The role of mutants in the study of vertebrate limb development: analysis of hypodactyly in the mouse and polydactyly in the chick.

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by
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Abstract

Vertebrate limb mutants are a valuable source of information on how the vertebrate limb normally develops. This thesis examines cellular and molecular aspects of limb development in the mouse mutant hypodactylly (Hd) and the chick mutant Talpid\(^{\prime}\) (ta\(^{\prime}\)).

Hypodactylly heterozygotes (Hd/+\) show a reduction of hindlimb digit 1, while homozygotes (Hd/Hd) have only one digit on all four limbs. I have analysed Hd/Hd and Hd/+ limb morphology in adulthood and throughout embryogenesis. Alterations in adult limb morphology are associated with reductions in size and shape of developing limb buds and in the number of digital blastemas that develop. The polarising region and apical ectodermal ridge (AER) are required for limb outgrowth and patterning. Hd mutant limbs have polarising activity and a well defined AER which persists for longer than in wildtype embryos. Sonic hedgehog (Shh) and fibroblast growth factor 4 (Fgf4), are expressed in the polarising region and apical ectodermal ridge respectively. In Hd/Hd mutants, expression of these genes is slightly prolonged. Hoxd genes are downstream targets of Shh, and are important in patterning the limb. Expression of both Hoxd13 and Hoxd11 are slightly altered in Hd/Hd limbs. In addition, loss of digits in hypodactylly is associated with an increase in mesenchymal cell death in developing limbs and an inability of limb mesenchymal cells to produce cartilage in culture.

Talpid\(^{\prime}\) (ta\(^{\prime}\)) homozygotes have polydactylous limbs with up to eight morphologically similar digits. I found that there is a graded distribution of polarising activity in ta\(^{\prime}\) limb bud mesenchyme, with ta\(^{\prime}\) anterior mesenchyme having very weak ectopic polarising activity. Shh is posteriorly localised in ta\(^{\prime}\) limbs and therefore not associated with weak polarising activity in anterior mesenchyme. In contrast, Bmp2 and Bmp7 are expressed uniformly across the antero-posterior axis, and Fgf4 transcripts are present throughout the entire apical ectodermal ridge.
Contents

Abstract 2
Contents 3
List of Figures 8
List of Tables 12
Acknowledgements 14

Chapter 1
A General Introduction to Vertebrate Limb Development

1.1 Embryonic Development 16
1.2 A Review of Vertebrate Limb Development 18
  1.2.1 Limb initiation 18
1.3 Specification of limb pattern 22
  1.3.1 The apical ectodermal ridge 24
  1.3.2 The progress zone 26
  1.3.3 The polarising region 26
  1.3.4 Limb ectoderm 32
1.4 Interspecies conservation of signalling in developing vertebrate limbs 32
1.5 Molecular basis of limb development 33
1.6 Signalling molecules in limb initiation and proximo-distal outgrowth 33
  1.6.1 Fibroblast growth factors 33
  1.6.2 Fibroblast growth factor receptors 36
1.7 Signalling molecules in antero-posterior patterning 36
  1.7.1 Retinoic acid 36
  1.7.2 Retinoid receptors and binding proteins 37
  1.7.3 The hedgehog gene family of signalling molecules 37
  1.7.4 Bone morphogenetic proteins 39
1.8 Dorso-ventral patterning 42
1.9 Signalling interactions along the three axes of the limb are coordinated 45
1.10 Regulatory genes expressed in the limb in response to signalling
    in the limb 45
  1.10.1 Vertebrate Msx genes 45
  1.10.2 Hox genes 47
  1.10.3 Even-skipped homologues 50
1.11 Aims of this thesis 53

Chapter 2
Materials and Methods used in the analysis of the mouse mutant Hypodactyly (Hd)

2.1 Mice 56
2.2 Chick embryos 56
2.3 Adult morphology 56
  2.3.1 External morphology of adult mice 56
  2.3.2 Skeletal analysis of adult mice through differential staining of cartilage and bone 56
  2.3.3 Scoring of the defects seen in the hindlimbs of Hd/-¥ adult mice 57

2.4 Embryonic Morphology 58
  2.4.1 Preparation of embryonic material 58
  2.4.2 Phenotyping of embryos 58
  2.4.3 External morphology of embryos 58

2.5 Analysis of limb polarising activity 58
  2.5.1 Fixation and staining of manipulated embryos 60

2.6 Analysis of gene expression in developing limbs 62
  2.6.1 Plasmid DNA 62

2.7 Elution of plasmid DNA from Whatmann filter paper 63

2.8 Transformation of competent cells 63

2.9 Small-scale preparation of plasmid DNA 64

2.10 Measuring the concentration of a DNA solution 65

2.11 Restriction digestion analysis of mini prep' plasmid DNA 65

2.12 Large-scale preparation of plasmid DNA 65

2.13 Purification of plasmid DNA through caesium chloride gradients 66

2.14 Removal of ethidium bromide from DNA purified by equilibrium centrifugation in caesium chloride-ethidium bromide gradients 67

2.15 Synthesis of Digoxigenin-labelled riboprobes 67

2.16 Preparation of embryonic material for non-radioactive wholemount in situ hybridisation 68

2.17 Non-radioactive in situ hybridisation with digoxigenin labelled probes 69
  2.17.1 Protocol according to Rosen and Beddington, (1993) 69
  2.17.2 Protocol according to Nieto et al., (1996) 71

2.18 Analysis of stained embryos 73

2.19 Estimation of area of distal limb that expresses the genes analysed 73

2.20 Cellular analysis of Hd mutants 73
  2.20.1 Analysis of mesenchymal cell death 73
  2.20.2 Skeletal analysis of the limbs of Hd mutant embryos 74
  2.20.3 Cell culture of limb mesenchymal cells in high density micromass cultures 75
  2.20.4 Preparation of conditioned medium and treatment of cultures with growth factors 75
  2.20.5 Fixing and staining of micromass cultures 76
  2.20.6 Estimation of the amount of cartilage produced in culture 76
2.20.7 Analysis of limb bud and apical ectodermal ridge morphology with scanning electron microscopy 76
2.20.8 Histological analysis of apical ectodermal ridge morphology in semi-thin araldite sections 77

Chapter 3

An introduction to the mouse mutant hypodactyly (Hd)

3.1 The mouse mutant hypodactyly (Hd) 78
3.2 Appearance of normal mouse limbs 78
  3.2.1 Adult limbs 78
  3.2.2 Morphology of limbs during murine development 79
  3.2.3 External appearance of limbs during development are correlated with final limb morphology 80
3.3 Outgrowth and patterning of the limb 80
  3.3.1 Shh and Fgf4 83
  3.3.2 Homeobox (Hox) genes 83
    3.3.2.1 Hoxd genes 84
    3.3.2.2 Hoxa genes 85
    3.3.2.3 Roles of Hox genes 86
3.4 Aims of this Chapter 86
3.5 Results 88
  3.5.1 Analysis of 3 live-born Hd/Hd, Hd/+, and +/+ littermates at 28 weeks 88
  3.5.2 External limb morphology of adult +/+, Hd/+, and Hd/Hd mice 96
  3.5.3 Skeletal analysis of +/+, Hd/+, and Hd/Hd littermates at 28 weeks 99
  3.5.4 The Hd/+ hindlimb phenotype is variable and shows degrees of left-right asymmetry within the same embryo 110
  3.5.5 External morphology of +/+, Hd/+ and Hd/Hd mice during embryogenesis 116
  3.5.6 Polarising activity of posterior mesenchymal cells in developing limbs of Hd mutant mice 128
  3.5.7 Gene expression in Hd mutant limb buds 130
    3.5.7.1 Expression of Shh 130
    3.5.7.2 Expression of Fgf4 131
    3.5.7.3 Expression of Hoxd13 131
    3.5.7.4 Expression of Hoxd11 132
3.6 Discussion and Conclusion 142
  3.6.1 Summary of results 142
3.6.2 Limb defects in *Hd/+* and *Hd/Hd* adult mice do not affect proximal structures and are more severe in the hindlimbs than in the forelimbs 143

3.6.3 There is evidence to suggest that *Hd* is not a null allele of *Hoxa13* 144

3.6.4 The hindlimbs are more susceptible to limb defects in both *Hd/+* and *Hd/Hd* mice 145

3.6.5 The *Hd/+* hindlimb phenotype is variable 147

3.6.6 Defects induced by the *Hd* mutation are not restricted to the limbs 148

3.6.7 *Hd/+* and *Hd/Hd* embryonic phenotypes 149

3.6.8 Polarising activity in *Hd/+* and *Hd/Hd* embryonic limbs 150

3.6.9 Gene expression in *Hd/+* and *Hd/Hd* embryonic limbs 152

**Chapter 4**

Analysis of cellular effects of the *Hd* mutation

4.1 Introduction 154

4.1.1 Programmed cell death 155

4.1.2 Chondrogenesis during limb development 156

4.1.3 Role of the apical ectodermal ridge in shaping the limb bud 159

4.2 Aims of this Chapter 160

4.3 Results 161

4.3.1 Mesenchymal cell death in the developing limbs of *Hd* mutants 161

4.3.2 Development of skeletal elements of the limb 165

4.3.3 *In vitro* chondrogenesis 174

4.3.4 The *Hd* mutation also affects the morphology of the apical ectodermal ridge 184

4.4 Discussion and Conclusion 202

4.4.1 Summary of results 202

4.4.2 Mesenchymal cell death is affected by the *Hd* mutation 202

4.4.3 Specification of mesenchymal condensations and subsequent chondrogenesis *in vivo* is affected by the *Hd* mutation 205

4.4.4 Chondrogenic potential of undifferentiated mesenchymal cells *in vitro* is affected by the *Hd* mutation 208

4.4.5 Apical ectodermal ridge morphology is affected by the *Hd* mutation 209

**Chapter 5**

Distribution of polarising activity and expression of *Fgf4*, *Shh*, and *Bmps* in the developing limbs of the polydactylous chicken mutant *Talpid*³

5.1 Introduction 211

5.2 Aims of this chapter 211
5.3 Materials and Methods

5.3.1 Chick embryos

5.3.2 Identification of homozygous \( \alpha^2 \alpha^3 \) mutants

5.3.3 Mapping polarising activity in developing limb buds of \( \alpha^2 \alpha^3 \) embryos

5.3.4 Grafting a normal polarising region to the anterior margin of a stage 20/21 \( \alpha^2 \alpha^3 \) limb bud

5.3.5 Fixation and staining of manipulated embryos

5.3.6 Retinoic acid treatment of \( \alpha^2 \alpha^3 \) limb buds

5.4 General molecular biology methods

5.5 Analysis of gene expression in limb bud of \( \alpha^2 \alpha^3 \) embryos

5.5.1 \( 35S \) in situ hybridisation

5.5.2 Non-radioactive, wholemount in situ hybridisation

5.6 Results

5.6.1 Distribution of polarising activity in \( \alpha^2 \alpha^3 \) limb buds

5.6.2 Response of \( \alpha^2 \alpha^3 \) limb mesenchyme to polarising region grafts and retinoic acid

5.6.3 Expression of Shh and Hoxd13 in \( \alpha^2 \alpha^3 \) limb buds

5.6.4 Expression of Fgf4 in \( \alpha^2 \alpha^3 \) limb buds

5.6.5 Expression of Bmp genes in \( \alpha^2 \alpha^3 \) limb buds

5.7 Discussion and Conclusion

Chapter 6

General Discussion and Conclusion

6.1 Summary of the finding of this thesis

6.2 General conclusions

6.3 Future work

Appendix 1

References
List of Figures

Chapter 1

**Figure 1.1** - Three dimensional, diagrammatic representation of a vertebrate limb bud consisting of a core of undifferentiated mesenchyme encased in an ectodermal jacket 21

**Figure 1.2** - Schematic representations of the early developing vertebrate limb bud indicating the important signalling centres involved in outgrowth and patterning 23

**Figure 1.3** - Final skeletal pattern of the normal chick wing and skeletal patterns induced after grafting polarising region cells to the anterior margin of a wing bud at stage 20 of development 29

**Figure 1.4** - Schematic representation of an early limb bud showing localisation of *Shh* transcripts to posterior mesenchyme in the same region as the polarising region 35

**Figure 1.5** - Schematic representation of a transverse section through the limb bud in figure 1.4 44

**Figure 1.6** - Structural organisation of the *Drosophila* HOM-C homeotic complex and its correspondence with genes of the four mouse *Hox* clusters based on regions of homology 49

**Figure 1.7** - Simplified representation of the nested expression domains of genes of the *Hoxd* and *Hoxa* cluster along the antero-posterior and proximo-distal axes respectively of an early developing vertebrate limb bud 51

**Figure 1.8** - A model for the role of *Hox* genes in limb development 52

Chapter 2

**Figure 2.1** - Stage 20 chick wing bud showing a loop in the anterior apical ectodermal ridge 61

Chapter 3

**Figure 3.1** - Line drawings of the mouse skeleton and the bones of the forelimbs and hindlimbs 82

**Figure 3.2** - Photographs of the three adult littermates 91

**Figure 3.3** - Photographs of the hindlimbs of three adult littermates shown in figure 3.2 93

**Figure 3.4** - Photographs of the hind-quarters of a homozygote (*Hd/Hd*) showing the malformed hindlimbs and the possible genital abnormalities 95

**Figure 3.5** - External morphology of the forelimbs and hindlimbs of three +/+ , *Hd/+*, and *Hd/Hd* littermates at 28 weeks 98
Figure 3.6 - Whole skeletons of three adult littermates at 28 weeks stained for cartilage and bone with alcian blue and alizarin red S

Figure 3.7 - Skeletal elements of forelimbs and hindlimbs of a wildtype (+/+) male mouse culled at 28 weeks and stained for cartilage and bone with alcian blue and alizarin red S

Figure 3.8 - Skeletal elements of forelimbs and hindlimbs of a male heterozygote (Hd/+) culled at 28 weeks and stained for cartilage and bone with alcian blue and alizarin red S

Figure 3.9 - Skeletal elements of forelimbs and hindlimbs of a male homozygote (Hd/) culled at 28 weeks and stained for cartilage and bone with alcian blue and alizarin red S

Figure 3.10 - Variability of the heterozygote (Hd/) hindlimb phenotype in a sample population of 110 adult female mice

Figure 3.11 - Scanning electron micrographs of 9.5 and 10.5 day post coitum (dpc) mouse embryos from matings of two heterozygotes (Hd/+)

Figure 3.12 - +/-, Hd/+, and Hd/Hd mice and limbs at 11.5 and 12.5 dpc

Figure 3.13 - +/-, Hd/+, and Hd/Hd mice between 13.5 and 16.5 dpc

Figure 3.14 - Wholemount in situ hybridisations of +/-, Hd/+, and Hd/Hd embryos between 10.5 and 12.5 dpc hybridised with species specific digoxygenin-labelled riboprobes to Shhd

Figure 3.15 - Wholemount in situ hybridisations of +/-, Hd/+, and Hd/Hd embryos between 11.5 and 14.5 dpc hybridised with species specific digoxygenin-labelled riboprobes to Fgf4

Figure 3.16 - Wholemount in situ hybridisations of +/-, Hd/+, and Hd/Hd embryos between 11.5 and 14.5 dpc hybridised with species specific digoxygenin-labelled riboprobes to Hoxd13

Figure 3.17 - Wholemount in situ hybridisations of +/-, Hd/+, and Hd/Hd embryos between 11.5 and 14.5 dpc hybridised with species specific digoxygenin-labelled riboprobes to Hoxd11

Chapter 4

Figure 4.1 - Hindlimbs of +/-Hd/+, +/- and Hd/Hd embryos at 11.5 and 12.5 dpc showing the shape of the hindlimb buds and the opaque patch of cells in Hd/Hd limb buds that colocalise to regions where mesenchymal cell death is detected

Figure 4.2 - +/-Hd/+, +/-, Hd/+ and Hd/Hd limbs at 11.5, 12.5 and 14.5 dpc showing regions of cell death as detected with Nile Blue Sulphate staining
Figure 4.3 - Alcian green stained limbs showing developing cartilage elements of +/+, Hd/+/, and Hd/Hd embryos at 12.5 and 13.5 dpc

Figure 4.4 - Alcian green stained limbs showing developing cartilage elements of +/+, Hd/+/, and Hd/Hd embryos at 14.5 dpc

Figure 4.5 - Alcian blue and Alizarin red S stained limbs of 15.5 dpc +/+, Hd/+/, and Hd/Hd embryos

Figure 4.6 - Alcian blue and Alizarin red S stained Hd/Hd and +/+ embryos at 16.5 dpc

Figure 4.7 - Histogram showing the average number of nodules in micromass cultures set up at normal density with mesenchyme from 11.5 dpc +/+/Hd/+ and Hd/Hd limb buds

Figure 4.8 - Histogram showing the average number of nodules in micromass cultures set up at normal density with mesenchyme from 12.5 dpc +/+, Hd/+ and Hd/Hd limb buds

Figure 4.9 - Cartilage nodules in 6 day micromass cultures of 12.5 dpc +/+ and Hd/Hd limb bud mesenchyme stained with Alcian blue for cartilage

Figure 4.10 - Histogram showing average nodule number in micromass cultures of 12.5 dpc +/+, Hd/+ and Hd/Hd mesenchymal cells that were fed with conditioned medium

Figure 4.11 - Histogram showing the number of cartilage nodules in micromass cultures set up with mesenchyme from 12.5 dpc +/+ and Hd/Hd limb bud mesenchyme, and in cultures consisting of a 50:50 mixture of +/+ and Hd/Hd mesenchymal cells limb buds

Figure 4.12 - Histogram showing the average number of nodules in micromass cultures set up at double density with mesenchyme from 11.5 dpc +/+/Hd/+ and Hd/Hd limb buds

Figure 4.13 - Histogram showing the average nodule number in micromass cultures of 12.5 dpc +/+, Hd/+ and Hd/Hd mesenchymal cells that were fed with different growth factors

Figure 4.14 - Scanning electron micrographs (SEM) of the limb buds of an 11.5 dpc Hd/Hd embryo
Figure 4.15 - Scanning electron micrographs (SEM) of the limb buds of an 11.5 dpc +/+Hd/+ embryo 188

Figure 4.16 - Scanning electron micrographs (SEM) of the limb buds of a 12.5 dpc +/+ embryo 190

Figure 4.17 - Scanning electron micrographs (SEM) of the limb buds of a 12.5 dpc Hd/+ embryo 192

Figure 4.18 - Scanning electron micrographs (SEM) of the limb buds of a 12.5 dpc Hd/Hd embryo 194

Figure 4.19 - Scanning electron micrographs (SEM) of the limb buds of 12.5 dpc +/+ and Hd/Hd embryos indicating the apical ectodermal ridge 196

Figure 4.20 - Scanning electron micrographs (SEM) of +/+, Hd/+ and Hd/Hd embryos at 13.5 dpc clearly indicating the differences in limb bud morphology between the three phenotypes 198

Figure 4.21 - Semi-thin araldite sections of the limbs of +/+ and Hd/Hd embryos at 11.5 and 12.5 dpc showing morphology of the apical ectodermal ridge 201

Chapter 5

Figure 5.1 - Photographs of a stage 20 and a stage 25 ta^/ta^ embryos in ovo 213

Figure 5.2 - Confirmation of of ta^3/ta^3 phenotype 217

Figure 5.3 - Schematic representation of a stage 20 chick wing bud showing a loop in the anterior apical ectodermal ridge 220

Figure 5.4 - Diagram showing how polarising activity was mapped in ta^3/ta^3 limb buds 221

Figure 5.5 - Skeletal patterns of 10-day chick wings that resulted from mesenchyme grafts from a stage 19/20 ta^3/ta^3 limb bud into the anterior margin of a normal stage 20/21 host 232

Figure 5.6 - Skeletal pattern of a 10-day control ta^3/ta^3 wing and contralateral ta^3/ta^3 wing after a polarising region graft from a stage 20 normal wing had been grafted to the anterior margin at stage 20 237

Figure 5.7 - Expression of Shh and Hoxd13 in unmanipulated ta^3/ta^3 limb buds, and in ta^3/ta^3 buds treated with retinoic acid beads 240

Figure 5.8 - Expression of Fgf4 in a stage 22 normal leg bud and a stage 22 ta^3/ta^3 leg bud 242

Figure 5.9 - Bmp2, Bmp4, and Bmp7 expression in normal and ta^3/ta^3 wing buds 245
List of Tables

Chapter 1
Table 1.1 - Developmental stage and positions along the main antero-posterior body axis in mouse, chick, and human embryos at which the fore- and hindlimb buds are initiated

Chapter 3
Table 3.1 - Number of live-born mice produced by the breeding colony of $Hd$ mice
Table 3.2 - External appearance of $+/+$, $Hd/+$, and $Hd/Hd$ littermates at 28 weeks
Table 3.3 - Regions of the adult skeleton affected by the $Hd$ mutation in $Hd/+$ and $Hd/Hd$ mice analysed at 28 weeks
Table 3.4 - Number of mice from a sample population of 110 adult female $Hd/+$/mice having different degrees of hindlimb digit I reduction, soft tissue syndactyly between digits II and III, and presence of an abnormal or absent terminal claw on hindlimb digit I on both left and right hindlimbs
Table 3.5 - Left to right asymmetry in the $Hd/+$/hindlimb phenotype in a sample population of 110 adult female $Hd/+$/mice having different degrees of hindlimb digit I reduction, soft tissue syndactyly between digits II and III, and the presence of an abnormal or absent terminal claw on hindlimb digit I
Table 3.6 - Summary the number of litters and phenotypes of the embryos in these litters from 112 $Hd/+$ X $Hd/+$/matings
Table 3.7 - Average crown to rump size of $+/+$, $Hd/+$, and $Hd/Hd$/embryos between 11.5 and 14.5 dpc
Table 3.8 - Average area of distal limb buds of $+/+$, $Hd/+$, and $Hd/Hd$/embryos at 11.5 and 12.5 dpc, the relative increase in limb buds size from 11.5 to 12.5 dpc, and the average difference in size of $+/+$ and $Hd/+$/limb buds in comparison to $Hd/Hd$/limb buds
Table 3.9 - Summary of results obtained from grafting posterior mesenchyme from $+/+$, $Hd/+$, and $Hd/Hd$/limb buds to the anterior border of a normal stage 20 chick wing

Chapter 5
Table 5.1 - Number of mesenchymal grafts from different regions of stage 19 - 23 and stage 23 - 24 $ta^3/ta^3$/wing and leg buds that were grafted to the anterior border of normal chick wings, and the identity of the most anterior additional digit formed
**Table 5.2** - Number of cases where skeletal alterations additional to the induction of additional digits were induced by grafted *Talpid* mesenchyme

<table>
<thead>
<tr>
<th></th>
<th>234</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Talpid</em></td>
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To my parents and my sister Louise, and in the memory of my dear Grandma and Grandad who I wish could have been here to share in this.
1. A General Introduction to Vertebrate Limb Development

1.1 Embryonic Development

Embryonic development results in the transformation of a single cell into a highly complex, multi-cellular organism consisting of many different cell types. During development, the structural organisation, or patterning of the embryo must be achieved. Pattern formation is a term used to describe this process and the concept of positional information provides a mechanism by which pattern formation can take place (Wolpert 1969; 1971). Cells in a developing system are instructed as to their relative positions (positional information) within a specific region of cells with fixed boundaries (for example the limb field, a region of the embryo from which limbs develop). Cells then interpret this information with reference to their genetic composition and developmental history (reviewed in Wolpert, 1994). Once cells are specified, the tissues and organs that they form part of are moulded into shape, a process known as morphogenesis, involving cell proliferation, cell movement, and programmed cell death.

