the developing handplate. In fact this domain does represent an previously unidentified domain of \textit{Wt1} activity as has later been confirmed by \textit{in situ} hybridization analysis (see Chapter 5).

One must be careful interpreting the NOR/NLS transgenic experiments. Whatever activity USWT1 has is weak and is easily subject to alteration by its site of integration into the host genome (position effects). It may be that transgenes in general, and NOR/NLS in particular, have increased susceptibility to position effects driving expression in certain organs or tissues, e.g. the limb and parts of the nervous system. Therefore preponderance of β-galactosidase expression in tissues is not a definitive indication of the ability of the USWT1 promoter fragment to drive reporter gene expression within them.

Since this study was completed Cohen \textit{et al.} 1997 and Hewitt \textit{et al.} 1996 have reported using about a 2 kb a region of the \textit{WT1} promoter linked to lacZ to generate 10 transgenic lines. They were unable to get transgene expression in any of these lines. The constructs they used contain only around 1 kb less 5’ sequence than USWT1 (Fig. 9). The integrated transgenes in their studies appear to have adopted an ‘inactive’ configuration within the host genome. pNOR/pNLS in this study did not. Reasons as to why this has happened are not at all clear. These constructs and USWT1 span similar regions of the putative 5’ proximal \textit{WT1} promoter; therefore they must contain similar elements for the binding of basal and tissue specific transcription factors. Increase copies of an inserted transgene may lead to increased transgene expression, e.g. (Schedl et al., 1996b). Neither Cohen \textit{et al.}, Hewitt \textit{et al.} nor myself have determined the numbers of copies integrated in any of these \textit{WT1} promoter-reporter transgenic lines. It seems unlikely however that I will have consistently got insertion of many copies of transgene leading to higher levels of
expression whilst they did not. Perhaps the extra chromatin inserted in the NOR/NLS transgenics as opposed to Cohen's and Hewitt's has helped to 'open up' a promoter which is weak in activity. The promoter is then activated to an extent where transcription from the locus is now detectable. This opening may simply be due the extra volume of DNA inserted in NLS/NOR animals; alternatively it could be due to a specific element encoded on USWT1 but missing in the Cohen and Hewitt constructs.
Fig. 13 β-galactosidase expression driven by USWT1 in E12.5 transient transgenic embryos.

T4 (a) and T5 (b) show transgene activity in regions of endogenous Wt1 activity. Abbreviations: (NT) neural tube, (IC) intercostal mesenchyme, (Liv) liver, (Pl) pleura, (Pe) peritonea, (UH) umbilicus, (Vb) vertebrae.
**Table 4 β-galactosidase expression in NOR/NLS transgenic embryos.**

<table>
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<tr>
<th></th>
<th>Wt1</th>
<th>NOR p21$^1$</th>
<th>NLS p52$^1$</th>
<th>T1$^2$ NOR</th>
<th>T2$^2$ NOR</th>
<th>T3$^2$ NOR</th>
<th>T4$^2$ NOR</th>
<th>T5$^2$ NOR</th>
<th>T6$^2$ NOR</th>
<th>T7$^2$ NLS</th>
<th>T8$^2$ NLS</th>
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<td>intercostal mesenchyme</td>
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1- Lines NORp21 and NLSp52 are stable transgenic lines

2- Embryos T1-8 are transient transgenic analyses

3-Whilst organs such as the mesonephros metanephros and gonads strongly express Wt1, the NOR and NLS transgenes are not expressed in them in any of the 10 transgenic lines analysed.

4- Wt1 expression detected by RT-PCR (Armstrong et al. 1992)
2.6 LacZ expression pattern in NOR and NLS transgenics.

2.6.1 Transgene expression in NORp21.

Expression of the NOR transgene in NORp21 transgenic embryos shows several similarities to that of endogenous Wt1. At E12.5 there is transgene activity in the pericardium, diaphragm, lateral body wall, body wall adjacent to the vertebrae and the tongue. Interestingly the visceral layer of the umbilical hernia expresses β-galactosidase, this tissue is continuous with the peritoneal mesentery of the gut which is a site of endogenous Wt1 activity. NOR transgene activity is evident in many other domains where endogenous Wt1 activity has not been reported. In the head, it is expressed in the mesenchyme behind the eyes, adjacent to the palate, at the back of the head, in the lower jaw and also in the nasal epithelium. NOR is expressed in parts of the third ventricle and forebrain. It is also expressed strongly in the notochord but interestingly not at the very tip of the tail. NOR is expressed in the apoptotic interdigital mesenchyme and in the developing musculature of the limb at the points where the limbs abut the body.

In the adult Wt1 activity is localized to the podocyte cells of the metanephros and the mesothelium. NOR is not expressed in the adult metanephros but is in the mesothelium overlaying the gonad.

2.6.2 Transgene expression in NLSp52.

At E12.5 NLS is expressed solely in the developing limb. It is confined to the dorsal side of the hand plate in both the posterior marginal zone and between digits 4 and 5. In the adult animal transgene expression has only been identified in the mesothelial coating of the gonad.

2.6.3 Transgene (NOR) expression in T1.

Transgene expression in T1 is solely in parts of the cerebral cortex and midbrain.
2.6.4 Transgene (NOR) expression in T2.

In T2 the NOR transgene is expressed on the surface of the umbilical hernia, intercostal mesenchyme, tongue and part of the lower jaw. There is transgene expression in all limb mesenchyme except that which is condensing to form the chondrogenic bone precursors. Part of the cerebellum, the mesenchyme of the tail and maxilla also show transgene activity.

2.6.5 Transgene (NOR) expression in T3.

In contrast to the transgene expression in T2, NOR in T3 is expressed in the condensing chondrogenic mesenchyme of the limbs and ribs, also in the notochord and part of the forebrain. The mesenchyme/peritoneum overlying the surface of the liver expresses NOR. This is a domain of high endogenous Wt1 activity.

2.6.6 Transgene (NOR) expression in T4.

T4 has transgene expression in the peritoneum/mesenchyme overlying the liver, pleura/mesenchyme surrounding the lungs, the interdigital mesenchyme and limb and body wall musculature. All of these tissues express Wt1 endogenously. Further transgene expression surrounds the thymus, tip of the tail and mesenchyme adjacent to the palatal shelves and pharyngo-tympanic tube.

The transgene is expressed in the ventral interneurons of the spinal chord, similar to the reported Wt1 expression domain. However at cervical regions it is also expressed in the motor neurones of the ventral horns where WT1 is not. In the rest of the nervous system transgene expression occurs in the hindbrain, a stripe across the midbrain, the optic nerve and the trigeminal ganglion.

Expression of the NOR transgene in T4 is illustrated in Fig. 13.
2.6.7 Transgene (NOR) expression in T5.

As with T4, T5 has NOR expression in the ventral spinal chord. There is further expression in what appears to be the developing musculature of the fore and hind limbs and the marginal zones of the handplate. The corpus striatum, pons/midbrain junction and in cells scattered throughout the tail also express NOR.

Expression of the NOR transgene in T5 is illustrated in Fig. 13.

2.6.8 Transgene (NOR) expression in T6.

T6 shows transgene expression in the mesenchyme around the eye, around the aorta and a hint of expression in the pelvic musculature.

2.6.9 Transgene (NLS) expression in T7.

There is transgene expression in the shoulder muscle and tongue of T7. Further expression occurs in the mesenchyme of the nose and palate and in part of the developing hypothalamus.

2.6.10 Transgene (NLS) expression in T8.

The transgene expression in T8 is very similar to that in some of the other embryos. There is expression of NLS in the interdigital mesenchyme, limb and shoulder muscles, intercostal mesenchyme, pericardium, pleura and the mesenchyme surrounding the paramesonephric duct as it approaches the urogenital sinus. All of these are domains of endogenous Wt1 expression. Like T4 and T5 there is β-galactosidase activity in the ventral spinal chord, and also the midbrain, medulla oblongata and dorsal root ganglia.
3. Creation of YAC transgenic lines.

3.1 Lines A, C and D.

Three of the transgenic lines, A, C and D, used in this study were donated by Dr Andreas Schedl (MRC HGU, Edinburgh, U.K.). Line A contains the construct WT470LZi and lines C and D WT280LZ. In all these lines a WTI exon 1/β-galactosidase fusion protein is expressed from the WTI promoter of the transgene.

The constructs used to create these lines were made as demonstrated in Fig. 14. A YAC (yIE5) was isolated spanning the human WTI locus (Fantes et al., 1995). yIE5 was introduced in yeast strain CGY2516. It was then retrofitted with an amplification vector (Schedl et al., 1993; Smith et al., 1993) to give rise to WT470. Alternatively yIE5 was truncated by homologous recombination with a fragmentation vector consisting of a 4.5 kb EcoRI-SalI fragment from 10 kb 3' of the WTI termination site cloned into pCGS990Hyglox (Smith et al., 1993). Truncation of yIE5 gave rise to a 280kb YAC, WT280. A lacZ gene was introduced into WT470 and WT280, by homologous recombination with the insert of pWT-lac. The recombination of pWT-lac with a WTI carrying YAC is shown in Fig. 15, (in this case the YAC is 800/4). This recombination gave rise to WT470LZi and WT280LZ respectively.

pWT-lac was constructed by cloning a 1.5 kb PstI-SalI fragment, spanning WTI exon 1, from λWT4 into a modified pUC18 vector (pUC18MCS). A lacZ gene was then inserted, in frame, into the XhoI site at the end of exon 1.

Lines A, C and D are all male sterile. The amplification vector used to retrofit yIE5 contains the Thymidine kinase (TK) gene which has been implicated in transgenic male sterility. In order to generate a construct which would allow male transmission,
yIE5 was retrofitted a second time, using a modified amplification vector in which the TK gene has been deleted (gift of Dr. S. Shen, MRC HGU, Edinburgh, U.K.), to give YAC construct 800/4.

### 3.2 Lines E, F, G and H.

To confirm the expression pattern seen in line A, further transgenic lines expressing β-galactosidase under the control of the WTI promoter were required. To avoid the problems of male transgenic sterility I constructed a new YAC construct, WT470LZii. This construct was created by homologous recombination between pWT-lac and 800/4 (Fig. 15). As with WT470LZi, a WTI exon1/β-galactosidase fusion protein under the control of the WTI promoter will be produced from WT470LZii. I then isolated WT470LZii DNA and used it to produce four more transgenic founders E, F, G and H.

#### 3.2.1 Construction of WT470LZii.

The replacement vector pWT-lac, digested with NotI, was used for transformation of yeast containing 800/4. Recombination between the WTI locus of 800/4 and pWT-lac inserts lacZ and Leu2 into the end of WTI exon 1. Transformed cells were selected by plating on -URA (Ura3 is present on the YAC short vector arm) -LEU media and 10 colonies picked. Pulse field gel (PFGE) analysis of plugs made from these colonies detected 9 containing a yeast artificial chromosome of the correct size (470 kb) (Fig. 16).

To detect if the lacZ gene has homologously recombined into WT470 (800/4) a Southern blot of EcoRI digested plugs was undertaken. This blot was hybridized with a WTI exon 1 specific probe (WTX). Insertion of the lacZ gene into the end of WTI exon 1 reduces the size of the exon 1 containing fragment from 15 kb to 8 kb (Fig. 15). Such a blot indicated the majority of colonies obtained from the transformation carry a mixture of recombined and non-recombined YACs (Fig. 17). This is an expected result, as due to the presence of the amplification vector on 800/4 each yeast cell carries several YACs. Only some of these may have undergone
homologous recombination. One colony (number 8) contains solely products of homologous recombination. For technical reasons this colony was not used for making yeast plugs to isolate YAC DNA for microinjection. Instead colony 10 was picked and then streaked out again under -LEU selection. All colonies picked from this plate contained only WT470LZii and no 800/4. One of these colonies was then used to as a source of YAC DNA for microinjection.

3.2.2 Isolation and microinjection of WT470LZii.

WT470LZii YAC DNA was isolated as described in the materials and methods chapter. Transgenic lines were derived by microinjection of this DNA into (C57BL/6 x CBA)F1 oocyte pronuclei. Some injections were carried out by myself, the majority by Ms Lesley McInnes. Four founder mice were obtained, E (female), F (male), G (male) and H (male). Only E and H were fertile and transmitted the transgene (through both male and female germlines).
Fig. 14 Scheme for the construction of WT470LZi and WT280LZ.
Fig. 15 Homologous recombination between 800/4 and pWT-lac introduces lacZ and leu2 into the YAC WTI locus.

The YAC 800/4 carries the human WTI locus. Recombination between this locus and the two regions of pWT-lac homologous to WTI exon 1 and to intron 1 inserts lacZ and leu2. The resulting YAC containing the fusion gene between WTI exon 1, lacZ and leu2 is named WT470LZii.
Fig. 16 Pulse field gel analysis of plugs made from colonies following transformation of 800/4 with pWT-lac.

(a) A pulse field gel of plugs made from colonies obtained after transformation of 800/4 with pWT-lac. The yeast chromosomes can be clearly seen including one of the correct size for WT470LZii (470kb). A Southern blot made from this gel is probed with (b) a WT/I cDNA (probe-WT33) and then stripped and probed with (c) a lacZ probe (ILZ). In (b) and (c) all lanes contain a chromosome of the correct size (470kb) which hybridizes with both WT33 and ILZ. The colony represented in lane 9 also carries chromosomes of the wrong size which hybridize both WT/I and lacZ. The wrong sized chromosomes in 9 are probably due to inter chromosomal recombination.
Fig. 17 Southern blot of EcoRI digested 800/4 plugs following transformation with pWT-lac.

Yeast plugs, made from colonies resulting from the transformation of 800/4 with pWT-lac are digested with EcoRI. This digest is Southern blotted and probed with WTI exon 1 (probe-WTX). Insertion of lacZ into WTI exon 1 reduces the size of the band detected with this probe from 15kb to 8kb. Only colony 8 contains solely the products of insertion of lacZ into WTI exon 1.
3.3 Molecular analysis of YAC transgenic lines.

The extent of YAC transgene integration in each transgenic line was determined by hybridizing EcoRI digested genomic Southern blots with a range of probes. The probes used were SVA and LVA (YAC short and long vector arms respectively), WT33 (WT1 cDNA) and L800 (a 800 bp fragment isolated from around 100kb 3' of the WT1 termination site). For a description of these molecular probes see materials and methods. The results of this genomic Southern analysis are shown in Fig. 18 and schematized in Fig. 19.

Lines A, C and D, all of which contain the TK gene in the LVA, are male sterile. All of these lines have the LVA integrated into the genome. The modified amplification vector used to make WT470LZii has TK deleted from the LVA. Two of the lines containing this transgene transmit it through both the male and female germline; neither of them have the LVA integrated. Two male founder mice, containing WT470LZii, F and G were sterile. Founder G had testes less than one quarter the size of wild type. It would be interesting to ascertain whether founders F and G have integrated a modified amplification vector. It seems likely that these mice will have an integrated WT470LZii LVA. If this is the case, the part of the TK gene responsible for male sterility is still present on the vector used to make 800/4.

A PCR based approach was used to estimate the number of copies of transgene integrated in each line. Primers J422 and J423 (materials and methods) amplify human and mouse WT1 exon 10 with identical efficiency to give rise to a 107 bp product. The region which they amplify spans a sequence difference between the human and mouse genes. A BstUI restriction site is present in the mouse, but not human, sequence. PCR products derived from the mouse genomic template will carry this site, those from the human YAC transgene template will not. Digestion of the mouse product with BstUI cuts it into 59 and 49bp fragments. PCR using J422 and J423 was carried out on genomic DNA from each transgenic line. Aliquots of
reaction product were removed during the exponential stage of sequence amplification (20–30 cycles). These products were then BstUI digested, run on a gel and Southern blotted. The blot was then hybridized with the oligonucleotide J420 (materials and methods). J420 hybridizes with equal efficiency to mouse template (59 bp) and human template (107 bp) derived PCR products. The ratio of human derived to mouse derived PCR products gives an indication of the number of human \textit{WT1} loci carried in the line (Fig. 20).

In order to answer questions raised elsewhere in this thesis (Chapter 5) the chromosomal location of transgene integration in lines A and H was determined via fluorescent \textit{in situ} hybridisation (FISH) (Fantes et al., 1992) (Fig. 21). The FISH analysis was carried out by Muriel Lee, on chromosome spreads of splenic haemopoietic cells. The probe used was cosmid B2.1 (Fantes et al., 1995).
Fig. 18 Southern blot of EcoRI digested genomic DNA from transgenic lines A, E, H, C and D.

Lanes A-D refer to their respective transgenic lines, (human) a wildtype human and (mouse) a Swiss strain mouse. The YAC DNA used in this blot came from WT280LZ.

(a) Southern blot probed with the WT1 cDNA. The lane containing human DNA represents the normal pattern of hybridization for the WT1 gene probed with its own cDNA. The numbers alongside the blot refer to the exons represented by each of these bands. Insertion of the lacZ gene into exon 1 of WT1 as has occurred in WT470LZ(i and ii) and WT280LZ adds an EcoRI site between exons 1 and 2. Due to this the exon 1 and 2 band is split into two (\* = exon 1, \** = exon 2). The human WT1 cDNA also hybridizes, less well, to the mouse Wti gene. This hybridization gives bands A-F, which can be seen in the mouse control lane, and also more clearly in lanes E and H. Lines A, H, C and D show fully intact WT1 loci integrated into the transgenic line. In line E however only bands from WT1 exon 1-3 are present, the rest of the gene has been lost.

(b) Southern blot probed with the YAC short vector arm (SVA). The short vector arm is integrated in lines A, E and H. The presence of multiple copies of SVA sequences in line H and their range of sizes implies the transgene has integrated in a tandem array of YAC with differing breakpoints 3' of the SVA.

(c) Southern blot probed with the YAC long vector arm (LVA). Transgenic lines C and D contain at least one copy of the LVA whilst line A shows bands of two differing sizes. A band in the human DNA lane must be due to contamination.

(d) Southern blot probed with L800. The L800 probe is a 800 bp fragment isolated from around 100kb 3' of the WT1 termination site. This region is not present on WT280LZ hence no band is seen in lines C and D. Lines A, E and H all contain at least one copy of this region. The band present in the human DNA lane must be sample contamination.

The contaminating band seen in the human control lane is of a similar size to that produced by hybridization of the L800 probe to WT470LZ. This raises the possibility that in the band in lanes A, E and H is also contamination. This is particularly a possibility in the analysis of line E. In line E the transgene is truncated 3' of WT1 exon3 and has no LVA. If the L800 probe is hybridizing to line E then a YAC intergration/rearrangement event similar to that postulated in fig. 19 must have occurred. Alternatively if the L800 probe is hybridizing to contamination in this lane the YAC may simply be truncated 3' of WT1 exon3.
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**WT33**

**SVA**

**LVA**

**L800**
Fig. 19 Schematic representation of the extent of transgene integration in transgenic lines.

Genomic Southern blots of each transgenic line were hybridized with a selection of probes: SVA and LVA (YAC short and long vector arms respectively), WT33 (WT1 cDNA) and L800 (a 800 bp fragment isolated from around 100kb 3' of the WT1 termination site).

A- Transgenic line A shows full integration of the transgene.

E- Transgenic line E is deleted from WT1 intron 3 until a point 5' of L800, it is further truncated 3' of L800. Alternatively the transgene may simply be truncated 3' of WT1 intron 3.

H- Transgenic line H shows full integration of the transgenes 5' of L800 they are then truncated 3' of this point.

C&D Transgenic lines C&D are both truncated at a point 5' of WT1 but contain full integration of transgene sequences 3' of the gene.
Fig. 19 Schematic representation of the extent of transgene integration in transgenic lines.
Genomic Southern blots of each transgenic line were hybridized with a selection of probes: SVA and LVA (YAC short and long vector arms respectively), WT33 (WT1 cDNA) and L800 (a 800 bp fragment isolated from around 100kb 3' of the WT1 termination site).

A- Transgenic line A shows full integration of the transgene.
E- Transgenic line E is deleted from WT1 intron 3 until a point 5' of L800, it is further truncated 3' of L800. Alternatively the transgene may simply be truncated 3' of WT1 intron 3.
H- Transgenic line H shows full integration of the transgenes 5' of L800 they are then truncated 3' of this point.
C&D Transgenic lines C&D are both truncated at a point 5' of WT1 but contain full integration of transgene sequences 3' of the gene.
The ratio of human derived to mouse derived PCR products gives an indication of the number of human WT1 loci carried in the line. Samples were removed from a PCR reaction at 25 (lane 1) 28 (lane 2) and 30 (lane 3) cycles. The figure shows the relative amount of human and mouse derived product at each of these stages. PCR was carried out on lines heterozygous for the transgene and homozygous for mouse Wt1. Hence the number of copies of transgene is equivalent to the ratio of human to mouse product multiplied by 2. Line A contains 1 copy of a transgenic WT1 locus, line H 9, line C 1 and line D 2. It is not possible to carry out this copy number analysis for line E as the transgene is deleted for WT1 exon 10. A control shown represents PCR carried out on DNA from a non transgenic Swiss mouse. The ratio and percentages quoted in this figure are both calculated directly from the intensity of the bands shown using the 'ImageQuant’ programme (Molecular Dynamics).

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Fig. 20 Estimation of the transgene copy number in each transgenic line.
Fig. 21 Fluorescent *in situ* hybridisation (FISH) mapping of transgene integration sites.

Computer enhanced chromosome spreads from lines A(a) and H(b). Spreads were probed with cosmid B2.1. In line A the transgene (green signal) has integrated into chromosome 4 in the region of (A3-B). Line H shows transgene (green signal) integration in the telomere of chromosome 4 (E).
3.4 Summary of the state of transgene integration in each line.

3.4.1 Line A.

Line A contains one copy of what appears to be a complete WT470LZi transgene. A Southern blot of genomic DNA from line A probed with WT33, indicates the WTI locus to be fully intact. Similarly both vector arms and L800 are integrated. There appear to be two copies of the LVA indicating a second partial integration of the 3’ end of WT470LZi. FISH analysis maps the transgene to chromosome 4, in the region from A3 through B.

3.4.2 Line E.

It was not possible to determine the WT470LZii transgene copy number in line E using the PCR based approach as the WTI locus has only partially integrated. It is only possible to make out bands corresponding to exons 1, 2 and 3 when a Southern blot of line E genomic DNA is probed with WT33. The SVA and L800 are present but not the LVA. This indicates that WT470LZii is deleted from WTI intron 3 until a point 3’ of WTI and 5’ of L800. It is then truncated further 3’ of L800. Potentially this transgene locus may not contain one deleted YAC but the cointegration of two truncated YAC fragments.

Alternatively it is possible that the L800 signal in the line E genomic blot is contamination. If indeed this is the case then the YAC transgene may simply be truncated 3’ of WTI exon3. Further analysis will be required to distinguish between these two possibilities.

3.4.3 Line H.

FISH analysis shows integration of many copies of WT470LZii. All of these are integrated in the vicinity of the telomere of chromosome 4 (band E). PCR analysis indicates 9 copies of the WTI locus are integrated in line H. A line H genomic
Southern blot hybridized with WT33 indicates that at least some of these are intact. There are many more than 9 copies of the SVA integrated, but none of the LVA. The EcoRI fragments which hybridize to SVA in the line H blot show a range of differing sizes. All these data imply that WT470LZii has integrated in a tandem array containing differing YAC breakpoints 3' of the SVA. This type of YAC transgene integration has been previously reported (Schedl et al., 1996b). It may be the result of a particular mechanism of assimilation into the host genome. By FISH analysis the transgene maps to the telomere of chromosome 4. Perhaps integration into a telomere could be a contributing factor in this mechanism, possibly due to the highly repetitive nature of this structure.

3.4.4 Line C.

Line C contains one copy of integrated transgene with an intact WTI locus and LVA. It however contains no SVA implying truncation of WT280LZ 5' of WTI.

3.4.5 Line D.

Two copies of the WTI locus are present in line D. These appear to be intact although due to low levels of DNA loaded on a genomic Southern blot it is difficult to make out the hybridization signal. The hybridization signal is weak but a long exposure suggests that line D contains two copies of the LVA. It contains no SVA sequences indicating the region downstream of the WTI locus is intact but that upstream it is truncated.
4. Analysis of the expression pattern of the YAC transgenes.

Three lines generated by microinjection with WT470LZ transmit the transgene. Lines A and H have identical embryological expression patterns which closely mirror that of endogenous WtI and highlight some potential unreported domains of expression. Line E has expression which is generally a subset of that of lines A and H but has some differences. Transgene expression in those adult tissues examined from the non-transmitting lines F and G was the same as lines A and H. I will first describe the expression pattern seen in lines A and H then describe the differences between this and that of line E.