Many different species are studied (from insects such as Drosophila melanogaster to mammals such as Mus musculus) in an attempt to understand the complexities of embryonic development, and it is becoming increasingly evident that, across species, similarities exist in the events that take place. Molecular techniques used to analyse how genes and molecules control embryonic development are identifying genes and gene families that are thought to be important during development, and that are conserved across species. Analysis of molecular mechanisms of development in Drosophila has been pivotal to the progress made in understanding molecular mechanisms of vertebrate development. Many of the genes known today to be important in development were first identified and cloned in Drosophila, and analysis of their function during Drosophila development has assigned putative functions to some of these genes. A significant finding has been that a number of these genes identified in Drosophila have similar counterparts or homologues in vertebrates, suggesting that similar molecular mechanisms of embryonic development may exist in many different species.
Vertebrate limbs are an excellent model system in which to study pattern formation; they develop from very simple structures (limb buds) into highly complex functional units (limbs), and, as in the rest of the embryo, cells must differentiate at the correct time and in the correct locations. The vertebrate limb is easily accessible to embryonic analysis and experimental manipulation, particularly in the developing chick embryo, and this has been the focus of much of the classical work on vertebrate limb development (reviewed in Tickle and Eichele, 1994). A wealth of information has also been gained from the study of murine limb development, and although experimental manipulations of post-implantation mouse embryos in utero are technically difficult, the murine genome is accessible to analysis, and many of the genes known to be important during vertebrate limb development have been mapped, cloned and sequenced (see The Jackson Laboratory Mouse Genome Database and references therein, http://www.jax.org). There are many genes still to be been cloned, as suggested by the large number (> 100), of spontaneous mouse mutants with limb defects (Grüneberg, 1952; Lyon et al., 1996) whose molecular basis have yet to be identified. In addition, with the development of transgenic technology, it is now possible to artificially induce mutations in the mouse through manipulation of the genome (Capecchi, 1994; Gatherer, 1993). Mouse mutants (both naturally occurring and artificially induced) are an extremely useful resource in the study of limb development as they provide an invaluable source of information about essential gene function and an opportunity to compare and contrast normal and abnormal development. Recent advances in transgenic technology have enabled the targeting of specific genes already known to be expressed in the developing limb (see Brandon, 1995a, 1995b, 1995c and references therein). Analysing the phenotypic outcome of such gene targeting experiments is enhancing the understanding of the roles specific genes and the molecules they encode play during murine limb development (discussed later in this thesis).

The developing mouse embryo is used as a model for human embryonic development, as many developmental mechanisms are thought to be conserved between different mammalian species, and many of the genes known to be important during murine limb development have also been identified in the human genome. Although the
chromosomal arrangement of these human genes is different, it has been suggested that they may still have similar functions to those in the mouse. Already possible mouse models of human inherited developmental defects have been identified (Belloni et al., 1996; Clarke, 1994; Darling, 1996; Darling and Abbott, 1992; Jacenko et al., 1994; Roessler et al., 1996; Winter, 1988). For example, very recently the targeted disruption of a murine gene called sonic hedgehog (Shh) has been generated (Chiang et al., 1996), resulting in a phenotype closely resembling that of a human developmental defect, holoprosencephaly (HPE) (Belloni et al., 1996; Roessler et al., 1996). Mutations in the human homologue of Shh have subsequently been shown to cause HPE in certain families (Belloni et al., 1996; Roessler et al., 1996). Thus, it is becoming increasingly evident that information gained from the study of development in both normal and mutant mice probably has direct relevance to both normal and abnormal human development.

1.2 A Review of Vertebrate Limb Development

1.2.1 Limb initiation

Vertebrate limbs develop from small, paired, bud-like outgrowths which emerge at specific times and at specific sites along the main antero-posterior body axis of the developing embryo, both of which vary between species (Hamburger and Hamilton, 1951; Kaufman, 1992; Milaire and Mulnard, 1984; O'Rahilly and Gardner, 1975; Table 1.1). However, the overall appearance of limb buds in different species is somewhat similar. Mechanisms involved in specifying limb position along the main antero-posterior body axis are not fully understood, but there is experimental evidence to suggest that they may involve expression of Hox genes along the developing main body axis (Cohn et al., 1997 in press).
Table 1.1

Developmental stage and positions along the main antero-posterior body axis in mouse, chick, and human embryos at which the fore- and hindlimb buds are initiated.

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<tr>
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<th>Mouse</th>
<th>Chick</th>
<th>Human</th>
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<td></td>
<td>Position</td>
<td>Stage</td>
<td>Position</td>
</tr>
<tr>
<td>Forelimb bud</td>
<td>Opposite somites</td>
<td>E9-9.5 days of gestation</td>
<td>Opposite somites</td>
</tr>
<tr>
<td></td>
<td>7-13</td>
<td></td>
<td>15 - 20</td>
</tr>
<tr>
<td>Hindlimb bud</td>
<td>Opposite somites</td>
<td>E10-10.5 days of gestation</td>
<td>Opposite somites</td>
</tr>
<tr>
<td></td>
<td>27 - 31</td>
<td></td>
<td>26 - 32</td>
</tr>
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</table>

Table 1.1

Table documenting the developmental stage and positions along the main antero-posterior body axis in mouse, chick, and human embryos at which the fore- and hindlimb buds are initiated.
The first morphological signs of limb development can be detected histologically as paired thickenings in the lateral plate mesoderm, the formation of which are correlated with a decrease in levels of cell proliferation in the intervening flank mesenchyme (Searls and Janners, 1971). Experiments have shown that prior to the appearance of the limb buds, prospective chick limb bud mesoderm has the ability to form limb structures when grafted to the flank of a host embryo (Reuss and Saunders, 1965; Saunders and Reuss, 1974). This indicates that cells in the presumptive limb regions of the embryo are specified to form limb structures even prior to the appearance of discrete buds.

Once induced, the paired thickenings grow out and form pronounced elevations, known as limb buds, which consist of a core of undifferentiated mesenchymal cells originating from the lateral plate mesoderm and the lateral somite, which give rise to the connective tissues and myogenic cells of the muscles respectively (Chevallier et al., 1977; Christ et al., 1977; Ordahl and Le Douarin, 1992), encased in a thin epithelial jacket (Figure 1.1). The limb buds are initially fairly simple, symmetrical structures, but as development proceeds, they grow out in a proximo-distal manner and become more complex and asymmetrical in shape.
Figure 1.1

Three dimensional, diagrammatic representation of a vertebrate limb bud consisting of a core of undifferentiated mesenchyme encased in an ectodermal jacket. The three axes of the limb are indicated at the bottom right of the diagram.
1.3 Specification of limb pattern

Limb development involves the intricate co-ordination of different developmental processes such as tissue-specific cell differentiation, control of cell growth and cell-cell interactions along three axes (proximo-distal, antero-posterior, and dorso-ventral; see Figure 1.1). Specification of antero-posterior and dorso-ventral axes of the limbs occurs prior to the appearance of limb buds (Hamburger, 1938; Saunders and Reuss, 1974). Experimental manipulations carried out in the chick have identified three major sets of signalling interactions in the developing limb bud that directly influence patterning and morphogenesis (reviewed in Tickle and Eichele, 1994). Signalling centres involved in these interactions are the specialised epithelium that runs antero-posteriorly along the distal edge of the bud, known as the apical ectodermal ridge, a region of posterior mesenchymal cells known as the polarising region (also known as the zone of polarising activity), and the non-ridge limb ectoderm (Figure 1.2A and 1.2B). They play roles in the specification of pattern along the proximo-distal, antero-posterior, and dorso-ventral axes of the limb respectively (reviewed in Robertson and Tickle, 1997; Tickle, 1994).

Signalling interactions across the three axes are at least partly interdependent of one another, thus ensuring that patterning and morphogenesis of the limb is tightly co-ordinated along all three axes of the limb (Yang and Niswander, 1995).
Figure 1.2

Schematic representations of the early developing vertebrate limb bud indicating the important signalling centres involved in outgrowth and patterning. (A) View of an early limb bud showing the localisation of the polarising region to posterior limb bud mesenchyme, the progress zone to distal tip mesenchyme, and the specialised epithelium that runs antero-posteriorly along the distal edge of the bud, known as the apical ectodermal ridge. (B) Transverse section of limb bud in (A) showing the non-ridge limb ectoderm on the dorsal and ventral aspects of the limb bud.
1.3.1 The apical ectodermal ridge

Soon after the initial appearance of the limb buds, the ectoderm at the distal tip of the bud undergoes morphological changes and forms a definite ridge running antero-posteriorly along the distal edge of the bud (Saunders, 1948; Figure 1.2A). This ridge, known as the apical ectodermal ridge, consists of tightly packed pseudostratified cells linked extensively by gap junctions (Fallon and Kelley, 1977). Experiments in the developing chick embryo have shown that an ectopic apical ectodermal ridge can be induced on the flank of a host embryo by grafting presumptive limb mesenchyme to this position (Dhouailly and Kieny, 1972; Reuss and Saunders, 1965; Saunders and Reuss, 1974; Tanaka et al., 1997), and limb mesenchyme loses this ability once the limb buds appear, indicating that the ridge is induced by transient signals from the underlying mesenchyme.

Recent evidence suggests that the dorso-ventral limb boundary organises the apical ectodermal ridge structure in the limb field of early-stage chick embryos (Tanaka et al., 1997). When new dorso-ventral boundaries are made by implanting dorsal tissue into the ventral limb field, an ectopic apical ectodermal ridge is induced on the ventral side of the host limb buds (Tanaka et al., 1997).

Once induced, the apical ectodermal ridge is maintained by signals from the underlying mesenchyme (Saunders, 1948; Searls and Zwilling, 1964; Zwilling 1956b, 1961, 1972). *In vivo* experiments have shown that the ridge degenerates within 48 hours of grafting it over non-limb mesenchyme. This degeneration however can be prevented by grafting small pieces of limb mesenchyme under the grafted ridge (Zwilling 1961, 1972). In addition, culturing apical ectodermal ridge dissociated from the underlying mesenchyme *in vitro* results in ectodermal cell death which can be prevented by co-culturing the apical ectodermal ridge with underlying limb mesenchymal cells (Boutin and Fallon, 1984; Searls and Zwilling, 1964). The ability of limb mesenchymal cells to maintain the apical ectodermal ridge is acquired at the same time as they lose their ability to induce an apical ectodermal ridge (Saunders and Reuss, 1974). Zwilling hypothesised that the ridge is maintained by an apical ectodermal maintenance factor (AEMF) which is selectively distributed throughout the limb mesenchyme (Zwilling and Hansborough,
1956), and the length, morphology, and duration of the apical ectodermal ridge may be related to the supply of this factor.

After the initial induction of the limb buds, further outgrowth and patterning along the proximo-distal axis of the limb bud is dependent on the presence of the apical ectodermal ridge. Removing the ridge leads to the loss of structures along the proximo-distal axis of the limb, the extent of which is dependent on the stage of development at which the ridge is removed, with more severely truncated limbs resulting when the ridge is removed early (Saunders, 1948; Summerbell, 1974). Grafting an ectopic apical ectodermal ridge to the dorsal or ventral surface of a host limb bud induces ectopic outgrowth of host limb mesenchyme at the site of the graft, resulting in the formation of ectopic limb structures (Saunders and Gasseling, 1968; Saunders et al., 1976).

Inductive interactions of the apical ectodermal ridge do not depend on ridge polarity with respect to underlying mesenchyme. When the ectoderm is rotated 180°, such that anterior mesenchyme is in contact with posterior apical ectodermal ridge, the bud continues to grow normally and the antero-posterior axis of the final limb is not affected (Zwilling, 1956a). Recombination experiments have also shown that the inductive interactions of the ridge are interchangeable within the same embryo and between embryos of different stages of development. The limb type that develops follows the mesenchymal component of the recombinant limb bud; wing mesenchyme recombined with leg apical ectodermal ridge results in formation of a wing (Zwilling, 1955) and young mesenchyme recombined with older ectoderm leads to development of a limb related to the age of the mesenchyme (Rubin and Saunders, 1972). These experiments show that the action of the apical ectodermal ridge on the underlying mesenchyme is permissive and not instructive; it allows limb outgrowth to occur without directly determining developmental fate.

There is evidence to suggest that in addition to its role during the proximo-distal outgrowth and patterning of the limb, the apical ectodermal ridge also influences the shape of the developing limb. It may act as a mechanical 'seam' ensuring that the bud is dorso-ventrally flattened (Hornbruch and Wolpert, 1970; Lee and Tickle, 1985). The length of the apical ectodermal ridge can be altered experimentally through the application
of a bead soaked in retinoic acid to the anterior margin of a developing chick wing bud. This also alters antero-posterior limb pattern and digit specification. High doses of retinoic acid result in a decrease in the length of the apical ectodermal ridge, the antero-posterior width of the bud, and the number of digits that develop (Lee and Tickle, 1985); low doses of retinoic acid lead to an increase in the length of the apical ectodermal ridge, a widening of the limb buds and the development of supernumerary digits (Lee and Tickle, 1985). This suggests that the ridge may play a role in determining the antero-posterior width of the limb bud, thus influencing the amount of mesenchyme available for digit development. Further evidence for this comes from the study of vertebrate limb mutants in which the size of the developing limb buds is associated with changes in length or presence of the apical ectodermal ridge (see later).

1.3.2 The progress zone
Bud outgrowth is associated with the maintenance of high levels of cell proliferation in a zone of undifferentiated mesenchymal cells underlying the apical ridge. This region is known as the progress zone (Summerbell et al., 1973; Figure 1.2A), and there is evidence to suggest that the length of time cells spend in the progress zone determines whether they will form proximal or distal limb structures. Cells that leave early, form proximal structures, whereas those that leave later, form more distal ones (Summerbell and Lewis, 1975). The loss of limb structures along the proximo-distal axis of the limb that results from removal of the apical ectodermal ridge is correlated with termination of cell proliferation in the progress zone after ridge removal (Saunders, 1948; Summerbell, 1974). Therefore, it is the interaction of the apical ectodermal ridge and the underlying cells of the progress zone that maintains the high levels of proliferation in this region of the limb allowing outgrowth and patterning of the limb to occur.

1.3.3 The polarising region
There are three digits in the chick wing, digits 2, 3, and 4, with 2 being most anterior and 4 most posterior (Figure 1.3A). When a block of posterior mesenchymal cells is grafted under a loop in the apical ectodermal ridge along the anterior margin of a stage 20 host
chick wing bud, respecification of host anterior mesenchyme occurs such that an extra set of digits is induced (Figure 1.3B). Instead of the normal 2, 3, 4 digit pattern, the manipulated wing has a mirror-image duplication, such that the digit pattern becomes 4, 3, 2, 3, 4, and the additional set of digits arises from the host anterior tissue (Saunders and Gasseling, 1968; Figure 1.3B bottom panel). The region of posterior mesenchymal cells capable of inducing these digits duplications is known as the polarising region, and signals produced by this region are thought to govern patterning across the antero-posterior axis of the developing limb (Saunders and Gasseling, 1968).

The polarising region has been mapped in the developing chick wing and leg by grafting blocks of mesenchymal cells taken from different antero-posterior locations in the limb to the anterior margin of a host chick wing, and analysing digit patterns that result (Hinchliffe and Sansom, 1985; Honig and Summerbell, 1985; MacCabe et al., 1973). These experiments have shown that polarising activity is asymmetrically distributed across the antero-posterior limb axis (Honig and Summerbell, 1985), and is at its highest in posterior-distal mesenchyme, just proximal to the progress zone. As the limb grows out, the polarising region continues to be located in posterior-distal mesenchyme, and is finally lost at a stage in development when the apical ectodermal ridge begins to regress and the hand/footplates are forming (Honig and Summerbell, 1985).

Polarising region signalling is dose-dependent; when small numbers of polarising cells (~ 30 cells) are grafted, a partial duplication with digit patterns 2, 2, 3, 4 (Figure 1.3B top panel) results whereas grafts of larger numbers of polarising cells (> 100 cells) give full duplications with digit patterns 4, 3, 2, 3, 4 (Tickle, 1981; Figure 1.3B bottom panel). In addition, the extent of duplications induced also depends on how long the grafted cells are left in place in the host wing; removing the graft after 15 hours leads to the induction of an additional digit 2 whereas grafts left in for 17 - 24 hours will specify a digit 3 (Smith, 1980). These experiments showed that the action of grafted polarising region cells on anterior host mesenchyme is both dose and time dependent, and increased support for the "morphogen model" of antero-posterior limb patterning (Tickle et al., 1975; Wolpert, 1989). Morphogens are signalling molecules involved in
pattern formation and morphogenesis (Brickell and Tickle, 1989), and this model suggests that the polarising region is the source of a diffusible morphogen, that forms a concentration gradient across the antero-posterior axis of the limb, the concentration of which instructs cells of their positional identities (Tickle et al., 1975; Wolpert et al., 1989). Thus, digits are specified in a concentration-dependent manner with high concentrations of morphogen specifying posterior structures and low concentrations specifying anterior structures (Tickle et al., 1975; Wolpert et al., 1989). Cells nearest the polarising region would be exposed to high concentrations and those furthest away, in the anterior limb bud, would be exposed to low concentrations giving rise to posterior and anterior structures respectively. Grafting experiments described above are easily explained by this model, as grafting fewer posterior mesenchymal cells or reducing exposure time to a polarising region graft would reduce the total concentration of morphogen anterior host cells are exposed to, leading to partial duplications in which ectopic anterior and not posterior digits are induced (Smith, 1980; Tickle, 1981).
Block of mesenchyme taken from the polarising region of a stage 20 donor chick wing or leg bud

-30 polarising region cells grafted to the anterior margin of the host wing bud

Loop in the apical ectodermal ridge along the anterior margin of a stage 20 host wing bud

>100 polarising region cells grafted to the anterior margin of the host wing bud
Figure 1.3

Diagrams showing final skeletal pattern of the normal chick wing and skeletal patterns induced after grafting polarising region cells to the anterior margin of a wing bud at stage 20 of development. (A) Normal chick wing with digit pattern 2, 3, 4 from anterior to posterior. (B) Digit pattern of chick wings after grafting different amounts of polarising region cells under a loop in the apical ectodermal ridge at the anterior margin of a stage 20 normal chick wing. The top limb has a digit pattern 2, 2, 3, 4 which is induced by grafting ~ 30 polarising region cells, whereas the bottom limb has a digit pattern 4, 3, 2, 2, 3, 4 which is induced by grafting more than 100 polarising region cells. A: anterior; P: posterior; Pr: proximal; D: distal; *: ectopic digit.
An alternative model has also been proposed to explain pattern formation during limb development. This model, known as the "polar coordinate model", suggests that cells of the limb have an intrinsic knowledge of where they are with respect to one another early in development; they have specific coordinates, or positional values (reviewed by Bryant and Gardiner, 1992). When the continuity of positional information in the limb is disrupted, for example by grafting posterior mesenchymal cells to the anterior margin, the cells at the site of the disparity respond by dividing and acquiring new positional values so as to fill in the discontinuity. This is known as intercalation.

However, there are several lines of experimental evidence suggesting that the morphogen model can fully explain the mechanisms of pattern formation in the limb, whereas the polar coordinate model cannot. The lack of induction of digit duplications by distal tip mesenchyme grafted anteriorly or anterior mesenchyme grafted posteriorly (Hinchliffe and Sansom, 1985; Honig and Summerbell, 1985; MacCabe et al., 1973) cannot be accounted for by the polar coordinate model which would predict intercalation and production of ectopic digits. In addition, this model cannot account for the differences in digit duplications induced by different numbers of polarising region cells, or when two polarising regions are grafted together at the anterior margin. The polar coordinate model would predict that no matter how many cells are grafted, or where the cells are grafted from and to, intercalation between posterior and anterior mesenchyme would take place leading to full digit duplications. This is clearly not the case when fewer cells are grafted (see above), or when two polarising regions are grafted together at the anterior margin which leads to the loss of digit 2 (Wolpert and Hornbruch, 1981). The loss of digit 2 can be explained by the morphogen model, as the anterior cells would be exposed to high concentrations of the polarising region morphogen, leading to induction of ectopic digits with posterior and not anterior identity (Tickle, 1981; Wolpert and Hornbruch, 1981).

Finally, separation of anterior and posterior mesenchyme with an impermeable barrier results in loss of anterior structures (Summerbell, 1979), suggesting that anterior limb mesenchyme requires signals from the posterior mesenchyme in order to allow normal development, thus supporting the morphogen model (Tickle et al., 1975; Wolpert, 1989).
1.3.4 Limb ectoderm

Epithelial-mesenchymal interactions control patterning across the dorso-ventral axis of the developing limb. When limb ectoderm is rotated so that, for example, dorsal ectoderm overlies ventral mesoderm, distal structures subsequently develop to conform with ectoderm polarity (MacCabe et al., 1974). Both dorsal and ventral ectodermal signals are required for patterning along the dorso-ventral axis of the limb; grafting an ectopic apical ectodermal ridge to either the dorsal or ventral surface of a host limb bud results in double dorsal or double ventral ectopic limb structures at the site of the graft (Saunders et al., 1976; Shellswell and Wolpert, 1977).

1.4 Interspecies conservation of signalling in developing vertebrate limbs

Developing limb buds of different vertebrate species are alike in appearance and it is thought that the processes of outgrowth and patterning occur via similar cellular and molecular mechanisms. Limb buds of amphibians, birds, rats, mice, and humans consist of a core of mesenchymal cells encased in an ectodermal jacket capped distally with an apical ectodermal ridge (Fallon and Kelly, 1977; Jurand, 1965; O'Rahilley et al., 1956; Saunders, 1948). The inductive interactions of limb ectoderm and apical ectodermal ridge are conserved between different avian species and between mammals and chick (Saunders, 1977 for references), such that the ectoderm of duck, mouse and rat limb buds can support outgrowth and development of a normal chick limb (Joquera and Pugin, 1971 cited in Saunders, 1977; Patou, 1968 cited in Saunders, 1977). In addition, blocks of posterior limb mesenchyme taken from several different vertebrate species have the ability to induce digit duplications when grafted to the anterior margin of a normal chick wing bud (Fallon and Crosby, 1977; MacCabe and Parker, 1976; Tickle, et al., 1976). These digit duplications are similar to those induced by a chick polarising region graft. Thus, signalling interactions involved in outgrowth and patterning of the vertebrate limb appear to be conserved between different species, although it is likely that species-specific differences do exist, and these will be responsible for differences in limb phenotypes observed in different vertebrate species.
1.5 Molecular basis of limb development

Molecular analysis of gene expression in normal and experimentally manipulated embryos has shown that outgrowth and patterning during limb development involves the expression of genes and molecules that appear to be part of intricate signalling cascades. Molecules involved in these cascades belong to various families, such as growth factors, transcription factors and retinoids (see below).

1.6 Signalling molecules in limb initiation and proximo-distal outgrowth

1.6.1 Fibroblast growth factors

The fibroblast growth factor (FGF) family consists of at least ten (FGF1 - FGF10) structurally related peptide signalling molecules (Yamasaki et al., 1996), and FGF2, FGF4, and FGF8 are thought to be involved in aspects of limb initiation, outgrowth and patterning.

Recently, it has been shown in chick embryos, that beads soaked in certain members of the FGF family can induce extra limbs when implanted into the lateral plate mesoderm of the presumptive flank (Cohn et al., 1995; Crossley et al., 1996; Ohuchi, 1995). The type of extra limb induced is dependent on the position of bead implantation; beads placed more anteriorly, near the prospective wing region, induce extra wings whereas those placed more posteriorly, near the prospective leg region, tend to induce extra legs (Cohn et al., 1995). These results suggest that the endogenous signal responsible for the induction of paired limb buds may be an FGF. The best candidate, at present is Fgf8, which is expressed strongly in intermediate mesoderm (the region of mesoderm that gives rise to the embryonic kidney) near the limb forming region (Crossley et al., 1996). Recent evidence suggests that retinoic acid is also required in conjunction with Fgf8 for initiation of the chick limb bud (Stratford et al., 1996; see also section 1.7.1 in this chapter).

There is good evidence that FGFs also mediate signalling by the apical ectodermal ridge. Ridge cells express at least three members of the FGF family. FGF2 protein and Fgf2 mRNA transcripts have been found in dorsal ectoderm, entire apical ectodermal ridge and subectodermal mesenchyme of the limb (Dono and Zeller, 1994;
Fallon et al., 1994; Savage et al., 1993; Savage et al., 1995; Figure 1.4). *Fgf4* transcripts have been found localised to the posterior apical ectodermal ridge (Niswander and Martin, 1992; Suzuki et al., 1992; Figure 4) and *Fgf8* transcripts are detected throughout the entire length of the ridge prior to the appearance of *Fgf4* (Crossley and Martin, 1995; Heikinheimo et al., 1994; Ohuchi et al., 1994; Figure 1.4). Experiments in chick embryos have shown that FGF2, FGF4 and FGF8 proteins can substitute for the ridge; limb outgrowth and patterning after ridge removal can be maintained by grafting beads soaked in these FGFs to the limb buds (Crossley et al., 1996; Fallon et al., 1994; Niswander et al., 1993; Vogel et al., 1996). FGF2 and FGF4 have also been shown to maintain the activity of the polarising region cells after removal of the posterior apical ectodermal ridge or in isolated *in vitro* culture (Anderson et al., 1993; Vogel and Tickle, 1993).
Figure 1.4

Schematic representation of an early limb bud showing localisation of Shh transcripts to posterior mesenchyme in the same region as the polarising region. Fgf2 and Fgf8 transcripts are localised throughout the entire length of the apical ectodermal ridge, and Fgf4 transcripts are restricted to the posterior apical ectodermal ridge. Dark blue: Shh in the posterior mesenchyme; purple: Fgf2, Fgf4, and Fgf8 in the posterior apical ectodermal ridge; pink: Fgf2 and Fgf8 in the anterior apical ectodermal ridge; A: anterior; P: posterior; Pr: proximal; D: distal.
1.6.2 Fibroblast growth factor receptors

FGFs exert their effects on cells via low affinity and high affinity cell surface receptors (Baird, 1994; Dionne et al., 1990; Klagsbrun and Baird, 1991; Partanen et al., 1991; Pasquale, 1990). Four high affinity fibroblast growth factor receptors have been identified, FGFR1 - FGFR4, three of which are expressed in the developing limb.

FGFR1 is expressed in the mesenchyme of the developing limb (Orr-Urteger et al., 1991; Patstone et al., 1993; Peters et al., 1991), FGFR2 is detected in the limb ectoderm including the apical ectodermal ridge (Orr-Urteger et al., 1991; Patstone et al., 1993), and FGFR3 is expressed later on, in the cartilage rudiments of developing bone and in the cartilage growth plates during endochondral ossification (Peters et al., 1993). The exact roles of these receptors during limb development are not fully understood, but mutations in both murine (Deng et al., 1994; Deng et al., 1996; Colvin et al., 1996; Yamaguchi et al., 1994) and human FGFRs lead to skeletal defects (Muenke and Schell, 1995), suggesting that they are required for skeletogenesis.

1.7 Signalling molecules in anteroposterior patterning

1.7.1 Retinoic acid

Retinoic acid has been detected in the developing limbs of chick and mouse embryos, where it is enriched posteriorly, in the region of the limb where the polarising region is found (Satre and Kochhar, 1989; Scott et al., 1994; Stratford et al., 1996; Thaller and Eichele, 1987, 1990). In the absence of an endogenous polarising region, beads soaked in retinoic acid are capable of directing limb patterning (Eichele, 1989; Tamura, 1990).

Furthermore, application of a retinoic acid-soaked bead to the anterior border of a normal chick wing can mimic the action of a polarising region grafted to the same position, and lead to induction of mirror-image digit duplications (Summerbell, 1983; Tickle et al., 1982, 1985). Effects of retinoic acid on limb pattern are dose- and position-dependent (Eichele et al., 1985; Tickle et al., 1985), and it has been found that retinoic acid induces an ectopic polarising region in mesenchyme distal to the bead (Wanek et al., 1991). This suggests that retinoic acid itself is not the morphogenetic signal produced by the polarising region during normal patterning of the limb, but confirms that it has the ability
to trigger the cascade of events leading to patterning of the limb, possibly by inducing the endogenous signal/signals that then provide(s) the positional information. Treatment of chick embryos prior to appearance of the wing buds with disulphiram, an inhibitor of didehydroretinoic acid synthesis, abolishes limb bud outgrowth and inhibits the induction of \textit{Shh} and \textit{Fgf4} expression, but does not affect expression of \textit{Fgf8} (Stratford et al., 1996). Application of a retinoic acid soaked bead can rescue bud outgrowth and expression of \textit{Shh}, suggesting that retinoic acid, in conjunction with \textit{Fgf8}, may be required for initiation of bud outgrowth and induction of \textit{Shh} and \textit{Fgf4} expression in the chick limb bud (Stratford et al., 1996).

1.7.2 Retinoid receptors and binding proteins
Retinoic acid exerts it's action through nuclear receptors belonging to the steroid/thyroid hormone nuclear receptor superfamily. These receptors act as ligand-dependent transcription factors that have the ability to regulate the expression of other genes. Two families of retinoid receptors have been identified, the retinoic acid receptors (RAR\(\alpha\), \(\beta\), and \(\gamma\)), and the retinoid X receptors (RXR\(\alpha\), \(\beta\), and \(\gamma\)), and members of both families are expressed in developing limbs suggesting that they may be involved in limb patterning, although their exact functions remain unclear (Dollé et al., 1994; Mangelsdorf et al., 1992; Rowe et al., 1991; Schofield et al., 1992; Smith and Eichele, 1991; Thaller et al., 1993). In addition to the retinoid receptors, cellular retinoic acid binding proteins (CRABP I and CRABP II) are expressed in developing limb buds (reviewed by Mendelsohn et al., 1992). Their precise roles during limb outgrowth and patterning remain unclear, but it is thought that they may play a role in controlling the concentrations of retinoic acid within the limb (Dollé, et al., 1989b; Maden, 1991).

1.7.3 The hedgehog gene family of signalling molecules
Sonic hedgehog (\textit{Shh}), a vertebrate homologue of the \textit{Drosophila} hedgehog gene (\textit{hh}) (reviewed in Hammerschmidt et al., 1997; Lee et al., 1992; Mohler and Vani, 1992) is expressed in the polarising region of the developing limb (Figure 1.4) and at other sites...
throughout the developing embryo (Chang et al., 1994; Echelard, et al., 1993; reviewed in Hammerschmidt et al., 1997; Johnson et al., 1994a; Riddle et al., 1993).

*hh* is implicated in short and long range signalling interactions at various sites during *Drosophila* development (reviewed in Hammerschmidt et al., 1997). During the establishment of segment polarity, it is thought to act primarily as a short-range signal, whereas during patterning of imaginal discs, it induces long-range effects via induction of other signalling molecules (reviewed in Hammerschmidt et al., 1997). *hh* is expressed in the posterior compartment of the imaginal discs and is involved in antero-posterior patterning, and subsequent proximo-distal outgrowth (Basler and Struhl, 1994; Tabata et al., 1992). Ectopic expression of *hh* in the anterior compartment leads to duplication of anterior wing structures with mirror-image symmetry (Fietz et al., 1994) reminiscent of wing duplications induced in the chick by polarising region grafts. It was suggested that *Shh* in vertebrates may carry out similar functions to *hh* in *Drosophila*, thus, making *Shh* a good candidate for the signal produced by the polarising region that directs antero-posterior limb patterning.

Experimental evidence supports this theory that *Shh* is involved in antero-posterior limb patterning; ectopic *Shh* expression, or the application of a bead soaked in SHH peptide to the anterior of a normal limb bud, mimic the action of a polarising region graft and induce a mirror-image set of digit duplications (Chang et al., 1994; Lopez-Martinez et al., 1995; Riddle et al., 1993). Recent evidence suggests that decreasing concentration thresholds of SHH peptide induce progressively more anterior digits (Drossopoulou, personal communication). Reduction of *Shh* expression is correlated with loss of the ulna and posterior digits in mice (Parr and McMahon, 1995) and mice with a targeted disruption in the *Shh* gene have limbs that are severely malformed, with distal structures being most affected (Chiang et al., 1996). These results suggest that SHH is an important signal produced by the polarising region and involved in antero-posterior patterning of the limb.

In *Drosophila*, transduction of *hh* signalling in target cells is thought to involve the genes patched (*ptc*) and smoothened (*smo*). *ptc* encodes a novel transmembrane protein, Ptc, with structural similarities to protein channels and transporter proteins
(Hooper and Scott, 1989; Ingham et al., 1991). Whereas smo encodes a transmembrane protein, SMO, with structural similarities to G-protein coupled receptors (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). A chicken patched-related gene (Ptc) has been isolated, and is expressed in regions associated with Shh expression (Marigo et al., 1996; Riddle et al., 1993). In the limb, it is restricted to the posterior mesenchyme overlapping the expression domain of Shh (Riddle et al., 1993).

In Drosophila, PTC and SMO are thought to function together in transducing the HH signal. SMO is proposed to be a constitutive activator of HH target genes, and the activity of SMO is normally repressed by PTC, and this repression is removed by HH binding to PTC or another intermediate receptor (reviewed in Hammerschmidt et al., 1997). Recently, direct biochemical evidence has shown that PTC binds SMO (Stone et al., 1996) and SHH (Marigo et al., 1996; Stone et al., 1996). In contrast, SMO has no SHH-binding activity (Marigo et al., 1996). Thus, PTC might be the binding component and SMO the intracellular signalling component of a receptor for SHH.

Although a vertebrate homolog of smo has yet to be isolated, the identification of a vertebrate ptc-related gene implies that, as in flies, Ptc may play a role in the transduction of the Shh signal during vertebrate limb development, and suggests conservation of signalling between flies and vertebrates.

1.7.4 Bone morphogenetic proteins

There is evidence that secondary signals are produced in response to Shh signalling and these are likely to be involved in mediating the effects of Shh in the limb. For example, during antero-posterior patterning of the Drosophila wing disc, reorganisation of the anterior compartment due to overexpression of hh involves induction of decapentaplegic (dpp), suggesting that dpp is a target of hh. Ectopic expression of dpp can also be induced by reducing the activity of ptc (Capdevila et al., 1994), and ectopic dpp expression alone is sufficient to give pattern alterations similar to those caused by ectopic hh (Basler and Struhl, 1994; Capdevila and Guerrero, 1994).

Bone morphogenetic protein 2 (Bmp2) is a vertebrate homologue of Drosophila dpp, and is a member of the transforming growth factor β (TGFβ)
superfamily of secreted signalling molecules (Kingsley, 1994). Seven Bmps have been identified, Bmp2 - Bmp8 (Kingsley, 1994; reviewed in Rosen and Thies, 1992), which are expressed at different times and in different locations throughout the developing embryo, suggesting that they have multiple roles during development (Hogan, 1996; reviewed in Kingsley, 1994). Several of the sites of expression include regions where epithelial-mesenchymal interactions take place, and in these regions, BMPs are thought to play key roles in the intercellular signalling that occurs between the epithelium and underlying mesenchyme (Francis et al., 1994; Jones et al., 1991a, b; Jones et al., 1992; Lyons et al., 1991; Vainio et al., 1993). Genetic evidence for the requirement of Bmps during development has come from recent studies on mice with targeted disruptions in Bmp7 (Hofmann et al., 1996; Luo et al., 1995; Lyons et al., 1995). These mice exhibit a variety of defects affecting structures known to require epithelial-mesenchymal interactions for their proper development (Hofmann et al., 1996; Luo et al., 1995; Lyons et al., 1995).

Bmp2, Bmp4, and Bmp7 are expressed in developing limbs of chicks and mice in complex and dynamic domains (Francis et al., 1994; Jones et al., 1991b; Jones et al., 1992; Lyons et al., 1991). During the early stages of limb development in both the chick and the mouse, Bmp2 and Bmp4 are expressed in the mesenchyme and overlying ectoderm; as development proceeds, Bmp2 expression becomes restricted to the posterior mesenchyme and apical ectodermal ridge (Francis et al., 1994; Bitgood and McMahon, 1995; Bellusci et al., 1996), and Bmp4 expression becomes restricted to anterior and posterior mesenchyme at the margins of the developing bud and the apical ectodermal ridge (Francis et al., 1994). In the chick, Bmp7 transcripts are differentially expressed in the wing and leg bud; in the wing Bmp7 is detected predominantly in the polarising region and the apical ectodermal ridge, whereas in the leg, transcripts are found in domains similar to those of Bmp4 (Francis-West et al., 1995). In the mouse, Bmp7 is expressed in the apical ectodermal ridge and diffusely throughout the limb mesenchyme (Luo et al., 1995; Lyons et al., 1995).

Expression of Bmp2 in posterior limb mesenchyme colocalises to the domain of high Ptc expression which is slightly larger than the expression domain of Shh
(Francis et al., 1994; Marigo et al., 1996). This overlapping expression of Shh, Ptc, and Bmp2 strongly suggests that they may be involved in the cascade of signalling events that leads to patterning of the limb. Evidence to support this theory comes from the finding that expression of Bmp2 can be induced in anterior ectoderm and mesenchyme by grafting a polarising region to the anterior margin of a host wing (Francis et al., 1994). In addition, Bmp2 expression can also be induced by Shh in the presence of FGF4 (Laufer et al., 1994). It may therefore be the case that, as in Drosophila where dpp acts as a downstream target of hh, Bmp2 may act in a similar way in the early developing vertebrate limb as a downstream target of Shh.

BMPs were first identified because of their ability to induce ectopic bone formation (Kingsley 1994 for a review; Rosen and Theis, 1992; Wozney et al., 1988) which implicated them in induction of bone and cartilage during skeletogenesis. Evidence to support this came from the finding that later on in limb development, when skeletal elements are being specified, expression of Bmp genes is associated with developing skeletal structures. Bmp2 is expressed in mesenchyme surrounding early cartilage condensations, Bmp4 is expressed in the perichondrium of developing cartilage elements, and Bmp7 is detected in interdigital mesenchyme and in cells around the cartilage rudiments (Duprez et al., 1996; Luo et al., 1995; Lyons et al., 1995). Genetic evidence for the involvement of Bmps in skeletal development has come from analysis of the short ear (se) mutation in mice which is a mutation in Bmp5 which affects the size of the ears, skull, and nose; and the number, shape, and size of some internal skeletal elements (King et al., 1994; reviewed in Kingsley 1994).