Following a description of the expression pattern of WT470LZ I will detail the less extensive analysis carried out on lines containing the WT280LZ transgene. Three lines, C, D and I, have been generated using the WT280LZ transgene. All transmit. However WT280LZ expression has been investigated in depth only in lines C and D.

Finally Dr. Andreas Schedl has used the WT280 YAC to generate three transgenic lines. I will briefly review the results of experiments he has conducted using these transgenic animals.

4.1 Early WT470LZ expression in E8.5-9.5 embryos (stage 12-15).

4.1.1 Expression in the pronephros and mesonephros.

The first expression of WT470LZ is detected in a six somite stage embryo (Fig. 22a). It occurs in single cells in the region of the forming intermediate mesoderm. The intermediate mesoderm forms from the proliferating coelomic epithelium at the junction between the splanchnopleuric and somatopleuric mesoderm (Bannister et al.,
1995). In the chick the first formation of intermediate mesoderm has been observed at the eight somite stage, lateral to the sixth somite (Saxen, 1987). In the WT470LZ transgenic mouse, the first cells to show expression of WT470LZ are immediately caudal to somite six of a six somite embryo. This expression domain potentially marks the first delamination of intermediate mesoderm cells from the coelomic epithelium. As the next somites form these β-galactosidase expressing cells become clustered (Fig. 22b). The first structure to be formed by the intermediate mesenchyme is the pronephros, which in the mouse consists of clusters of cells (Saxen, 1987). Hence these clusters of WT470LZ cells are likely to mark the pronephros.

Subsequently to pronephros formation, the pronephric duct forms caudally and independently of the pronephros and does not express the transgene. By 15-18 somites (stage 14) the intermediate mesenchyme has given rise to a mesonephric condensation of cells; these cells express WT470LZ and extend caudally as the tail elongates concurrent with somitogenesis (Fig. 22c). In different embryos it is possible to see transgene expression either more caudal than the last somite which has been formed or up to two somites rostral to this. Therefore the differentiation of mesonephric tissue is to some degree independent of somitogenesis. Furthermore although the mesonephros is a segmental organ it does not show the segmentation seen in the paraxial mesenchyme. At the 22 somite stage mesonephric tubules begin to form and can be seen as a thickening in the caudal part of the β-galactosidase positive mesonephric ridge (fig. 22d). At the rostral end of the 22 somite embryo mesonephric ridge transgene expression has spread into the proliferating coelomic epithelium overlying the mesonephric tissue (Fig. 22f).

Using this reporter gene it has now been possible to deduce the exact timing of initial Wt1 expression. The gene is first active in the pronephric cell clusters. In contrast previous in situ analyses have been unable to detect Wt1 activity earlier than the 13 somite stage. In these studies Wt1 mRNA expression detected was in the more substantial mesonephric tissues (Armstrong et al., 1992; Rackley et al., 1993).
4.1.2 Expression in the septum transversum.

Simultaneous to the switching on of WT470LZ expression in the pronephros a second domain of β-galactosidase positive cells appears caudal to the embryonic heart. This domain of expression is the septum transversum (Fig 22b). The septum transversum is a second tissue which forms by proliferation of the (caudal pericardial) coelomic epithelium (Bannister et al., 1995). It is originally a single block of cells but at the 16 somite stage becomes split by invasion of the endodermal hepatic bud. This split into two parts, the pars diagrammatica/pars pericardialis and the pars mesenterica, can clearly be seen in transgenic embryos (Fig. 22c,d and e). By the 22 somite stage a third domain of β-galactosidase expression appears, in the mesenchyme adjacent to the cardinal vein as it passes the developing forelimb buds.

*In situ* studies have not previously reported expression of Wt1 in the septum transversum. Chick and alligator WT1 whole mount *in situ* hybridizations indicate WT1 expression in ‘vitelline veins’ caudal to the heart (Kent, 1994; Kent et al., 1995). These domains could well refer to expression in the septum transversum. The septum transversum forms tissues which express Wt1 at later stages of development, e.g. parts of the pericardium, diaphragm, and gut mesenteries (Armstrong et al., 1992; Rackley et al., 1993). Hence it is very likely that lacZ expression in the septum transversum represents a *bona fide* region of Wt1 expression.

The intermediate mesoderm and the septum transversum are spatially distinct. There is no evidence that developmental signals regulating the timing of their formation are shared. We have observed E8.5 embryos in which either the septum transversum, or the intermediate mesoderm, but not both is expressing the transgene. However we have yet to quantify these observations.

The proliferating coelomic epithelium gives rise to two types of progeny, mesenchymal and epithelial cells (Bannister et al., 1995). Both the intermediate mesoderm and the septum transversum are localized mesenchymal cell populations.
produced by the proliferating coelomic epithelium. At a later stage of development another mesenchymal organ, the spleen, derives from the splanchnopleuric coelomic epithelium dorsal to the stomach and expresses \textit{Wt}t (Armstrong et al., 1992; Rackley et al., 1993). Hence, \textit{Wt}t appears to be expressed in mesenchymal derivatives of the proliferating coelomic epithelium. Mesenchymal cells derived from the coelomic epithelium may stay mesenchymal or undergo a mesenchymal to epithelial transition. The pronephric/mesonephric duct is an example of an epithelial tissue which arises directly from the coelomic epithelium without going through a mesenchymal stage (Bannister et al., 1995; Saxen, 1987), it does not express \textit{Wt}t (Pritchard Jones et al., 1990; Armstrong et al., 1992; Rackley et al., 1993) nor the WT470LZ transgene (Fig. 22f).
Fig. 22 Early expression pattern of the WT470LZ transgene.

(a) 6 somite mouse embryo (line A). Expression of WT470LZ can be seen in the intermediate mesoderm forming at just caudal to the sixth somite. (b) 10 somite mouse embryo (line A). Expression of the transgene can be seen in clusters of cells representing the forming pronephros and in the septum transversum. (c) In the 17 somite mouse embryo (line A) the mesonephric cord/mesonephros is forming which expresses WT470LZ. WT470LZ continues to be expressed in the pronephros and the septum transversum. (d) 22 somite mouse embryo (line A), the septum transversum has been split by invasion of the hepatic bud into the pars pericardialis and pars mesenterica, (* plane of section for panel f). (e) Saggital section (x50) of E9.5 mouse embryo (line A). (f) Transverse section of E9.5 mouse embryo (x100) (line A).

Abbreviations: (H) heart, (IM) intermediate mesoderm, (ST) septum transversum, (P) pronephros, (Ms) mesonephros, (PP) pars pericardialis, (PM) pars mesenterica, (HP) hepatic primordium, (Clm) coelom, (NT) neural tube, (C) caudal, (Rs) rostral, (A) aorta, (S) somite, (CE) coelomic epithelium, (GT) gut tube, (SpM) splanchnopleuric mesoderm, (SmM) somatopleuric mesoderm, (MsD) mesonephric duct, (D) dorsal, (V) ventral.

Note: In fig 22f the arrow labelled CE is not pointing to the coelomic epithelium but to the mesonephric cord (mesonephric mesenchyme - Ms). The coelomic epithelium is the single epithelial cell layer laying ventral to the mesonephric cord.
4.2 WT470LZ expression in E 9.5-11.5 embryos (stages 16-18).

4.2.1 Transgene expression in the mesonephros.

By E9.5 the mesonephric ridge has expanded rostrally as far as the septum transversum and caudally to the cloaca. Formation of the mesonephros is beginning (Saxen, 1987; Kaufman, 1992). Since the completion of this study Sainio et al. have reported that the mesonephros contains two different types of nephron which form via different mechanisms (Sainio et al., 1997). This study did not investigate the difference in transgene expression between the two different nephron types. In the mature mesonephric nephron WT470LZ is not expressed in the tubule, but is expressed in the glomerulus (Fig. 23a). This expression pattern exactly mirrors that reported for the endogenous Wt1 (Pritchard Jones et al., 1990; Armstrong et al., 1992; Rackley et al., 1993). The pronephric/mesonephric duct continues not to express the transgene.

At stages 15-16 transgene expression encompasses the whole mesonephric ridge and hence includes the region of the adrenogenital primordia (Hatano et al., 1996). As development proceeds (stages 18-19) (Fig 23) the adrenal and genital primordia split. The genital primordia becomes localized to the ventrosagittal part of the ridge where the level of transgene expression remains strong. In contrast, dorsomedially where the adrenal primordia localizes the transgene expression begins to weaken and is lost by stage 18.

4.2.2 Transgene expression in other mesoderm derived tissues.

Between E9.5 (stage 15) and E10.5 (stage 16) there is rapid expansion of transgene expression. The somatopleuric and splanchnopleuric proliferating coelomic epithelium as well as a thin layer of mesenchyme beneath the epithelium begin to express the transgene (Fig. 23). This layer is new mesenchymal tissue produced by the coelomic epithelium (Bannister et al., 1995). It has a more fibrous appearance.
than the mesenchyme which it overlays and will go on to form a number of tissues including the pleura, pericardium and peritoneum. All these tissues are continuous with each other, the septum transversum and mesonephric mesenchyme. \textit{Wt1} is expressed in all of the mesenchyme derived from coelomic epithelia throughout the embryo in addition to specific localized regions. Much of the reported expression pattern of \textit{Wt1} can be explained by following the development of tissues formed from this mesenchyme using transgene expression as a marker.

By E10.5 the common sinuatrial chamber of the heart which was embedded in the septum transversum has risen up into the pericardial cavity. As it does this it carries with it the transgene expressing pars pericardialis which forms the epicardium. Later between E10.5 and E11.5 sclerotomal rib precursors migrate into and split the somatopleuric mesenchyme of the pericardium (Bannister et al., 1995). Transgene expression continues in the mesenchyme of the parietal pericardium on the proximal side of the ribs and partially surrounds them (Fig. 23b).

The forming gut derived from the splanchnopleuric endoderm is covered in a layer of \(\beta\)-galactosidase expressing peritoneal tissue derived from the proliferating splanchnopleuric coelomic epithelium and the pars mesenterica of the septum transversum (Bannister et al., 1995).

The endodermal hepatic diverticulum continues to grow into the \(\beta\)-galactosidase positive septum transversum. Soon after the formation of the endodermal bud the endodermal cells begin to proliferate. The basement membrane which separates the mesenchyme of septum transversum from its overlying (coelomic) epithelium breaks down. The epithelial cells migrate into the mesenchyme guiding the endodermal tissue of the hepatic diverticulum. This endodermal tissue forms columns into the mesenchyme. The migration of these epithelial and endodermal cells gives rise to the hepatic sinusoids (Bannister et al., 1995). These sinusoids are a site of haematopoiesis from E10, containing extra vascular haematopoietic foci. The epithelial tissue lining the sinusoids is derived from the epithelium overlaying the
septum transversum (Le Douarin, 1975). WT470LZ is expressed on those cells lining the sinusoids (Fig. 24). Later, as the liver increases in size the lateral plate mesoderm and its overlying epithelium are also incorporated into the liver (Bannister et al., 1995; Le Douarin, 1975).

Blood formation takes place in the blood islands of the extra embryonic yolk sac de novo from E8. Transgene expression has not yet been investigated in this tissue. The AGM region (aorta, gonads and mesonephros), i.e. the region of the urogenital ridge, has de novo haematopoietic activity from E9. It is not clear where the site of haematopoiesis is in the AGM, however haematopoietic foci have been observed within the dorsal aorta. For a review of haematopoiesis in the mouse embryo see Dzierzak and Medvinsky, 1995. The mesonephric ridge expresses the transgene, but WT470LZ does not appear to be expressed within the dorsal aorta (Fig. 23).

The hepatic sinusoids are a site of haematopoiesis from E10. It is not clear what tissue within the liver these haematopoietic stem cells derive from. One proposal is that the stem cells do not originate de novo within the liver but migrate into it. This migration is potentially in two waves, first from the yolk sac and subsequently the AGM (Dzierzak and Medvinsky, 1995).

WT470LZ is expressed in both the AGM and the hepatic sinusoids. WTI has been implicated in the process of haematopoiesis and has been detected in adult bone marrow by RT-PCR (Inoue et al., 1994; Fraizer et al., 1995; King Underwood et al., 1996). However the transgene is not clearly expressed in any haematopoietic foci in either the AGM or the hepatic sinusoids. It remains to be ascertained if the transgene is expressed at any point during the differentiation of haematopoietic cells.
Fig. 23 Expression of WT470LZ in urogenital development (E11.5 mouse embryo).

(a) transverse section at level of the forelimbs (x50). WT470LZ is expressed in the mesenchyme of the intermediate mesoderm including the genital ridge. It is also expressed in the mesenchyme underlying the coelomic epithelium in the region of the somites. It is not expressed in the mesonephric tubules or the mesonephric duct but is expressed in the mesonephric glomerulus. The germ cells within the genital ridge do not express the transgene. (b) transverse section at the level of the hind limbs (x30). WT470LZ is expressed in the mesenchyme produce lying below the proliferating coelomic epithelium and the metanephric blastema. WT470LZ is not expressed in the ureteric bud and the rib primordia. Abbreviations: (A) aorta, (Rb) rib precursors, (SmM) splanchnopleuric mesoderm, (SpM) somatopleuric mesoderm, (UB) ureteric bud, (MtB) metanephric blastema, (DM) dermomyotome, (SCV) subcardinal vein, (MnD) mesonephric duct, (MnT) mesonephric tubule, (G) mesonephric glomerulus, (GR) genital ridge, (GC) germ cells, (HL) hind limb, (NT) neural tube.

Both embryo sections are from line A.
Fig. 24 WT47OLZ expression in hepatic sinusoids of a E12.5 mouse embryo. (x200). The transgene is expressed in the epithelial lining on the sinusoid and the mesothelial coat of the liver both of which are derived from the septum transversum. Abbreviations: (BC) blood cells, (M) mesothelium, (Sin) sinusoid, (SinE) epithelium lining the sinusoid. Line H.
4.3 WT470LZ transgene expression between E12.5 and 15.5 and in the adult.

4.3.1 Transgene expression in mesonephric ridge and gonads.

By E12.5 the mesonephros is regressing. WT470LZ is still expressed in a gradient, high in the genital ridge (ventrolateral) and very low in the mesonephric mesentery (dorsomedial). The epithelial paramesonephric duct invaginates from the coelomic epithelium at the lateral margins of the mesonephros between E12.5 and E13.5 (Kaufman, 1992). Similar to the mesonephric duct the paramesonephric duct does not express WT470LZ (Fig. 25b).

From E11.5 to E13.5 whilst the growing gonad is still indifferent WT470LZ is expressed in the somatic interstitial cells of the gonad but not the germ cells (Figs. 23a and 25). Following sex cord formation in the developing testes (E13.5) transgene expression becomes localized in the interstitial cells surrounding the cords (Fig. 25). Surprisingly it is not expressed in the Sertoli cells constituting the wall of the cords; this is in contradiction to the expression pattern of Wt1. Wt1 is expressed in Sertoli cells from foetal into adult life (Pritchard Jones et al., 1990; Armstrong et al., 1992; Mundlos et al., 1993).

In both testis and ovaries transgene expression is lost from the interstitial cells at the centre of the developing gonad after E13.5, but remains longer at the periphery. There is strong transgene expression however in the rete testis (and female rete ovari) (Fig. 25c and d). Wt1 expression has previously been reported in these structures (Mundlos et al., 1993). The origin of these structure is not known, they are a relatively new evolutionary addition to the gonad and are only found in tetrapod vertebrates (Hildebrand, 1988). The rete testis links the cranial mesonephric tubules which will form the lobules at the head of the epididymis with the seminiferous tubules of the gonad. It is epithelial and formed within the mesenchymal stroma of the gonad. It may form by extension of mesonephric tubules adjacent to the
developing gonad, or could differentiate *de novo* within the gonadal mesenchyme. Satoh describes the formation of primordial sex cords which extend out from the mesonephric tubules before the rapid expansion of the gonadal mesenchyme out into the coelomic cavity (Satoh, 1985; Satoh, 1991). He proposes that these cords will give rise to both the *rete testis/ovarii* and the secondary folligulogenous and seminiferous sex cords. If the *rete testis* does form by extension of the mesonephric tubules, it may be formed by recruitment of mesenchymal cells lying distal to the mesonephric tubules to undergo a mesenchymal to epithelial transition. This process would be similar to that seen during nephrogenesis. Alternatively migration of epithelial cells from with the mesonephric tubule may give rise to the *rete testis/ovarii*. WT470LZ is highly expressed in the *rete testis* however, whilst the mesonephric tubules do not express the transgene. As this is the case it seems unlikely that the *rete testis/ovarii* form by clonal expansion of the mesonephric tubules. The glomerular cells of the mesonephros do show a high level of WT470LZ expression. Extension of these glomerular cells to give the *rete testis* however also seems unlikely as Satoh describes primordial sex cord formation by bifurcation of the mesonephric tubule and not extension of the glomerulus.

There is a large degree of contention as to the origin of the secondary sex cords. Satoh argues that it is extension of the primordial (mesonephric derived) sex cords which gives rise to the secondary sex cords (Satoh, 1985; Satoh, 1991). The thickening of the coelomic epithelium over the gonad to form primary sex cords merely facilitates the rapid extension of the gonad into the coelomic cavity. A number of investigators believe that the secondary sex cords arise from the coelomic epithelium of the gonad and not from the mesonephros (Bannister et al., 1995). The coelomic epithelium thickens prior to the expansion of the gonad into the coelomic cavity forming primary sex cords. These are proposed to extend to subsequently form the secondary sex cords and hence differentiate into the Sertoli cells of the testis and granulosa cells of the ovary. A previous Wt1 expression study lends strength to this hypothesis. Mundlos *et al.*, 1993 show cords of Wt1 positive cells running from the epithelium surrounding the developing ovary into its centre. These cords may well
represent the forming granulosa cells, however the authors do not discuss this data. Buehr et al., 1993 showed that mesonephric cells were required for testis development. They did not appear to contribute to the testicular Sertoli cell or ovarian granulosa cell populations only to the peritubular myoid cells. Interestingly, although WT470LZ is not expressed in the testicular Sertoli cells, it is in the testicular rete and the peritubular cells both of which are of potential mesonephric origin (Fig. 25 c and e).

Given the high level of WtI expression in the coelomic epithelium and Sertoli/granulosa cells but not the interstitial cells of the gonad WtI may represent a very informative marker for following the development of different gonadal cell populations. Careful immunohistochemical analysis of the dynamics of WtI expression in the developing gonad may lead to further information about the origin of granulosa and Sertoli cells. Such analysis may be able to demonstrate whether these cells and the rete testis/ovarii are derived from the mesonephros, coelomic epithelium, or a mixture of both.

WT470LZ is expressed in both the capsule (of the epididymis) which envelops the testis, and the ovarian capsule. In the male, transgene expression becomes concentrated around the mesonephric duct and the remnants of the mesonephric tubules, which become the lobules at the head of the epididymis (Fig. 25a). In the female expression similarly becomes concentrated around the paramesonephric duct (future oviduct) (Fig. 25 b and d). In the adult female the stroma of the endometrium and myometrium both express the transgene. These tissues develop from the mesenchyme surrounding the genital cord. The genital cord is formed by the caudal fusion of the paramesonephric ducts. Therefore the mesenchyme forming the endometrium and myometrium is originally derived from the coelomic epithelium of the urogenital ridge. WtI is expressed endogenously in the endometrium and myometrium (Armstrong et al., 1992; Rackley et al., 1993; Zhou et al., 1993).
In adult testes expression of WT470LZ is in the peritubular but not the Sertoli cells (Fig. 25e). In the adult ovary transgene expression occurs in the interstitial cells. WT470LZ is not clearly expressed in the follicular granulosa cells. A hint of expression in these cells may be artefactual (Fig. 25f). Further examination of WT470LZ in ovarian tissue is required. This study should use ovaries from young animals as \textit{WtI} is not expressed in mature follicles but only in primordial, primary and secondary follicles (Hsu et al., 1995).

The WT470LZ transgene is expressed throughout embryogenesis in a pattern mirroring that of \textit{WtI}. However it is not expressed in either the testicular Sertoli cells and probably not in the ovarian granulosa cells. Both the Sertoli and granulosa cells are sites of endogenous \textit{WtI} expression. A possible explation for the inability of WT470LZ to drive reporter gene expression in Sertoli and granulosa cells is because it is derived from genomic DNA surrounding the human \textit{WTI} locus rather than from the murine \textit{WtI} locus. DNA sequences which play a role in sexual differentiation are often highly divergent between species, for example the \textit{Sry} gene (sex determining region \textit{Y}) is evolving rapidly and may even be subject to adaptive selection (Whitfield et al., 1993). \textit{Sry} is expressed in the pre Sertoli cells of the genital ridge switching them to a Sertoli fate from a default female granulosa cell state (Ramkissoon and Goodfellow, 1996). Perhaps those regulatory DNA sequences required to drive \textit{WtI} expression in the sex specific Sertoli and granulosa cells have undergone species specific divergence.
Fig. 25 Expression of WT470LZ in the developing gonad.

(a) E13.5 testis (x50) (line H). Expression of WT470LZ mimics that of endogenous Wt1 except there is no expression in the Sertoli cells. (b) E13.5 ovary (x100) (line H). Wt1 WT470LZ is expressed in the gonadal mesenchyme but not the primordial germ cells. (c) E15.5 testis (x100) (line H). WT470LZ expression is upregulated in the rete testis. (d) E15.5 ovary (x200) (line H). WT470LZ expression is upregulated in the rete ovarii. (e) Adult testis (x200) (line F). Expression is only in the peritubular cells. (f) Adult ovary (line A) (x200). There is WT470LZ expression in the interstitial cells surrounding the follicles but not the granulosa cells.

Wt1 is expressed endogenously in both the Sertoli and granulosa cells however there is no X-gal staining in these cells in both line A and line H. It is likely that this means there is no WT470LZ expression in these cells. However it is possible that the reason that there is no staining in these cells is because there is a failure of X-gal penetration. In particular in panels e and f the tissue was stained whole and then fixed and sectioned afterward. To test this possibility adult gonads should be cryosectioned and then stained as were the embryonic organs (panels a-d). One way to test if the lack of staining is due the failure of transgene expression rather than X-gal penetration is to stain organs such those from the mouse strain ROSA26 with a constitutively active transgene.

Abbreviations: (TA) tunica albuginea (which is formed by condensation of mesenchyme beneath the peritoneal epithelium), (SmT) seminiferous tubules, (MsD) mesonephric duct, (IC) interstitial cells, (MsT) mesonephric tubules, (CptE) caput epididymis, (CpsE) corpus epididymis, (CaE) cauda epididymis, (PGC) primordial germ cell (Note: this is mislabelled (PcG) in 25b), (PMsD) para-mesonephric duct, (SC) Sertoli cell, (rt) rete testis, (ro) rete ovarii, (PTC) peritubular cell, (Sz) spermatozoon, (St) spermatid, (Sg) spermatogonium, (GrC) granulosa cells, (O) oocyte.
4.3.2 Transgene expression in the adrenal glands.

There is no transgene expression in the cortex or medulla of the foetal adrenal gland following its split from the genital primorida (Fig 23a). The developing foetal adrenal gland is quickly enveloped ventrally and later dorsally by a mesenchymal capsule derived from the mesonephros. This capsule expresses the WT470LZ transgene which again mirroring the reported Wt1 expression pattern (Rackley et al., 1993)(Fig. 26 a and b).

The gonad and adrenal cortex both derive from the proliferating coelomic epithelium of the urogenital ridge. However whilst the cells of the gonad continue to express Wt1/WT470LZ, those of the adrenal cortex do not. The interstitial cells of the gonad stay mesenchymal in nature whilst the gonad expands rapidly into the coelomic cavity. In contrast the cells constituting the foetal adrenal cortex are highly differentiated. The forming adrenal is invaded by neural crest cells which form the adrenal medulla. Subsequently capillaries arise from the adjacent mesonephric arteries and invade the cortex radially. The foetal adrenal cortical cells remain in small subcapsular nests. These nests proliferate forming cords which penetrate deep between the sinusoids and capillaries. These cells which make up the foetal adrenal cortex degenerate as they move toward the medulla (Bannister et al., 1995).