Thus it appears that BMPs have several functions during vertebrate limb development: in the early limb, in association with Shh and Ptc, they may be involved in early patterning events (Bellusci et al. 1996; Bitgood and McMahon, 1995; Goodrich et al., 1996); whereas in the later limb, they may be involved in specification of cartilage and bone (Kingsley, 1994; Vortkamp, 1997).
1.8 Dorso-ventral patterning

Wnt7a, Lmx1, and En1 genes are differentially expressed across the dorso-ventral axis of the limb, making them good candidates for genes involved in signalling interactions along this limb axis.

Wnt7a is a member of the Wnt gene family whose members encode secreted glycoproteins thought to act as relatively short-range signalling molecules involved in cell-cell signalling throughout the developing embryo (McMahon, 1992). During limb development Wnt7a is restricted to the dorsal limb ectoderm in both chicks and mice (Dealy et al., 1993; Parr et al., 1993; Parr and McMahon, 1995; Figure 5), suggesting that it may have a role in pattern formation along the dorso-ventral limb axis. Transgenic studies have shown that functional inactivation of Wnt7a results in duplication of ventral limb structures on the dorsal aspect of the limb (Parr and McMahon, 1995), confirming that this gene is required for dorso-ventral patterning of the limb.

Dorsal chick limb mesenchyme expresses Lmx1, a gene encoding a LIM homeodomain transcription factor (Riddle et al. 1995; Vogel et al., 1995; Figure 1.5). Experimental evidence shows that ectopic expression of Wnt7a in ventral limb ectoderm in vivo can induce and maintain ectopic Lmx1 expression in underlying ventral mesenchyme (Riddle et al., 1995). In addition, WNTa signalling can maintain and induce Lmx1 expression in vitro (Riddle et al., 1995), indicating that Lmx1 is a possible downstream target gene of Wnt7a (Riddle et al., 1995). Further experiments have shown that ectopic expression of Lmx1 alone is sufficient to induce dorsalisation of ventral mesenchyme (Riddle et al., 1995; Vogel et al., 1995). Thus, both Wnt7a and Lmx1 are important signals in the molecular pathways that specify dorso-ventral limb pattern.

It has been proposed that there may be other genes involved in dorso-ventral limb patterning which may have reciprocal expression patterns to both Lmx1 and Wnt7a, and that these genes may be involved in specification of ventral cell fate. A homeodomain containing transcription factor, engrailed 1 (En1), was identified due to its similarity to the Drosophila engrailed (en) segment polarity gene (Joyner et al., 1985; Joyner and Martin, 1987). During development, En1 is expressed at several sites throughout the embryo, including neural tissues and limb buds (Davis and Joyner, 1988). In
Drosophila, en has been shown to be involved in neurogenesis and segmentation (DiNardo et al., 1985; Kornberg, 1981; Schmidt-Ott and Technau, 1992), suggesting that Enl may have similar functions in vertebrate embryos. In the developing vertebrate limb, Enl transcripts are localised to ventral limb ectoderm (Davis and Joyner, 1988; Figure 1.5). Mice with a targeted deletion in Enl have multiple defects including dorsal transformations of ventral paw structures and subtle alterations in patterning along the proximo-distal axis of the limb (Loomis et al., 1996). Thus, in the limb, Enl may function in part by repressing dorsal differentiation. In addition, Enl appears to be required for formation of the apical ectodermal ridge, since loss of Enl function also induces subtle alterations in patterning along the proximo-distal axis of the limb (Loomis et al., 1996). Recent evidence suggests a possibility that aspects of dorso-ventral polarity in the limb mesenchyme are established as early as stage 9 - 10, prior to the expression of factors such as Wnt7a and Lmx1 (Tanaka et al., 1997).
**Figure 1.5**

Schematic representation of a transverse section through the limb bud in figure 1.4. 

*Wnt7a* and *Lmxl* transcripts are restricted to the dorsal ectoderm and dorsal mesenchyme respectively, and *Enl* transcripts are localised to the ventral ectoderm. **Grey dotted shading:** *Shh* in the posterior mesenchyme; **purple:** *Fgf2, Fgf4, and Fgf8* in the posterior apical ectodermal ridge; **pink:** *Fgf2 and Fgf8* in the anterior apical ectodermal ridge; **red:** *Enl* in the ventral ectoderm; **yellow:** *Lmxl* in the dorsal mesenchyme; **green:** *Wnt7a* in the dorsal ectoderm; **A:** anterior; **P:** posterior; **V:** ventral; **Ds:** dorsal.
1.9 Signalling interactions along the three axes of the limb are coordinated

Cellular and molecular interactions that take place across the three axes of the limb during development are not fully independent of one another. Molecular feedback loops exist allowing outgrowth and patterning of the limb to be tightly coordinated (Laufer et al., 1994; Niswander et al., 1994). As previously discussed, signalling of the apical ectodermal ridge and the polarising region are pivotal in directing limb development, and FGF4 and SHH respectively are endogenous signals produced by these regions. Experiments have shown that Fgf4 expression in the apical ectodermal ridge can be regulated by Shh, and Shh expression itself can be activated in mesenchymal cells by retinoic acid in the presence of FGF4. Once Shh expression is induced in the mesenchyme, it's expression can be maintained by FGF4 alone, thus establishing a feedback loop between the polarising region and the apical ectodermal ridge (Laufer et al., 1994; Niswander et al., 1994). Further evidence has shown that in addition to FGF4, the dorsal ectoderm and WNT7a are required to maintain Shh expression, thus forming a link between the mechanisms that control patterning across the dorso-ventral and other axes of the limb (Yang and Niswander, 1995).

1.10 Regulatory genes expressed in the limb in response to signalling in the limb

1.10.1 Vertebrate Msx genes

Msx1 and Msx2 are vertebrate homeobox-containing genes (Coelho et al., 1991; Hill et al., 1989; Monaghan et al., 1991; Robert et al., 1989; Robert et al., 1991; Suzuki et al., 1991; Yokouchi et al., 1991) related to the Drosophila muscle-segment homeobox (msh) gene (Gehring, 1987). Msx1 and Msx2 have complex and dynamic expression patterns at many different sites throughout the developing embryo, and show strong associations with regions where epithelial-mesenchymal interactions occur (Coelho et al., 1993; Davidson et al., 1991; reviewed by Davidson and Hill, 1991; Hill et al., 1989; Jowett et al., 1993; Robert et al., 1991; Satokata and Maas, 1994; Wang and Sasoon, 1995).

In the early developing mouse limb bud, Msx1 is expressed throughout the mesenchyme and ectoderm, whereas Msx2 is predominantly expressed in the ectoderm
and ventral mesenchyme (Hill et al., 1989; Hill and Davidson, 1991; Robert et al., 1989). As the limb buds grow out, ectodermal expression of Msxl becomes restricted to the apical ectodermal ridge (Hill and Davidson, 1991), and mesenchymal expression becomes localised to cells in the progress zone. As these changes in Msxl expression occur, Msx2 continues to be expressed in the ventral and distal ectoderm and in the distal, ventro-anterior mesenchyme (Hill et al., 1989; Hill and Davidson, 1991; Robert et al., 1989). During the later stages of limb development, when the digits are developing and interdigital zones are clearly visible, both genes are expressed in marginal and interdigital mesenchyme (Hill and Davidson, 1991).

Expression of Msxl and Msx2 in the limb is position-dependent (Davidson et al., 1991). When non-expressing cells are grafted distally in the limb in close proximity to the progress zone and apical ectodermal ridge, Msxl and Msx2 are rapidly activated in the transplanted mesenchymal cells (Davidson et al., 1991). Transcripts are detected in a graded distribution, with the region of highest expression being underneath the apical ectodermal ridge. This suggested that signals from the apical ectodermal ridge may influence the expression of these genes (Davidson et al., 1991). Removal of the apical ectodermal ridge leads to a decrease in cell proliferation in the progress zone, inhibition of bud outgrowth, and downregulation of Msxl expression in distal mesenchyme. However, bud outgrowth and Msxl expression can be maintained after ridge removal by FGF4 and FGF2, both in vivo and in vitro (Ros et al., 1993; Watanabe and Ide, 1993; Vogel et al., 1995a). This provides further evidence to support the idea that the apical ectodermal ridge plays a role in maintaining Msxl expression in underlying mesenchyme, and that Msxl expression is required to maintain the progress zone.

The molecular pathways which Msx genes are involved in, and the exact control mechanisms involved in regulation of Msx expression throughout the embryo are not fully understood. However, the correlation of Msx gene expression with regions of the embryo where inductive epithelial-mesenchymal interactions occur, suggests that they could function in the early response of cells to inductive signals that influence growth and/or differentiation. Msx expression domains in the limb, and at other sites in the embryo, overlap with expression domains of members of the Bmp gene family (Bellusci
et al. 1996; Bitgood and McMahon, 1995; Francis et al., 1994; Jones et al., 1991; Luo et al., 1995; Lyons et al., 1995), and Msx expression can be activated by BMP2 and BMP4 protein (Hogan et al., 1994; Vanio et al., 1993; Watanabe and Le Douarin, 1996). This suggests that Msx and Bmp genes may be involved in the same molecular pathways, with Bmp genes influencing the expression of Msx genes. However, not all regions of the embryo that express Msx genes express Bmp genes, which may indicate that there are both common and more site-specific mechanisms of regulation, depending on the region of the embryo concerned (reviewed in Davidson, 1995). Recent molecular evidence suggests that some of the spatial aspects of MsxI expression are separately controlled by different enhancer elements present in the MsxI promoter (Mackensie et al., 1997). These enhancer elements are thought to act both independently and in concert to generate the complex MsxI expression patterns (Mackensie et al., 1997). In addition, studies of the Msx2 promoter have found apical ectodermal ridge-specific enhancer domains (Liu et al., 1994; Sumoy et al., 1995), and further evidence suggests that MsxI may have the ability to autoregulate its own expression (reviewed in Davidson, 1995), however, this has not yet been substantiated.

1.10.2 Hox genes

Vertebrate Hox genes belong to a family of regulatory genes characterised by the presence of a short, 183 base pair sequence, known as the homeobox (Gehring et al., 1987; McGinnis et al., 1984). The homeobox encodes a 61 amino acid DNA-binding region, known as the homeodomain. Hox genes are thought to act by binding to DNA sequences in order to regulate transcription of target genes (Gehring et al., 1987; McGinnis et al., 1984). Hox genes show sequence similarity to Drosophila homeotic selector genes, first identified through the analysis of mutants; mutations in homeotic genes cause transformations of one body part into another, a phenomenon known as homeosis. In Drosophila, homeotic selector genes are located within a single complex, the homeotic gene complex, which is split into two clusters, Antennapedia (ANT-C) and Bithorax (BX-C) clusters (reviewed Akam, 1989; Figure 1.6). Genes of these clusters
are involved in controlling segment identity and identify structures along the antero-posterior axis of the embryo (reviewed Akam, 1989).

There are approximately 40 Hox genes organised into four clusters of linked genes Hoxa, Hoxb, Hoxc, and Hoxd. In the mouse these clusters are located on chromosomes 6, 11, 15 and 2 respectively (Figure 1.6). Sequence comparisons between vertebrate Hox, and Drosophila homeotic selector genes shows that these genes can be aligned into paralogous groups (reviewed by Duboule, 1994; Figure 1.6). The conservation of sequence, and expression patterns of Hox genes in developing vertebrate embryos, has led to the suggestion that gene function may also be conserved between the species.

During vertebrate development, Hox genes are expressed in cells along the antero-posterior body axis and in developing limbs in a highly ordered spatio-temporal manner (Gaunt et al., 1989; Holland and Hogan, 1988; Kessel and Gruss, 1990), suggesting that, as in Drosophila, they may be involved in specification of positional identity. A remarkable feature of vertebrate Hox genes is that the position of a gene within a cluster reflects the timing and anterior boundary of its expression. Their expression is said to follow the rules of spatial and temporal colinearity such that genes at the 3' end of the cluster are expressed prior to, and in domains that extend more anteriorly to, the more 5' genes within the cluster (Duboule, 1992, 1994; Hunt and Krumlauf, 1992; McGinnis and Krumlauf, 1992).
Figure 1.6

Figure showing the structural organisation of the *Drosophila* HOM-C homeotic complex and its correspondence with genes of the four mouse *Hox* clusters based on regions of homology. The top part of the figure (above the red line) shows the two homeotic Bithorax (BX-C) and Antennapedia (ANT-C) complexes of the *Drosophila* HOM-C complex. Large vertical arrows indicate the cognate relationships between these genes and the genes of the mouse *Hox* clusters. The bottom part of the figure (below the red line) shows the four mammalian *Hox* clusters, *Hoxa*, *Hoxb*, *Hoxc*, and *Hoxd*, located on murine chromosomes 6, 11, 15 and 2 respectively. Paralogous genes are located on the same vertical line. Genes are represented by open boxes (*Drosophila*) or black boxes (mouse). The large arrow at the bottom of the figure indicates the colinear relationship between gene order and the relative antero-posterior boundaries and temporal order of gene expression. This figure is base on Duboule, (1992) and McGinnis and Krumlauf, (1992).
During early limb development, genes of the Hoxd and Hoxa clusters are expressed in spatially restricted domains along the antero-posterior and proximo-distal limb axes respectively (Figure 1.7). On the basis of the timing and spatial restriction of their expression, they have been considered good candidates for downstream targets of signalling from the polarising region and apical ectodermal ridge which is suggestive of them playing a role in both proximo-distal and antero-posterior patterning (Dollé et al. 1989a, 1991a; Dollé and Duboule, 1989; Duboule and Dollé, 1989; Haack and Gruss, 1993; Oliver et al., 1989; Yokouchi et al., 1991; Yokouchi et al., 1995; Figure 1.7).

Further evidence suggests that Hox genes of cognate groups 9 - 13 work in unison to specify limb bone formation in a proximal to distal direction (Davis et al., 1995). Group 9 cognate genes are thought to be intimately involved in specification of the proximal limb girdle, whereas group 13 cognate genes are thought to act more distally in the limb specifying the digits (Figure 1.8). The roles of Hox genes in limb development will be discussed in more detail in the introduction to Chapter 3 of this thesis.

1.10.3 Even-skipped homologues

Two related vertebrate genes that are homologous to the pair rule gene even-skipped (eve) in Drosophila (Harding et al., 1986), Evx1 and Evx2 (Dush and Martin, 1992), lie at the 5' end of the Hoxa and Hoxd gene clusters, respectively (Figure 1.6). In Drosophila, eve controls expression of the segment polarity genes wingless (wg) and hedgehog (hh). In vertebrates, Wnt genes and Shh are homologues of wg and hh, but it is not known if Evx1 or Evx2 regulate their expression. Evx1 is expressed in the posterior limb mesenchyme and its expression requires signals from the apical ectodermal ridge (Niswander and Martin, 1993). It has been suggested that Evx1 may play a role in maintaining the progress zone. Alternatively, even though Evx genes are transcribed in the opposite direction to the Hox genes, they could function as additional members of the Hoxa and Hoxd clusters (Bastian et al., 1992).
Figure 1.7
Simplified representation of the nested expression domains of genes of the (A) Hoxd and (B) Hoxa cluster along the antero-posterior and proximo-distal axes respectively of an early developing vertebrate limb bud. Red: cells expressing Hoxd9 - d13; orange: cells expressing Hoxd9 - d11; yellow: cells expressing Hoxd9; dark green: cells expressing Hoxa9 and Hoxa13; lime green: cells expressing Hoxa9 and Hoxa11; pale green: cells expressing Hoxa9.
**Figure 1.8**

Diagram of the model for the role of *Hox* genes in limb development proposed by Davis et al. (1995). The *Hox* cognate groups 9-13 (boxes) represent paralogous genes of the *Hox* loci and are drawn in the 3' to 5' direction. It is proposed that cognate genes work in union to specify limb bone formation in the proximal to distal direction (left to right). In addition to these paralogous interactions, *Hox* genes in the same linkage group or across groups may also interact during limb development. Figure adapted from Davis et al. (1995).
Aims of this thesis

The aims of this thesis were to analyse cellular and molecular aspects of limb development in two vertebrate limb mutants, hypodactyly (Hd) in the mouse and talpid3 (ta3) in the chick. These mutants have an apparent disruption of normal limb patterning, such that there is severe loss of digits in hypodactyly and gain of digits in talpid3. It was hoped that by studying limb development in these mutants, it would be possible to make direct comparisons between normal and abnormal development, and by doing so, gain a better understanding of the nature of these defects and further unravel the sequence of events, both cellular and molecular, that result in patterning of the normal limb.

At the time this study was commenced, very little work had been carried out on hypodactyly. The only information available was from the initial brief reports of Hummel (1963, 1970), which documented the skeletal defects observed in the limbs of adult mice. Heterozygotes (Hd+/) were reported to have a reduced digit I on the hindlimbs, whereas homozygotes (Hd/Hd) have a single digit on all four limbs. The work described in the first section of Chapter 3 of this thesis, was carried out to confirm the findings of Hummel (1963, 1970) in regards to the morphology of the adult phenotypes. No previous studies have examined the morphology of Hd mutant mice throughout the stages of embryogenesis when the limbs are forming. Therefore, in order to discover potential clues as to what the defects are and how they arise, it was of vital importance to carry out an embryological study in order to analyse the morphology of embryonic mutant limbs and pinpoint the time at which the defects are first visible.

The polarising region and apical ectodermal ridge are required for outgrowth and patterning along the antero-posterior and proximo-distal axes of the limb, and the endogenous polarising region and ridge signals involved are proposed to be Shh and Fgf4. One could predict that the loss of digits in hypodactyly might be attributable to the loss or reduction of expression of Shh and/or Fgf4 during the stages of development when the hands and feet are being specified. Chapter 3 also addresses the signalling ability of posterior Hd/Hd limb mesenchyme, and the expression of Shh and Fgf4 in this mutant.
The *Hd* gene maps to murine chromosome 6 (Mock et al., 1987), and very recently the molecular basis of *Hd* has been identified as a deletion in the *Hoxa13* gene (Mortlock et al., 1996). *Hox* genes of paralogous group 13 are thought to be involved in specification of the distal-most regions of the limbs; the hands and feet (Fromental-Ramain et al., 1996). The results described here add further evidence to support this possible role of *Hoxa13* in specification of hands and feet. However, inactivation of *Hoxa13* in the *Hoxa13<sup>-/-</sup>* knockout mouse, does not produce a comparable phenotype to that of *Hd/Hd* mice, instead *Hd/Hd* mice more closely resemble *Hoxa13<sup>-/-</sup>Hoxd13<sup>-/-</sup>* double knockout mice. These findings suggested the possibility that *Hoxd13* expression in the limbs of *Hd/Hd* mice may be altered, and so the final section of Chapter 3 analyses the expression of *Hoxd13* and another *Hoxd* gene, *Hoxd11*. Knowing that *Hd* is caused by a mutation in *Hoxa13*, makes this mutant a valuable tool for analysis of the downstream targets and functions of the *Hox* genes during vertebrate limb development. Chapter 4 discusses alterations in cell behaviour and additional morphological defects in the limbs of *Hd* mutant mice, and discusses the findings in relation to their possible contribution to the final phenotypic outcome of the mutation.

The chicken mutant Talpid<sup>3</sup> (*ta<sup>3</sup>*, has multiple defects that are widespread throughout the embryo (Ede and Kelly, 1964a, 1964b). Limbs of *ta<sup>3</sup>/ta<sup>3</sup>* embryos are polydactylyous, with up to 8-10 morphologically similar digits, many of which are partially fused and form a pattern which lacks any obvious antero-posterior polarity (Ede and Kelly, 1964b; Hinchliffe and Ede, 1967; reviewed by Hinchliffe and Johnson, 1980). The limb phenotype in *ta<sup>3</sup>/ta<sup>3</sup>* embryos is associated with several different defects in the limb: there is a broadening of the developing limb buds; a lack of mesenchymal cell death and a defect in the mechanism that establishes antero-posterior polarity in the limbs (Ede and Kelly, 1964b; Hinchliffe and Ede, 1967; reviewed by Hinchliffe and Johnson, 1980; Izpisúa-Belmonte et al., 1992).

The absence of antero-posterior polarity in the digit pattern of *ta<sup>3</sup>/ta<sup>3</sup>* limbs is associated with a lack of discrete patterns of *Hoxd* gene expression in the developing limbs, which could account for the lack of specific digit identity (Izpisúa-Belmonte et al., 1992). It is not known how this altered pattern of *Hoxd* expression arises, but it could be
be due to a change in either signal distribution in the bud or the response of \(ta^3/ta^3\) limb cells to polarising region signals (Izpisúa-Belmonte et al., 1992). The work on Talpid\(^3\) described in Chapter 5 investigates different possible causes of the lack of antero-posterior polarity in the limbs of \(ta^3/ta^3\) embryos by mapping the distribution of polarising activity in \(ta^3/ta^3\) limbs, and analysing the response of \(ta^3/ta^3\) limb cells to a polarising signal. In addition, it documents the expression patterns of other genes known to be involved in signalling and response in the developing limb. Some of the work described in Chapter 5 of this thesis was carried out in collaboration with Dr. P. Francis-West and Dr. J-C. Izpisúa-Belmonte.
Chapter 2
Materials and Methods used in the analysis of the mouse mutant
Hypodactyly (Hd)

2.1 Mice
Mice carrying the Hd mutation on a (C57BL/6J x C3HeB/FeJLe-a) F1 background were obtained from the Jackson Laboratory, Bar Harbor, Maine, USA and maintained at University College London. Timed matings were set up between male and female heterozygotes (Hd/+). Embryos were staged according to the appearance of a vaginal plug. Matings between Hd/+ animals were checked in the morning for presence of a vaginal plug as evidence of coitus; midday on the day of plug appearance was taken as 0.5 days post coitum (dpc). Pregnant dams were culled on the required day of gestation by cervical dislocation or carbon dioxide asphyxiation.

2.2 Chick embryos
Fertilised wildtype chicken eggs (White Leghorn) were obtained from Poyndon Farm, Waltham Cross, Hertfordshire, U.K. Eggs were incubated at 38 ± 1°C and staged according to Hamburger and Hamilton (1951).

2.3 Adult morphology
2.3.1 External morphology of adult mice
Photographs of live adult mice were taken, and external morphology was studied with the aid of a dissection microscope and an attached camera after mice were culled.

2.3.2 Skeletal analysis of adult mice through differential staining of cartilage and bone
To allow visualisation of the skeleton, mice were fixed and processes for differential staining with alcian blue and alizarin red S which stain cartilage and bone respectively. The protocol used was that of C. Arnott (Kaufman, 1992). Specimens were fixed in
80% ethanol for a minimum of 24 hours before removing the skin and viscera and dehydrating in 96% ethanol for 24 hours. In order to remove any fat from the skeletons, they were placed in 100% acetone for 3 days, then rinsed for 2 hours in 96% ethanol. Skeletons were stained in a solution of 0.005 % alizarin red S and 0.015 % alcian blue in 70 % ethanol containing 1 % acetic acid for 6 hours, at 37 °C. After staining, skeletons were rinsed in 96% ethanol for 1 hour before being washed in running tap water for 2 hours prior to treatment with 1 % aqueous potassium hydroxide (KOH) for 24 hours. As soon as the skeletons were clearly visible, the soft tissues were cleared through a series of graded solutions of glycerine in 1% aqueous potassium hydroxide (20 % glycerine in KOH, 50 % glycerine in KOH, 80 % glycerine in KOH). Each step was carried out for approximately three weeks and specimens were stored, analysed and photographed in 100% glycerine.

2.3.3 Scoring of the defects seen in the hindlimbs of Hd/+ adult mice:
The external appearance of the hindlimbs of a sample population of 110 adult female mice exhibiting the Hd/+ phenotype as assessed in respect the severity of the hindlimb defect. Limbs were scored in terms of digit I morphology, soft tissue syndactyly between digits II and III, and the presence and morphology of the terminal claw on digit I.

Digit Score -

- 0 - total loss of digit I, no digital structure visible externally.
- -I - small, nodule-like digit I.

Soft tissue syndactyly score -

- 0 - no soft tissue syndactyly between digits II and III.
- 1/2 - soft tissue syndactyly approximately 1/2rd of the way up the interdigital space between digits II and III.
- 2/3 - soft tissue syndactyly approximately 2/3rds of the way up the interdigital space between digits II and III.
- full - full soft tissue syndactyly between digits II and III.
Digit I terminal claw score -
  0 - no terminal claw on digit I.
  r - round terminal claw on digit I.
  h - hooked terminal claw on digit I.

2.4 Embryonic Morphology

2.4.1 Preparation of embryonic material
Embryos were removed from the uterus under sterile conditions and placed into Minimum Essential Medium containing 10% foetal calf serum, 100 units ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin, 0.25 μg ml⁻¹ fungizone and 2mM L-glutamine. Embryos were dissected out from their surrounding membranes under a dissecting microscope prior to being fixed/processed for further analysis.

2.4.2 Phenotyping of embryos
Embryos were phenotyped and assigned a putative genotype on the basis of the appearance of their developing limbs; by working back from later stages of gestation to earlier stages embryos exhibiting abnormal limbs closely resembling those of the reported adult phenotypes have been identified. Embryos with narrow and somewhat pointed limb buds were assumed to be $Hd/Hd$ embryos and those with an apparent loss of tissue along the anterior margin of the hindlimb buds were assumed to be $Hd/+$. embryos.

2.4.3 External morphology of embryos
The external morphology of embryos was studied with a Zeiss dissecting microscope fitted with a camera or camera lucida. Photographs and drawings of embryos were made to record the embryonic morphology of the three phenotypes.

2.5 Analysis of limb polarising activity
In order to test for polarising activity, a block of posterior limb mesenchyme from wildtype or mutant limb buds was grafted to the anterior margin of a normal chick wing.
Manipulated embryos were allowed to develop for a further six days, after which they were fixed and stained for skeletal analysis.

Host embryos were prepared by windowing normal, stage 20 chick embryos, a hole was made with fine forceps in the extra-embryonic membranes surrounding the embryo around the area of the right wing bud to allow access to this bud. Using a sharpened tungsten needle, the ridge ectoderm along the anterior margin of the limb bud was carefully separated from the underlying mesenchyme to produce a loop in the ridge (Figure 2.1) under which blocks of mesenchyme were to be grafted.

Donor tissue was prepared from the limbs of 10.5 to 12.5 dpc +/-, \( H^d/ + \), and \( H^d/H^d \) embryos. Embryos were removed from the uterus under sterile conditions and placed into sterile growth medium (Appendix 1), their necks were severed and limb buds removed from the body using sharpened tungsten needles. Buds from individual embryos were kept separately from one another. The isolated buds were marked with a small cut along the anterior margin and pricked several times before being placed into 2% trypsin on ice at 4°C for 30 minutes in order to remove the ectoderm. After trypsinisation, buds were washed twice in ice-cold growth medium, and ectoderms were carefully removed by teasing them away from the underlying mesenchyme with the aid of tungsten needles and fine forceps. The mesenchymal cores of the limb buds were placed into fresh medium, and a block of mesenchyme was cut out from the posterior mesenchyme and stored in sterile medium on ice prior to grafting.

In order to graft the donor mesenchyme into the host wing buds, the isolated blocks of mesenchyme were gently pipetted into the host egg with a little sterile medium, being careful not to pipetted to vigorously and lose the piece of mesenchyme. The aim was to place the block of mesenchyme onto the body of the embryo just proximal to the right wing. The donor tissue was then eased into position under the prepared loop in the apical ectodermal ridge along the anterior margin of the hosts right wing bud. The egg was resealed, and the manipulated embryo allowed to develop for a further 6 days at 38 ±1 °C.
2.5.1 Fixation and staining of manipulated embryos

After 6 days incubation, manipulated embryos were removed from the egg and culled by decapitation. The trunks were washed in 1X phosphate-buffered saline (PBS) and lower limbs and guts removed prior to fixing overnight at room temperature in a solution of 5% trichloroacetic acid (TCA) in H\textsubscript{2}O. The next day, the fixative was removed and replaced with a staining solution of 0.1% Alcian Green in acid alcohol (70% ethanol, 0.1% hydrochloric acid). The embryos were stained for 24 hours at room temperature, after which they were washed overnight in acid alcohol overnight to remove stain differentially from non-cartilaginous structures. Prior to clearing, specimens were dehydrated in 100% ethanol overnight, and then cleared in methyl salicylate allowing visualisation of cartilaginous elements of the manipulated limb skeleton. Embryos were analysed and photographed with the aid of a dissection microscope and attached camera.
Figure 2.1
Diagram of a stage 20 chick wing bud with a loop cut in the anterior apical ectodermal ridge under which block of mesenchyme were grafted.
2.6 Analysis of gene expression in developing limbs

2.6.1 Plasmid DNA

Plasmids containing murine Fgf4, Shh, Hoxd11, and Hoxd13 cDNA were obtained from G. Martin (Fgf4), A. McMahon (Shh), and D. Duboule (both Hoxd11 and Hoxd13) and have all been described previously; Shh (Echelard et al., 1993), Fgf4 (Niswander and Martin, 1992), Hoxd11 (Izpisúa-Belmonte et al., 1991) and Hoxd13 (Dollé et al., 1991).

2.6.1.1 Mouse Fgf4 plasmid

The plasmid clone, K2, contains the full length coding region of K-FGF (~620 base pairs) derived from a PCR clone of Fgfk cDNA inserted into a bluescript vector (2959 base pairs). The vector-insert junctions are at the SmaI site which is non-preserved and the insert is cut out with EcoRI and BamHI. An antisense riboprobe for in situ analysis was produced by linearising with BamHI and transcribing with T3 RNA polymerase.

2.6.1.2 Mouse Shh plasmid

This plasmid clone, Hh - 16.1, contains a partial cDNA of murine Shh (~ 642 base pairs, with ~ 300 base pairs missing from 3' end and ~ 200 base pairs missing from 5' end) inserted into a bluescript BSII -SK plasmid vector (3.0 Kilobases) at the EcoRI site. An antisense riboprobe for in situ analysis was produced by linearising with HindIII and transcribing with T3 RNA polymerase.

2.6.1.3 Mouse Hoxd11 plasmid

This plasmid clone contains approximately 300 bp of the murine Hoxd11 cDNA inserted into pGEM1 (AccI/BamH1 fragment) and was provided by D. Duboule. Large scale plasmid preparations were carried out by Dr. J. Brown. The plasmid was linearised with HindIII and transcribed with T7 RNA polymerase to produce an antisense riboprobe for use in in situ analysis. Linearising with BamHI and transcribing with SP6 yields a sense RNA riboprobe.
2.6.1.4 Mouse Hoxd13 plasmid

This plasmid clone contains approximately 1300 bp of the murine Hoxd13 cDNA inserted into Bluescript/pGEM1 (HindIII fragment) and was provided by D. Duboule. Large scale plasmid preparations were carried out by Dr. J. Brown. The plasmid was linearised with PvuII and transcribed with T7 RNA polymerase to produce an antisense riboprobe for use in in situ analysis.

2.7 Elution of plasmid DNA from Whatmann filter paper

The Fgf4 and Shh plasmids were sent as spots absorbed onto Whatman filter paper. The disk of filter paper containing to DNA was placed into a sterile microfuge tube and 100μl of 10mM Tris pH7.5 was added, the tube vortexed and then left at room temperature for 5 minutes in order to rehydrate the DNA. The tube was then spun briefly in a bench top centrifuge and the supernatant removed and placed into a clean tube. This was then used to transform competent E. coli DH5α cells.

2.8 Transformation of competent cells

Competent cells were prepared from the lab stock using the method of Sambrook et al., (1989). 5μl of circular plasmid DNA at a concentration of 0.01μg/μl was added to 100μl of competent DH5α cells and incubated on ice for 1-2 hours. The mixture was then heat shocked at 42°C for 2 minutes to facilitate uptake of plasmid DNA by the bacterial cells, and placed on ice for 2 minutes before adding 1.0 ml of LB-medium. Cultures were incubated at 37°C for 45 minutes, after which they were briefly centrifuged, most of the supernatant was removed and the bacterial pellet resuspended in the remaining medium. The transformed DH5α cells were plated on LB-agar plates containing 0.1 mg/ml ampicillin and incubated overnight at 37°C to select for bacteria which had taken up the plasmid DNA. The next day single bacterial colonies were visible on the plates. As a positive and negative control, DH5α cells were also transformed with 5 ng of pBluescript plasmid DNA (1.0 ng/μl) or not transformed at all and plated out on LB-agar plates containing 0.1 mg/ml ampicillin and incubated overnight at 37°C. The
positive control gave multiple single colonies, whereas the negative control showed no colonies at all.

2.9 Small-scale preparation of plasmid DNA

Single bacterial colonies was picked from overnight agar-plate cultures and used to inoculate 5.0 ml of LB-medium containing 0.1 mg/ml ampicillin in a 15.0 ml screw capped Universal tube. The inoculated medium was incubated overnight at 37°C with vigorous shaking. After overnight incubation, 1.5 ml of the culture was placed into a microfuge tube and spun at 13,000 rpm for 1 minute at 4°C. The supernatant was removed and discarded leaving the bacterial pellet as dry as possible. The pellet was resuspended with vortexing in 100 μl of ice-cold solution I (Appendix I), 200 μl of freshly prepared solution II was added and the contents of the microfuge tube mixed thoroughly by inverting the tube five times forming a viscous bacterial lysate. 150μl of ice-cold solution III was added and the tube gently vortexed in an inverted position for 10 seconds to disperse solution III through the viscous bacterial lysate. This mixture was then incubated on ice for 5 minutes then centrifugation at 13,000 rpm for 5 minutes. The supernatant containing the DNA was transferred to a clean microfuge tube and the DNA was cleaned by adding an equal volume of phenol:chloroform and mixing thoroughly by vortexing. The tube was spun at 13,000 rpm for 2 minutes, the supernatant transferred to a fresh tube and the double-stranded DNA precipitated by addition of 1/10 volume 3M sodium acetate pH 5.2 and 2.5 volumes of ethanol at -20°C. The precipitated DNA was pelleted by centrifugation at 13,000 rpm for 15 minutes at 4°C, the resulting supernatant was removed by aspiration and all drops of residual fluid removed from the sides of the tube. The pellet was rinsed with 1.0 ml of 70% ethanol at 4°C, all supernatant was removed as previously described and the pellet air dried at room temperature for 10 minutes. The DNA pellet was redissolved in 50.0 μl of TE (pH 8.0) containing DNAase-free pancreatic RNAase (20 μg/ml) with brief vortexing and stored at -20°C.
2.10 Measuring the concentration of a DNA solution

Using a spectrophotometer, the optical density of DNA solutions obtained from the small scale preparation of plasmid DNA were measured at wavelengths of 260 and 280. The average sample $\frac{260}{280}$ ratio should be ~ 1.8, and the concentration in $\mu$g/$\mu$l is the reading at OD$_{260} \times 10$.

2.11 Restriction digestion analysis of mini prep' plasmid DNA

In order to check that the correct DNA has been synthesised and isolated from the mini prep's, plasmid DNA was digested with the appropriate restriction enzymes and recommended buffer in order to obtain linearised DNA and to release the insert to check it's size. 10 $\mu$g of DNA was digested at the appropriate temperature for 2 hours with 10 units of restriction enzyme, in a total reaction volume greater than 10 times the volume of the enzyme used. Digests were analysed on horizontal agarose gels containing between 0.7 and 1.0% (w/v) agarose in 1X TBE (Tris-Borate-EDTA) with 0.5 $\mu$g/ml ethidium bromide. A 1$\mu$l aliquot of the digestion reaction was mixed with 9 $\mu$l H$_2$O and 1 $\mu$l of gel loading dye and loaded into a well of the gel. ~ 0.1$\mu$g of DNA marker (1Kb marker or HI HindIII) was also loaded into an adjacent well in the gel, and the gel were electrophoresed at approximately 10 volts/cm in 1X TBE (Tris-Borate-EDTA) buffer. DNA fragments were visualised using an ultraviolet transilluminator and photographed with Polaroid film (Type 667). The size of the insert and plasmid vector DNA can be seen from the gel, and if in agreement with the correct sizes for that clone, the remainder of the mini prep' culture was used to inoculate a large culture in order to obtain a large quantity of DNA.

2.12 Large-scale preparation of plasmid DNA

250 ml of LB-medium containing 0.1 mg/ml ampicillin was inoculated with 3.5 ml of mini prep cultured (prepared as described above) and incubated overnight at 37°C with vigorous shaking. The culture was poured into a 500 ml centrifuge bucket and spun at 4000 rpm at 4°C for 15 minutes. The supernatant was removed and the tube inverted to allow excess fluid to drain away, then the pellet was resuspended in 100 ml of ice-cold
STE. This was spun at 4000 rpm at 4°C for 15 minutes to pellet the bacterial cells, the
supernatant was removed and excess fluid allowed to drain away. The bacterial pellet was
resuspended in 18.0 ml of solution I, 2.0 ml of freshly prepared lysozyme (10 mg/ml in
10mM Tris HCl pH 8.0) and 40.0 ml of freshly prepared solution II were added. The
contents of the centrifuge bucket were thoroughly mixed by gently inverting the bottle
several times. 20.0 ml of ice-cold solution III was added, the solutions mixed by shaking
to allow a white precipitate to form, and the mixture centrifuged at 400 rpm at 4°C for 15
minutes in order to pellet the precipitate. The supernatant containing the DNA was
removed and placed into a fresh centrifuge tube, 63.0 ml of isopropanol was added and
the tube stored at room temperature for 10 minutes. Nucleic acids were recovered by
centrifugation at 5000 rpm at room temperature for 15 minutes. The supernatant was
carefully decanted off the pelleted DNA, and the bottle inverted to allow all excess fluid to
drain away. The pellet was rinsed with 70% ethanol and then allowed to air-dry at room
temperature. The dried pellet was dissolved in 3.0 ml of TE pH 8.0.

2.13 Purification of plasmid DNA through caesium chloride gradients
The 3.0 ml of resuspended plasmid DNA was purified through caesium chloride
gradients; 3.0 g of caesium chloride was added and mixed at room temperature until all
the solid had dissolved. 0.24 ml of 10 mg/ml ethidium bromide in H2O was added to the
DNA/CsCl solution, this was then centrifuged at 8000 rpm at room temperature for 5
minutes. The clear red solution was transferred into a Beckman ultracentrifuge tube and
filled with 1:1 (w/v) solution of CsCl/Te pH 8.0. A 1/100 dilution of Triton X100 was
added to the tube to compact the RNA, and the tube was heat sealed with a metal cap. The
tube was centrifuged at 85 K at 20°C for 7.5 hours. Two bands of DNA in the centre of
the gradient are visible, the upper band consists of linear bacterial (chromosomal) DNA
and nicked circular plasmid DNA; the lower band consists of closed plasmid DNA. The
depth red pellet on at the bottom of the tube consists of ethidium bromide/RNA
complexes. The material at the top of the tube is protein. The bands of DNA were
collected by inserting a 21 gauge hypodermic needle into the top of the tube to allow air to
enter the tube. In order to minimise the chance of contamination of plasmid DNA, the top
band of chromosomal DNA was collected first; tape was placed along the length of the
tube in order to prevent leakage, an 18 gauge needle was inserted, bevelled edge facing
up, so that the tip is positioned just below the DNA band. The DNA was collected in a
tube, and the end of the needle sealed with a ball of plasticine. Leaving this needle in
place, a second 18 gauge needle was inserted into the tube with the bevelled edge up and
the point just bellow and parallel to the lower band of DNA and the DNA was collected
into a clean tube.

2.14 Removal of ethidium bromide from DNA purified by equilibrium
centrifugation in CsCl-Ethidium bromide gradients

An equal volume of butan-1-ol was added to the DNA/ethidium bromide solution, and
the two phases mixed by vortexing. The tube was then centrifuged at 6500 rpm at room
temperature for 3 minutes. The lower phase was transferred to a clean tube and the
process was repeated 4 times until the ethidium bromide has visibly been removed
(solution no longer looks pink). Overnight dialysis was carried out to remove the CsCl;
the DNA/CsCl solution was pipetted into pre-soaked, autoclaved dialysis tubing and both
ends sealed with dialysis clips. This was then placed into a beaker containing 500 ml of
TE pH 8.0 at 4°C. After dialysis, the DNA solution was removed from the dialysis tube
and placed into a clean microfuge tube. 1/10 volume of 13M sodium acetate and 2
volumes of 100% ethanol were added and the mixture incubated at -20°C for 3 hours.
The mixture was spun at 13000 rpm at 4°C for 10 minutes, the precipitated DNA was
dissolved in 1.0 ml of TE pH 8.0. The OD_{260} of the final DNA solution was measured,
and the concentration of the DNA calculated. The concentration of the plasmid DNA was
calculated as above, and then stored in aliquots at -20°C. The DNA was analysed by
restriction digestion and agarose electrophoresis, as described above.