The fetal cortex undergoes a rapid degeneration in neonates and is replaced by the adult cortex. The adult cortex consists of three zones the thin outer zona glomerulosa, inside this is the thick zona fasciculata and between the zona fasciculata and the medulla lies the thinner zona reticularis. Cells of the adult adrenal cortex unlike those of the foetal cortex have high metabolic activity. They secrete hormones such as mineralocorticoids (zona glomerulosa), glucocorticoids (zona fasciculata/reticularis) and, in many mammals including humans, but not rodents, sex hormones (zona reticularis). The zona glomerulosa is derived from the small nest of cortical cells which lie just beneath the adrenal capsule in the foetal gland. The zona fasciculata and zona reticularis both form from cords which emenate from the zona glomerulosa as the foetal cortex is replaced by that of the adult (Bannister et al., 1995).
Variegated WT470LZ expression is seen in the adult adrenal gland in columns running through the cortex (Fig. 26e). Lines transgenic for a lacZ reporter under the control of the mouse *steroid 21-hydroxylase* gene promoter show the same variegated columnar expression pattern in the adult adrenal cortex (Morley et al., 1996). This pattern is a reflection of a variagated distribution of *steroid 21-hydroxylase/lacZ* transgene mRNA. Cryptic repression of *steroid 21-hydroxylase/lacZ* transgene is occurring in a subset of the cells of the zona glomerulosa. The cells of zona glomerulosa then proliferate centripetally giving rise to clonal columns in the adrenal cortex in which the transgene is either expressed or repressed. The same mechanism is likely to be responsible for the columnar expression of WT470LZ.

*Wt1* expression has not been reported in the adult adrenal cortex, WT470LZ activity within this tissue indicates that it may be. There are a number of similarities between the adrenal gland and other organs which express the *Wt1* gene. The adrenal cortex is derived from the mesenchyme of the intermediate mesoderm and shares its primordium with the gonad. This primordium is identifiable by its expression of the product of the *Ftz-F1* gene - steroidogenic factor 1 (SF1) (also known as adrenal 4-binding protein (Ad4BP)) (Hatano et al., 1996). SF1 is an orphan nuclear receptor implicated in the regulation of genes encoding the steroid hydroxylases. Knockout of the *Ftz-F1* gene in transgenic mice leads to lack of gonads and adrenal glands (Luo et al., 1994; Sadovsky et al., 1995).

The adrenal cortex furthermore has the rare ability to regenerate after damage. The subcapsular cortical cells continue to produce cortical tissue throughout adult life (Bannister et al., 1995). The adrenal cortex shares this ability to regenerate with other tissues which are known to express *Wt1*, such as the endometrium of the uterus and the metanephros. If one metanephros is damaged the other is able to undergo a new programme of growth and differentiation to take on the function of the damaged organ. *Wt1* has been demonstrated to be upregulated during this process in the pig (Kushner et al., 1992). In the axolotl (*Amblystoma mexicanum*) it is believed that Sertoli cells have a bipotentiality and may differentiate into duct cells which upon
encountering a germ cell engulf it and redifferentiate into Sertoli cells. *WTI* is expressed during this process of redifferentiation into Sertoli cells (Del Rio-Tsonis et al., 1996). It is interesting to speculate that *WTI* may be involved in retaining the ability of a cell to dedifferentiate and undergo a new programme of proliferation and redifferentiation.

To attempt to detect *Wtl* activity in the adult adrenal cortex I carried immunohistochemical staining of the murine adult adrenal cortex. This staining only detected protein within the mesothelial coat of the organ, not the cortex. Therefore *Wtl* may not be expressed at all in the adult adrenal cortex or may be expressed at levels too low to be detected by immunohistochemical techniques. On the other hand perhaps *WTI* is expressed in the human adult adrenal cortex and sequences on the human WT470LZ YAC drive transgene expression in the murine tissue. However the equivalent expression of *Wtl* in the murine adult adrenal cortex may not occur.

### 4.3.3 Transgene expression in the developing metanephros.

β-galactosidase expression in the developing metanephros of WT470LZ transgenic lines exactly mirrors that reported for *Wtl* (Pritchard Jones et al., 1990; Armstrong et al., 1992; Rackley et al., 1993; Mundlos et al., 1993; Phelan et al., 1994) (Fig. 26). At E11.5 the ureteric bud branches from the caudal portion of the mesonephric duct. It grows into the tissue of the metanephric blastemal stems cells (Fig. 23b). There is no transgene expression in the ureteric bud but there is expression in the metanephric blastemal stem cells. The ureteric bud grows and branches within these cells and transgene expression is increased in those cells which condense around the tip of these branches. Expression is further upregulated in the proximal part of the S shaped body which form the podocyte cells of the mature glomerulus. As nephrogenesis proceeds radially in the developing kidney β-galactosidase expression is lost in the more proximal blastemal cells of the kidney which differentiate into stroma.

Transgene activity is also present in the peritoneal tissue which envelopes the organ.
In the adult metanephros WT470LZ is expressed in the podocyte cells of the glomerulus (Fig. 26d). This expression pattern is consistent with published Wt1 in situ data. However β-galactosidase activity can also sometimes be seen in the renal pelvis and collecting ducts. These structures do not express the endogenous Wt1 gene. Furthermore these structures do not express the transgene in the developing foetal metanephros. Perhaps lacZ activity driven by the WT470LZ YAC is being upregulated in the adult collecting ducts and renal pelvis. This seems unlikely and does not reflect the very close similarity between the WT470LZ activity and Wt1 expression. The podocyte cells of the glomerulus are involved in the filtration of the blood in the glomerulus and the deposition of solutes into the nephron. Hence a second explanation for the β-galactosidase activity in the renal pelvis and collecting ducts is that the podocytes are secreting some of the high levels of WT1/β-galactosidase fusion protein contained within them into the metanephric nephrons. The presence of this protein then gives rise to the β-galactosidase activity seen in the ducts; either because it is trapped in the ducts during staining or because some of it is reabsorbed back into the walls of the ducts and pelvis.
Fig. 26 Expression of WT470LZ in the metanephros and adrenal gland.

Refer also to Fig. 3. (a) E15.5 metanephros and adrenal gland (x50) (line H). WT470LZ is expressed in the mesothelial tissue surrounding the organs, it is expressed at a higher level in the blastema and is upregulated in the comma shaped bodies and glomeruli. (b) E17.5 metanephros and adrenal gland (x30) (line H). WT470LZ is lost from the kidney stroma but continues to be expressed at the periphery of the kidney where nephrogenesis is proceeding. (c) Close up of comma shaped body from plate b (x100) (line H). WT470LZ expression can be seen in a band of metanephric blastemal cells alongside the bifurcating ureteric bud. (d) Adult glomerulus (x100) showing expression of WT470LZ in the glomerular podocyte cells but not those of the capillary (line H). (e) Vibratome section through an adult adrenal gland (x30) (line A).

Abbreviations: (Ad) adrenal gland, (CB) comma shaped body, (IG) immature glomerulus, (G) glomerulus, (St) stroma, (CD) collecting duct - bifurcation of the ureteric bud, (M) mesothelium, (P) podocyte, (Cap) capillary, (GT) glomerular tuft, (AdM) adrenal medulla, (AdC) adrenal cortex, (MtB) metanephric blastema.
4.3.4 transgene expression in the pleura, pericardium and peritoneum.

The internal organs of the embryo undergo a large degree of transformation and expansion from E12.5 onwards. Throughout this period of development WT470LZ continues to be expressed in the pleural, peritoneal and pericardial tissues of the embryo which surround these internal organs (Fig. 27). By following the processes of organ development the significance of the later expression pattern of the transgene becomes clearer.

Expansion of the lungbuds in the pericardial/peritoneal canals causes displacement of the pleura causing it to invade, and split, areas of the body wall. This invasion splits away the (WT470LZ expressing) coelomic epithelium derived mesenchyme from the underlying mesoderm (Bannister et al., 1995); this process is illustrated in Fig. 27c. Structures formed by this process continue to express the transgene. Pleural tissue invades the region dorsal to the lungs and heart coming to lie over the surface of the sclerotome derived vertebrae and ribs (Fig. 27c 1 and 2). At E13.5 when the intercostal muscles are beginning to form WT470LZ is still expressed intercostally. Rostral expansion of the apical part of the lung forces pleural tissue rostrally (Fig. 27c 1) so that it comes to rest on the lateral aspect of the common cardinal vein (Fig. 27a and b). Further expansion of the lateral parts of the pleural cavity splits the somatopleuric mesenchyme bordering the pericardial cavity. The tissue peeled away from this mesenchyme now lies between the pleural and pericardial cavities forming the dorsal visceral pericardia; it continues to express WT470LZ (Fig. 27c 3). The dorsal part of the lungs expand caudally. This dorsocaudal expansion peels away part of the (somatopleuric) mesenchyme dorsal to the mesonephric ridge such that this mesenchyme contributes to part of the diaphragm (Fig. 27c 2). In addition to contribution from the somatopleuric mesenchyme the diaphragm also derives from the WT470LZ positive tissue of the pars diagrammatica and dorsal mesentery of the oesophagus.
The development of the peritoneal cavity is extremely complex. WT470LZ expression is found on the serosal visceral surfaces of the gut and other abdominal organs. This is similar to that described for WtI (Armstrong et al., 1992; Rackley et al., 1993). The serosal visceral surface of the gut is derived from pars mesenterica of the septum transversum and the splanchnopleuric coelomic epithelium (Bannister et al., 1995) both of which continue to express WT470LZ.

As foetal development proceeds the thickness of the pleural, peritoneal and pericardial tissues becomes greatly reduced. In neonates and adults a single layer of visceral epithelia (the mesothelium) is left on the surface of the body organs. In adult WT470LZ transgenic animals transgene expression is evident in the mesothelial tissue surrounding organs such as the heart, testes, ovaries and adrenal glands. Fig. 28 shows WT470LZ expression in the pericardial mesothelium of the adult heart.

The diaphragm, parietal and visceral pericardia, mesenchyme adjacent to the developing sclerotome, mesenchyme of the abdominal wall, gut mesentery and the visceral coating of other foetal organs have all previously been shown to express WtI (Armstrong et al., 1992; Rackley et al., 1993; Phelan et al., 1994; Mundlos et al., 1993). Similarly the adult mesothelium is a postulated site of WtI activity and it expresses WT470LZ. All these tissues share a common embryological origin, they are derived from the coelomic epithelium or mesenchyme produced from it (Bannister et al., 1995). As WtI is also expressed in organs formed from the intermediate mesoderm, derivatives of the coelomic epithelium comprise a major site of WtI expression.
Fig. 27 Expression of WT470LZ in the developing pleura, pericardium, peritoneum and diaphragm.

(a) Transverse section E10.5 mouse embryo (x25) (line A). There is transgene expression in a layer below the cardinal vein, and a thin layer of expression in the pleura, visceral and parietal pericardia. (b) Transverse section E12.5 mouse embryo (x25) (line A). The volume of the pleura, pericardia and peritonea has increased from E10.5. The parietal pericardium is histologically distinct from the overlaying fibrous pericardium. The pleura has invaded the dorsal mesenchyme and lays adjacent to the vertebrae. (c) Sagittal section E13.5 mouse embryo (x25) (line H). WT470LZ is expressed in the pleura, pericardium, peritoneum and diaphragm. The metanephros has not stained in this section due to the presence of an intact capsule through which X-Gal cannot penetrate. 1) the pleura expands apically. 2) the pleura expands caudally splitting away part of the somatopleuric mesoderm to form part of the diaphragm. 3) expansion in lateral part of the pleural cavity. (d) Transverse section E12.5 Wt/h homozygous null mouse embryo (x25). The heart and lungs are malformed. There may be a reduction in the volume of pleura and visceral and parietal pericardium. Abbreviations: (Pl) pleura, (At) atrium, (Ve) ventricle, (NT) neural tube, (CV) cardinal vein, (L) lungs, (VP) visceral pericardium, (PP) parietal pericardium, (FP) fibrous pericardium, (Mu) muscle, (Vb) or (V) vertebrae, (D) diaphragm, (Mt) metanephros, (Li) liver, (Pe) peritoneum, (CS) corpus striatum, (MB) midbrain, (P) in fig 27c P refers pericardium, in 27d P refers to pleura
Fig. 28 Expression of WT470LZ in adult heart.

WT470LZ is expressed in the mesothelium (visceral pericardium (VP)). (At) atrium, (Ve) ventricle.

Line F.
4.3.5 Transgene expression in differentiating musculature.

In situ analysis of Wt1 expression previously identified expression in differentiating body wall musculature (musculature of the trunk) between E12 and E13 (Armstrong et al., 1992). The WT470LZ transgene is similarly expressed in differentiating body wall musculature. However expression of the transgene occurs in all developing epaxial muscles. All epaxial muscle are formed by premitotic myoblast cells which migrate from the ventrolateral edge of the somitic myotome differentiating into muscle at their destination (Bannister et al., 1995). The regions where epaxial muscle formation occurs by migration of somitic myoblasts are the limbs, trunk and diaphragmatic muscles. The muscles of the tongue are formed in the same way; single myoblasts migrate from the ventrolateral part of the occipital somites into the base of the tongue. However the formation of other muscles in the head is more complex (Bannister et al., 1995). The extrinsic ocular muscles, i.e. those muscles involved in movement of the eye, develop from prechordal mesenchyme at the rostral tip of the notochord process. The great majority of striated musculature of the head derives from the somitomeres (Bannister et al., 1995). Somitomeres contain the paraxial mesenchyme but do not undergo a process of segmentation similar to that of somitogenesis. Mesenchyme from the somitomeres migrates into the branchial arches early in foetal development. Subsequently premitotic myoblasts derived from this mesenchyme migrate back out of the arches to form the striated musculature of the head.

In E10.5 embryos β-galactosidase expressing cells surround the ventrolateral edge of the somitic myotome (Fig. 29a). The picture is clearer in E12.5 embryos, strong domains of transgene expression being present both rostral, (in the developing deltoids) and caudal to the base of the forelimb (Fig. 29b). These rostral and caudal domains of expression are continuous with β-galactosidase expression in the dorsal and ventral myogenic zones of the forelimbs (Fig. 29c and d). A similar pattern is repeated in the hindlimbs which develop about half a day behind the forelimbs. Further transgene expression is seen in all differentiating trunk muscles. Between E13.5 and E15.5 a low level of transgene expression can be seen in the tongue (Fig.
30b), in which Wt1 expression has previously been reported by RT-PCR (Armstrong et al., 1992). Further WT470LZ expression is seen in other developing striated muscle of the head.

The cell type expressing WT470LZ is unclear. WT470LZ is not expressed in the somitic myotome. It is however expressed in sparsley separated cells within the developing epaxial muscles. Where these developing muscles pass through a field of mesenchyme which is expressing the transgene the number of cells expressing the transgene in the muscle is much higher (Fig. 29b and c). Possibly this indicates that it is not myoblasts themselves which are expressing the transgene but other mesenchymal cells caught up within the developing myotubes. Transgene expression is not seen in fully formed myotubes. As WT470LZ is expressed in the mesenchyme into which epaxial muscle can migrate e.g. the limbs and intercostal regions, WTI may somehow be involved in the process of myoblast migration.

The significance of WT470LZ expression within the developing epaxial muscle of the body remains unclear. The role if any of Wt1 in the process of myogenesis requires a great deal of clarification. However studies carried out by Dr Kiyoshi Miyagawa have implicated Wt1 in the process of myogenesis. He demonstrated a correlation between the state of the WTI gene in Wilms' tumours and the histology of the tumour (Hastie, 1994). Tumours with a mutation in the WTI gene contained a large amounts of muscular tissue whereas this tissue was not seen in tumours containing no WTI mutation. Furthermore when WT1 protein was added to a culture of myoblastic (C2P9) cells it inhibited myotube formation by those cells.
4.3.6 Transgene expression within the limb.

Further to the expression of WT470LZ within the developing limb musculature, expression of the transgene is also seen in regions of limb mesenchyme undergoing apoptosis.

WT470LZ expression is switched on at E12.5 in the anterior and posterior marginal zones and the base of the mesenchyme web between the third and fourth digits. Over the next twenty four hours β-galactosidase expression is very dynamic first appearing between the second and third digit and then spreading in stripes either side of the condensing digits. Simultaneously expression begins in the webs between the first and second digit and the fourth and fifth digits, and, by E13.5 zones of WT470LZ expression run alongside each developing digit (Fig. 29c). At E15.5 thin domains of expression sit in the remnants of the interdigital web and run alongside each digit. Further transgene expression occurs around the developing metatarsals and between the condensing radius and ulna (Fig. 29e).
Fig. 29 Expression of WT470LZ in the developing limb and body wall musculature.

(a) Transverse section of region of mesonephros and somite in E10.5 mouse embryo (x100) (line A). WT470LZ is expressed in the mesonephros and in a layer of tissue below the coelomic epithelium. This domain of expression wraps around the ventral end of the myotome. There is no expression of WT470LZ within the myotome itself. (b) Expression of WT470LZ in the deltid muscle of a E12.5 mouse embryo - transverse section (x100) (line A). WT470LZ is expressed within the somatopleuric mesenchyme in which the muscle is forming and within the muscle itself. (c) Thick sagittal section E13.5 mouse embryo (line A). WT470LZ is expressed in the deltoids, intercostal regions, interdigitally and between the radius and ulna (* plane of section for plate d). (d) Coronal section of E13.5 mouse forelimb (x50) (line H). (e) Sagittal section of E15.5 mouse hind limb (x50) (line H). WT470LZ is localized along side and between the developing digits and bones.

Abbreviations: (NT) neural tube, (Myt) myotome, (SmM) somatopleuric mesenchyme, (CE) coelomic epithelium, (Clm) coelom, (Ms) mesonephros, (Del) deltid, (IC) intercostal mesenchyme, (BW) body wall, (ID) interdigital, (OZ) opaque zone, (VMZ) ventral myogenic zone, (DMZ) dorsal myogenic zone, (Rd) radius, (Ul) ulna, (D) digit, (Li) liver, (Pe) peritoneum.
4.3.7 Transgene expression in the head.

At E12.5 the transgene is expressed in the mesenchyme adjacent to the pharyngo-tympanic (Eustachian) tube. It is not however, expressed in the ectodermal epithelial lining of the tube. This domain of expression occurs surrounding the whole length of the tube but is particularly pronounced at the distal end where the tubo-tympanic recess will form. A further domain of WT470LZ expression occurs distal to this, laying beneath the acoustic meatus. By E15.5 expression below the external acoustic meatus is less pronounced, however strong transgene expression occurs in the mesenchyme surrounding the tubo-tympanic recess. A similar expression in the head mesenchyme occurs at the lateral margin of the vertically directed palatal shelves and weak expression occurs at the base of the forming eyelids (data not shown).

Throughout development the pharyngo-tympanic tube and the palatal shelves undergo extensive morphological changes. The expression WT470LZ in the mesenchyme surrounding these tubes may be associated with this remodelling. At the distal end of the pharyngo-tympanic tube the tubo-tympanic recess will form by hollowing out a region of this mesenchyme, a similar process will occur at the acoustic meatus. Given that WT470LZ is expressed in regions of apoptosis in the limb the tissue expressing the transgene is these domains may also be involved in apoptosis.

In the adult animal the acinar cells of the submandibular glands express the transgene. The submandibular glands arise from an ectoderm mesenchyme interaction however no transgene activity is seen during foetal development.

4.3.8 Transgene expression in ectoderm derived tissues.

4.3.8.1 Transgene expression in the forebrain and midbrain.

At E10.5 the WT470LZ is switched on in a non mesodermal lineage. It is expressed in part of the ectodermally derived forebrain. This is potentially a new site of Wt1 expression. By E12.5 transgene expression in the forebrain of the developing embryo
has become localized to several cell layers on the medial aspect of the rapidly expanding corpus striatum (Fig. 30a and b). Expression continues in this domain between E12.5 and E15.5 by which time it has waned. The transgene is also expressed at E13.5 in very few cells scattered throughout the roof of the neopallial cortex. As WT470LZ expression extends right into the forebrain (corpus striatum) the gene does not appear to be involved in delineating brain segmental boundaries.

At E12.5 WT470LZ expression appears in individual cells in the intermediate zone of the dorsal part of the roof of the midbrain. The number of these cells increases over the next 24 hours. The cells are large and round in shape. By virtue of the shape and position of these cells they are potentially trigeminal mesencephalic cells (T. Jessel pers. comm.). These cells are neural crest in origin but fail to migrate out of the brain; they express the neuronal marker \( \text{Lim}1 \). To confirm that these \( \beta \)-galactosidase expressing cells are trigeminal mesencephalic cells they will be checked for \( \text{Lim}1 \) immunoreactivity.

A preliminary investigation of WT470LZ expression in the adult has localized transgene expression to two domains in the midbrain. The first domain constitutes a group of cells in the region of the thalamus which may represent cells derived from the medial ganglionic eminence (corpus striatum mediale) (Bannister et al., 1995). The second group is found in a region of the deep white layer of the superior colliculus. Trigeminal mesencephalic cells contribute to the cells of the superior colliculus (Bannister et al., 1995). Hence both these WT470LZ expressing domains may be linearly derived from WT470LZ neurons generated during early brain formation.

4.3.8.2 Transgene expression in the cerebellum.

Wt1 activity has previously been detected in the roof of the fourth ventricle of the foetal brain (Armstrong et al., 1992). Cells in the roof of the fourth ventricle begin to strongly express WT470LZ at E12.5. They continue to do so at E13.5 (Fig. 31) and E15.5. At E13.5 and E15.5 cells in the intraventricular portion of the cerebellar
primordium are also expressing the transgene. Neurons derived from both the roof of the fourth ventricle and the cerebellar primordium are involved in cerebellar development.

Three sets of precursor neurons are involved in cerebellar development, reviewed by Hatten and Heintz, 1995; Hatten et al., 1997. The cerebellar primordium forms between the mesencephalon (midbrain) and the metencephalon. Between E10 and E14 the cerebellar primordium grows by proliferation of the cerebellar deep nuclei precursors within its ventricular zone. From E13 onwards precursors for Purkinje cells, Golgi cells astroglia and the interneurons of the molecular layer (basket and stellate cells) begin to proliferate in this ventricular zone. These exit the cell cycle and migrate into the centre of the cerebellar primordium forming a zone of immature neurons. Both these populations of neurons are derived from the mesencephalon (Hallonet et al., 1990). Cells derived from the metencephallic roof of the fourth ventricle of the embryonic brain begin to migrate into the cerebellar primordium over the rhombic lip of the metencephalon at E13. These cells give rise to a germinal zone known as the external granular layer (EGL). The EGL overlies the ventricular zone giving rise to all other neurons of the cerebellum. After birth a rapid proliferation of the EGL increases its size from one to eight cell layers thick. At postnatal day 15 EGL proliferation stops and the cells of the EGL migrate through the molecular and Purkinje cell layers into the cerebellar primordium to form the internal granular cell layer (IGL) of the adult cerebellum.

The adult cerebellum consists of four main layers. An outer molecular layer containing the basket and stellate interneurons. Immediately proximal to the molecular layer is a single cell layer of Purkinje cells. Proximal to the Purkinje cell layer is the inner granular layer (IGL) containing very high numbers of granular neurons outnumbering the Purkinje cells around 250:1 (Hatten and Heintz, 1995). Two other cell type are present within the granular layer, Golgi and unipolar brush cells (Mugnaini and Floris, 1994). The inner layer of the cerebellum consists of the dense cerebellar deep nuclei.
WT470LZ is weakly expressed in the adult cerebellum. This domain of expression consists of a layer of transgene expressing cells on the distal border of the IGL at the level of the Purkinje cell layer. Proximal to this layer further scattered cells are expressing the transgene. WT470LZ is possibly marking the Purkinje cells. In the IGL it is possible that the transgene is marking only a subset of the granular neurons; however all granular cells of the IGL are believed to be genetically homogenous (Hatten et al., 1997). Whichever cells of the granular layer WT470LZ is marking they may be the direct descendants of cells expressing WTI/WT470LZ in the roof of the fourth ventricle at E13.5. Further analysis of WT470LZ during cerebellar development is required.

4.3.8.3 Transgene expression in the spinal cord.

A further domain of WT470LZ expression spans the most rostral part of the myelencephalon and the cervical part of the spinal cord. It is switched on in a medially placed domain of the dorsal myelencephalon and cord at E12.5. By E13.5 the most rostral neurons of this zone have sent out axons. These axons have β-galactosidase activity hence it is possible to trace their route through the cord (Fig. 32d). At E15.5 there also appears to be some expression of β-galactosidase in the roof plate of the cord. No expression of WTI has previously reported in the dorsal cord so this is a potentially novel site of activity. However even if WTI is not expressed in this dorsal cord domain the expression pattern is still very interesting. Whilst columns of motor neurons in the ventral half of the cord have discrete boundaries of expression along the rostro-caudal axis of tube similar rostro-caudal boundaries of expression have not been reported for neurons in the dorsal cord (Lumsden and Krumlauf, 1996). WT470LZ is marking a domain of in the dorsal cord which has a caudal boundary in the cervical region.