2.15 Synthesis of Digoxigenin-labelled riboprobes

A standard labelling reaction was carried out in which 8.5 µl of sterile RNAase-free
water, 4 µl of 5x transcription buffer, 2.0 µl of 0.1 M dithiothreitol, 2 µl of 10x
digoxigenin-labelled nucleotide mix, 1.5 µg of linearised plasmid DNA, 0.5 µl of
placental ribonuclease inhibitor (100 U/µl) and 1.5 µl of RNA polymerase (SP6, T7 or T3) were added to a microfuge tube on ice. The reagents were mixed and centrifuged briefly prior to incubation at 37°C for 2 hours. After 2 hours, a 1 µl aliquot of the synthesis reaction was removed and run out on an 0.7% (w/v) agarose in 1X TBE (Tris-Borate-EDTA) gel containing 0.5 µg/ml ethidium bromide to estimate the amount of probe synthesised. An RNA band ~10-fold more intense than the plasmid band should be seen, indicating that ~15 µg of probe has been synthesised. 2 µl of RNAase-free DNAase I (1 U/µl) was then added and the tube incubated for a further 15 min at 37°C. The reaction was stopped on ice by adding 2 µl of 0.2M EDTA solution, pH 8.0, then 130 µl of RNAase-free distilled water, 50 µl 10M ammonium acetate, and 400 µl 100% ethanol were added. The tube was then incubated at -20°C for 30 minutes. The tube was spun for 10 minutes in a microfuge, the pellet washed with 70% ethanol, then air dried. The pellet was then redissolved in 150 µl of ice-cold water, 50 µl of 10M ammonium acetate and 400 µl of 100% ethanol were added, the tube incubated at -20°C for 20 minutes, then spun in a microfuge for 20 minutes. The pellet was washed with 70% ethanol and the pellet air dried. The pellet was then redissolved in ice-cold TE at ~0.1 µg/µl and stored at -20°C to -70°C. Probes were diluted 1-5:1000 in hybridisation mix for in situ analysis.

2.16 Preparation of embryonic material for non-radioactive wholemount in situ hybridisation

Embryos were removed from the uterus and placed into sterile DEPC-treated, phosphate buffered saline (PBS-depc) before being dissected out from their surrounding membranes and phenotyped. Putative Hd/Hd and Hd/+ mutants and +/- littermates were identified on the basis of their limb morphology as previously described (Robertson et al, 1996; Chapter 3). Embryos were considered to be putative Hd/Hd embryos if they had narrow, pointed limb buds as opposed to the expected paddle shaped limb buds from 11.5 dpc onwards. Embryos were washed in ice cold PBS-depc prior to being fixed overnight at 4°C in fresh 4% paraformaldehyde in PBS-depc. After fixation embryos were dissected into two halves, left and right, by splitting them down the midline using a sharpened
tungsten needle and fine forceps. This allowed for in situ analysis of two different genes within the same embryo. After fixing, embryos were washed twice in ice-cold PBT, dehydrated through a series of 25%, 50%, 75% and 2x in 100% Methanol: PBS for 5 minutes each and stored at -20°C in 100% methanol for up to one month.

2.17 Non-radioactive in situ hybridisation with digoxigenin labelled probes

Non-radioactive wholemount in situ analysis of embryos was carried out using two different protocols according to Nieto et al. (1996) and Rosen and Beddington (1993). These two protocols use different pretreatment methods to permeabilise the tissue and different hybridisation conditions. It is noticeable that for the tissue that I have used, these protocols have resulted in different levels of efficiency of hybridisation and levels of non-specific backgrounds. I have found that the Nieto et al. (1996) protocol gives optimal results as the levels of non-specific background are low. However, irrespective of which protocol is used, there is a difference in the levels of non-specific background at different developmental stages and depending on how long the embryos were stored prior to hybridisation.

Important notes on all washes:

In situations where individual embryos are to be probed, washes and hybridisations are carried out with small volumes of solutions in sterile 2 ml, round bottomed Eppendorfs or cryotubes. When changing solutions, a small amount of fluid is always left in the bottom of the tube to avoid embryos drying out. Unless otherwise stated, all washes are carried out with gentle rocking.

2.17.1 The following protocol is according to Rosen and Beddington, (1993):

Pretreatment:

Embryos were rehydrated on ice through 75%, 50%, 25% methanol:PBS before washing them three times for 5 minutes at room temperature in PBS containing 0.1% Tween 20 (PBT). Embryos were permeabilised to allow optimal probe penetration into the tissue by washing three times for 30 minutes each at room temperature in RIPA (150
mM NaCl, 1% NP-40, 0.5% sodium deoxycholate (DOC), 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 8.0; use autoclaved solutions of everything except DOC, SDS and NP-40). Embryos were then refixed for 20 minutes at room temperature in 4% paraformaldehyde, 0.2% glutaraldehyde in PBS to prevent disintegration of tissues during later stages of the protocol. After refixation, embryos were washed twice in PBT prior to prehybridisation.

Prehybridisation and Hybridisation:
The PBT was replaced with a 1:1 solution of hybridisation buffer:PBT and embryos were allowed to sink to the bottom of the tubes. The 1:1 solution was then replaced with 0.5-1.0 ml of hybridisation buffer containing 100 µg/ml tRNA and 100 µg/ml sheared, heat denatured herring sperm DNA (both the tRNA and herring sperm DNA should be phenol extracted and RNAase-free) and embryos were prehybridised at 70°C for 1-5 hours. After prehybridisation, the solution was removed and replaced with 0.5-1.0 ml of fresh hybridisation solution containing around 0.1 µg/ml of digoxygenin-labelled riboprobe. Embryos were hybridised overnight at 70°C in a heating block placed on a rocking platform.

Posthybridisation Washes:
After hybridisation, embryos were washed twice in fresh, prewarmed hybridisation solution for 5-10 minutes at 70°C, before being washed twice for 5 minutes at 65°C in a solution of 2X SSC, 50% formamide, 0.1% Tween-20. Following this, the embryos were subjected to three 30 minute washes at 65°C in the same solution. Embryos were allowed to cool to room temperature, then washed three times at room temperature with 1X TBST (dilute from 10X stock of TBST).

Antibody Conjugate binding:
Embryos were blocked for 1-3 hours in 10% heat-inactivated lamb serum/TBST at room temperature. The serum was removed and replaced with 0.5-1.0 ml of 10% heat-
inactivated lamb serum/TBST containing a 1/5000 dilution of anti-digoxigenin antibody conjugate. Embryos were incubated in this solution overnight at 4°C with gentle rocking.

Post-Antibody Conjugate Washes and Colour Detection:

The antibody solution was removed and embryos were washed three times at room temperature in TBST (5 minutes each), followed by three 1 hour washed in the same at room temperature. Prior to colour detection embryos were washed three times for 10 minutes each with freshly made alkaline phosphatase buffer (APB: 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween-20, 100 mM Tris pH 9.5; make fresh from stocks). The APB was removed and replaced with 0.4-0.5 ml of colour detection solution, either AP substrate precipitation solution (Boehringer Mannheim) or APB containing 4.5μl/ml NBT and 3.5μl/ml BCIP/X-phosphate. The colour detection was carried out in the dark at room temperature for anything from 30 minutes to several hours (depending on the strength of the probe used and the abundance of the RNA species being detected; more abundant species will be detected faster than less abundant ones). The colour reaction was stopped by rinsing the embryos three times in PBT, 1 mM EDTA, before being fixed and stored in 4% paraformaldehyde at 4°C.

2.17.2 The following protocol is according to Nieto et al., (1996):

Post-fixation:

After fixation, embryos were washed twice in ice-cold PBS, using tungsten needles the heads were removed and embryos split into two by carefully cutting down the midline. Embryos were then rinsed once in PBS, 0.1% Triton-X 100 (PBT) and dehydrated by washing for 10 minutes each on ice in a graded methanol series diluted in PBT (25% methanol, 50% methanol, 75% methanol). Finally embryos were washed twice for 10 minutes in ice-cold 100% methanol and stored at -20°C for up to three months.

Pretreatment:

Pretreatment of embryos was carried out by rehydrating through a series of graded methanol in PBT (75% methanol, 50% methanol, 25% methanol), washes were for 10
minutes each on ice with rocking, followed by two washes in PBT at room temperature. Treatment in a solution of 20µg/ml proteinase K in PBT was carried out for 5-20 minutes at room temperature, with gentle rocking. Embryos were then rinsed twice in PBT before being refixed in freshly prepared 4% PFA, 0.2% glutaraldehyde in PBT at room temperature for 20 minutes.

Prehybridisation and Hybridisation:
After refixation embryos were rinsed twice in PBT, the PBT was removed and replaced with 0.5 ml of prehybridisation solution (Appendix 1) and embryos were allowed to sink to the bottom of the tube. Embryos were then prehybridised in 0.5 - 0.75 ml of fresh prehybridisation solution at 65°C with gentle rocking for 2 hours to overnight. Prehybridised embryos were stored at -20°C for up to 6 months. Embryos were hybridised overnight at 65°C with gentle rocking in hybridisation solution containing approximately 0.1 - 0.2 µg/ml of digoxygenin-labelled riboprobe.

Posthybridisation Washes:
Post-hybridisation washes of moderate stringency at 65°C were carried out with gentle rocking to remove any unhybridised probe; three 20-30 minute washes in 2X SSC, 0.1% CHAPS, followed by three 20-30 minutes washes in 0.2X SSC, 0.1% CHAPS. Embryos were rinsed twice in KTBT (see Appendix 1) at room temperature with gentle rocking prior to blocking in a solution of 20% sheep serum in KTBT (heat denatured at 70°C for 30 minutes and quenched on ice prior to use) for 2-3 hours at 4°C.

Antibody Conjugate binding:
Embryos were incubated overnight at 4°C with gentle rocking in a 1/2000 dilution of anti-DIG antibody (Boehringer) in 20% sheep serum in KTBT.

Post-Antibody Conjugate Washes and Colour Detection:
After overnight antibody incubation, embryos were washed six times for 1 hour at room temperature in KTBT with gentle rocking, then overnight in the same at 4°C. Prior to
colour detection, embryos were washed twice for 15 minutes at room temperature in NTMT (Appendix 1). Colour detection was carried out at room temperature in the dark in a solution of NTMT containing 4.5μl/ml of stock nitroblue tetrazolium salt (NBT; Appendix 1) and 3.5μl/ml of stock 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Appendix 1). Embryos were left for at least 30 minutes, then periodically checked for signal. The colour reaction was stopped by washing several times in NTMT then refixing in 4% PFA in PBT.

2.18 Analysis of stained embryos

Embryos were analysed with the aid of a Zeiss dissecting microscope, and photographs were taken on Kodak T64 film.

2.19 Estimation of area of distal limb that expresses the genes analysed

In order to estimate the percentage of the total distal limb area of Hd mice that expresses Hoxd11, Hoxd13, and Shh, individual limbs were dissected away from the body walls of the embryos and re-photographed at a magnification of x32 onto Ilford FP4 black and white film. Photographed limbs were traced and the area of expression marked, these images were then scanned using a Hewlett Packard deskjet scanner and digitised and analysed using NIH image analysis 5.8 in order to obtain an estimation of the area of each limb bud expressing the specific genes detected.

2.20 Cellular Analysis of Hd mutants

2.20.1 Analysis of mesenchymal cell death

Cell death was assessed by Nile blue sulphate staining. Embryos were dissected out from their membranes in Tyrodes solution prewarmed to 37°C then sacrificed by severing the spinal chord with sharpened tungsten needles. After two quick rinses in prewarmed Tyrodes, embryos were stained in a solution of 0.005% Nile blue sulphate in Tyrodes at 37°C for 10-15 minutes. After staining, the embryos were rinsed quickly in Tyrodes at 37°C then overnight at 4°C. The following day limbs were then photographed using
Kodak T64 slide film or drawn with the aid of a dissecting microscope and attached camera or camera lucida.

2.20.2 Skeletal analysis of the limb of *Hd* mutant embryos

The developing skeletons of mice were analysed by staining with alcian green and clearing in either methyl salicylate or glycerol. Embryos to be cleared in methyl salicylate were removed from their extra-embryonic membranes, washed in 1X phosphate-buffered saline (PBS) and fixed overnight in a solution of 5% trichloroacetic acid (TCA). The next day the fixative was removed and replaced with a staining solution of 0.1% Alcian Green in acid alcohol (70% ethanol, 0.1% hydrochloric acid). The embryos were stained for 24 hours at room temperature, after which they were placed in acid alcohol overnight to remove stain differentially from non-cartilaginous structures. Prior to clearing, the embryos were dehydrated in 100% alcohol then cleared in methyl salicylate allowing visualisation of cartilaginous elements of the developing skeletal system. Embryos were analysed and photographed with the aid of a dissection microscope and attached camera. Methyl salicylate is a volatile and harmful substance and so a second method devised by A. Sheasby (personal communication) was carried out that uses glycerol as the clearing agent.

Embryos were fixed in 5% TCA or 80% ethanol, dehydrated for an hour in 100% ethanol before being washed in acetone for 24 hours to remove any fat from the skeletons. The next day embryos were rinsed in 100% ethanol for an hour, stained in 0.1% Alcian Green in 70% ethanol, 0.1% hydrochloric acid for three hours, then washed in running tap water for an hour. Embryos were treated with 1% aqueous potassium hydroxide (KOH) for an hour and cleared through a series of 20%, 50%, and 80% glycerol in KOH before being stored in 100% glycerol. Again, embryos were analysed and photographed with the aid of a dissection microscope and attached camera.

To allow visualisation of the skeleton of older mice, they were fixed and processed for differential staining with alcian blue and alizarin red S which stain cartilage and bone respectively (as described above).
2.20.3 Cell culture of limb mesenchymal cells in high density micromass cultures

Limb buds were removed from embryos and washed in sterile growth medium (Appendix 1). Isolated buds from each embryo were transferred into separate dishes containing ice-cold 2% trypsin in calcium and magnesium free HBSS, and incubated on ice for 30 minutes. After trypsinisation, limbs were placed into ice-cold growth medium and stored on ice for 10 minutes before teasing away the ectoderm from the underlying limb mesenchyme with sharpened tungsten needles. After removal of the ectoderm, limb bud mesenchyme was placed into sterile microfuge tubes containing 1.0 ml of growth medium. Mesenchymal cells were then dissociated into a single cell suspension by gentle pipetting of the contents of the tube up and down a sterile glass pipette. The number of cells in each of the tubes was counted using a haemocytometer and the cell suspension subsequently adjusted to 2x10^7 cells/ml by centrifugation and addition the appropriate volume of culture media (CMRL containing 10% foetal calf serum, 100 units ml^-1 penicillin, 100μg ml^-1 streptomycin, 0.25μg ml^-1 fungizone and 2mM L-glutamine). Individual micromass cultures were set up in separate wells of NUNC 4-well culture dishes; 10μl of the cell suspension, containing 2x10^5 cells, was pipetted as a single drop into the centre of each well. The mesenchymal cells contained in these 10μl drops were allowed to settle and adhere to the tissue culture dish by incubating for 1-1.5 hours at 37°C. The wells of the culture dishes were then flooded with 300 μl of culture medium and incubated for six days at 37°C in 5% CO₂. Cultures were fed daily with fresh or conditioned medium.

2.20.4 Preparation of conditioned medium and treatment of cultures with growth factors

Conditioned medium was prepared from cultures of 12.5 dpc putative +/-, Hd/+, and Hd/Hd mesenchymal cells. Cultures were set up as normal. However after 24 hours of incubation, the medium was removed and mixed with fresh medium to a concentration of 50-75%, this was then used to feed other cultures. Micromass cultures of 12.5 dpc limb bud mesenchyme were set up as described, and fed daily with medium containing
10 ng/ml of TGFβ1 (obtained from P. Martin), FGF2 or FGF4 (obtained from R and D Systems).

2.20.5 Fixing and staining of micromass cultures
Culture medium was removed, each well rinsed with 500μl of PBS, and then 500μl of fresh, filtered 4% paraformaldehyde in PBS (PFA) was added. Cultures were fixed overnight at 4°C, after which the PFA was removed and cultures were washed in filtered PBS. In some instances, cultures were photographed at high magnification using an inverted phase contrast microscope prior to staining for cartilage.

Cultures were stained by replacing the PBS with a staining solution of 1% alcian blue in 0.1N hydrochloric acid for 4 hours at room temperature. The staining solution was removed and stained cultures were washed three times in fresh, filtered PBS, before being dehydrated through a series of 50%, 70%, 90%, and 100% alcohol (5 minutes each). Cultures were photographed under a dissecting microscope and then stored under 100% glycerol at 4°C.

2.20.6 Estimation of the amount of cartilage produced in culture
To estimate the amount of cartilage produced by each of the cultures, the number of cartilage nodules in each culture as shown by alcian blue staining was counted. The number of nodules in cultures originating from the same embryo were added together and then divided by the number of cultures to give an average value for that embryo.

2.20.7 Scanning Electron Microscopy
9.5-14.5 dpc Hd/Hd, Hd/+ , and +/- embryos were washed in 1X PBS after dissection from the uterus and removal of extra-embryonic membranes. Embryos were then rinsed in filtered Tyrodes solution and fixed and stored at 4°C in modified Tyrodes (Tyrodes containing 1% glutaraldehyde). After post-fixation in 1% osmium tetroxide in 0.1M phosphate buffer for 1 hour at 4°C, the specimens were dehydrated in graded alcohols and rinsed thoroughly in amyl acetate. Specimens were then subjected to critical point drying, mounted on metal studs and sputter coated with gold particles. Prior to sputter
coating, the distal tips of several limb buds were removed in order to see the mesenchyme, by tapping the distal end of the limb bud with a tungsten needle. These specimens were then sputter coated with gold particles as normal. All specimens were analysed using a Hitachi S-530 scanning electron microscope and photographed onto medium format, Ilford FP4 black and white film.

2.20.8 Araldite Histology

Embryos or isolated limbs were fixed in half strength Karnovsky's (Appendix I) overnight at 4°C. Prior to embedding in araldite resin, specimens were washed for 20 minutes each in 50% and 70% alcohol, then stained for 45 minutes in 0.5% alcian green in acid alcohol, washed for 30 minutes in 90% alcohol, then washed four times in 100% alcohol each for 15 minutes. Once fully dehydrated, specimens were cleared in propylene oxide (3 X 10 minute washes) then soaked for 45 minutes in 50:50 propylene oxide: araldite resin. Specimens were then rotated overnight in araldite resin at room temperature before being embedded in fresh resin and placed at 60°C overnight to harden the resin. Limbs were sectioned at 1μm using an ultracut microtome, approximately 5 out of every 10 sections were taken until the limbs were fully sectioned. Photographs were taken using a Zeiss Axiovert 405M microscope with an inbuilt camera and Ilford FP4 film.
Chapter 3
An introduction to the mouse mutant hypodactyly (Hd): a morphological study of adult and embryonic limb phenotypes, and a molecular analysis of expression of genes known to be important in patterning the vertebrate limb

3.1 The mouse mutant hypodactyly (Hd)
Hypodactyly (Hd) is a semidominant, lethal mutation in the mouse that is characterised by reduction or loss of digits (Hummel, 1970; Hummel and Chapman, 1963). Homozygotes (Hd/Hd) usually die in utero, but those surviving to term are infertile and have a single digit on all four limbs. Heterozygotes (Hd/+) are less severely affected; they are fully viable and fertile and exhibit a reduction of hindlimb digit I (Hummel, 1970). Hd maps to a region of murine chromosome 6 close to the Hoxa cluster of vertebrate Hox genes (Mock et al., 1987). Little is understood about how the observed defects arise, however very recent work has identified a 50 base pair deletion in a member of the Hoxa cluster, Hoxa13, as the molecular basis of the Hd mutation (Mortlock et al., 1996).

3.2 Appearance of normal mouse limbs
3.2.1 Adult limbs
Mouse limbs can be divided into three regions which I will refer to as proximal, middle, and distal (also known as stylopod, zeugopod, and autopod respectively). In the forelimb the proximal region includes the shoulder girdle and humerus, middle region includes radius, ulna and proximal carpals, and the distal region includes the small and long bones of the hands (Figure 3.1). In the hindlimb the proximal region includes the pelvic girdle and femur, middle region includes the tibia, fibula and proximal tarsals, and the distal region includes the small and long bones of the feet (Figure 3.1).

Previous studies have indicated that limb defects caused by the Hd mutation are confined to the hands and feet which normally consist of five digits, digits I, II, III, IV, and V, with digit I being most anterior and digit V most posterior. Digit I in the forelimb
is naturally truncated and its external appearance is much reduced when compared to other forelimb digits. Both hands and feet are covered dorsally with hairs and ventrally with soft tissue walking pads; 5 digital pads on the hands and 5 digital, 4 metatarsal, and 2 tarsal pads on the feet (Kaufman, 1992).

3.2.2 Morphology of limbs during murine development

In the mouse, forelimb buds are first evident at 9 - 9.5 days post coitum (dpc) with hindlimb buds appearing a day later, at 10 - 10.5 dpc (Kaufman, 1992). Throughout the early stages of limb development, forelimb development is approximately 0.5-1.0 day in advance of the hindlimbs. By 11.5 dpc, the limb buds are relatively symmetrical in shape and consist of a core of undifferentiated mesenchyme encased in an ectodermal jacket. The apical ectodermal ridge is present along the antero-posterior distal margin of the buds. A prominent marginal vein is located in the subapical mesenchyme in both forelimbs and hindlimbs. At 12.5 dpc, the distal regions of both forelimb and hindlimb buds expand out into polygonal structures which are known as the developing hand- and footplates respectively. In the forelimb it is clear where digits will subsequently differentiate since the surface of the handplates are corrugated due to the presence of ridges, known as digital rays. These digital rays are separated by distinct interdigital zones. By 13.5 dpc, hindlimb digital rays are clearly evident and interdigital zones are highly indented. In the handplates, interdigital zones are more indented than in the footplates and, by 14.5 dpc, little of the webbing between forelimb digits remains.