WTI activity has been described in the ventral spinal cord. WTI comes on at E11.5 in a small domain adjacent to the ventricular zone immediately ventral to the sulcus limitans. The position of WTI activity changes moving ventrally as the cord matures (Armstrong et al., 1992). WT470LZ is expressed in a pattern mirroring that of WTI. WT470LZ is expressed in the ventral part of the cord at E12 (Fig. 32a). The
transgene expression runs the length of the spinal cord from cervical regions to beyond the hindlimbs. It is particularly strong in the lumbar region of the embryo. By E13.5 the domain of transgene expression encompasses a large area of the ventromedial part cord (Fig. 32b). At E15.5 the domain of transgene activity lies at the most ventromedial part of the cord and the level of expression has become much weaker (Fig. 32c).

The expression pattern of WT470LZ in the ventral spinal cord appears to be very similar to the interneuron subset of the Lim3 gene expression pattern (T. Jessel pers. comm.). In the chick Lim3 is expressed along the whole length of the ventral spinal cord. It is expressed the motor neurons of the median motor column (MMC) (Tsuchida et al., 1994; Tanabe and Jessell, 1996). By analogy to the timing of Lim3 expression in the chick, expression of Lim3 in the mouse would be expected to begin at around E10.5. WT470LZ is not seen as early as E10.5. E11.5 embryos have not been properly examined for WT470LZ activity in the spinal chord. However Wt1 expression has been described in E11.5 spinal cord. It remains to be determined whether Wt1 is marking the same neurons which also express Lim3 or whether it marks a population of neurons generated half a day to a day later.

4.3.8.4 Transgene expression in the eye.

A further ectodermally derived tissue in which Wt1 expression has been detected (by RT-PCR) is the eye (Armstrong et al., 1992). WT470LZ expression is switched on in individual cells scattered throughout the outer germinal layer of the neuroretina at E13.5 (Fig. 33a). At E15.5 expression is much stronger and is localized to the inner (non germinal) layer of the proximal neuroretina immediately adjacent to the optic nerve (Fig. 33b). Light X-gal staining of the hyaloid tissue and back of the lens is seen which is due to endogenous β-galactosidase activity. As with the eye the neuroepithelium of the nose is derived from an ectodermal placode. At E15.5 a number of sparsely dispersed cells in the vomeronasal organ express WT470LZ.
**Fig. 30 WT470LZ expression in the forebrain and midbrain.**

(a) Transverse section E12.5 mouse embryo (x25) (line A). WT470LZ is expressed in the ventricular layer if the corpus striatum medial. (b) Sagittal section E13.5 mouse embryo (x10) (line A). WT470 is expressed in the corpus striatum, cerebellar primordium, neural tube as well as pericardium, pleura, peritoneum and tongue. (c) E13 mouse embryo - dorsal view (line A). WT470LZ expression is seen in the midbrain (putative trigeminal mesencephalic nuclei), roof of the fourth ventricle, neural tube and limb. (* plane of section for plate d). (d) Transverse section of the midbrain at the level of the third ventricle (E12.5) (x400) (line A). Large round cells expressing the WT470LZ transgene are potential trigeminal mesencephalic nuclei. Abbreviations: (CSL) lateral corpus striatum, (CSM) medial corpus striatum, (NT) neural tube, (D) dorsal, (V) ventral, (CN) caudal neural tube, (LN) lumbar neural tube, (CP) cerebellar primordium, (CS) corpus striatum, (T) tongue, (Viv) fourth ventricle, (Viii) third ventricle, (Lb) limb, (MB) midbrain, (VL) ventricular layer, (TgM) putative trigeminal mesencephalic nuclei.
Fig. 31 Expression of WT470LZ at the metencephalic/mesencephalic junction.

(a) Lateral sagittal section from E13.5 embryo (x50). (b) Medial sagittal section from E13.5 embryo (x50). Abbreviations: (T(VZ)) ventricular zone of the tectum, (CP) choroid plexus, (CB) cerebellar primordium, (RViv) roof of the fourth ventricle.

Both embryo sections are from line A.
Fig. 32 Expression of WT470LZ in the spinal cord.

(a) Transverse section through the lumbar region of the spinal cord in E12.5 mouse embryo (x100) (Line A). WT470LZ is expressed adjacent to the ependymal layer adjacent to the suculus limitans. (b) Transverse section lumbar region of the spinal cord in E13.5 mouse embryo (x50) (Line H). The domain of transgene expression has moved ventrally. (c) Transverse section lumbar region of the spinal cord in E15.5 mouse embryo (x50) (Line H). (d) Transverse section cervical region of the spinal cord in E13.5 mouse embryo (x50) (Line H). The transgene is expressed in the dorsal part of the cord and axons extend from this region through the white matter and express b-galactosidase.

Abbreviations: (D) dorsal, (V) ventral, (CC) central canal, (EL) ependymal layer, (SL) suculus limitans, (DRG) dorsal root ganglion, (WM) white matter, (GM) grey matter, (AX) axon, (RP) roof plate.
Fig. 33 Expression of WT470LZ in the developing eye.

(a) E13.5 mouse eye (x100). WT470LZ expression is switched on in individual cells scattered throughout the outer germinal layer of the neuroretina. (b) E15.5 mouse eye (x50). WT470LZ expression is localized to the inner layer of the proximal neuroretina adjacent to the optic nerve. Abbreviations: (P) pigmented epithelium, (N) neuroretina, (Ls) lens, (OD) optic disc, (IN) inner layer of neuroretina, (ON) outer layer of neuroretina.

Embryo sections are from line A.
4.4 Transgene expression in line E.

Transgene expression in line E occurs at a much lower level than A and H (Fig. 34a). It first appears in the mesonephros (E9.5). By E12.5 expression encompasses the whole urogenital ridge and in the adult it is expressed in the metanephric glomerula podocytes. As in lines A and H, WT470LZ expression occurs in the pleura, peritoneum and pericardium at a much reduced level. The transgene is expressed in the developing limb/body wall musculature but no activity occurs interdigitally.

There is no transgene activity in the nervous system equating with that occurring in line A and H. However ectopic expression is seen in the ciliary body of the eye and in a stripe along the midline of the midbrain roof.

4.5 Expression of WT280LZ in line C.

The β-galactosidase expression pattern in line C throughout embryonic development is very similar to that of lines A and H (Fig. 34b). Between E9.5 and E11.5 line C, A and H embryos, have transgene expression in the pericardium, pleura, peritoneum, mesonephric ridge, base of the limbs and the corpus striatum. However at E11.5 ectopic domains of expression peculiar to line C become apparent. There is transgene expression in the dorsal root ganglia running the length of the body, the branchial arches and in a bar running above the eye.

The similarity between the transgene expression pattern in line C and lines A and H continues throughout foetal development. At E13.5 in lines C, A and H there is transgene activity in the pericardium, pleura and peritoneum, interdigital regions and muscle along the dorsal and ventral sides of the developing limb. The transgene is expressed within both the mesonephros and the gonad; but the level of β-galactosidase expression in the gonad is greatly reduced compared to that of the mesonephric ridge. This does not occur in lines A and H. Furthermore line C, in contrast to in lines A, E and H, shows no transgene expression in the developing
metanephros, or adult metanephros. At E13.5 line C transgenic embryos also show very similar transgene expression in the head to those of line A and H. There is strong expression in the developing head muscles, tongue, lower jaw and in the mesenchyme adjacent to the oral and nasal cavities. Interestingly in line C there is also low transgene expression within the developing submandibular glands.

In line C WT280LZ expression in ectodermally derived tissues comes on at E10.5 in the corpus striatum. Later it is expressed in the corpus striatum, the roof of the fourth ventricle and the eye, as occurs in lines A and H. In addition, ectopic expression of the transgene is seen in the optic nerve, dorsal root ganglia and peripheral nervous system. No transgene expression occurs in the myelencephalon or spinal cord.

4.6 Expression of WT280LZ in line D.

In line D WT280LZ is expressed weakly in the pericardium and adjacent to the cardinal veins (Fig. 34c). It is expressed more strongly in the mesonephros. The foetal and adult gonad express WT280LZ. As in line C the expression level of the transgene in the developing gonad is greatly reduced compared to the level in the mesonephros. Additionally, as in line C, there is no expression of WT280LZ in the foetal or adult metanephros.
Fig. 34 Expression of WT280LZ/WT470LZ in lines C, D and E.

(a) Sagittal section of line E embryo E11.5 (x12.5). WT470LZ is expressed in the mesonephric ridge and pleura and at low levels in the pericardium. There is ectopic expression of the transgene in a stripe across the roof of the midbrain. (b) E11.5 line C embryo. WT280LZ is expressed in the same domains as endogenous Wt1 as well showing ectopic expression in the dorsal root ganglia, branchial arches and around the eye. (c) Dissection of a line D embryo E10.5. There is only WT280LZ expression in the mesonephros (where is localised to the glomeruli) and the mesenchyme surrounding the cardinal vein. Abbreviations: (Pl) pleura, (MsR) mesonephric ridge, (MB) midbrain, (P) pericardium, (BA) branchial arch, (CS) corpus striatum, (E) eye, (DRG) dorsal root ganglion, (CVM) mesenchyme surrounding common cardinal vein, (MsG) mesonephric glomerulus.
4.7 The WT280 YAC can partially rescue the WTI knockout phenotype.

Dr Andreas Schedl has used the WT280 YAC to generate three transgenic lines. These lines were crossed onto the Wti knockout background. The WT280 transgene was able to rescue those defects in the Wti knockout which lead to embryonic death. Neonates homozygous for the Wti null allele and carrying the WT280 transgene were recovered at the expected frequency. They had no edema or disruption of the thoracic organs. These neonates however died within 48 hrs of birth. A post-mortem indicated defects in kidney development to be the probable cause of death.

In the majority of partially rescued mice no branching of the ureteric bud occurs and consequently no subsequent development of the metanephros. This metanephric phenotype is the same as is seen in Wti knockout animals (Kriedberg et al., 1993). In a subset of partially rescued mice however metanephric development begins but does not proceed to completion. In these animals in the initial stages of nephrogenesis occur normally; branching of the ureteric bud occurs and comma and S-shaped bodies are formed. Mature glomeruli are not observed in the partially rescued kidneys at E18.5. Detailed analysis of the S-shaped bodies present in these partially rescued kidneys (E18.5) indicates that the flattening of the proximal part of the S-shaped bodies which gives rise to mature podocytes does not occur. Throughout the epithelial structures of the partially rescued kidney apoptosis is significantly increased to a level four times that of wildtype. The partially rescued phenotype demonstrates a second role for WT1 in nephrogenesis. As well as being required for the initial stages of kidney induction WT1 is also necessary at a later stage in the epithelial maturation of the glomerular podocyte cells.

In partially rescued animals in addition to the failure of metanephric development, gonad formation is also blocked. At E15.5 a small region of adrenal cortex is present however neonates have no evidence of adrenal glands. No Wti expression in the developing adrenal has been detected in Wti in situ hybridization studies or with the
WT470LZ transgene. However in addition to a requirement for Wt1 in gonad and metanephric formation, the gene is also required for adrenal formation.
5. Analysis of WtI expression and the phenotype of the WtI knockout mouse.

Study of the expression patterns of the WT470LZ and WT280LZ transgenes reveals expression in tissues and organs where WtI activity has not been previously reported. In particular expression of the transgene in the limb and dorsal spinal cord represents potentially newly discovered sites of WtI activity. We carried out WtI whole mount mRNA in situ analysis on E12.5 and E13.5 embryos and further radiactive mRNA in situ analysis on sections from E12.5 embryos to attempt to confirm some of these potentially new expression patterns.

Using the data provided by the analysis of the WT470LZ and WT280LZ as a guide the WtI knockout mouse was reexamined. This was done to attempt to detect any subtle defects not picked up in the original analysis of the animal.

5.1 WtI is expressed in the limb.

In lines transgenic for either WT280LZ or WT470LZ β-galactosidase is expressed in the anterior, posterior and interdigital apoptotic zones. Whilst WtI expression has not previously been reported in the mouse limb, it has in alligator. This study reported WTI expression at the base of the limb, in the developing musculature and anterior and posterior marginal zones (Kent, 1994; Kent et al., 1995). These regions in the alligator are apparently homologous to those expressing the transgene in the mouse, with the exception of the interdigital apoptotic zones (Fig. 35f).

Whole mount WtI mRNA in situ analysis carried out on E12.5 embryos highlighted a clear domain of WtI expression at the base of the webbed mesenchyme between digits 3 and 4 (Fig. 35a and b). At E13.5 expression of WtI, whilst less strong, runs in a stripe either side of the developing digits (Fig.35d). WtI expression in the
musculature at the base of the limb was confirmed by radioactive Wtl in situ analysis on sections of an E13.5 mouse embryo (data not shown). The domain is continuous with that already described in the body wall (Armstrong et al., 1992).

5.2 Wtl is not expressed in the dorsal part of the spinal cord.

WT470LZ is expressed in the dorsal part of the spinal cord at cervical levels. Wtl whole mount in situ on E13.5 decapitated embryos shows expression of the gene in ventral parts of the spinal cord at this level (Fig. 35e). This ventral domain of expression is the same as that already described in the cord at lumbar levels (Armstrong et al., 1992). No expression was seen in the dorsal part of the cord. Transgene expression in this domain may be due to ectopic expression of β-galactosidase caused by cryptic elements in the transgene. Similar ectopic expression in the dorsal part of the cord at caudal regions has been seen in embryos carrying other transgenic constructs, e.g. the Trp1 promoter linked to lacZ (I. Jackson pers. comm.)
Fig. 35 Whole mount Wt1 mRNA in situ analysis.

(a) Wt1 whole mount in situ analysis on a E12.5 mouse embryo. Wt1 expression can be seen interdigitally, in the musculature at the base of the limb and in the flanks. (b) Section (x50) through hand plate of the E12.5 embryo (plate a) demonstrating Wt1 expression interdigitally at the base of the digits. (c) E12.5 mouse embryo in situ ‘sense’ control demonstrating that signal within the head in due to trapping of the probe in the cerebral ventricles. (d) Wt1 whole mount in situ analysis on a limb from E13.5 mouse embryo demonstrating weak Wt1 expression in the interdigital region. (e) Cervical region of a decapitated Wt1 whole mount in situ E13.5 mouse embryo. There is Wt1 expression in the ventral but not in the dorsal neural tube. (f) Stage 12-13 alligator whole mount Wt1 mRNA in situ analysis demonstrating Wt1 expression in the developing handplate, limb musculature, body wall and hindbrain (from Kent et al. 1995). (i) front view, (ii) rear view.

Abbreviations: (NT) neural tube, (Pl) pleura, (ID) interdigital mesenchyme, (D) digit, (Rs) rostral, (C) caudal, (P) pericardium, (L) limb, (Mu) musculature, (HP) hand plate, (MB) midbrain, (HB) hindbrain, (BW) body wall.
5.3 *Wti* is not expressed in the somite.

In WT470LZ transgenic embryos the transgene is expressed in myotome derived body wall, limb and intercostal muscles. Kent *et al.* suggested that *Wti* expression may be expressed in part of the sclerotome in stage 21 chick embryos (Kent *et al.*, 1995). This finding seems surprising. β-galactosidase expression is not seen in sclerotome derived tissues in WT470LZ transgenics and *Wti* expression has not been reported in these tissues. We re-examined *Wti* and *Myogenin in situ* carried out by Kent *et al.* on stage 21 chick. These show chick *Wti* expression abutting the somitocoel, in what appears to be the ventral part of the somite (data not shown).

We examined the expression of the WT470LZ transgene in embryos of E10.5-E11.5 to ascertain whether the transgene is being expressed within the somite. β-galactosidase expression envelops the ventral end of the somitic myotome but is not clearly within the somite derived tissue (Fig. 29a). To further investigate the nature of somitic *Wti* expression radioactive *in situ* analysis of this region on sections of an 10.5 mouse embryo were undertaken using *Pax1*, *Pax3* and *Wti* molecular probes. *Pax1* is a marker of sclerotome derived cells and *Pax3* a marker for myotome derived cells. In the axial region of the E10.5 embryo domains of cells expressing either *Wti*, *Pax1* or *Pax3* abut, but are mutually exclusive (Fig. 36). *Wti* is not being expressed within any somite derived cell populations. This analysis was carried out in mouse and not chick. However the stages of somitic development between those *in situ* carried out by Kent *et al.* and myself are comparable. It is possible that in some anterior somites, at one stage of development, *Wti* is expressed in the cells of the chick somite. This however seems unlikely. A more likely explanation is that the expression of *Wti* observed by Kent *et al.* is in cells of the intermediate mesoderm which have invaded a region originally occupied by somitic cells. This could occur whilst the somite undergoes morphogenetic changes during the formation of the sclerotome and dermomyotome. This invasion may be possible because although the somite lays down a basement membrane as it forms, the membrane is not continuous and is broken in vicinity of the intermediate mesoderm (Christ and Ordahl, 1995).
There may be no functional relationship between the $WtI$ expressing tissue of the intermediate mesoderm and the somite. Alternatively invasion of $WtI$ expressing cells may limit the process of myogenesis. Intermediate mesoderm co-cultured with somitic mesoderm prevents myogenic differentiation within this tissue (Gamel et al., 1995). Loss of $WTI$ expression in Wilms' tumours appear to be correlated with a large amount muscle formation within them, furthermore WT1 protein can repress myotube formation in myoblast cell culture (Hastie, 1994). Could a role of $WTI$ in the suppression of myogenesis explain the expression of the gene in the epaxial myogenic regions of the embryo?
Fig. 36 In situ hybridization analysis of the junction between the intermediate mesoderm and the somite.

(A) - Wt1. (B) - Pax3. (C) - Pax1. (all x100). Wt1 abuts the edge of the ventral myotome and wraps around it but the two domains of expression remain distinct. Abbreviations: (nt) neural tube, (mt) mesonephric tubule, (md) mesonephric duct, (my) myotome, (sc) sclerotome, (a) aorta, (ug) urogenital ridge, (g) gut, (mth) mesothelium/coelomic epithelium, (lb) limb bud. Image enhanced using the X-overlay and XV computer packages (MRC Human Genetics Unit, Edinburgh).
5.4 Analysis of Wt1/- embryos.

With knowledge of the expression pattern of the WT470LZ and WT280LZ transgenes the phenotype of homozygous null Wt1 embryo was re-examined (gift of J. Kreidberg Harvard Medical School, Boston, USA and R. Jaenisch, Whitehead Institute for Biomedical Research, Cambridge, USA) (Kreidberg et al., 1993). E12.5 and E13.5 Wt1 knockout embryos were isolated, sectioned and haematoxylin/eosin (H&E) stained. No obvious morphological effects of the loss of Wt1 were seen in any ectodermally derived tissues. Similarly no effects were apparent in those in areas of transgene expressing head mesenchyme.

5.5 A Wt1 null phenotype in the pleura, pericardia and diaphragm.

The phenotype in the developing urogenital system of homozygous Wt1 null mice has already been reported (Kreidberg et al., 1993). The same investigation notes that these embryos have a small malformed heart, lungs and discontinuities of the diaphragm. Pericardial bleeding and edema also occur which are postulated to be due to cardiac malfunction. There is no expression of the transgene within the myocardium. These abnormalities are however, consistent with the transgene/Wt1 expression in the visceral and parietal pericardia, pleura and diaphragm.

Wt1 knockout embryos were isolated, sectioned and examined at E12.5 and E13.5. In these embryos there was an apparent reduction in the volume of the outer pericardium and the pleura. The reduction of the pericardium is consistent with loss of part of the WT470LZ/Wt1 positive parietal pericardial layer (Fig. 27d). The Wt1 negative fibrous pericardia which lies distal to this layer may not be affected. E12.5 and E13.5 knockout embryos show edema. Therefore it is difficult to tell whether the pericardial and pleural tissues are simply present and displaced by the swelling of the embryo or reduced in cell number. A large number of apoptotic bodies and phagocytes are present within the pericardium and pleura of the E12.5 and E13.5
knockout embryos. This implies that a high level of cell death is taking place in these tissues. Even if the cell death occurring in these tissues is above than that in wildtype embryos however, it may be as a consequence of embryonic edema and death rather than loss of local \( WtI \) expression.

5.6 No obvious \( WtI \) null phenotype in the limb.

Both WT470LZ and WT280LZ are strongly expressed in the apoptotic interdigital mesenchyme of the developing limb (Fig. 29). Cell death in these regions is essential for correct limb digit patterning to occur (Konig et al., 1993). Expression of \( WtI \) within these domains was confirmed by whole mount \textit{in situ} analysis (Fig. 35). We therefore undertook a study of any potential differences in \( WtI \) knockout and wild type limb patterning. Fore and hind limbs were removed from knockout mice and wild type litter mates at E12.5 and E13.5. The pattern of apoptosis within these limbs was examined by TUNEL analysis and H&E staining (Fig. 37). No differences in the patterns of apoptosis between wild type and knockout embryos could be identified. Similarly there was no apparent difference in limb musculature between wild type and knockout embryos.

The lack of \( WtI \) expression in the forming limb may not give rise to any phenotype. Alternatively the \( WtI \) knockout mice limb phenotype may be very subtle and not obvious at the early stage of development (E13.5) when homozygous null embryos die. \( WtI \) has a very transient expression pattern during limb development and it would be unsurprising if the gene had only a very subtle function in limb patterning. The regulatory element responsible for driving \( WtI \) expression in the developing limb is carried on the 5kb region of promoter present in pUSWT1. If this element could be further defined and then disrupted in WT280, neonate mice partially rescued with this YAC would not have expressed \( WtI \) in the limb during development. In these animals a limb phenotype, if one occurs, may be easier to observe.
Fig. 3.7 Comparison of forelimb patterning in wild type versus homozygous Wt/l null E13.5 mouse embryos.

(a) Wild type E13.5 forelimb (x50). (b) Homozygous null E13.5 forelimb (x50). (c) Close-up of region from plate b (x200). There is no obvious difference in patterning of the digits or regions of apoptosis between the wildtype and Wt/l knockout limbs. Abbreviations: (D) digit, (ID) interdigital mesenchyme, (ApC) apoptotic cells.

In plate c apoptotic cells are not TUNEL labelled but H&E staining of the section strongly labels apoptotic cell bodies.
5.7 Crossing Wtl knockout mice with WT470LZ (line A) transgenic mice.

To better determine the effect of the loss of Wtl activity tissues which normally express the endogenous gene, we attempted to cross the WT470LZ transgene onto a Wtl homozygous null background. Line A transgenic mice were mated with Wtl heterozygous null (+/-) mice. As line A is male sterile only female offspring from this cross were used for further matings. These were mated to Wtl +/- males in order to generate Wtl-/- mice carrying the WT470LZ transgene.

Thirty four E12.5 embryos were isolated from this cross. 6 were homozygous null for Wtl and 16 expressed the WT470LZ transgene. Surprisingly no embryos which were Wtl null expressed the transgene although half would be expected to do so. Given this surprising result it seemed possible that the transgene had integrated onto the same chromosome, (chromosome 2), as the endogenous Wtl locus. FISH analysis (Fantes et al., 1992) was carried out with a cosmid probe specific for the human WTI locus (B2.1(Fantes et al., 1995)) to determine if this was indeed the case. WT470LZ in line A is integrated on chromosome 4 (Fig. 21a). Hence it should be possible to isolate Wtl homozygous null embryos which carry the transgene. Besides the possibility of simply being unlucky there seemed two other likely explanations for the inability to do so.

The WT470LZ transgene may not be able to be expressed on the Wtl knockout background. Rupprecht et al. 1994 have footprinted several Wtl binding sites in the Wtl promoter and propose that Wtl negatively autoregulates itself. However as WT470LZ potentially fails to be expressed on the Wtl null background it may be that Wtl protein is actually required for Wtl expression. If this is the case positive autoregulation of the Wtl locus is occurring. Alternatively tissues which normally express the transgene may be lost from E12.5 and E13.5 Wtl knockout embryos.
We postulated that, if transgene activity is lost because of the loss of transgene expressing tissues at E12.5, it may be possible to detect activity at earlier stages of embryonic development. E9.5 and E10.5 embryos were isolated and decapitated. The heads were washed 4 times in PBS before being genotyped by PCR analysis (Grindley et al., 1995 and materials and methods). The bodies of these embryos were stained for β-galactosidase activity. Of 20 embryos isolated 6 were knockout but only one of these (which was an E9.5 embryo) carried the WT470LZ transgene. In this embryo the transgene was expressed. Another 6 embryos were not WtI homozygous null but carried and expressed the transgene. Again there were fewer embryos which were WtI knockout and carried the transgene than expected. One embryo did however, indicating that the transgene can be expressed on a knockout background, at least at this early stage of development. It is still not possible to explain why so few WtI knockout embryos express the transgene. Possibly tissue expressing WT470LZ in WtI knockout embryos does undergo apoptosis as development proceeds. An E9.5 day embryo expresses WT470LZ in the mesonephric mesenchyme which is largely unaffected in a WtI homozygous null embryo. Organs developing later such as the metanephros, gonad and pericardia are affected by loss WTI activity.

The more recently characterized line H also contains the WT470LZ transgene. FISH analysis of this line also localized the transgene integration site to chromosome 4 (Fig. 21b). Furthermore this line does not have problems of male sterility. In order to further analyze the expression of WT470LZ on the WtI null background a cross is presently being undertaken between line H and the WtI knockout line.
6. Discussion.

6.1 Tissue specific regulation of the WTI gene.

The WTI gene is expressed in a complex tissue-specific manner during development. Attempts to study how the gene is regulated have met with some difficulty. Hence, to date, all information on WTI gene regulation has been generated in cell lines (Fraizer et al., 1994; Cohen et al., 1997; Hofmann et al., 1993; Rupprecht et al., 1994; Wu et al., 1995; Hewitt et al., 1995a). We have attempted to study WTI promoter activity in vivo in transgenic animals. The results of similar transgenic studies have recently been reported by other groups (Cohen et al., 1997; Hewitt et al., 1996). We have been able to go much further than these groups towards elucidating WTI promoter activity. Firstly, we have been able to use YAC based transgenic constructs to mimic the endogenous expression pattern of the Wtl gene. Secondly, we have compared the expression pattern of a lacZ reporter gene driven by three different WTI promoter/reporter constructs in lines where the constructs have either integrated intact or are rearranged. By carrying out this comparative analysis we have been able to begin to localize tissue specific regulatory elements within the WTI promoter.

(Table 5 summarizes reporter gene expression patterns driven by WT470LZ, WT280LZ and USWT1 and compares this to the previously reported endogenous Wtl pattern.).
Table 5 β-galactosidase expression in WT470LZ, WT280LZ and NOR/NLS transgenic embryos.

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1 - Expression level is reduced compared to other transgenic lines.
2 - Wt1 expression detected by RT-PCR.
3 - Expression domain different to that of endogenous Wt1 or other lines.
4 - Expression of WT280LZ has been seen in 3/3 transient transgenic lines analysed.
6.1.1 Plasmid (USWT1) transgenic analysis.

The putative 5' proximal \textit{WTI} promoter encompassed by USWT1 is very weak and subject to position effects in transgenic animals. However analysis using this construct has demonstrated that tissue-specific enhancer elements responsible for driving \textit{WTI} expression in the spinal cord, handplate, epaxial musculature and pericardium, pleura and peritoneum may all localize to the \textit{WTI} promoter sequence in USWT1. No reporter gene expression driven by USWT1 has been detected in the urogenital system of 10/10 NOR/NLS transgenic embryos examined.

It is to a degree surprising that no expression of lacZ in the NOR/NLS transgenic embryos was seen in the urogenital system, whilst weak expression was identified in the pericardium, pleura and peritoneum. All of these tissues consist of mesenchyme produced originally by the proliferating coelomic epithelium. One explanation is that there may be low levels of reporter gene expression in the mesonephros and the gonad in some of the NOR/NLS transgenic embryos. This low level of expression however, is masked by endogenous β-galactosidase activity. Alternatively, the regulatory elements required for the expression of \textit{WTI} during the development of the metanephric and gonadal mesenchyme may be different from those required for generalized mesenchymal expression. This second hypothesis seems more likely as line C and D embryos also show differential transgene activity between the urogenital system and the pleura, pericardia and peritoneum.

The \textit{WTI} promoter region contained on USWT1 is reported to have promiscuous promoter activity in all cell lines in which it has been tested (Fraizer et al., 1994; Cohen et al., 1997) and Table 2). This same promoter has potential tissue specific activity when assayed \textit{in vivo}. This difference between promoter activity in cell lines and transgenics illustrates the importance of carrying out the analysis of any putative promoter elements in an \textit{in vivo} transgenic assay. Cohen \textit{et al.} 1997 and Hewitt \textit{et al.} 1996 made a total of 10 transgenic animals with a construct a similar to USWT1 (Fig. 9). They found no transgene expression at all. The Cohen and Hewitt constructs seem to have integrated in an ‘inactive’ chromatin configuration whilst those in
NOR/NLS transgenics have not. The NOR or NLS transgenes themselves are very susceptible to position effects. One of the implications of these results is that the chromatin structure of the WT1 promoter region may be regulated during development. USWT1 may contain specific sequences, which serve to open up the promoter into a more active configuration, sequences which may be missing from the Cohen and Hewitt constructs.

6.1.2 Expression of β-galactosidase in lines A and H driven by WT470LZ mimics that of the endogenous Wt1 locus.

The expression pattern of the lacZ reporter gene in WT470LZ transgenic animals is very similar to that of the endogenous Wt1 gene. It is expressed in the same spatio-temporal pattern during embryonic development as endogenous Wt1, except for the Sertoli cells of the testes and potentially the granulosa cells of the ovary. It appears that nearly all the correct regulatory sequences are present on this YAC construct to get proper expression of the WT1 locus in vivo.

6.1.3 Localization of potential tissue specific regulatory elements.

WT280LZ in lines C and D is truncated somewhere 5' of the WT1 gene. These lines show no transgene expression in the developing metanephros or mature podocyte cells. Transient transgenic analysis with WT280LZ shows that this construct can drive β-galactosidase in the same cells as endogenous WT1 during nephrogenesis. Elements required to drive WT1 expression in the developing metanephros appear to have been deleted in lines C and D and are likely to lie 5' of the WT1 locus. Lines A, H and E which do not show any disruption of the YAC transgene 5' of WT1 have a normal β-galactosidase expression pattern during nephrogenesis. The USWT1 construct is unable to drive β-galactosidase expression during metanephric nephrogenesis hence elements controlling this expression may be located some distance further 5' of WT1 than the area covered by USWT1. Long distance, tissue specific, regulatory elements are quite common. A number of human genetic diseases
are proposed to be caused in some patients, by the displacement of such elements by chromosomal translocation. Examples of diseases where this has been reported are aniridia (*PAX6*), X-linked deafness (*POU3F4*) and Campomelic dysplasia (*SOX9*) (Bedell et al., 1995). Similarly, several mouse mutants can be ascribed to DNA rearrangements far from the gene locus. For example tissue specific defects in the expression of steel factor (SCF) from the *Sl* locus are caused by DNA rearrangements over 100 kb 5' of the *Sl* gene (Bedell et al., 1996).

Expression from USWT1 is very weak and susceptible to position effects. In the WT280LZ/WT470LZ YAC transgenics a much larger region of the promoter is integrated. These show strong, tissue specific expression of the transgenic construct. The WT470LZ transgenes in line A (1 copy integrated into euchromatin) and line H (12 copies integrated into a heterochromatic telomere) show position independent, copy number dependant expression. These YAC transgenes may simply set up the correct chromatin domain for transcription because they contain all correct tissue specific *WT1* enhancer elements. Alternatively, encoded somewhere on the YACs may be an LCR.

The proximal promoter sequences of genes such as tyrosinase (Montoliu et al., 1996), *CD2* (Festenstein et al., 1996) and those of the globin locus (Martin et al., 1996; Townes and Behringer, 1990) have only weak, but tissue specific activity in transgenic animals. A locus control region (LCR) spatially separate from these proximal elements acts to activate strong, position independent, copy number dependant expression from these promoters when also included on transgenic constructs. The LCR of the *β* globin locus was identified because deletions upstream of the *β* globin complex, leaving the *β* globin genes intact, cause haemoglobinopathies associated with loss of globin function (Townes and Behringer, 1990). No mutations have been identified in Wilms' tumour patients (Grubb et al., 1995; Campbell et al., 1993) which directly point to the *WT1* locus having an LCR.
Whilst no naturally occurring mutations indicate the presence of a WTI LCR, lines D and E and to some extent in line I retain the ‘normal’ WTI expression pattern but show a very large reduction in transgene activity. Line E expresses lacZ in the pericardium, pleura and peritoneum, however this expression level is greatly reduced compared expression in lines A and H. The elements required to drive tissue specific transgene expression in the pericardium, pleura and peritoneum probably lie immediately 5’ of the WTI locus as lacZ expression can be driven by USWT1 in these tissues. The level of this USWT1 mediated expression however is low and susceptible to position effects. Other elements which are present on the YAC constructs may be needed to stabilize the transcription it mediates. Such elements may be missing from the integrated transgene in line E. A closer analysis of the state of the transgene integration within this line is required.

6.2 Future work on WTI regulation.

WT470LZ, and possibly WT280LZ, express a β-galactosidase reporter gene in the same spatio-temporal pattern as WTI during embryonic development. Transgenic lines containing WT280LZ and WT470LZ in which potential tissue specific enhancers elements have been manipulated can be made. Such lines could provide a powerful method of assessing element function. Some of the putative tissue specific elements identified in cell culture experiments provide possible candidate sequences for analysis in this fashion, e.g. the CR element (Dehbi et al., 1996; Dehbi and Pelletier, 1996) and the CTC repeat (Cohen et al., 1997).

The CR element lies around 400 bp upstream of the main WTI transcription initiation start site. It contains two paired box binding sites and has been implicated in the repression of the WTI promoter by co-transfected Pax2 and Pax8. These molecules are strong candidates for interaction with WTI. They are co-expressed with WTI during the early stages of nephrogenesis and later adopt a mutually exclusive expression pattern to it (Eccles et al., 1995). The CR element lies within the putative proximal WTI promoter element contained in USWT1. However NOR/NLS
transgenics show no expression of β-galactosidase during nephrogenesis. This does not mean that the CR element definitely has no role to play in the nephrogenic expression of \textit{WTI}. Elements lying outside USWT1 may be required to stabilize or synergize its activity.

Sequence comparison of \textit{WTI} promoters from different species may highlight elements involved in gene regulation. 400bp of the proximal chick \textit{WTI} promoter has been cloned (Kent, 1994). It does not show extensive homology to the mouse (47\% identity) or human (45\% identity) \textit{WTI} promoter. A few regions are more highly conserved. None of these regions of conservation match with regions so far proposed to have a function in \textit{WTI} regulation. They may serve as pointers for future promoter dissection. The Sp1 binding CTC repeat is not in a highly conserved domain. It could none-the-less be functional in the chick, as the equivalent sequence in the analogous area of the chick promoter is 5' GTC CAC CCC CTC 3'. Recently a cosmid including the puffer fish (\textit{Fugu rubripes}) \textit{WTI} locus has been isolated (Dr. C. Miles pers. comm.). \textit{Fugu rubripes} provides a useful system to dissect gene and promoter function. It has on average only 12\% the amount of DNA at any one locus compared to mouse, human and many other higher vertebrates (Elgar et al., 1996). Sequences which are conserved between \textit{Fugu} and other species are likely to be functional. The \textit{Fugu WTI} cosmid will be sequenced, and used in transgenic assays to analyse both gene and promoter function.

\section{Why is WT280 unable to rescue \textit{Wti} homozygous null mice?}

Andreas Schedl crossed the WT280 transgene onto the \textit{Wti} knockout background. \textit{Wti} expression from three different WT280 transgenic lines, when crossed onto the \textit{Wti} knockout background, was able to rescue the defects of the thoracic organs which cause embryonic death. It was however, unable to rescue adrenal glands, gonads or full nephrogenesis. In these partially rescued animals nephrogenesis was never able to proceed from the S-shaped body stage to form a mature glomerulus.
The failure of a full rescue implies that WT280 does not express WTI in way that can fully compensate for the loss of endogenous Wt1.

Both WT280LZ and WT470LZ transgenic animals show reporter gene expression during urogenital development which mirrors that of endogenous Wt1. Although it must be pointed out that WT280LZ expression in the urogenital system has only been seen in transient transgenic embryos. It is likely therefore that only a subtle difference between the activity of the WT280 transgene and the endogenous locus leads to failure of urogenital development in the partially rescued mice. Functional differences between the YAC and the endogenous locus seem more likely to be in gene regulation rather than protein function. The amino acid sequence of the mouse and human is very highly conserved (>96% identical) (Buckler et al., 1991), on the other hand, species specific differences in WTI regulation exist. The WT470LZ (and probably the WT280LZ) transgene, for example, is not expressed in the testicular Sertoli cells (Fig. 25 a and c). The WT280LZ transgene does not however seem to be expressed in a pattern obviously different to that of Wt1 in the rest of urogenital development. Perhaps therefore, it is subtle differences in a process, such as the control of the ratio of alternative WTI splice forms, which cause the partially rescued phenotype. Alternatively the level of WTI protein in different tissues may be critical. Podocyte formation, in particular, is associated with an upregulation of the level of Wt1 expression (Armstrong et al., 1992; Pritchard Jones et al., 1990) (Fig. 3). In the WT280 transgenic kidney this upregulation may not occur.
6.4 Developmental role of the *WTI* gene.

The expression of WT470LZ in transgenic mice mirrors that of the endogenous *Wtl* gene. Hence, investigation of the expression pattern of WT470LZ throws new light on potential developmental roles of the *Wtl* gene.

6.4.1 *Wtl* is expressed in mesenchymal, but not direct epithelial, derivatives of the proliferating coelomic epithelium.

At E8.5 WT470LZ is expressed in the intermediate mesoderm. This tissue gives rise to the organs of the urogenital system and the adrenal glands (Saxen, 1987; Bannister et al., 1995). At the same time WT470LZ expression is also detected in the septum transversum (Fig. 22). This structure will give rise to parts of the diaphragm, pericardium and gut mesentery (Bannister et al., 1995). The intermediate mesoderm is formed by proliferation of the coelomic epithelium at the junction of the splanchnopleuric and somatopleuric mesoderm. The septum transversum also represents a localized mesenchymal cell population derived from the proliferating coelomic epithelium. The splenic capsule which forms later in development (E15.5), is another structure formed in this way. This tissue also expresses *WTI* (Armstrong et al., 1992).

By E10 expression of the WT470LZ transgene has spread throughout the coelomic epithelium. The coelomic epithelium is beginning to proliferate and gives rise to mesenchymal cells sandwiched between the coelomic epithelium and the underlying mesoderm (Fig. 27). WT470LZ is expressed in this tissue. This tissue will form different serosal structures, e.g. pleura, pericardia, peritoneum or diaphragm, dependent upon the location. The cells of this newly formed mesenchyme are histologically distinct from those which constitute the splanchnopleuric and somatopleuric mesoderm. Although WT470LZ/Wtl is expressed in the coelomic epithelium as it undergoes proliferation, it is not a prerequisite for this proliferation.
WT470LZ is expressed in the intermediate mesoderm before it is expressed in the overlying coelomic epithelium.

In addition to giving rise to mesenchymal cells, the coelomic epithelium also invaginates to form epithelial structures, e.g. the mesonephric duct and the paramesonephric duct. These tissues do not express WtI or WT470LZ (Figs. 22 and 23). The coelomic epithelium itself however does express WtI/WT470LZ implying that WtI expression must be lost during the process of invagination to form these tissues. In short, WT470LZ/WtI is expressed in the mesenchymal cells, but not in the epithelial cells, which derive directly from the coelomic epithelium. This relationship is schematized in Fig. 38.

Occasionally extra-renal Wilms’ tumours are found in the retroperitoneal space and gonads (Aterman, 1989). These tumours may be formed by misplaced metanephric tissue. Alternatively, all the mesenchymal tissue derived from the coelomic epithelium may have the same original developmental potentiality. This tissue would include the mesonephric cord, gonads and peritoneum. Mutation of WT1 or other Wilms’ tumour genes in these tissues, before their developmental potentiality is restricted, could potentially give rise to a tumour histologically similar to Wilms’ tumour. Similarly metanephric blastemal cells have a greater developmental potentiality than just the formation of nephrons and stroma. Mutation of WT1 or other Wilms’ tumor genes in the metanephric blastema can give rise to foci of ectopic bone, muscle and adipose tissue.

Desmoplastic small round cell tumour (DSRCT) is associated with the fusion of the N terminus of the EWS gene to the C terminus of WT1. This tumour also occurs in tissues derived from the proliferating coelomic epithelium such as the gonad, peritonea and pleura (Gerald et al., 1995; Ladanyi and Gerald, 1994). One explanation for sites of DSRCT generation by the chimeric protein is that the tumour is generated where WT1 is normally active. The EWS/WT1 protein may interact with the members of the same cellular pathway as WT1. One case of DSRCT has been
reported in the brain; perhaps this tumour was generated from a region which is expressing WT470LZ and hence potentially \textit{Wtl} (Tison et al., 1996).

\textbf{Fig. 38} \textit{W7}l \textit{is expressed in the mesenchymal, but not the epithelial cells which derive directly from the proliferating coelomic epithelium.}
6.4.2 WTI in metanephric organogenesis.

The WT470LZ and WT280LZ transgenes show the same pattern of expression as the WTI gene during metanephric nephrogenesis. During the process of nephrogenesis the proximal part of the S-shaped body matures into the podocyte cells of the glomerulus. This process is associated with upregulation of WTI/WT470LZ/WT280LZ expression. It is this stage which is blocked during metanephros formation in WT280 partially rescued mice. As metanephros formation occurs normally until this stage, the partially rescued kidney demonstrates a role for the WTI gene in nephrogenesis which differs from that demonstrated in WTI knockout mice. WTI is required for the final maturation of the glomerular podocytes epithelium during nephrogenesis. Although epithelial structures form in Wilms' tumours which represent different stages of nephrogenesis no structures corresponding to mature glomerulus form (Pritchard Jones and Fleming, 1991). Perhaps the glomeruloid bodies seen in some Wilms' tumours are equivalent to the furthest stages of nephrogenesis seen in the WT280 partially rescued kidneys.

6.4.3 WTI function in the adrenogenital primordium.

The adrenogenital primordium lies within the mesonephric ridge (Hatano et al., 1996). WT470LZ, and by implication WTI, is expressed in the adrenogenital primordium from its origin at E9.5. As the primordium splits, WT470LZ expression is lost in the adrenal part remaining only in the gonadal part (Fig. 23). Loss of WTI expression in the developing foetal adrenal cortex may be involved in specifying the differences between adrenal and gonadal developmental pathways.

In WTI knockout mice, partially rescued with the WT280 transgene, formation of the gonad or adrenal gland does not take place. WTI is expressed in the adrenogenital primordium but is no longer expressed at later stages of adrenal formation. WTI function in the adrenogenital primordium is therefore required for later adrenal gland formation. It is not clear what happens to the adrenogenital primordium in WTI homozygous null mice. It may undergo apoptosis or simply fail to proliferate and
remain as a rudimentary structure. By analogy to the phenotype seen in the metanephric blastema of a Wt1 homozygous null embryo, expression of Wt1 may be required to allow the primordium to transduce and respond to a proliferative signal. Alternatively Wt1 may simply be required for primordium proliferation itself.

Given the requirement for Wt1 in the adrenogenital primordium to allow adrenal formation, the loss of Wt1 expression from this primordium would also be expected to prevent gonadal development. However this may not be the case. Reinterpretation of the Wt1 knockout mouse data provided by Kreidberg et al., 1993 indicates that the gonad starts to extend into the coelomic epithelium, and may begin to form some internal structure, before it is lost. If the gonadal mesenchyme does begin to form before receding, there may be parallels between the effects of Wt1 loss on the development of the gonad, and on the metanephros. Perhaps the gonad begins to develop but undergoes apoptosis in the absence of an inductive signal. Such a signal may come from the development of the rete testis/ovarii or alternatively the primary sex cords. On the other hand it may not be the lack of a signal which causes the apoptosis but the failure of the gonadal mesenchyme to respond to it.

6.4.4 The role of the Wt1 gene in sex determination.

Severe genital and gonadal abnormalities are associated with (both XY and XX) patients suffering from Denys-Drash syndrome. In addition to malformed or ambiguous external genitalia patients may have streak gonads or ovo-testes. In these patients the process of primary sex determination i.e. Sertoli cell formation has been disturbed. Circumstantial evidence that WT1 may play a role in the formation of Sertoli cells comes from a study of WT1 gene expression in the urodele amphibian testis (Del Rio-Tsonis et al., 1996). In urodeles, in regions termed regenerating cysts, testicular duct cells appear to engulf germ cells and differentiate into Sertoli cells. WT1 is expressed in these duct cells during this process.

WT470LZ is not expressed in the Sertoli cells (and possibly the granulosa cells) whereas endogenous Wt1 is (Fig. 25). The sequences required to drive β-
galactosidase expression in these cells must be missing from the transgene. WT470LZ contains the human WTI gene and promoter. The species specific differences between the WTI promoter of humans and mice may be responsible for the inability of the WT470 promoter to drive lacZ in Sertoli cells. Support for this hypothesis comes from the study of pUSWT1 activity in cell lines. This human promoter was able to drive reporter gene expression in human granulosa cells but not in murine Sertoli cells.

The mammalian SRY gene (Ramkissoon and Goodfellow, 1996) provides a good example of a gene in which the rapid evolution of species specific differences is occurring. It is evolving very rapidly outside its DNA binding domain, and appears to undergoing species specific divergence which is faster that the rate of natural drift at that locus (Whitfield et al., 1993). SRY is involved in sex determination. It is the switch required to force precursor cells to form Sertoli cells and hence trigger all subsequent secondary male sexual differentiation. In the absence of SRY a default female pathway is taken. A murine Sry transgene introduced into XX mice causes gonadal sex reversal by driving the differentiation of Sertoli cells in the XX gonad (Koopman et al., 1991). However a human SRY transgene is unable to do the same although it is expressed at the right place and time. Rapid species specific evolution of genes involved in sex determination may aid the process of speciation by causing intraspecific hybrid sterility.

Wti is expressed in the gonadal ridge before the onset of Sry expression. However perhaps Sry binding on the Wti promoter and its association with other species specific factors may be responsible for the Wti expression in Sertoli cells. The murine Wti promoter contains a putative Sry/SRY binding site AACAAT (Harley et al., 1994) (Fig. 8) although no such sequence is present in the human promoter.

The Sox9 gene is co-expressed with both Wti and Sry in the gonadal ridge and is involved in sex reversal and Campomelic dysplasia (Ramkissoon and Goodfellow, 1996). Following male gonadal sex determination Sox9 expression becomes localized
to Sertoli cells. A human derived YAC covering the SOX9 locus, as with WT470LZ, was unable to drive expression of a lacZ reporter in Sertoli cells (V. Wunderle pers. comm.). This raises the possibility that Sry, Wt1, and Sox9 could all be part of a species specific co-evolving group of genes.

6.4.5 Wt1 in limb development.

*Wt1* is expressed in the limb musculature and throughout developing epaxial musculature of the embryo (Fig. 29). WT470LZ is expressed in these domains although it is unclear in which cell type. The transgene does not mark all myogenic cells. Perhaps *Wt1* expressing tissue is repressing the myogenic pathway, as has been discussed by Hastie, 1994. The anterior, posterior and interdigital apoptotic zones of the developing limb also express *Wt1* (Fig. 35). They represent sites where apoptotic cell death occurs during the process of limb morphogenesis (Zakeri and Ahuja, 1994). Expression of *Wt1* in these domains suggests that the gene may be involved in patterning the developing limb. WT470LZ does not appear to be expressed in the interdigital undergoing apoptosis. Its expression appears to be in cells adjacent to those which are apoptotic. It is first expressed in the proximal part of the interdigital mesenchyme whilst the distal part undergoes apoptosis. WT470LZ then becomes localized alongside the developing digits as the apoptosis of the interdigital mesenchyme spreads proximally. *Wt1* may be involved in limiting the region of death. Its expression comes on first in the proximal interdigital mesenchyme which is last to die. Furthermore, in *Wt1* knockout and partially rescued mice, apoptosis appears to be the default pathway taken by some tissues which would normally express the gene. Interestingly when cell death in the interdigital regions of chick embryos is blocked the default pathway for these cells is chondrogenesis (Zou and Niswander, 1996). In correlation with this finding tumours loss of *WT1* expression in some Wilms’ can lead to the aberrant differentiation of metanephric blastemal stem cells into muscle and cartilage.
6.4.5.1 Are any limb defects related to WTI mutation in humans?