The developing hindlimb is relatively symmetrical throughout its development, and at 14.5 dpc digit I is only slightly shorter than digit V. The hand plate however gradually becomes asymmetrical from 14.5-15.0 dpc onwards; digit I develops to a lesser degree than the other digits and by 16.5 dpc, the forelimb digit I is significantly smaller than other forelimb digits. In this sense, forelimb digit I appears somewhat vestigial compared to the others and the nail that develops has a flat plate rather than distinct claw as seen on the other digits.
3.2.3 External appearance of limbs during development is correlated with final limb morphology

Some vertebrate limb mutants have alterations in size and shape of developing limb buds, and these changes are thought to influence the final limb pattern (reviewed in Hinchliffe and Johnson, 1980; Johnson, 1986). Increases in limb bud size invariably result in development of supernumerary digits (polydactyly) as in polydactylous mouse mutants such as Strong's Luxoid ($L^{D}$), recombination induced mutant 4 ($Rim4$), hemimelic extra toes ($Hx$) and extra toes ($Xr$) (Green, 1989; Johnson, 1967; reviewed in Johnson, 1986; Masuya et al., 1995). Decreases in limb bud size are associated with digit reduction or fusion, possibly due to a reduction in the amount of space, or amount of cells available for digital development. Digital blastemas can become squashed together resulting in fusion of digits (syndactyly) as in the mouse mutation syndactylism ($sm$) (Grüneberg 1956), or they can be lost and/or reduced resulting in the subsequent loss or reduction of digits, as seen for example in the mouse mutant postaxial hemimelia ($px$) (Searle, 1964) and limb deformity ($ld$) (Green, 1968; Kleinbrecht et al., 1982; Woychik et al., 1985; Zeller et al., 1989). $px$ is an autosomal recessive mutation causing a post-axial mesenchymal deficiency in the developing fore- and hindlimb buds. This leads to a complete absence of digits IV, V, and occasionally III, corresponding carpals, metacarpals, and a reduction or absence of the ulna in the forelimbs, and a reduction of digit V, syndactyly, and reduction of the fibula in the hindlimbs (Searle, 1964). $ld$ is an autosomal recessive mutation causing a reduction in the distal antero-posterior limb axis giving limb buds a more ‘pointed’ morphology than normal, this leads to fusion of the radius/ulna and tibia/fibula, loss of digits and syndactyly of the bones of hands and feet (Zeller et al., 1989).

3.3 Outgrowth and patterning of the limb

Previous experiments have shown that specification of digits requires interactions between the apical ectodermal ridge and cells of the polarising region and progress zone (see Chapter 1). Polarising activity of mesenchymal cells can be measured by grafting them ectopically under a loop in the anterior apical ectodermal ridge of a chick wing bud.
Cells with high polarising activity will induce full mirror-image digit duplications, whereas cells with low polarising activity will induce only partial duplications (Hinchliffe and Sansom, 1985; see Chapter 1). Inappropriate distribution of polarising activity in the limb can result in patterning defects; polydactyly can result from ectopic polarising activity, as in \( Is^{P} \), \( Rim4 \), \( Hx \) and \( Xr \) (Chan et al., 1995; Masuya et al., 1995), whereas loss of digits can result from a reduction of polarising activity as in limb deformity (\( ld \)) (Haramis et al., 1996). Limb defects observed in \( Hd/Hd \) embryos occur relatively late during limb development, and are suggestive of defects in antero-posterior patterning. It is conceivable that there may be a reduction of polarising activity in posterior limb mesenchyme during specification of the hand and footplates. Maintenance of polarising activity in the polarising region requires interactions with the apical ectodermal ridge. \( Shh \) and \( Fgf4 \) are major signals of the polarising region and apical ectodermal ridge, and a molecular feedback loop between these two genes maintains the functioning of the polarising region and apical ectodermal ridge (see Chapter 1).
Figure 3.1
3.3.1 Shh and Fgf4

In the developing mouse limb, Shh is first expressed in a small group of posterior forelimb bud mesenchymal cells at 9.75 dpc (Echelard et al., 1993; Chang et al., 1994). By 10.5 dpc, both forelimb and hindlimb buds show strong expression in posterior, distal mesenchyme, and by 11.5 dpc, posterior mesenchymal expression of Shh is restricted to hindlimb buds. No expression is detected in the limb buds beyond 12.5 dpc (Echelard et al., 1993). Fgf4 is differentially expressed throughout the apical ectodermal ridge; in the mouse it is first expressed at 10.0 - 10.5 dpc in the posterior apical ectodermal ridge of the forelimb bud. As development proceeds, expression levels gradually reduce, and by 12.0 dpc expression is no longer detectable in the ridge. Hindlimb expression is similar to that in the forelimb, but is seen approximately 0.5 - 1.0 days later (Niswander and Martin, 1992).

As mentioned in the previous section, polydactyous mutants 1stD, Rim4, Hx and Xr exhibit ectopic polarising activity, and this is known to be associated with ectopic expression in the anterior limb of both Shh and Fgf4 (Chan et al., 1995; Masuya et al., 1995). In comparison, reduction of polarising activity observed in the ld mutant, which leads to loss of limb structures, is associated with a premature termination of expression of Shh and Fgf4 in posterior limb mesenchyme and apical ectodermal ridge respectively (Haramis et al., 1996). A reduction in Shh expression is also associated with loss of posterior digits in the Wnt7a knockout mouse (Parr and McMahon, 1995). Thus, misexpression of Shh and Fgf4 in vertebrate limb mutants provides further evidence for the involvement of these genes in polarising region and apical ectodermal ridge signalling during outgrowth and patterning of the limb.

3.3.2 Homeobox (Hox) genes

Within the last year, studies have shown that Hypodactyly is caused by a mutation in a homeobox (Hox) gene, Hoxa13 (Mortlock et al., 1996). Hox genes (see Chapter 1) are thought to be downstream target genes of Shh, and in the developing limb, 5' members of the Hoxd and Hoxa clusters are expressed in complex and dynamic patterns that suggest
they may be involved in determining cell behaviour and patterning of the limb (reviewed in Duboule, 1992).

3.3.2.1 *Hoxd* genes

5' members of the murine *Hoxd* complex are sequentially activated during vertebrate limb development, and in the early limb bud they are expressed in overlapping domains across the antero-posterior axis (Figure 1.7 in Chapter 1). The timing and spatial restriction of gene expression within the early limb bud reflects the position of a gene on the chromosome; the more 5' the position of a gene within the chromosomal cluster, the later and more postero-distal the expression domain (Dollé et al., 1989; Dollé and Duboule, 1989; Duboule and Dollé, 1989; Oliver et al., 1989). At 9.0 dpc, *Hoxd9, Hoxd10, Hoxd11*, and *Hoxd12* are strongly expressed in mouse limb mesenchyme with *Hoxd12* expression being weaker and more postero-distally restricted than the expression of *Hoxd9 - d11*. At 9.75 dpc, *Hoxd13* is detected in very posterior distal mesenchyme within the domain of *Hoxd12* expression, and by 10.5 dpc, *Hoxd13* expression is expressed in a slightly larger domain in the posterior mesenchyme, but still within that of *Hoxd12*. These nested domains of *Hoxd* gene expression across the antero-posterior axis of mouse limbs are maintained, but by 11.5 dpc, the domains, especially of the more 5' genes, have increased in size and expanded anteriorly.

*Hoxd* gene expression within the developing mouse limb is complex and dynamic, and by 12.5 dpc, expression is confined to the precartilagenous digital blastemas, with more 5' genes being restricted to more proximal blastemas. As development proceeds, the levels of expression of more 3' members within the *Hoxd* group decrease, such that, at 14.5 dpc, transcripts of *Hoxd13* are detected abundantly in the mesenchyme surrounding the phalangeal cartilage anlage in the hindlimbs, whilst the levels of *Hoxd12* and *Hoxd11* have decreased and that of *Hoxd10* is at background levels (Dollé et al., 1989; 1991; Dollé and Duboule, 1989; Duboule and Dollé, 1989; Oliver et al., 1989).
### 3.3.2.2 *Hoxa* genes

Expression studies have shown that 5' members of the *Hoxa* cluster are expressed in restricted, overlapping domains across the proximo-distal axis of the early limb buds (Haack and Gruss, 1993; Yokouchi et al., 1991), suggesting that they play important roles in patterning across this limb axis. In the developing mouse limb, expression patterns of *Hoxa10, Hoxa11,* and *Hoxa13* across the proximo-distal axis of the limb are established between 9.5 and 12.5 dpc (Haack and Gruss, 1993) prior to the appearance of segmented limb structures.

*Hoxa10* and *Hoxa11* expression is first seen at 9.75 dpc in a small region of posterior distal cells, whereas *Hoxa13* is first detected almost a day later, at 10.5 dpc in dorsal mesenchymal cells at the posterior boundary of the limb. Over the next 48 hours, there is a progressive change in expression domains such that an anterior extension is detected in the distal limb bud resulting in expression across the entire distal antero-posterior axis (Haack and Gruss, 1993). The domains are restricted along the proximo-distal axis such that *Hoxa13* is more distally restricted than both *Hoxa11* and *Hoxa10,* and *Hoxa11* is more distally restricted than *Hoxa10.* An interesting finding has been that expression domains of *Hoxa13* and *Hoxa11* are mutually exclusive; at the time when *Hoxa13* is activated in distal mesenchyme, *Hoxa11* is switched off in this region, suggesting that *Hoxa13* directly downregulates *Hoxa11* (Haack and Gruss, 1993).

Exactly how these restricted patterns of expression of *Hoxa* genes are achieved during limb development are still unknown. It has been proposed that they could arise due to specific positional signals leading to sequential activation at the posterior distal tip of developing limb buds, with elaboration of pattern occurring as a result of cell movements and growth of the limb (Haack and Gruss, 1993). However, recent experiments in the chick have shown that cells marked well anterior to the initial anterior expression boundary of *Hoxa13* give rise to daughter cells that are later found within the *Hoxa13* expression domain (Nelson et al., 1996). This result implies that sequential activation of *Hoxa13,* coupled with growth of the limb at the distal tip are not solely responsible for establishing the expression boundary of *Hoxa13* in the developing limb (Nelson et al., 1996), this may also be true for other *Hox* gene expression domains in the limb.
Irrespective of how the exact expression domains are specified during development, the expression patterns of \textit{Hoxa} genes in the developing limb are suggestive of them being involved in segmentation events that occur along the proximo-distal limb axis (Haack and Gruss, 1993; Yokouchi et al., 1991).

3.3.2.3 Roles of \textit{Hox} genes

It was initially proposed that specific combinations of \textit{Hox} genes may specify the identity of different skeletal elements in the limb (Ispizúa-Belmonte et al., 1991; Morgan and Tabin, 1993). However, there are several lines of genetic evidence indicating that this is not the case \textit{per se}, but instead, \textit{Hox} genes appear to be important in controlling the amount of tissue available for skeletal development. Targeted disruptions of members of the \textit{Hoxa} and \textit{Hoxd} clusters do not result in limb homeosis, that is they do not result in the transformation of one limb part into another. Instead they give rise to regional malformations in the shapes, sizes, and segmentation of bones in the limb, suggesting that \textit{Hox} genes control the localised growth and/or recruitment of cells that contribute to the formation of the appendicular skeleton (Davis and Cappechi, 1994, 1996; Davis et al., 1995; Dohlé et al., 1993; Favier et al., 1995, 1996; Fromental-Ramain et al., 1996; Small and Potter, 1993).

3.4 Aims of this Chapter

At the time of commencing this study, very little work had been carried out on the hypodactyly mutation. The only information available was from the original brief reports of Hummel (1963, 1970), in which the morphology of adult mice was briefly discussed. In order to try and understand how and when the defects arise in \textit{Hd} mice, the observations of Hummel (1963, 1970) on the morphology of adult mutant mice were confirmed. The embryology of hypodactyly mutants had not been described, and so the second aim of this chapter was to analyse the external morphology of mutant phenotypes throughout the stages of development when limbs are forming. This has allowed documentation of the morphology of developing mutant limbs and identification of the stage in development when limb defects are first detected. The third aim was to
investigate mechanisms of limb patterning by analysing the signalling ability of posterior
Hd/Hd limb mesenchyme, and the expression of Shh and Fgf4. A 50 base pair deletion in
the Hoxa13 gene has been found in hypodactyly (Mortlock et al., 1996) and Hox genes of
paralogous group 13 are thought to be involved in specification of the distal-most regions
of the limbs, the hands and feet (Fromental-Ramain et al., 1996). Therefore, in the final
section of this chapter, expression of Hoxd13 and another Hoxd gene, Hoxd11 was
compared in the limbs of Hd/Hd, Hd/+, and +/+ mice during the stages of development
when the limbs are forming.
3.5 Results

3.5.1 Analysis of 3 live-born *Hd/Hd, Hd/+*, and *+/+* littermates at 28 weeks.

A total of 612 live-born offspring from 103 litters have been produced by the breeding colony of mice carrying the *Hd* mutation, the average litter size being approximately 6 mice (Table 3.1). Of these 612 mice, 241 (113 female and 128 male) were morphologically normal, 370 (193 female and 177 male) had defects of the both hindlimbs, and only 1 had defects on all four limbs. The morphologically normal mice were all assumed to be wildtype (*+/+*), whereas those with hindlimb defects were assumed to be heterozygotes (*Hd/+*) and the mouse with defects of all four limbs was assumed to be a homozygote (*Hd/Hd*). The *Hd/Hd* mouse analysed in this study was male, and, in addition to the limb defects, it proved to be infertile.

In order to analyse the morphological phenotype of adult *Hd/Hd, Hd/+*, and *+/+* mice in more detail, the *Hd/Hd* mouse was culled at 28 weeks along with *+/+* and *Hd/+* littermate (Figure 3.2). All three mice were of similar size; from the tip of the snout to the tip the tail they were found to be 18.70 ± 0.68 cm (Table 3.2). External defects of the *Hd/+* mouse were restricted to the hindlimbs, and no genital defects were observed. The hindlimb defects were restricted to the anterior region of the feet, the distal-most region of the limbs (Table 3.2). In comparison, the *Hd/Hd* mouse showed morphological defects restricted to the hands and feet all four limbs (Figure 3.2D and 3.3C), and, in addition, it appeared to have some form of genital abnormality visible externally (Figure 3.4A and B). The glans penis and two small flaps of skin protrude from the preputial cavity (Figure 3.4A), and the whole genital area appears somewhat inflamed (Figure 3.4B). No other external morphological defects were detected (Table 3.2).
### Table 3.1
**Summary of offspring from 103 litters**

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>128</td>
<td>113</td>
<td>241</td>
</tr>
<tr>
<td>Hd/+</td>
<td>177</td>
<td>193</td>
<td>370</td>
</tr>
<tr>
<td>Hd/Hd</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>306</td>
<td>306</td>
<td>612</td>
</tr>
</tbody>
</table>

*Table 3.1*

Table showing the number of live-born mice produced by the breeding colony of *Hd* mice. A total of 612 mice were produced from 103 litters, with the average number of live-born offspring per litter being approximately 6.

### Table 3.2
**External Morphology of 3 littersmates (+/+, Hd/+ and Hd/Hd) at 28 weeks**

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>Hd/+</th>
<th>Hd/Hd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total length of animal</td>
<td>18.70 cm</td>
<td>19.40 cm</td>
<td>18.02 cm</td>
</tr>
<tr>
<td>Body length from tip of snout to base of tail</td>
<td>10.00 cm</td>
<td>10.20 cm</td>
<td>9.02 cm</td>
</tr>
<tr>
<td>Tail length from base to tip of tail</td>
<td>8.70 cm</td>
<td>9.20 cm</td>
<td>9.00 cm</td>
</tr>
<tr>
<td>Craniofacial morphology</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Limb morphology</td>
<td>Normal</td>
<td>Forelimbs are normal, hindlimbs have a reduction defect of digit I</td>
<td>Forelimbs and hindlimbs have a reduction defect. Only one digit present on all four limbs</td>
</tr>
<tr>
<td>Other morphological defects</td>
<td>None</td>
<td>None</td>
<td>Possible defects around genital region</td>
</tr>
</tbody>
</table>

*Table 3.2*

Table summarising the external appearance of +/+, *Hd/+*, and *Hd/Hd* littermates at 28 weeks. Defects are restricted to the hindlimbs of the *Hd/+* mouse and to all four limbs, and possibly the genitalia of the *Hd/Hd* mouse.
**Figure 3.2**

Photographs of the three adult littermates. (A): wildtype (+/+ ) male mouse at 28 weeks; (B): heterozygous (Hd/+ ) male mouse at 28 weeks; (C) and (D): homozygous (Hd/Hd) mouse at 28 weeks. White arrows in D indicate the single digit on both Hd/Hd Scale bars = 1.0 cm
Figure 3.3
Photographs of the hindlimbs of three adult littermates shown in figure 3.2. (A): Wildtype (+/+); (B): Heterozygote (Hd/+), indicating the reduced hindlimb digit I (white arrows); (C): Homozygote (Hd/Hd), indicating the severely malformed hindlimbs (white arrows) and the genital abnormality (small black arrow). I: hindlimb digit I; II: hindlimb digit II; III: hindlimb digit III; IV: hindlimb digit IV; V: hindlimb digit V.
Figure 3.4
Photographs of the hind-quarters of a homotygate (Hd/Hd) showing the malformed hindlimbs and the possible genital abnormalities. (A): the glans penis and two small flaps of skin protrude from the preputial cavity (black arrows); (B): the entire genital area looks red and somewhat inflamed (black arrow). (A) and (B) are photographs of the same animal taken from slightly different angles.
3.5.2 External limb morphology of adult +/+, Hd/+, and Hd/Hd mice

In the mouse, the hands and feet normally consist of five digits, digits I, II, III, IV, and V running from anterior to posterior. Digit I in the forelimb is very small and its external appearance is much reduced compared to other forelimb digits. External forelimb morphology of the Hd/+ mouse is identical to that of its +/- littermate; both the +/- and Hd/+ forelimbs have hands with a small nodule-like anterior digit I and well developed digits II to V, all of which are capped with hooked terminal claws (Figure 3.5A and B; Table 3.3). The hands are covered dorsally with hairs and ventrally with soft tissue walking pads (Figure 3.5A and B). In comparison, Hd/Hd forelimbs have defects that appear externally to be restricted to the hands; both left and right hands have only one, centrally placed digit (Figure 3.5C and D; Table 3.3). This single digit, capped by a hooked terminal claw, is well developed and has the resemblance of a normal forelimb digit, however it is not possible to identify which digit it is.

The feet of +/- mice have five well developed digits, numbered I to V from anterior to posterior, all of which are capped with a hooked terminal claw (Figure 3.5E and F; Table 3.3). The feet of both Hd/+ and Hd/Hd mice differ from those of +/- mice, with those of Hd/Hd being more severely affected than Hd/+ (Table 3.3). Hd/+ feet have four well developed and identifiable digits, digit II to V, and a reduced digit I (Figure 3.5G and H; Table 3.3). In the Hd/+ mouse analysed here, digit I is reduced in size and capped with a terminal claw of abnormal morphology; it is rounded instead of hooked. The dorsal surface of the hindlimbs are covered with hair and ventrally there are soft tissue walking pads (Figure 3.5G and H; Table 3.3). In contrast, the Hd/Hd littermate has a single digit on both feet (Figure 3.5I and J; Table 3.3). However, unlike the single forelimb digit on each of the Hd/Hd hands, the single digit-like structure on each of the Hd/Hd feet are noticeably less well developed, and does not resemble normal hindlimb digits. The exact identity of the single hindlimb digits cannot be determined from external morphology alone; they appear very stump-like and rudimentary, with the result that both feet resemble a large soft tissue pad. Additionally, the morphology of the left and right feet is not identical; the digit-like structure on the right foot has no terminal claw, whereas on the left foot it has a small, but rounded terminal claw (Table 3.3).
Figure 3.5

Photographs of the external morphology of the forelimbs and hindlimbs of three +/+, Hd/+ and Hd/Hd littermates at 28 weeks. (A): +/+ forelimbs; (B): Hd/+ left forelimb; (C): lateral view and (D) ventral view of the Hd/Hd left forelimb with a single, well formed digit (arrows); (E): dorsal view and (F) ventral view of the +/+ right hindlimb with a well formed hindlimb digit I (arrow); (G) dorsal view and (H) ventral view of the Hd/+ right hindlimb with a reduced digit I (arrows); (I) Hd/Hd right hindlimb showing the lack of well formed digits (arrow); (J) lateral view of the Hd/Hd right hindlimb showing the lack of well formed digits (arrow)
3.5.3 Skeletal analysis of +/+, Hd/+ and Hd/Hd littermates at 28 weeks.