The observed interdigital expression of WtI described in this thesis may be of clinical relevance. A strong correlation between sporadic Wilms' tumours and exostoses in patients has been noted (Hartley et al., 1994). Exostoses are benign enchondromata which consist of cartilage capped osseous projections especially from the long bones of a patient. They are rarely present at birth but develop within the first decade of life. The expression pattern of WT470LZ laying alongside the developing bones of the limb and thorax raises the interesting possibility that WTI may be involved in the suppression of exostoses development.

Recently a gene (EXT2) involved in hereditary multiple exostoses was isolated from 11p12 (Wuyts et al., 1996). In at least one case of a Wilms' tumour patient with exostoses the individual had a deletion of 11p11.2-p14.2 (Bartsch et al., 1996). Hence in this case ETX2 lies within a contiguous deletion including other loci involved in the WAGR syndrome. However disruption of EXT2 is unlikely to explain exostoses formation coincident with a number of sporadic Wilms' tumour cases.

6.4.6 Potential developmental roles for WtI in mesodermally derived organs.

6.4.6.1 WtI may be involved in the mesenchymal to epithelial cell type transition.

The expression of WtI in a number of tissues which undergo a mesenchymal to epithelial cell type transition lead to the suggestion that it plays a role in this process (Armstrong et al., 1992). In support of this theory, data from A. Schedl (this thesis) demonstrates that WtI is required for the final stages of epithelial maturation in the glomerular podocyte cells.

A general role for WtI in the mesenchymal to epithelial cell type transition process seems unlikely given the expression pattern of the WT470LZ transgene. The
glomerular podocytes and the rete testis/ovarii represent the only structures which express WT470LZ when undergoing this process. Interestingly both the glomerular podocytes and the rete testis/ovarii are derived from tissue which is already expressing Wt1; the level of Wt1 expression is upregulated during their formation.

Other epithelial tissues derived from mesenchymal cells e.g. the cornea or coelomic epithelium (mesothelium) do not express Wt1 whilst forming and form in the Wt1 knockout mouse (Kreidberg et al., 1993). WTI cannot be involved in the mesenchymal to epithelial cell type transition in these tissues. The idea that WTI does not play a role in this transition in the mesothelium is supported by Langerak et al. They found no correlation between WTI expression levels and epithelial characteristics in mesothelial cell lines (Langerak et al., 1995).

The role of WTI in controlling a mesenchymal to epithelial cell type transition during nephrogenesis is also not clear. Epithelial structures are found within Wilms' tumours which have lost both copies of the WTI gene. Although in these cases it does remain unclear as to whether such structures have undergone the full epithelial maturation programme. No structures representing mature glomeruli are found in Wilms' tumours (Pritchard Jones and Fleming, 1991), supporting a role for WTI in the final podocyte maturation step.

If WTI does not have a general role in mesenchymal to epithelial transitions what else might it be doing? Perhaps WTI may play a role in mediating the processes of proliferation and apoptosis. The WT470LZ transgene is expressed in all the mesenchymal derivatives of the proliferating coelomic epithelium (Fig. 38). It is also expressed in the proliferating coelomic epithelium itself. Potentially, the gene could be promoting the proliferation of these tissues, although these tissues do form in the Wt1 homozygous null mice. Expression of Wt1 in the interdigital regions of the embryo suggests that WTI has a role in mediating the process of apoptosis. In support of this hypothesis, the developing metanephros undergoes apoptosis in the
WT1 knockout mice. In these animals furthermore, the lack of WT1 expression in the pericardium, pleura and septum transversum may also lead to increased apoptosis.

6.4.6.2 WT1 may interact with the Bone Morphogenetic Proteins.

Bone morphogenetic proteins, (BMPs), are presently the molecules most favoured to play a major role in controlling apoptosis and patterning in the developing vertebrate limb. BMPs are secreted signalling molecules which are members of the TGFβ superfamily. Bmp2, Bmp4 and Bmp7 are all expressed in the developing mouse limb. Bmp7 and Bmp2 in particular become localized alongside the developing digits in a very similar expression domain to that of Wt1 (Hogan, 1996). Studies of BMP signalling in the chick limb also imply that this process has an important role in control of both interdigital cell death and chondrogenesis (Yokouchi et al., 1996; Macias et al., 1997; Zou and Niswander, 1996; Kawakami et al., 1996). Bmp7 and WT1 potentially interact in mediating metanephric nephrogenesis (see introduction). Perhaps WT1 and Bmp7 also interact in controlling apoptosis in the interdigital regions.

In addition to WT1 interaction with BMP molecules during nephrogenesis and limb patterning, BMP molecules could potentially induce WT1 expression in mesodermal patterning. BMP4 plays a major role in mesoderm formation and patterning in Xenopus embryos (Hogan, 1996) where xWT1 may mark the site of intermediate mesoderm formation (Carroll and Vize, 1996). In chick BMP4 may also play a role in patterning the mesoderm (Takahashi and Tonegawa, 1997), and WT1 again marks the site in intermediate mesoderm development (Kent et al., 1995). In mice, the majority of homozygous null Bmp4 embryos die around the time of gastrulation without making any embryonic mesoderm (Winnier et al., 1995). A few Bmp4 knockout embryos survive to the forelimb bud stage. In these embryos, the mesoderm is highly disorganized and it is not clear if an intermediate mesoderm forms. As the WT470LZ transgene is a marker of the intermediate mesoderm, crossing it onto Bmp4 knockout mice would give an indication of whether intermediate mesoderm formation is occurring.
Examination of transgenic embryos expressing the WT470LZ and WT280LZ transgenes has identified \textit{Wt}1 activity in several lineages. It is expressed in the mesenchymal derivatives of the coelomic epithelium, the somatopleuric mesenchyme of the limbs, in developing epaxial muscles of the embryo and in the ectodermal tissue of the CNS. Evolutionarily all of these expression domains are conserved at least as far as alligator and chick (Kent et al., 1995). Is there any way of rationalising why the gene may be expressed in such a diverse range of cell types? If \textit{Wt}1 is part of a BMP mediated signalling pathway, then acquisition of elements responsive to BMP may help to explain the expression pattern of the gene in the higher vertebrates. The acquisition of responsiveness to BMP signalling has previously been suggested to be responsible for the recruitment of the \textit{Msx} genes to limb pattern formation (Davidson, 1995) and perhaps the same may be true for \textit{Wt}1. In addition BMP signalling could drive gene expression during the formation of the intermediate mesoderm and in parts of the nervous system (for example the neural crest (trigeminal mesencephalic cells) and the eye).

### 6.4.7 A potential role for \textit{Wt}1 in patterning.

Expression of \textit{Wt}1 during the process of nephrogenesis and in the developing nervous system implies that it may have a role in the process of patterning.

Following condensation of the metanephric blastema at the tip of the branches of the ureteric bud, the \textit{Wt}1 positive renal vesicle forms. The vesicle is already patterned at this early stage of nephrogenesis. Cells adjacent to the ureteric bud express \textit{E-cadherin} whilst more distal cells express \textit{K-cadherin}. By the S-shaped body stage, proximal cells express \textit{E-cadherin}, distal cells \textit{Wt}1, and those in the middle \textit{K-cadherin}. Mesenchymal cells which do not form epithelium lose \textit{Wt}1 expression and express the forkhead transcription factor \textit{BF2} (Lechner and Dressler, 1997).

An interaction between \textit{Wt}1 and \textit{Pax}2 has been proposed to occur during nephrogenesis. \textit{Wt}1 and \textit{Pax}2 are co-expressed in the renal vesicle and then \textit{Pax}2 is down regulated in the distal part of the S-shaped body concurrent with \textit{Wt}1.
upregulation in this structure (Eccles et al., 1995). An interaction between Pax2 and Wt1 may also occur during patterning of the spinal cord. Pax2 is expressed throughout the neural tube from E10 except in a small domain in the ventral part (Nornes et al., 1990) This ventral domain seems to be convergent with the domain of the cord in which Wt1 is expressed. Double staining WT470LZ with anti Pax2 antibodies will allow this relationship to be investigated further.

In the developing spinal cord WT470LZ/Wt1 is expressed in similar fields to the lim domain genes (Tsuchida et al., 1994; Tanabe and Jessell, 1996). Expression in these domains implies that Wt1 is not involved in the generation of neurons but in their subsequent patterning. Wt1 may be co-expressed in a domain of Lim3 positive interneurons. Lim3 expressing neurons arise in the ventricular zone of the neural tube expressing Pax6. They fail to develop in homozygous Pax6 mutant embryos (Seyed) (Ericson et al. 1997). Given this information we are now undertaking a study to look at the state of Wt1 expression in the neural tube of Seyed embryos.

Interactions between lim domain genes and Wt1 may also be involved in patterning the embryonic mesoderm. Lim1 is expressed within the presumptive lateral and intermediate mesoderm at E7.5 of mouse development, it becomes localized to the intermediate mesoderm a day later (Barnes et al., 1994). At later stages Lim1 is expressed during mesonephric and metanephric nephrogenesis (Fujii et al., 1994). Expression of Lim1 precedes that of Wt1 within this structure. In the absence of Lim1 expression, no formation of the pronephros or the mesonephros occurs and the embryos die at about E10 (Shawlot and Behringer, 1995).

In the eye WT470LZ is again expressed in a Pax6 positive domain of neuron generation (Mugnaini and Floris, 1994). This putative Wt1 eye expression domain in the proximal part of the neuroretina, is also adjacent to Pax2. At E14 Pax2 is expressed in the optic stalk and most proximal part of the neuroretina. At E18 Pax2 expression is found on the innermost cells of the proximal neuroretina (Nornes et al., 1990).
In the developing cerebellum \textit{Wt1} is expressed in cells of the roof of the fourth ventricle at E12.5. Here again \textit{Wt1} is probably co-expressed with \textit{Pax6} and in a domain adjacent to but exclusive from \textit{Pax2} (Stoykova and Gruss, 1994). WT470LZ activity is seen in the Purkinje layer of the adult cerebellum and in a few scattered cells of the granular layer. \textit{Pax6} continues to be expressed in the internal granular layer in the adult whilst the Golgi cells which come from the same precursors as the Purkinje cells do not express WT470LZ but do express \textit{Pax2} (Stoykova and Gruss, 1994). Since the completion of the study for this thesis we have detected strong expression of \textit{Wt1} within the cells of the Purkinje layer by immunohistochemistry. In keeping with the scattered expression of lacZ positive cells in the granular cell layer, \textit{Wt1} is also present but to a much lesser extent in these cells. The localisation of the \textit{Wt1} protein in both the Purkinje and granulosa cells is not nuclear but solely cytoplasmic. This unusual cellular localisation for the \textit{Wt1} protein raises the exciting possibility that \textit{Wt1} may be involved in RNA metabolism in these cells.

6.5 Future work on the developmental role of \textit{WTI}.

One of the most important future pieces of work to carry out is the evaluation of WT470LZ expression in the brain of both the lines A and H. The transgene expression seen in these lines then needs to be correlated with that of the endogenous \textit{Wt1} locus by a mixture of mRNA \textit{in situ} hybridization and immunohistochemical analysis.

I also wish to further investigate the expression of \textit{Wt1} in the spinal cord. This will require double labelling of the spinal cord with either \textit{Wt1} or \(\beta\) galactosidase antibodies and markers of a number of neuronal populations within the cord (e.g. Lim1, Lim3 and Pax2) (Tanabe and Jessell, 1996). The target of any axonal projections which come from the population of \textit{Wt1} positive neurons in the spinal cord and other neurons in the brain can be investigated by the use of a tau-lacZ cassette targeted in the WT470 \textit{Wt1} locus. The protein encoded by this tau-lacZ
construct binds microtubules and is transported down the axons of labelled neuronal cells (Mombaerts et al., 1996).

The WT470LZ transgene is a good marker of cells in an embryo either expressing WTI or which are able to express the gene. Hence the WT470LZ transgenic animal provides a useful tool to analyze the fate of these cells on different genetic backgrounds. I am crossing the WT470LZ transgene onto the Wtl knockout background. Preliminary results (Chapter 5) indicate that the transgene may not be expressed on this background at midgestational stages. If this is the case WTI may be positively autoregulated at these stages of development. If the WT470LZ transgene is expressed on the WTI knockout background then it will be a useful marker to follow the fate of the cells which normally express the gene. This may be particularly useful in determining what happens to the pleura and pericardial tissues in the Wtl knockout animal. It is defects in these tissues which may be responsible for the Wtl homozygous null embryonic lethality (Kreidberg et al., 1993). Furthermore it will be interesting to investigate any alterations which occur in the development of the spinal cord and brain in the Wtl homozygous null embryo. Any such alterations are not apparent by normal morphological analysis. Subtle alterations may be difficult to detect even using the WT470LZ marker. For example En1 interneurons do not require En1 function for their early survival or differentiation (Matise and Joyner, 1997). In the same way Wtl expression in neurons may not be required for their generation or maintenance but instead for a later stage of development such as correct axonal targeting. In this eventuality a tau-lacZ transgene will be required to ascertain any neural defects in the Wtl knockout animal.

Neurons which express Wtl are generated in the Pax6 positive domain of the spinal cord. The patterning of the spinal cord is altered in Sey (Pax6 null) animals. In particular Lim3 positive neurons which arise in the Pax6 positive domain in wildtype animals have an altered fate in Pax6 null animals (Ericson et al. 1997). Hence I am presently crossing the WT470LZ transgene onto the Sey°d mutant background. However, as with the cross of the transgene onto the Wtl knockout background, any
alterations in neuronal patterning may be easier to ascertain using a tau-lacZ containing YAC transgene.

A reporter gene driven by WT470 mimics the endogenous expression pattern of WTI. Hence WT470 may be used as a promoter to investigate the lineage of cells in organs in which Wti is expressed. A lacZ cassette in which the first 289 bp of the lacZ gene is duplicated (LaacZ) has been inserted into 800/4 (C. Miles pers. comm.). Duplication of the first 289 bp of lacZ creates a stop codon and the LaacZ gene is unable to produce an active product. With a very low frequency in mammalian cells (1-2 x 10^6) intragenic recombination between the direct repeat in this cassette occurs. Recombination within the LaacZ gene produces reconstitutes an active lacZ locus and hence an active β galactosidase enzyme (Bonnerot and Nicolas, 1997; Nicolas et al., 1996). This WT470LaacZ transgene will be useful for investigating the distribution of cells produced by the proliferating coelomic epithelium in the pericardium, pleura and peritoneum. It may also help investigate whether cells and at different point in kidney and gonad development become restricted in the set of tissue types which they can form. Such a restriction has been proposed to operate in the kidney (Qiao et al., 1995). This transgene will also be to address the level of contribution of mesonephric tissues to the developing gonad. A question which has proved intransigent to study (Buehr et al., 1993; Satoh, 1985; Satoh, 1991).

WT470LZ is not expressed in the Sertoli cells of the gonad. Similarly a SOX9 YAC transgene driving a lacZ reporter is not expressed in these cells. This raises the possibility that there has been a species specific evolution of regulatory sequences which drive expression of these genes in the Sertoli cells. The Sry/SRY gene, responsible for male sex determination in mammals is expressed in the Sertoli cells. Sry contains an HMG (High Mobility Group) box capable of sequence specific binding. Introduction of mouse Sry gene can cause sex reversal in female mice. The human SRY gene however is unable to cause the same effect (Koopman et al., 1991). Recent evidence implies that this difference between the mouse and human Sry genes in this assay is inherent to a region of the protein in the C terminal domain outwith
the HMG box (Bowles et al., 1997). We intend to cross mice expressing the human SRY gene onto lines carrying WT470LZ or the SOX9 transgene. If ß galactosidase is then expressed in the Sertoli cells of the resultant offspring of these crosses it would demonstrate that the regulatory framework of the WTI and SOX9 gene is co-evolving with the SRY protein.

YACs are very useful for transgenic experiments because of their large insert size and the ease with which inserts can be altered by homologous recombination. However they have a number of drawbacks including a tendency to re-arrange and be chimeric. The reason that a human YAC covering the WTI locus was used in this study was because a YAC of the homologous mouse locus was unstable. Bacterial artificial chromosomes (BACs) have a large insert size up to 350 kb (Peterson et al., 1997). They are however, very stable. A recent technological advance means that it is now possible to carry out accurate homologous recombination in BACs at a high frequency (N. Heintz pers. comm.). Therefore it may be easier in the future to use a BAC construct for the type of transgenic analysis detailed in this thesis. To this end we shall screen a mouse BAC library to attempt to get BACs spanning the WTI locus.
7. Materials and methods.

7.1 Bacterial Cell Culture and Plasmid DNA Preparation.

7.1.1 Media and solutions.
All chemical were supplied by BDH unless otherwise stated. All media were sterilised by autoclaving. Solution and media are made up as described in Sambrook et. al., 1989 unless otherwise stated.

L-broth (LB): In 1 litre water dissolve 10g tryptone, 5g yeast extract, 10g NaCl, 2.46g MgSO₄.
L-agar: In 1 litre water dissolve 10g tryptone, 5g yeast extract, 10g NaCl, 2.46g MgSO₄, 15g agar (Oxoid Ltd).
Ampicillin (Sigma): Make stock at 50mg/ml in dH₂O, filter sterilise. Store at -20°C. Use at final concentration 50µg/ml.
X-Gal (Sigma): Make stock at 20mg/ml in DMF (Sigma). Store protected from light at -20°C. Use at final concentration of 40µg/ml.
IPTG (Sigma): Make stock at 100mM. Store protected from light at -20°C. Use at final concentration of 0.5mM.

7.1.2 Growing bacterial cells on agar plates.
The desired volume (up to ~200µl) of bacterial cells is pipetted onto the surface of the L-agar, and is then spread by a sterile bent glass rod until the liquid has thoroughly soaked into the agar. (If more cells than are contained in 200µl are required, the cells may be concentrated by 1 min centrifugation in a microfuge followed by resuspension of the pellet in a smaller volume). The plates are then inverted and incubated for 12-16 hours at 37°C. Cells were viable from plates stored at 4°C for several weeks.
7.1.3 Preparation of plasmid DNA.

**Small scale** - The desired colony on an agar plate was used to inoculate 6ml of LB plus ampicillin and was grown at 37°C overnight with continuous shaking at 250rpm. These cells were then harvested and purified with a QIAprep Spin Plasmid Kit (Qiagen) as per manufacturers instructions.

**Large scale** - The selected bacterial colony was grown overnight at 37°C in a 400ml for a further overnight culture. The culture was then centrifuged in 50ml volumes at 4000rpm for 20mins at 4°C. These cells were then harvested and purified with a Qiagen Plasmid Kit (Qiagen) as per manufacturers instructions.

7.2 Enzymatic Manipulation of DNA.

7.2.1 Solutions.

TE: 10mM Tris, 1mM EDTA.

7.2.2 Restriction enzyme digestion of genomic DNA.

Digestions of DNA with restriction endonucleases were carried out in the appropriate buffer at the recommended temperature. Restriction enzymes were supplied by Boehringer Mannheim, NEB and Gibco BRL. 1μg of DNA was digested in 10-20μl using 1-2 units of enzyme for 1½ hours. Genomic DNA was digested overnight with the addition of 1/20 volume 0.1M spermidine (Sigma). If two different enzymes were used, both of which required the same buffer, the digests were carried out simultaneously. Otherwise, after digestion with one enzyme, the sample was ethanol precipitated and then resuspended. The appropriate buffer was then added and the second digestion carried out. When necessary, reactions were terminated by heating to 68°C or 80°C for 15 minutes, according to the heat sensitivity of the enzyme.
7.2.3 Dephosphorylation of 5' termini.

Calf intestinal phosphatase (CIP) was used to dephosphorylate the 5' ends of the vector molecules before cloning. This prevents recircularisation of vector molecules during the ligation step.

7\mu g DNA was dephosphorylated with 0.1 unit CIP (Boehringer Mannheim) in 50\mu l 1x CIP buffer (10mM Tris.HCl, pH 8.3, 1mM ZnCl2, 1mM MgCl2) at 37°C for 30 mins. The reaction was stopped by the addition of 1\mu l of 0.5M EDTA. The dephosphorylated DNA was then extracted with 1:1 phenol:chloroform and ethanol precipitated. The pellet was resuspended in 10\mu l TE.

7.2.4 Ligation of cohesive termini.

On ice, the insert DNA and the vector DNA were mixed, typically in a ratio of 5:1 vector to insert. 10-100ng of vector DNA was normally used. The reaction was carried out in 10-50\mu l using 0.1 unit of bacteriophage T4 DNA ligase (Boehringer Mannheim). in 1x ligase buffer (66mM Tris.HCl, pH 7.5, 5mM MgCl2, 1mM DTT, 1mM ATP). The ligation mixture was then incubated at 16°C overnight, or at RT for 2 hours.

7.3 DNA electrophoresis.

7.3.1 Electrophoresis solutions.

20x TBE: 1M Tris.HCl, pH8.0; 20mM EDTA; 1M boric acid, pH8.3.

20x TAE: 0.8M Tris.HCl, pH 8.0; 20mM EDTA; 0.4M acetic acid.

10x DNA Loading Buffer: 20% Ficoll (Pharmacia), 100mM EDTA, orange G (Sigma).
7.3.2 Agarose gel electrophoresis.

DNA molecules were separated according to their size on horizontal agarose medium gels (SeaKem LE, FMC Bioproducts). The percentage of agarose used to make the gel depended on the size range of the DNA molecules to be resolved. Digested genomic DNA or plasmid DNA was commonly run on 0.8% agarose gels, whereas smaller fragments, such as most PCR products, were run on 1-2% agarose gels. All agarose gels were made with and run in 1x TAE. 1/10 of the sample volume of 10x loading buffer was added to the DNA prior to loading the sample on the gel. Either mini gels (30ml agarose) or midi gels (120ml agarose) were used depending on requirements. Gels were run at 25-80V, in Hybaid gel tanks, depending on resolution and run-time required. 250ng of the appropriate size marker was used per gel. Size markers used were: λ DNA digested with Hind III (Boehringer Mannheim) or φX174 digested with Hae III (Boehringer Mannheim).

After electrophoresis, DNA was stained by gently agitating the gel in running buffer containing 250μg/ml ethidium bromide for 20 minutes. DNA fragments were visualised on a mid range UV transilluminator and photographed using a video copy processor (Mitsubishi).

7.3.3 Purification of DNA from agarose.

Following a run, gels were viewed on a mid range UV transilluminator and the required fragment was excised using a sterile scalpel blade. This was done as quickly as possible to minimise UV nicking of the DNA. Care was taken to ensure that a minimum of agarose was excised with the required DNA band. DNA was isolated from the gel slice using the QIAEX Gel Extraction Kit (Qiagen) as per manufacturers instructions.
7.3.4 Pulsed field gel electrophoresis.

Agarose plugs were washed once in TE and equilibrated to the running buffer (0.25x TBE) over three hours. A 1% agarose (MP agarose, Boehringer Mannheim) gel was made up in 0.25x TBE and the plugs inserted into the wells. The wells were sealed with 1% LMP agarose and the gel run in pre-cooled sterile 0.25x TBE at 12°C. Gels were run using the Chef-DR II pulse field tank and control module (Biorad). PFGE conditions used for WT470, WT470LZ, WT280 and WT280LZ: Initial time 22, end time 30, voltage 200V.

7.4 Cloning of DNA Molecules into Plasmid Vectors.

7.4.1 Strain of bacteria used.

The competent cells were made from *E.coli*, strain XL1-Blue (Stratagene). The genotype of XL1-Blue cells is: recA1 endA1 gyrA96 thi-1 hsdR17 supE4 relA1 lac [F’ proAB lacIq ZΔM15 Tn10 (Tet’)]

7.4.2 Preparation of competent cells.

Using a sterile loop, XL1-Blue bacterial cells from a frozen stock were streaked out onto a fresh L-agar plate and grown at 37°C overnight with continuous shaking at 250rpm. One of the resulting colonies was then used to inoculate 10mls of LB plus tetracyclin, which was left at 37°C in a shaking incubator overnight. This whole culture was then used to inoculate 1l of LB plus tetracyclin, and this was grown shaking at 37°C. The absorbance at 600nm of the culture was continually checked on a spectrophotometer.

The cells were harvested when the Abs$_{600}$ was between 0.5 and 1.0, which is still in the log phase of growth. The flask containing the culture was then chilled on ice for 15-30 mins then the contents were centrifuged in ice cold 50ml Falcon tubes for 15 mins at 4000rpm at 4°C. The supernatant was discarded and the pellets resuspended
in a total of 11 of sterile water before centrifugation as before. The pellets were resuspended in a total of 500ml sterile water and re-centrifuged. Pellets were then resuspended in 20ml of sterile distilled water and centrifuged again. The final resuspension was in 2-3ml of sterile distilled water or 10% glycerol. This cell suspension was then aliquoted into 40μl volumes in 1.5ml eppendorf tubes. Cells resuspended in water were used immediately. Cells in glycerol were snap frozen on dry ice plus methanol (Fisons) and stored at -70°C.

7.4.3 Plasmid vectors.

The plasmids vector used were pBluescribe (pBS; Stratagene) and pUC18. pBluescribe is a 2746bp plasmid derived from (Yanisch-Perron et al., 1985). pUC18 is the same as pUC19 but with the multiple cloning site inverted.

7.4.4 Electro-transformation of competent cells.

An aliquot of competent cells was thawed on ice, and then the transforming DNA (usually 1μl of the ligation reaction) was added, mixed with the cells and left on ice for 1min. This was then transferred to an ice-cold cuvette (Flowgen) and subjected to a pulse of 2.47kV in a BioRad Gene Pulser. The cells were swiftly mixed with 1ml of LB, transferred to an eppendorf tube and left at 37°C for 1hr to enable the cells to begin to express the ampicillin resistance gene conferred by the transformed plasmid. Aliquots of several different volumes were then spread onto L-agar plus ampicillin plates and incubated overnight at 37°C.

7.4.5 Selection for colonies that contain recombinant plasmids.

Selection was by a simple process of blue/white selection. The polylinker of the vector pBS interrupts the β-galactosidase producing gene, lacZ. However the disruption is in-frame and results in a harmless insertion of a few amino acids into the β-galactosidase gene. Expression of this gene within bacterial cells can result in the production of a blue colour if the medium on which the cells are grown contains
the chromogenic substance X-Gal and the derepressor of the lac operon, IPTG. If however the polylinker site in the plasmid is interrupted by an insert of foreign DNA, a functional β-galactosidase enzyme cannot be transcribed and hence the resultant colonies are white.

7.4.6 Ethanol precipitation.

To concentrate DNA and remove salts, a 1/10 volume of 2M NaAc, pH 5.5 was added to the DNA solution, followed by 2-2½ volumes of 100% ethanol at -20°C (alternatively 0.7 vols of isopropanol can be used). The contents of the tube were mixed and then chilled at -20°C for 1 hour to overnight. The tube was then centrifuged at 11,000rpm for 15 mins at 4°C. The supernatant was poured off, and the pellet dried under vacuum. The pellet was then resuspended in the desired volume of dH₂O or TE.

7.5 Manipulation of Yeast.

All protocols in this section are adapted from Schedl et. al. 1996a.

7.5.1 Media and solutions.

**SE**: 1M Sorbitol, 20mM EDTA pH8

**TENPA**: 10mM Tris pH7.5, 1mM EDTA pH8.0, 1000mM NaCl, 30μm spermine, 70μm spermidine.

**Microinjection buffer (IB)**: 10mM Tris pH7.5, 0.1EDTA pH8, 100mM NaCl, 30μM spermine, 70μM spermidine.

For 1l of medium: 20g glucose, 6.7g D.O.B. 2% raffinose (BIO 101 Inc)-Yeast nitrogen base for use with drop out media, amino acids (Sigma) added minus those required for selection as described in Markie, 1996. Autoclave the resulting mixture. If plates are required instead of liquid media add 1% Bacto-agar (Oxoid) before autoclaving and pour plates whilst the agar is still molten.
7.5.2 Lithium Acetate yeast transformation.

Yeast was grown to approx. $2 \times 10^7$ cells/ml, 10mls of cells are required for each transformation. The cells were spun for 2000rpm 5mins then washed in 1/2 vol. of 0.1MLiOAc in TE pH7.5. They were the resuspended in 1/100 volume of 0.1M LiOAc in TE pH7.5 and gently agitated for 1hr. Following this the cells were aliquoted and 1µg of transforming plasmid DNA added per 0.1ml of cells, 10µl of sheared deproteinized carrier DNA was also included in the reaction. These cell were then incubated for 30mins at 30$^\circ$C. Next 0.7ml of 40% PEG 3300, 0.1MLiOAc in TE pH7.5 and was added and mixed well. (40% PEG is made by autoclaving a stock of 44% PEF and adding 1/10 volume of 1MLiOAc in TE pH7.5 when the solution is cool). The cells were then incubated 1hr at 30$^\circ$C followed by heat shock of 42$^\circ$C in a water bath for 5 mins. The cells were then harvested by spinning 2000rpm for 5mins (or microfuge 5s) and washed in TE. The cells were then resuspended in 0.2-0.4ml of TE or sterile water and 0.2ml plated on a selective plate. The plates were incubated at 30$^\circ$C for 2-3 days whilst transformants grew up.

7.5.3 Preparation of yeast plugs.

5ml of SD medium was inoculated with yeast and grown until late log phase (2-3 days, 30$^\circ$C, 250rpm agitation). A solution of 1% Seaplaque GTG LMP agarose (FMC) in SE buffer containing 14mM $\beta$ mercaptoethanol was made up and kept at 42$^\circ$C until use. Cells were harvested at 4000rpm for 5min (Sorvall RT6000) and the pellet resuspended in 50ml of SE buffer. The cell suspension was transferred into a 50ml Falcon tube and washed 2x in SE (4000rpm 5min). Meanwhile the bottom of insert moulds (Pharmacia) were sealed with strips of autoclave tape, they were then placed on ice. Following the final wash of the yeast cells the supernatant was decanted off. All traces of liquid were removed by cleaning the inside of the tube with a paper towel. 200µl of SE buffer was added to the cells which were then resuspended with a cut off yellow tip. 0.5 aliquots were transferred to an eppendorf tube and kept at 37$^\circ$C. Just before use 10mg Zymolyase-100T (ICN Biomedical) was dissolved in 2.5ml of the LMP agarose and 0.5ml of this solution was transferred to
the yeast cell suspension. This was mixed thoroughly by pipetting up and down with
a cut off blue tip, this suspension was kept at 42°C. Using a cut off yellow tip pipette
100μl aliquots of the mixture into were transferred to the plug formers on ice and left
to set for 10mins. Once set the plugs were transferred to SE buffer + 14mM β
mercaptoethanol and 1mg/ml Zymolase and incubated 4-6hrs 37°C. After this period
the buffer was replaced with 0.2M EDTA, 0.1M Tris pH8, 0.5M NaCl, 1%SDS,
0.5M β mercaptoethanol and 1mg/ml proteinase K (BCL Biochemical) using at least
0.5ml/plug. The plugs were then incubated at 37°C overnight. Following this
incubation the plugs were washed for 6x30mins in TE pH8 until no bubbles from
SDS solution were left. Plugs were stored in 0.5M EDTA at 4°C.

7.5.4 Restriction enzyme digestion of plugs.

Around 1/3 of a plug (20-40μl) was melted at 65°C with 4μl of restriction enzyme
buffer in an eppendorf tube. This tube was then transferred to 37°C (or temperature
required for enzyme of choice) and allowed to cool. Then restriction enzyme was
added and the solution left to incubate overnight. For loading on an agarose gel the
mixture was heated up to 65°C and then loaded.

7.5.5 Isolation of YAC DNA for microinjection.

The process of the isolation of YAC DNA for microinjection is schematized in Fig.
39. Stages from the purification of WT280LZ YAC DNA for microinjection are
demonstrated in Fig. 40.

PFGE was carried out on yeast plugs loaded in tandem next to one another in the
preparative lane. After the gel had run the marker lanes on either side of the
preparative lane including about 0.5cm of the preparative lane were cut off and
stained in Ethidium Bromide. The position of the YAC was marked under UV with a
sterile scalpel blade (Fig. 39a, Fig. 40a). The gel was reassembled an the position of
this mark used as a guide to excise the part of the preparative lane containing the
YAC. A slice from above the YAC and another below it were also excised to serve as
marker lanes for the second gel run. The gel slices were positioned on a minigel tray
with the YAC slice in the middle and a 4% NuSieve GTG LMP agarose (FMC) gel (0.25xTAE) cast around them (Fig. 39b). This is done so that this second gel runs at 90° to the original PFGE run. The gel was run for 6-8hrs at 4V/cm in 0.25xTAE, circulating the buffer. The two marker lanes were cut off and stained to enable the localization of the required YAC DNA (Fig. 39c). The concentrated DNA from the corresponding position of the YAC DNA lane was then excised. This slice was equilibrated on a rocking platform in 20ml of TENPA buffer for >1.5hrs. All traces of buffer were then removed and the agarose melted for 3min at 68 °C. This was the centrifuged for 10s, incubated for an additional 5min at 68 °C and then transferred to 42°C for 5min. 2U agarase (New England BioLabs) per 0.1ml of molten gel slice was then added and a further incubation of 3hrs at 42°C undertaken. The DNA solution was dialysed for 1hr on floating dialysis membrane (Millipore, pore size 0.05μm) against microinjection buffer (IB). DNA concentration was determined by checking 1-2μl on a thin 0.8% agarose gel with small slots using λ DNA of known concentration as standard. DNA integrity was checked on a PFGE gel (Fig.40b).
Fig. 39 Schematic drawing of the two step gel isolation procedure for YAC DNA to use for microinjection.

After Schedl et. al. 1996. (A) After preparative PFGE both sides of the gel are cut off, stained in ethidium bromide (hatched areas), and the position of the YAC DNA is marked under UV light using a scalpel blade. The gel is reassembled and the region of the gel containing the YAC in the preparative lane (hatched box), as well as two marker slices containing yeast chromosomes (black boxes) are excised. (B) Gel slices are positioned on a gel chamber, embedded in 4% agarose, and standard gel electrophoresis is performed at a 900 angle to the PFGE run. (C) Marker lanes are stained to localize the concentrated DNA and the areas corresponding to the YAC is excised from the centre lane.
Fig. 40 Steps in the purification of WT280LZ for microinjection.

(a) Following preparative PFGE marker lanes are cut off and stained and the position of WT280LZ marked but cutting out a small piece of gel with a scalpel. (b) Following final purification the integrity of the WT280LZ DNA for microinjection is checked by PFGE.
7.6 DNA isolation from murine tissues.

7.6.1 DNA extraction from tail tips.

Once tail tip had been obtained it was immediately placed into 0.5ml of tail tip buffer (1% SDS, 0.3M NaAc, 10mM Tris pH7.9, 1mM EDTA, 200μg/ml proteinase K (BCL Biochemical). Samples were incubated overnight at 55°C. DNA was then extracted from the tail tips by ethanol precipitation (section 7.4.6). Alternatively for PCR analysis the tips were frozen at -20°C and then spun 10min at 4°C in a microcentrifuge. 0.2μl of the supernatant from this spin could then be used as a DNA template in a PCR reaction.

7.6.2 DNA extraction from adult tissues.

A kidney was homogenized in buffer (100mM EDTA, 50mM Tris pH8, 1% SDS, 0.5mg/ml) and further buffer then added up to 25ml. The homogenate was digested overnight at 50°C. The DNA was extracted once with an equal volume of phenol, once with 1:1 phenol:chloroform and once with chloroform. The DNA was then ethanol precipitated, spooled and washed in 70% EtOH. After air drying for 15mins the DNA was resuspend overnight in ~1ml TE.

7.7 Transfer of DNA and RNA to membranes.

7.7.1 Southern transfer.

DNA was transferred from gels to nylon membranes by capillary blotting. This method was adapted from Southern, 1975. Gels were photographed next to a ruler to allow for future sizing of DNA fragments. The DNA was depurinated by gently shaking the gel in 0.25M HCl for 20 min. The acid was then neutralised by gently shaking the gel in 0.4M NaOH 3x 15mins. A
large strip of 3MM filter paper (Whatman) was soaked in 20 X SSC (3M NaCl, 0.3M Na$_3$C$_6$H$_5_7$.2H$_2$O, pH 7.0) and placed on a board. The ends of the paper were placed in a reservoir of 20x SSC, forming a wick. The gel was placed inverted on top of the wet filter paper, then a correctly sized piece of nylon membrane (Biodyne B, Pall), was placed directly onto the gel. Four pieces of 3MM blotting paper, pre-soaked in 20x SSC, were placed on top of the membrane. Air bubbles were removed carefully. Any exposed wick was screened off with Saran wrap (Dow Chemical Company), then a weighted stack of paper towels was placed on top. Gels were blotted for overnight. After blotting, the membranes washed with 2xSSC and then DNA was bound to the filter by baking the membranes at 80°C for 1-2 hours. Membranes were stored in Saran wrap at 4°C.

7.7.2 Transfer of bacterial colonies to filters.

Nylon (Hybond-N, Amersham) circular filters were laid carefully on the surface of the agar plate, so as to avoid air bubbles. The filter was left on the surface for approximately 10s. At this point the orientation of the filter on the dish was marked by stabbing a needle containing waterproof ink through the filter into the agar in an asymmetric pattern.

One sheet of 3MM paper was soaked in each of the following solutions:

a) 10% SDS
b) denature - 1.5M NaCl, 0.5M NaOH
c) neutraliser - 1.5M NaCl, 0.5M Tris.HCl (pH 7.5)
d) 2x SSC

The excess was then poured off and the sheets of 3MM placed in trays. The filters were placed face up on the sheets of 3MM and left for 1 min in SDS, then 3min in denature, 3min in neutraliser and 30s in SSC. The filters were left to air dry and were then baked at 80°C for 1 hour.
7.8 Radiolabelling of DNA.

7.8.1 Random Priming of DNA probes.

This method is adapted from Feinberg and Vogelstein, 1984. A labelling reaction with \([\alpha-^{32}\text{P}]-\text{dCTP}\) involves random priming from hexanucleotides and then polymerization along the DNA strand catalysed by the Klenow fragment of *E.Coli* polymerase 1. A radiolabelled base is incorporated at every C nucleotide.

DNA to be used as a probe was either in solution or contained within a gel slice. 25-50ng was labelled for a single hybridization. Before use in a labelling reaction, the slice was remelted at 70°C. For oligonucleotide probes, 25-50ng of the precipitated oligonucleotide was used.

The DNA strands were firstly denatured by heating to 100°C for 10min. DNA in solution was then kept on ice to prevent reannealing of the strands, DNA in a gel slice was cooled to 37°C. The labelling reaction was carried out using a Random Prime kit (Boehringer Mannheim). 11μl total of DNA plus water was mixed with 3μl 10μCi/μl \([\alpha-^{32}\text{P}]-\text{dCTP}\) (Amersham), 1μl (2 units) Klenow enzyme, 1μl each of dATP, dTTP and dGTP and 2μl reaction buffer. The reactions were then incubated at 37°C for 30-40min.

The percentage incorporation of the radiolabelled nucleotide was checked by TCA precipitation of ~0.5μl of the reaction mix on a GF/B circular filter (Whatman). If the incorporation was 50% or above, the unincorporated nucleotides were removed by running the probe through a Sephadex G-50 Nick column (Pharmacia Biotech). The storage buffer was removed and then the column was washed by running through 1ml TE. The probe was then added to the top of the column, followed by 400μl TE. The probe was then eluted with a further 400μl TE. The probe was then denatured by heating to 100°C for 10min, and was kept on ice until adding it to the prehybridization solution in the bottle.
7.8.2 End-labelling of DNA oligonucleotides.

This labelling reaction involves the transfer of the radiolabelled terminal phosphate group of $[\gamma ^{32}\text{P}]\text{ATP}$ to the terminal $5'$-OH group of the oligonucleotide.

10pmoles oligonucleotide DNA was mixed with 5µl (30µCi) $[\gamma ^{32}\text{P}]\text{ATP}$ (Amersham) and 2µl (20 units) PNK (Boehringer Mannheim), in a total of 20µl 1x PNK buffer (5mm Tris.HCl, pH 8, 1mM MgCl$_2$, 0.5mM DTT). The reaction was incubated at 37°C for 30min, then the probe was added to the hybridization bottle.

7.8.3 Hybridization of filters.

Hybridization buffer: 0.25M Na$_2$HPO$_4$ pH7.2, 0.175% SDS, 1mM EDTA

Wash buffer (DNA probes): 20mM Na$_2$HPO$_4$ pH7.2, 1% SDS, 1mM EDTA

Wash buffer (Oligo probes): 2xSSC, 1% SDS, 1mMEDTA

Under a solution of 50mM Na$_2$HPO$_4$ pH7.2, the filter was placed between two slightly larger sheets of gauze. For hybridization to multiple filters with a single probe, up to 3 layers of filters could be placed in the same bottle, with layers of gauze separating them. Sheets of gauze were not always used. Air bubbles trapped between the filter and the gauze were removed. The filter and gauzes were then rolled up together, transferred to a glass hybridization bottle (Hybaid) and unrolled onto the surface of the bottle. Around 12ml of prehybridization solution was added to a small bottle and about 18ml to a large bottle, and the bottle rotated in an hybridisation oven (Hybaid) at 68°C for a minimum of 1 hour before adding the probe.

For oligonucleotide probes, prehybridization (and hybridization) was performed at a temperature ~10°C lower than the $T_m$ of the oligonucleotide, commonly 48°C.

For random-primed probes, hybridization was carried out at 68°C overnight. For oligonucleotide probes, the hybridization was overnight at a temperature 10°C lower than the $T_m$ of the oligonucleotide (commonly 48°C).
7.8.4 Washing conditions.

Following hybridization, the filters were removed from the bottle and separated from the layers of gauze. Filters were washed on a rotating platform in a waterbath. For random primed probes the filters were washed with 3x30min changes of ~500ml washing solution. The temperature of the wash (~65°C) depended on the washing stringency required. Filters hybridized with oligos were washed 2x5min RT, 1x10min 45°C. The filters were then wrapped in Saran wrap (Dow Chemical Company), avoiding creases.

7.8.5 Detection of hybridization signal.

 Autoradiography The filters were placed in a light-tight cassette with a signal enhancing screen. They were then exposed to X-OMAT x-ray film (Kodak) for a length of time dependent on the amount of radiolabelled probe left bound to the filter (several minutes to several days). Filters hybridised to 32P-labelled probes were exposed at -70°C, those labelled with 35S were exposed at RT. Stratagene Glogos II luminescent markers were used for alignment. The film was developed on an automatic x-ray film processor RGII (Fuji).

Phosphorimaging Alternatively, the filters were exposed to a phosphor screen (Molecular Dynamics) for hours to several days. The screen was then scanned on a PhosphorImager (Molecular Dynamics), where a laser beam converts the radioactive signal into a digital image, with variations in the pixel value proportional to the amount of radioactive signal present. The grey-scale image was adjusted as desired and was then printed on a laser printer.

7.8.6 Removal of radioactive probe from filters.

Filters may be used several times with different hybridisation probes, so it was sometimes necessary to remove radioactive probes from filters. The filters were washed in 0.2M NaOH at RT for 30min, to denature the probe DNA strand from the
surface of the filter. The neutral pH was then restored by a 2x15min wash in 25mM Na$_2$HPO$_4$ also at RT. The filters were then exposed to x-ray film overnight to check that all the probe had been removed.

7.9 Polymerase Chain Reaction (PCR) protocols.

7.9.1 Oligonucleotide Synthesis.

Oligonucleotides were synthesised (by Agnes Gallagher) as ammonium stocks on an Applied Biosystems 381A oligonucleotide synthesiser. Oligonucleotides were precipitated from ammonium stocks by ethanol precipitation of 350µl of the stock. The precipitated DNA was resuspended in 500µl water and the concentration (C) (µmole/ml) assessed by measuring the Abs$_{260}$. Abs$_{260}$= εxC. The extinction coefficient ε of the oligonucleotide is calculated by adding up individual extinction coefficient for each base in the molecule. The extinction coefficient for each base is: dGTP 11.7ml/µmole, dCTP 7.3ml/µmole, dATP 15.4ml/µmole and dTTP 8.8ml/µmole.

7.9.2 Standard PCR reaction mix.

dNTPs (2mM each)(Advanced Biotechnologies) 2.5µl, 1% triton X (Sigma) 2.5 µl (optional), PCR buffer (Perkin Elmer Cetus) 2.5 µl, MgCl$_2$ 1.5mM (Perkin Elmer Cetus) 1.5µl, Amplitaq (Perkin Elmer Cetus) 0.1 µl, primers 10pM of each at10pmoles/µl, ddH$_2$O up to 25 µl. Template DNA added (30-50ng). Reaction overlaid with a drop of mineral oil (Sigma).
7.9.3 Oligonucleotide sequences.

Table 6 Oligonucleotide sequences and descriptions.

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<tr>
<th>Name</th>
<th>Sequence 5' - 3'</th>
<th>Description</th>
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<td>C911</td>
<td>ACT TCA CTC GGG CCT TGA TAG</td>
<td>WTI, intron 9, sense</td>
</tr>
<tr>
<td>C912</td>
<td>GTG GAG AGT CAG ACT TGA AAG</td>
<td>WTI, exon 10, antisense</td>
</tr>
<tr>
<td>J420</td>
<td>TG CAT GTT GTG ATG GCG GAC</td>
<td>WTI, exon 10, antisense</td>
</tr>
<tr>
<td>J422</td>
<td>TGA AAA GCC CTT CAG CTG TC</td>
<td>WTI, exon 10, sense</td>
</tr>
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<td>J423</td>
<td>GGA GTT TGG TCA TGT TTC TCT</td>
<td>WTI, exon 10, antisense</td>
</tr>
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<td>PT65-2</td>
<td>GGA GAT AAG CCC CAA AGT TA</td>
<td>WtI, 5' proximal promoter, sense</td>
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<td>PT65-6</td>
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<td>WtI, 5' proximal promoter, antisense</td>
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</tr>
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<td>H214</td>
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7.10 Protocols for genotyping mice.

7.10.1 PCR based genotyping.

Genotyping NOR/NLS transgenics and line E.

Primers: 443 and 444 detect the lacZ gene
Protocol: 94°C 3min + 30 cycles of (92°C 30s, 55°C 45s, 72°C 1min) + 72°C 10min

For genotyping Line A,C,D,H mice.

Primers: C911 and C912 detect human WTI genomic sequences. It is possible to include an internal control in this react by adding additional primers H213 and H214 for Pax6 and removing an equivalent amount of water from the reaction mix.
Protocol: 94°C 2min 30s + 35 cycles of (94°C 1min, 58°C 1min, 72°C 1min) + 72°C 9min
For detection of null alleles in Wtl knockout mice.

Primers: E489 and E490 detect the neoR cassette in the targetted Wtl null allele (fig41a).

Protocol: 93°C 2min + 9 cycles of (60°C 1min, 72°C 30s, 93°C 10s) + 9 cycles of (60°C 1min, 72°C 1min, 93°C 10s) + 12 cycles of (60°C 1min, 72°C 1min 30s, 93°C 10s) + 72°C 10min.

7.10.2 Genotyping of Wtl homozygous null transgenic mice.

The presence of a Wtl null allele can be detected by using a PCR reaction which amplifies the neoR cassette which has been introduced into the Wtl gene during targeting (Fig. 41a). This method is however, unable to distinguish between a heterozygous and homozygous null animal.

7.10.2.1 Southern hybridization method.

Extra embryonic tissue was taken from isolated embryos and digested overnight at 55°C in 100μl tail tip buffer. DNA was prepared from this by ethanol precipitation and resuspended in 20 μl TE. All of this DNA was then digested overnight with BglII. The digestion product was run overnight on a 1% agarose gel in 1xTAE (Fig. 41b) and then blotted overnight onto filter. The blotted filter was then probed with the WT300 molecular probe. WT300 hybridizes to sequences in Wtl exon 1; hence a lane where WT300 hybridizes to a 5kb band represents a mouse in which at least one copy of the wildtype Wtl locus is present, e.g. Fig40c B1-3. A lane where hybridization does not occur represents an animal with no copies of Wtl exon 1 and which is therefore Wtl homozygous null, e.g Fig. 41c lane B6.

This technique is very labour intensive. Furthermore it is problematical in that the amounts of DNA used are very small and can be lost e.g. Fig. 41b lane B4 and B8. A lack of signal in lanes B4 and B8 on the hybridized filter (Fig. 41c) does not mean that these embryos are homozygous null but rather that the DNA from these animals
is not present on the filter. In order to overcome these problems a PCR based method was designed to detect \textit{WtI} null animals.

7.10.2.2 \textbf{PCR based method}

Primers were designed to amplify part of the mouse 5' UTR which would not cross react with the human 5' UTR. This allows embryos carrying a human derived \textit{WTI} transgene, e.g. WT470LZ, to be typed for the lack of endogenous \textit{WtI} mouse alleles. Due to the high GC content of the \textit{WtI} 5' region standard Perkin Elmer Cetus PCR buffer was unable to amplify sequences correctly. Therefore AM buffer (gift of Dr. G. Bates, Guy's Hospital, London) was used instead. AM buffer: 670mM Tris, 166mM NH\textsubscript{4}SO\textsubscript{4}, 20mM MgCl\textsubscript{2}, 1.7mg/ml BSA. Solution in TEpH8.0

Primers: PT65-2, PT65-6 amplify mouse 5' sequences (370bp band); H213, H214 amplify \textit{Pax6} as an internal control (150bp band).

Protocol: 94°C 5mins + 30 cycles of (94°C 30s, 56°C 30s, 72°C 45s) + 72°C 10min.

Fig41d shows an example of the result of a PT65-2/Pt65-6/H213/H214 PCR based analysis of potential \textit{WtI} knockout embryos. Lanes A1 and B6 have an internal control band but no \textit{WtI} band and hence represent homozygous null embryos. Lanes B5 and B7 have the same band but also a \textit{WtI} specific band (370bp). These lanes represent animals which have at least one copy of the wildtype \textit{WtI} locus.
Knockout of the endogenous Wt1 locus is carried out by replacing Wt1 exon 1 and part of intron 1 with a PGK-neo cassette (Kreidberg et al., 1993). (b) BglIII digested DNA from the extra embryonic membranes of potential homozygous Wt1 null embryos. (c) Hybridization of a blot of DNA from b with WT300 a probe which hybridizes to Wt1 exon1. No band is present in lane B6 indicating that the DNA in this lane came from a Wt1 homozygous null embryo. The lack of bands in lanes B4 and B8 are due to the lack of blotted DNA in these lanes. (d) An example of a result from a PCR based method of genotyping homozygous Wt1 null animals. Lanes A1 and B6 have an internal control band (150bp) but no Wt1 band and hence represent homozygous null embryos. Lanes B5 and B7 have the same band but also a Wt1 specific band (370bp). These lanes represent animals which have at least one copy of the wildtype Wt1 locus.
a

Wild type allele

Knockout allele

b

MW (kb)

23.1
20.5
9.4
6.6
2.3
2.0

B1 B2 B3 B4 B5 B6 B7 B8 B9

5 kb -

A1 B5 B6 B7

370bp -
150bp -
7.11 Transgenic methodology.

YAC and plasmid constructs were introduced into fertilized mouse oocytes by pronuclear microinjection. Mice were superovulated either by myself or by members of the BRF/transgenic facility support staff. Oocytes were collected by myself. Following microinjection oocytes were cultured overnight and those which reached the two cell stage were then transferred into foster mothers. New born pups were typed to see if they were transgenic by either PCR or Southern analysis. The methodology was essentially the same as that detailed extensively elsewhere (Hogan et al., 1986).

7.11.1 Microinjection of mouse oocytes.

Plasmid DNA was linearized by restriction enzyme digestion and purified by Qiaex extraction. A concentration of plasmid DNA of 1ng/μl diluted in T0.1 (10mM Tris pH7.5, 0.1mM EDTA pH8) was used for microinjection. YAC DNA was purified and concentrated as described in section 7.5 and injected in microinjection buffer (IB) at 5ng/μl. Injection were carried out using a IM3000 microinjector (Narashige). Injection was done using constant ‘balance’ pressure slightly higher than that required to counteract capillary action. In this way a constant flow of DNA solution is coming from the end of the microinjection needle.

7.12 Analysis of mouse embryos.

X-fix solution: Made up in 0.1M phosphate buffer, (made by adding 126ml 0.1M NaH₂PO₄·2H₂O to 400ml 0.1M Na₂HPO₄), 2% formaldehyde (added as Millory’s 10% neutral buffered formalin - 10% formaldehyde in 0.1M NaH₂PO₄), 0.2% gluteraldehyde, 2mM MgCl₂, 5mM EGTA pH8.

Detergent wash: Made up in 0.1M phosphate buffer, 2mM MgCl₂, 0.1% sodium deoxycholate, 0.02% Nonidet P40 (Boehringer Mannheim), 0.05% bovine serum albumen (BSA).
**X-gal stain solution**: Made up in detergent wash, 0.085% NaCl, 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 0.1% X-gal in DMF.

**BSA/gelatin**: 100ml PBS add 0.5g gelatin heat in water bath (50°C) to dissolve. Cool to room temp then dissolve in 15.5g BSA when this has dissolved add and dissolve 20g sucrose. Sodium azide 0.1% can be used as a preservative for this mix.

**Aqueous mountant**: 10g gelatin, 60ml dH₂O, 70ml glycerine, 250μl phenol. Dissolve gelatin in water in a conical flask 50°C and mix well. Add glycerine and phenol and mix well again. Aliquot into 10ml amounts and store at 4°C. Heat to 50°C before use. When making up the mountant heat this in a water bath/oven not in a microwave.

### 7.12.1 Isolation of mouse embryos.

The day of the vaginal plug following mating was designated E0.5. Pregnant females were killed by cervical dislocation and the embryo dissected from the uterus into ice cold PBS (Oxoid). Any further embryo dissection was also carried out in ice cold PBS. If tissue was to be cryosectioned it was then washed in a drop OCT compound (Miles Inc.) and then transferred into a drop of OCT on a piece of 3MM paper (Whatman) and snapfrozen in liquid nitrogen. If tissues was to be whole mount X-Gal stained it was transferred to X-fix. If tissue was for mRNA *in situ* hybridization embryos were transferred to 4% PFA in PBS overnight at 4°C.

### 7.12.2 Whole mount X-gal staining of mouse embryos and tissues.

Embryos/tissues were placed in excess X-fix (at least 1ml per embryo) for 1hr-overnight at 4°C. The fix was drained off and then the embryos were washed 3x20min in detergent wash, RT. The final wash was replaced by X-gal stain and the embryos incubated at 30°C for a few hours to overnight depending on the level of signal. Incubation at this temperature as opposed to 37°C takes longer but significantly reduces the level of background endogenous β galactosidase activity in mouse tissues. Following staining the embryos were wash 3x20min in PBS and then fixed in 4% PFA in PBS overnight at 4°C. Staining must be carried out in glass and not plastic containers.
7.12.3 X-gal staining of cryosections.

10μm Cryosections were cut on a JUNG CM3000 cryostat (Leica) and mounted onto TESPA’d slides. The sections were then allowed to warm up to RT, this takes around 20mins. The slides were placed in a coplin jar in X-fix for 20mins then washed 3x5min in detergent wash. The wash was decanted off and replaced with X-gal stain and the coplin jar sealed with nescofilm (Brandon Chemical Ind. Ltd.). The slides were incubated at 30°C for a few hours to overnight depending on the level of signal. Following staining the slides were then washed 3x20min in PBS and fixed in 4% PFA for 1 hour at 4°C. These slides then could be counterstained and mounted as required.

7.12.4 Vibratome sectioning of embryos.

Embryos were washed in PBS. They were then equilibrated in 4%sucrose/PBS overnight, equilibrated in 20%sucrose/PBS overnight and then equilibrated in BSA-gelatin mix overnight. Embryos were then removed from the BSA/gelatin mixture and any excess mix sticking to them blotted off. The embryos were then fixed in 25% gluteraldehyde for 1min. Meanwhile 3ml BSA/gelatin mix was added to a bijoux and 300ul 25% gluteraldehyde added to it. This was mixed quickly by inversion a few times. Embryos were added to this mix and orientated with fine forceps until the mixture began to set. After leaving the mixture to set for a few mins the block was removed from the bijoux. This block was then cut to size and blotted on tissue. For sectioning the block was stuck to a cutting dish with superglue (Super Attak, Loctite) and covered with another dish to prevent drying whilst the superglue was setting (~5mins). The dish was placed in the vibratome (Vibratome series 1000, Technical Products International) which was filled with luke warm water to above the level of the block. The settings used were: speed 2, amplitude 8, taking 150um sections. The sections were floated onto a normal slide and the excess water blotted from around the edges of the sections. They were then mounted with aqueous mountant and left on a hotplate for a hour or so to prevent bubbles forming in the aqueous mountant as it dried.
7.12.5 Wax embedding and sectioning of embryos.

Embryos were fixed overnight in 4% PFA at 4°C and then washed in PBS 3x20min. They were then transferred to a Tissue-Tek VIP (Miles Inc.) for processing. Programme: PBS 1x1hr, 30% EtOH 1x1hr 40°C, 50% EtOH 1x1hr 40°C, 70% EtOH 1x1hr 40°C, 85% EtOH 1x1hr 40°C, 95% EtOH 1x1hr 40°C, 100% EtOH 2x1hr 40°C, 100% xylene 2x1hr 40°C, paraffin wax 4x1hr 60°C. The embryos were then embedded in further melted paraffin wax contained in a mould. The wax was set by holding the mould on the surface of a bath a water at RT. For in situ hybridization protocols all solutions need to be sterile to ensure no RNAse activity.

Embryos of E9.5 or younger and small pieces of tissue were processed by hand. The process was similar to that above with the incubations done at RT instead of 40°C and the length of incubation cut to 2x15-20min.

7μm sections were cut on a standard microtome e.g. Riechert-Jung 2030. A new sterile blade was used for sections required for in situ hybridization. Sections were floated out into (sterile) distilled water at 40°C. Section required for in situ hybridization were floated onto TESPA’d slides and those for standard histological analysis onto untreated slides. Slides were baked overnight at 60°C to seal the sections onto the slide. Those slides required for in situ hybridization were stored in clean sealed boxes with silica gel desiccant.

TESPA protocol - New clean slides were used straight out of the box from the manufacturer. The slides were washed 10%HCl in 70% EtOH for 20s, then washed in sterile distilled water for 20s. The slides were then washed in 100% acetone for 20s and air dried. When dry the slides were placed in 2% TESPA in acetone for 20s (TESPA : 3-aminopropyl-triethoxy silane, C₉H₂₃NO₃Si (Sigma)). After this treatment they were air dried and stored in clean sealed boxes with desiccant.
7.12.6 Histological staining of sections.

**Dewaxing:** Wax sections need to be dewaxed before staining. The slides were washed in the following solutions: 2x5min xylene, 2x5min 100% EtOH, 1x 5min 90% EtOH, 1x 5min 70% EtOH, 1x 5min 50% EtOH, 1x 5min 30% EtOH, few min in water.

**TUNEL analysis:** Detection of apoptotic cells was carried out using an In Situ Cell Death Detection Kit, AP (Boehringer Mannheim) in accordance with the manufacturers instructions. Substrate for Alkaline Phosphatase was provided using SIGMA FAST Fast Red TR/Naphthol AS-MX Phosphate tablets (Sigma) in accordance with the manufactures instructions.

**Haematoxylin and eosin (H&E) staining:** Slides were placed in haematoxylin (Surgipath) for 4-5min. They were then wash well in running tap water. The washed slides were then transferred to 1%HCl in 70% EtOH a few seconds and washed in running tap water. Following this wash the slides were transferred to saturated lithium carbonate solution for 10-15 seconds and then washed in running tap water for at least 3min. The slides were then placed in eosin solution (3 parts of 1% aqueous eosin (Eosin, Yellowish (TAAB)) : 1 part 1% EtOH, 0.05% acetic acid) for 2-5min depending on the tissue type. The slides were then rinsed quickly in water and processed as follows: 100% EtOH 1x15s then 2x1min, xylene 1x5min. Slides were transferred to fresh xylene whilst mounting. To mount the slides the xylene was drained off, a line of DPX (BDH) added over the section and a coverslip and placed over it.

In many cases sections with X-gal staining were only counterstained with eosin to increase contrast between the X-gal and counter stain. This practice however reduces histological resolution in the sections.

Sections were analyzed and photographed with either a Ziess Axioplan 2 with a Yashica 108 multiprogram camera or a Wild Heerbrugg Photomakroskop M400 with
a Wild Leitz WPS Photoautomat. The photographic film used was 64 ASA colour film (Fuji)

7.12.7 Immunohistochemical staining of cryosections.

Cryosection were left to dry at RT 1hr and then fixed in 1:1 methanol:acetone at -20°C for 10min. They were then dried at RT for 10-20mins followed by rehydration in PBS for 5min. Next the sections were incubated in blocking solution (BS) for 15min (BS: 2% BSA (Sigma), 2% sheep serum, 7% glycerol, 0.2% Tween 20 (ICN Biochemicals)). Following this the sections were incubated sections with primary antibody diluted in BS 1hr. The primary antibodies used were a 1/100 dilution of C19 anti Wt1 (Santa Cruz Biotechnology) or 1/100 α-Tubulin (Sigma). Following incubation the sections were washed 3x3min in PBS in a coplin jar. The rest of the procedure was carried out in a dark chamber. The sections were incubated with a secondary antibody diluted in PBS for 45min (secondary antibody Donkey anti-Rabbit- FITC (Jackson Immunolabs)). They were then washed 3x3mins in PBST (PBS + 0.1% Tween20 (ICN Biochemicals). The slides were mounted in VectaMount (Vector Labs) containing 1/1000 propidium idodide and the coverslip sealed with TipTop Vulkanisierfluessigkeit. Slide were analyzed using a Ziess Axioplan microscope with a Photometrics CCD camera and Digital Scientific software.

7.12.8 mRNA radioactive in situ hybridsation.

Some of the in situ analyses presented in this thesis were done entirely by myself other with the help of E. Graham and L. McInnes.

Probe labelling - A $^{35}$S labelled ssRNA probe was transcribed from a dsDNA template via the following method.

Add to a sterile eppendorf in this order: 3μl 5x transcription buffer (Boehringer Mannheim - for either T3 or T7 polymerase), 1μl 10mM rATP, 1μl 10mM rCTP, 1μl
10mM rGTP, 1µl 1M DTT (Dithiothreitol), 6µl water, 12µl $^{35}$SUTP
(>1mCi/100ml)(Amersham), 5µl template DNA template linearized with the
appropriate restriction enzyme (0.5-1mg/5ml), 1.2µl RNase inhibitor (Boehringer
Mannheim), 0.8µl T7 or T3 polymerase (Boehringer Mannheim) as appropriate.
Incubate 37°C 25min. Add a further 0.8µl of polymerase. Incubate a further 25 min.
Add 2µl tRNA (10mg/ml), 1 µl RNAse free DNAse (Boehringer Mannheim).
Incubate 37°C 10mins. To stop reaction add : 2µl 100mM EDTA, 167µl TE (see X)
with 50mM DTT.

The mixture was then stuck on ice, transferred to a Microcon column (Amicon) and
spun in a bench centrifuge for 10mins. The reaction tube was rinsed with a further
200µl of TE which was then spun in the column 5min. 25µl of TE/DDT was added to
the column which was incubated on ice for 15mins The column was the inverted in a
new tube and spun 5min. The ice incubation step and spin were repeated. The
solution spun off the column was taken as the probe. A 1µl aliquot was taken for
scintillation counting.

Scintillation counting - 1µl of probe was added to 19µl TE/DDT and mixed. 10µl
was added to each of two Whatmann GF/B filters. One filter was washed 3x TCA
(trichloroacetic acid solution - (Sigma)) then once in ethanol. The TCA was allowed
to stand on the filter for a few seconds to allow precipitation of the RNA before
being drawn through under vacuum. The filters were left to dry. Unwashed and
washed filters were counted separately in scintillation vials with 10µl Ecolite+
scintillation fluid (ICN) assuming 50% counting efficiency. Counting was done in a
Packard Tri-Carb 1500 Liquid Scintillation Analyser.

(precipitate count)/(total count) x 100 = % incorporation which should be at least
10%. The probe was then diluted with TE/DTT so that when hybridization mix was
added in a 1:9 ratio, the final count is $1.1 \times 10^5$ dpm/ml (dpm = disintergrations per
min).
50x Denhardt's solution: 5g Ficoll, 5g Polyvinyl pyrolidine, 5g BSA. Made up to 500ml with distilled water, filter sterilized and stored at -20°C

Hybridization mix: In 1x Denhardt's solution: 50% formamide, 10% dextran sulphate, 20mM Tris pH8, 0.3M NaCl, 5mM EDTA, 10mM sodium phosphate, 0.5mg/ml tRNA. Immediately before use add 50mM DTT.

Prehybridization treatment of slides - Slides were taken through the following solutions in batches of 20: Xylene 2x5min, 100% EtOH 2x2min, (90%, 70%, 50%, 30% EtOH) 2mins each, PBS 2mins with agitation and finally 4% PFA in PBS pH 7.2 10min. At this point slides were split into two racks to ensure no PFA remains trapped between them. PBS 2x2min, 20mg/ml Proteinase K (BCL Biochemicals) in 50mM tris, 5mM EDTA 7.5mins, PBS 1min, 4% PFA 2min, distilled water 10s, 0.1M TEA (triethanol amine) pH8 30s, 0.1M TEA + 0.3125 % acetic anhydride 2x5min with stirring, PBS 2min, 0.85% NaCl 2min, (30%, 50%, 70%, 90% EtOH) 1min each, 100% EtOH 3x5min. Finally the slides were dried in a dust free box.

Hybridization - Probe/hybridization mix was heated to 80°C, rapidly cooled on ice and then ~50μl added per slide. Clean coverslips were put over the slides. The slides were then placed horizontally in a sealed box including a tissue soaked in 5mls of 50% formamide 5XSSC. The box was heat sealed inside two plastic bags and submerged in a water bath at 55°C overnight.

Post hybridization washes. These were carried out in a Hybaid Omnislide Wash Module. The washing protocol was: 5xSSC 55°C a few seconds, 5xSSC 10mM DTT 55°C 15-30min (until the coverslips fall off), high stringency wash 65-68°C 30min (high stringency wash 50% formamide, 2xSSC, 0.1M DTT (add DTT just before use)), NTE (0.5M NaCl 10mM Tris 5mM EDTA pH7.5) 37°C 2x5min, NTE + 2μm/ml RNAse A 37°C 30min, NTE 37°C 30min, high stringency wash 65-68°C 30min, 2xSSC RT 4x10min, 0.1xSSC RT 4x5min, 30% 50% 70% 90% EtOH 1min each, 100% EtOH 2x5min and finally allow the slides to air dry.
In the dark, slides were then dipped in 1:1 sterile distilled water:Ilford K5 emulsion at 40°C. They were then left to air dry for 3 hours. Following this they were transferred to a sealable box with desiccant, covered with foil (light tight) and left 4°C for 2-3 weeks before developing. Slides were developed by immersing in Kodak D19 developing solution for 4min, washed in sterile distilled water 10s, fixed in a 1:2 solution of AMFIX:water for 5min and then finally washed in water 2x10min. The slides were then counterstained by dipping in 1% methyl green (Sigma), air dried and mounted with DPX.

7.12.9 Whole mount in situ analysis.

Preparation of probe: 1μg of template DNA was taken. Added to this was: 4μl of polymerase buffer (T7 or T3), 4μl of DIG mix (10mM dATP, dCTP, dGTP 6.5mM dUTP, 3.4mM DIG-UTP (Boehringer Mannheim)), 1μl 1M DTT, 2μl RNAse inhibitor, 2 μl polymerase (T3 or T7) and sterile water to 40 μl. the reaction mix was incubated for 1hr at 37°C. After this step a further 2 μl polymerase was added and the incubation repeated. After this incubation 20U of DNAseI (Boehringer Mannheim) and 1μl tRNA (10μg/ml) were added. A further incubation for 15min at 37°C was carried out. The reaction was stopped with 0.2mM EDTA pH8.0. The RNA was then precipitated by adding 5 μl 4M LiCl, 150 μl EtOH and incubating for -20°C for at least 2hrs. The tube was the spun in microcentrifuge a 15mins and the pellet washed 2x with 70% EtOH. The pellet was then resuspended in 100ul DEPC treated water with 1μl RNAse inhibitor added. This probe was then stored at -20°C until use. 10μl of the probe used for in situ.

Hybridization: The whole mount in situ hybridization was carried out by Jacob Hecksher-Sorenson as detailed in In situ hybridisation a practical approach ed. Wilkinson, 1992.
7.13 Eukaryotic cell culture.

7.13.1 Culture conditions.

Table 7 Cell culture conditions.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media</th>
<th>Temperature and CO₂ concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC29</td>
<td>RPMI (GIBCO BRL), 10% FCS - foetal calf serum (GIBCO BRL)</td>
<td>37°C, 5% CO₂</td>
</tr>
<tr>
<td>COV434</td>
<td>1:1 FlO (GIBCO BRL):DMEM - Dulbecco modified Eagle's medium (Flow Labs)</td>
<td>37°C, 5% CO₂</td>
</tr>
<tr>
<td>M15</td>
<td>DMEM, 10%FCS</td>
<td>32°C, 7.5% CO₂</td>
</tr>
<tr>
<td>PyT45</td>
<td>DMEM, 10%FCS</td>
<td>37°C, 5% CO₂</td>
</tr>
<tr>
<td>10T1/2</td>
<td>DMEM, 10%FCS</td>
<td>37°C, 5% CO₂</td>
</tr>
<tr>
<td>C2</td>
<td>DMEM, 10%FCS</td>
<td>37°C, 5% CO₂</td>
</tr>
</tbody>
</table>

Cells were cultured in 80ml tissue culture (Nunc) flasks. They were split when they reached confluence. All manipulation were performed in a laminar flow air cabinet.

Splitting protocol: The medium was aspirated from the cells, 6ml of Trypsin versene 1:10 (0.2% trypsin, 0.04% EDTA in Dulbecco ‘A’) was added for 5mins. 4mls of growth medium was then added and the cell spun at 2000rpm 4°C. The supernatant was aspirated and the cell resuspended in 10ml of fresh medium, about 2mls was used to seed a new flask.

7.13.2 Transfection of eukaryotic cells.

Cells were grown in 25cm² round culture dishes (Nunc). Transfection was carried out on cells of about 70% confluency. 50μl of plasmid (10ng/μl), 15μl Lipofectamin (GIBCO BRL) (2μg/μl) and 35μl medium without FCS were taken and mixed in a sterile tube. This mixture was incubated at room temperature for 45mins. Meanwhile the growth medium was aspirated and the dish quickly washed 3 times in medium without serum. 2ml of medium without serum was carefully added to the DNA/lipofectamin mix and this was added to the culture dish. The cell were then incubated as normal for that cell type for 12hrs. After this period of incubation the
medium was aspirated and replaced with normal medium containing serum. The cells were grown a further 24hrs or so. Then the medium was decanted and cells washed once in PBS. 2.5mls of X-fix was added to the dish which was incubated on ice for 5mins. It was then washed with PBS and 2.5ml of X-gal staining solution added. The dish was now incubated at 37°C for 1hr or for as long as it took for the blue X-gal staining to appear.

7.14 Molecular probes.

WT33 - WTI cDNA as first isolated by Call et al., 1990
WTX - EcoRI/XhoI genomic fragment spanning WTI exon 1.
WT300 - 300bp fragment of WTI exon 1 isolated by EcoRI/NcoI digestion of PKS2.
PKS2 - 610bp fragment of WTI 3' UTR described in Mundlos et al, 1993
SVA - YAC short vector arm probe, 1.4 kb SalI/StuI fragment isolated from pYAC4
LVA - YAC long vector arm probe, 1.1 kb EcoRI/Pst1 fragment isolated from pBR322.
L800 - An 800bp single copy clone isolated from WT470 DNA. This clone was isolated from the region of the EagI site lying around 100kb downstream of WTI (Fantes et al., 1995) via a linking library approach (A. Schedl pers. comm.).
TRPLZ - ~6kb XbaI/BamHI fragment of plasmid UY1 (gift of H. Smith MRC HGU Edinburgh). UY1 consists of the 1.4 kb of the TRP1 promoter (-1335 to +107), TRP1 exon 1 and a lacZ reporter gene (Lowings et al., 1992).
ILZ - 3.6kb HindIII/BamHI fragment of UY1 containing the lacZ cassette.
Pax1 - Plasmid name - 'pmprd HincII-SacI'. 313bp genomic HincII/SacI fragment from containing pair box region of Pax1. (gift of J.Moss MRC HGU Edinburgh)
Pax3 - Plasmid name - 'pc pax3 PstI/HindIII'. 519bp PstI/HindIII fragment from 3' end of Pax3 cDNA containing part of the homeobox. (gift of J.Moss MRC HGU Edinburgh).
8. Bibliography.