To examine skeletal structures, the +/+, Hd/+ and Hd/Hd littermates described in the above section were processed and stained for cartilage and bone. Morphological defects in the skeletons of Hd/+ and Hd/Hd mice are restricted to the appendicular skeleton; the cranial and axial skeletons are unaffected (Table 3.3; Figure 3.5). As previously reported, the bones of the +/+ forelimb are the humerus, radius and ulna, eight carpals, five metacarpals, five first (proximal) phalanges, four second (middle) phalanges, and five third (distal) phalanges with terminal claws at their tips. The preaxial digit, digit I is shorter than the other forelimb digits due to the fact that it lacks a middle phalange and has a very short distal one (Figure 3.7a). The bones of the hindlimb are the femur, tibia and fibula, seven tarsals, five metatarsals, and the same number of phalanges as in the forelimbs (Figure 3.7b). The Hd/+ mouse has normal forelimbs (Figure 3.8a) and a preaxial defect in the hindlimbs; digit I is reduced in size due to the absence of the proximal and distal phalanges, in addition to the presence of a rounded instead of hooked terminal claw (Figure 3.8b).

The Hd/Hd adult mouse exhibits gross morphological defects in the distal aspects of both left and right fore- and hindlimbs. The forelimbs are similar in morphology; a single, relatively well formed digit is present and consists of two small bones that resemble a metacarpal and a phalange capped with a hooked terminal claw (Figure 3.9a). This digit articulates centrally with the more proximal carpal-like structures, of which there are five in the right and four in the left hand. The left and right hindlimbs exhibit a more variable phenotype and will therefore be considered individually. The right hindlimb has one digit-like structure consisting of a long fused bony element, there is no terminal claw (Figure 3.9b). The tarsals are almost completely missing, a calcaneum-like structure is present along with a small tarsal-like bone, but there is no evidence of an intermedium, tibiale, centrale or the five distal tarsals. Four small sesamoid-like bones are evident on the medial aspect of the right foot; these may be the remnants of the tarsals. In addition, two boney structures are evident in the soft tissue proximal to the calcaneum at the level of the base of the tibia and fibula. The left hindlimb also has one digit-like structure, which consists of two bony elements; one long bone possibly representing a
metatarsal-like structure, the other smaller bone resembling a reduced phalange. This digit is capped by a rounded terminal claw. More proximally, four tarsal-like bones are present in addition to boney structures in the soft tissues at the base of the tibia and fibula (Figure 3.8b).
Table 3.3
Skeletal morphology of 3 adult littermates

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>Hd/+</th>
<th>Hd/Hd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial skeleton</td>
<td>No detectable defects</td>
<td>No detectable defects</td>
<td>No detectable defects</td>
</tr>
<tr>
<td>Axial skeleton</td>
<td>No detectable defects</td>
<td>No detectable defects</td>
<td>No detectable defects</td>
</tr>
<tr>
<td>Appendicular skeleton:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoulder girdle</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Humerus</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Radius</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ulna</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Carpals</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Metacarpals</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phalanges</td>
<td>+</td>
<td>+</td>
<td>- (1)</td>
</tr>
<tr>
<td>Terminal claw</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pelvic girdle</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Femur</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tibia</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fibula</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tarsals</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Metatarsals</td>
<td>+</td>
<td>+</td>
<td>- (1)</td>
</tr>
<tr>
<td>Phalanges</td>
<td>+</td>
<td>Missing on digit I</td>
<td>Missing on digit I</td>
</tr>
<tr>
<td>Terminal claw</td>
<td>+</td>
<td>Missing on digit I</td>
<td>Round terminal claw</td>
</tr>
</tbody>
</table>
+ : present and normal, - : absent, (1) : number of possible structures present.

Table 3.3
Table listing regions of the adult skeleton affected by the Hd mutation in Hd/+ and Hd/Hd mice analysed at 28 weeks. Defects are specifically restricted to the bones of the feet, or both hands and feet in Hd/+ and Hd/Hd mice respectively.
Whole skeletons of three adult littermates at 28 weeks stained for cartilage and bone with alcian blue and alizarin red S. (a): Homozygote ($Hd/Hd$); (b): Heterozygote ($Hd/+); (c): Wildtype (+/+).
Figure 3.7

Skeletal elements of forelimbs (a) and hindlimbs (b) of a wildtype (+/+) male mouse culled at 28 weeks and stained for cartilage and bone with alcian blue and alizarin red S.
Figure 3.8

Skeletal elements of forelimbs and hindlimbs of a male heterozygote (Hd/+), culled at 28 weeks and stained for cartilage and bone with alcian blue and alizarin red S. (a): Hd/+ mice have normal forelimbs, but a reduced hindlimb digit I is shown in (b), arrows indicate reduced hindlimb digit I on left and right hindlimbs. This digit has no phalanges or hooked terminal claw.
Figure 3.9

Skeletal elements of forelimbs and hindlimbs of a male homozygote (Hd/Hd) culled at 28 weeks and stained for cartilage and bone with alcian blue and alizarin red S. (a): Hd/Hd mice have a single, well formed forelimb digit (arrow heads) consisting of a metacarpal and a phalange capped with a hooked terminal claw on both left and right forelimbs.  

(b) Hd/Hd hindlimbs have a more rudimentary single digit-like structure consisting of a lone fused boney element. Arrow: digit-like structure on the right hindlimb; arrow heads: digit-like structure on the left hindlimb.
3.5.4 The *Hd/+* hindlimb phenotype is variable and shows degrees of left-right asymmetry within the same embryo.

On analysis of other *Hd/+* adult mice, it is evident that the *Hd/+* hindlimb phenotype is variable and can range from a total loss of digit I including the terminal claw to a slight reduction in the length of digit I and the presence of an abnormal, rounded terminal claw (Figure 3.10). It is also observed that there are varying levels of soft tissue syndactyly between digits II and III (Figure 3.10). The variability of the external *Hd/+* hindlimb defect was assessed in a sample population of 110 adult female *Hd/+* mice. Individual hindlimbs were scored in terms of digit appearance, level of soft tissue syndactyly between digits II and III and the appearance of the terminal claw (Table 3.4). Out of a total of 220 hindlimbs taken from these 110 adult female *Hd/+* mice, 40.9% had no digit I visible on external analysis, and 59.1% had a reduced and stumpy digit I that was visible externally. 72.3% of hindlimbs showed no soft tissue syndactyly between digits II and III, 21.4% showed soft tissue syndactyly approximately half way up the interdigital space between digits II and III, 5.4% of hindlimbs showed soft tissue syndactyly approximately two thirds of the way up the interdigital space between digits II and III, and finally, only 0.9% showed full soft tissue syndactyly between digits II and III. On analysis of the terminal claw morphology on hindlimb digit I, 45% of limbs had no terminal claw on the reduced or absent digit I, 53.1% had a terminal claw of abnormal morphology with it being round instead of hooked, and only 1.8% of limbs had a hooked terminal claw.

It is also evident that within each individual animal, the morphology of left and right hindlimbs are not always identical; there is left-to-right asymmetry in hindlimb morphology (Table 3.5). Taking each of the three criteria on which the hindlimbs were scored separately, 12.7% of the mice have asymmetry in the appearance of hindlimb digit I, with the right hindlimb being more affected. 29.1% of mice have asymmetry in the levels of soft tissue syndactyly between digits II and III, with the right hindlimb being only slightly more affected than the left. Finally, 15.5% have asymmetry in the appearance of the terminal claw on digit I, and the right hindlimb is only very slightly more affected than the left.
Figure 3.10

Photographs demonstrating the variability of the heterozygote \((Hd/+)\) hindlimb phenotype in a sample population of 110 adult female mice. (a - c): indicate level of reduction of hindlimb digit I and corresponding digit score in brackets; (d - g): indicate level of soft tissue syndactyly between digits II and III, and corresponding soft-tissue syndactyly score in brackets; (h - j): indicate terminal claw morphology on hindlimb digit I, and corresponding terminal claw score in brackets. (a): left hindlimb with total loss of digit (digit score = 0); (b): right hindlimb with small nodule-like digit I (digit score = -1); (c): normal, wildtype right hindlimb with well formed digit I capped with a hooked terminal claw; (d): left hindlimb with no soft tissue syndactyly between digits II and III (syndactyly score = 0); (e): left hindlimb with soft tissue syndactyly 1/2 the way up the interdigital space between digits II and III (syndactyly score = 1/2); (f): left hindlimb with soft tissue syndactyly approximately 2/3rds of the way up the interdigital space between digits II and III (syndactyly score = 2/3); (g): left hindlimb full soft tissue syndactyly between digits II and III (syndactyly score = full); (h): no digit I terminal claw (terminal claw score 0); (i): rounded digit I terminal claw (terminal claw score = r); (j): hooked terminal claw (terminal claw score = h).
Table 3.4

Variability of \( Hd/+ \) hindlimb phenotype

<table>
<thead>
<tr>
<th></th>
<th>Left</th>
<th>Right</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digit I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>48 (21.8%)</td>
<td>42 (19.1%)</td>
<td>90 (40.9%)</td>
</tr>
<tr>
<td>-I</td>
<td>62 (28.2%)</td>
<td>68 (30.9%)</td>
<td>130 (59.1%)</td>
</tr>
<tr>
<td>Syndactyly</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>78 (35.5%)</td>
<td>81 (36.8%)</td>
<td>159 (72.3%)</td>
</tr>
<tr>
<td>1/2</td>
<td>25 (11.4%)</td>
<td>22 (10.0%)</td>
<td>47 (21.4%)</td>
</tr>
<tr>
<td>2/3</td>
<td>6 (2.7%)</td>
<td>6 (2.7%)</td>
<td>12 (5.4%)</td>
</tr>
<tr>
<td>full</td>
<td>1 (0.45%)</td>
<td>1 (0.45%)</td>
<td>2 (0.9%)</td>
</tr>
<tr>
<td>Terminal claw</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>52 (23.6%)</td>
<td>47 (21.4%)</td>
<td>99 (45.0%)</td>
</tr>
<tr>
<td>round</td>
<td>54 (24.54%)</td>
<td>63 (28.64%)</td>
<td>117 (53.1%)</td>
</tr>
<tr>
<td>hooked</td>
<td>4 (1.8%)</td>
<td>0</td>
<td>4 (1.8%)</td>
</tr>
</tbody>
</table>

Table 3.4

Table showing the number of mice from a sample population of 110 adult female \( Hd/+ \) mice having different degrees of hindlimb digit I reduction, soft tissue syndactyly between digits II and III, and the presence of an abnormal or absent terminal claw on hindlimb digit I on both left and right hindlimbs. **0:** no digit I, no soft tissue syndactyly or no terminal claw, **-I:** reduced digit I, **1/2:** soft tissue syndactyly approximately half way up the interdigital space between digits II and III, **2/3:** soft tissue syndactyly approximately two thirds of the way up the interdigital space between digits II and III, **full:** soft tissue syndactyly all the way up the interdigital space between digits II and III, **round:** round terminal claw, **hooked:** hooked terminal claw, **:** figures in brackets represent percentage of total number of limbs.
Table 3.5
Left right asymmetry in Hdo/+/hindlimb phenotype

<table>
<thead>
<tr>
<th>Hindlimb</th>
<th>Number of cases</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left</td>
<td>Right</td>
</tr>
<tr>
<td>Digit I</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>-I</td>
<td>-I</td>
</tr>
<tr>
<td>No asymmetry total</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>-I</td>
</tr>
<tr>
<td></td>
<td>-I</td>
<td>0</td>
</tr>
<tr>
<td>Asymmetry total</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

Severity of digit morphology when asymmetry is observed, is more affected in the right hindlimb than the left; 71%:29%

<table>
<thead>
<tr>
<th>Syndactyly between digits II and III</th>
<th>0</th>
<th>1/2</th>
<th>2/3</th>
<th>full</th>
<th>No asymmetry total</th>
<th>70.9%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>2/3</td>
<td>full</td>
<td>1</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>1/2</td>
<td>1/2</td>
<td>2/3</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>No asymmetry total</td>
<td>13</td>
<td>15</td>
<td>2</td>
<td>1</td>
<td>32</td>
<td>29.1%</td>
</tr>
</tbody>
</table>

Severity of syndactyly when asymmetry is observed, shows no major difference in severity of left vs. right hindlimbs; 53%;47%

<table>
<thead>
<tr>
<th>Terminal claw on digit I</th>
<th>0</th>
<th>round</th>
<th>No asymmetry total</th>
<th>93</th>
<th>84.5%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>round</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>round</td>
<td>0</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hooked</td>
<td>round</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asymmetry</td>
<td>17</td>
<td>15.5%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Severity of digit morphology when asymmetry is observed, is more affected in the right hindlimb than the left; 100%:0%
Table 3.5

Table showing the left to right asymmetry in the $Hd/+\,$ hindlimb phenotype in a sample population of 110 adult female $Hd/+\,$ mice having different degrees of hindlimb digit I reduction, soft tissue syndactyly between digits II and III, and the presence of an abnormal or absent terminal claw on hindlimb digit I. 0 :- no digit I, no soft tissue syndactyly or no terminal claw, -I :- reduced digit I; 1/2 :- soft tissue syndactyly approximately half way up the interdigital space between digits II and III, 2/3 :- soft tissue syndactyly approximately two thirds of the way up the interdigital space between digits II and III, full :- soft tissue syndactyly all the way up the interdigital space between digits II and III; round :- round terminal claw, hooked :- hooked terminal claw.
3.5.5 External morphology of +/+, Hd/+ and Hd/Hd mice during embryogenesis

In order to gain an understanding of the embryological development of Hd/+ and Hd/Hd limb phenotypes, the external morphology of embryos was studied during the critical stages of embryogenesis when the limbs are forming. A total of 1017 embryos from 112 litters between 9.5 and 17.5 dpc were analysed, the average number of embryos in a litter being 9 (Table 3.6). This is interesting in light of the fact that the average litter number of the live-born litters was 6, thus showing that an average of 3 embryos per litter die in utero. It is probable that the embryos that die are Hd/Hd embryos. The crown to rump size of Hd/Hd, Hd/+ , and +/+ embryos was not visibly different when analysed with the naked eye. However, when the crown to rump length of a sample population of embryos between 11.5 and 14.5 dpc was measured more accurately, Hd/Hd embryos appear to be marginally smaller than Hd/+ and +/+ embryos from 11.5 dpc onwards (Table 3.7).

During the early stages (9.5-10.5 dpc) the limbs of all embryos in litters from matings of two Hd/+ mice appeared normal both under the light microscope and under a high power scanning electron microscope (108 embryos from 13 litters; Table 3.6; Figure 3.11). Assuming that within these litters there were Hd/Hd embryos, it thus appears that at these early stages, Hd/Hd limb development proceeds as normal; limb buds are initiated, appear as elevations on the flank of the developing embryo, and develop into symmetrical buds. No external morphological differences were observed until 11.5 dpc.
Table 3.6

Numbers of embryos analysed from $Hd/+ \times Hd/-$ intercrosses

<table>
<thead>
<tr>
<th>Age in dpc</th>
<th>Nº of litters</th>
<th>Nº of embryos</th>
<th>$+/+$</th>
<th>$Hd/+ \times Hd/-$</th>
<th>$Hd/Hd$</th>
<th>Dead</th>
<th>?</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5</td>
<td>2</td>
<td>19</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>11</td>
<td>89</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>11.5</td>
<td>26</td>
<td>259</td>
<td>166</td>
<td>71</td>
<td>71</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>28</td>
<td>256</td>
<td>89</td>
<td>72</td>
<td>78</td>
<td>(4)</td>
<td>17</td>
</tr>
<tr>
<td>13.5</td>
<td>13</td>
<td>126</td>
<td>27</td>
<td>57</td>
<td>41</td>
<td>(2)</td>
<td>1</td>
</tr>
<tr>
<td>14.5</td>
<td>6</td>
<td>58</td>
<td>13</td>
<td>22</td>
<td>23</td>
<td>(4)</td>
<td>4</td>
</tr>
<tr>
<td>15.5</td>
<td>9</td>
<td>75</td>
<td>25</td>
<td>43</td>
<td>8</td>
<td>(4)</td>
<td>-</td>
</tr>
<tr>
<td>16.5</td>
<td>8</td>
<td>74</td>
<td>27</td>
<td>34</td>
<td>13</td>
<td>(7)</td>
<td>-</td>
</tr>
<tr>
<td>17.5</td>
<td>5</td>
<td>43</td>
<td>15</td>
<td>18</td>
<td>10</td>
<td>(4)</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3.6

Table summarising the number of litters and phenotypes of the embryos in these litters from 112 $Hd/+ \times Hdf/+ mounts. Embryos were analysed between 9.5 and 17.5 dpc. Prior to 11.5 dpc, no differences in limb bud morphology are detected in litters of mice from mating between two $Hd/+$ mice. At 11.5 dpc, a number of embryos in a litter do not have paddle-shaped limb buds, but instead have more pointed limb buds. These embryos are considered to be $Hd/Hd$ embryos. $Hd/+$ embryos and $+/+$ embryos are not distinguishable from one another until a day later, at 12.5 dpc, when putative $Hd/+\times Hdf/+$ embryos have hindlimb buds that are less angular along the anterior margin of the developing footplates. (): number of embryos found dead upon removal of embryos from the uterus. It was not possible to phenotype these embryos as many of them were resorptions, too young, or decaying badly; ?: embryos of unknown phenotype.
Table 3.7
Average embryo size of different phenotypes at different developmental stages

<table>
<thead>
<tr>
<th></th>
<th>+/-</th>
<th>Hd/+</th>
<th>Hd/Hd</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5 dpc</td>
<td>6.35 ±0.59 mm</td>
<td>6.5 ±0.58 mm</td>
<td></td>
</tr>
<tr>
<td>(n=20)</td>
<td>(n= 4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.5 dpc</td>
<td>8.0 ±0.82 mm</td>
<td>8.43 ±0.54 mm</td>
<td>7.6 ±1.14 mm</td>
</tr>
<tr>
<td>(n= 4)</td>
<td>(n=7)</td>
<td>(n=5)</td>
<td></td>
</tr>
<tr>
<td>13.5 dpc</td>
<td>10.75 ±1.04 mm</td>
<td>10.61 ±0.85 mm</td>
<td>10.33 ±0.49 mm</td>
</tr>
<tr>
<td>(n=8)</td>
<td>(n=18)</td>
<td>(n=12)</td>
<td></td>
</tr>
<tr>
<td>14.5 dpc</td>
<td>11.68 ±0.5 mm</td>
<td>11.78 ±0.67 mm</td>
<td>10.88 ±0.35 mm</td>
</tr>
<tr>
<td>(n=9)</td>
<td>(n=9)</td>
<td>(n=8)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.7
Table showing the data average crown to rump size of +/-, Hd/+, and Hd/Hd embryos between 11.5 and 14.5 dpc
Figure 3.11

Scanning electron micrographs of 9.5 and 10.5 day post coitum (dpc) mouse embryos from matings of two heterozygotes (Hd/+). (a): 9.5 dpc embryo, the well developed left forelimb bud is clearly visible (arrow); (b): forelimb bud of a 10.5 dpc embryo; (c): hindlimb bud of a 10.5 dpc embryo. In all photographs anterior is to the top.
At 11.5 dpc, the developing limb buds of some embryos within a litter are slightly different from normal buds and do not exhibit the normal rounded morphology, but are instead narrower (71 embryos out of 259; Table 3.6). The embryos exhibiting these narrower limb buds are considered to be putative \textit{Hd/Hd} embryos (Figure 3.12 b and c). The total area of the distal limb buds of a sample population of \textit{Hd/Hd}, \textit{Hd/+} and +/- limbs were analysed using NIH image analysis 5.8, and the results show that at 11.5 dpc there is a detectable reduction in the size of \textit{Hd/Hd} limbs when compared to \textit{Hd/+} and +/- limbs (Table 3.8); \textit{Hd/Hd} limb buds are approximately 18% smaller than those of +/- or \textit{Hd/+} littermates. In addition to the narrower shape and smaller size of these limb buds, there is a region of distal mesenchyme towards the tip of the buds that appears more opaque and white than the surrounding mesenchyme or the mesenchyme in the limb buds of other embryos within the litter. A well developed apical ectodermal ridge is present running antero-posteriorly along the distal edge of the putative \textit{Hd/Hd} limb buds. This ridge appears more pronounced than normal (see section on apical ectodermal ridge).

One day later in development, at 12.5 dpc, limb buds of some embryos are even narrower and begin to assume a pointed morphology (78 out of 256; Table 3.6; Figure 3.12f, j, and i). There is no reduction in overall proximo-distal length of these pointed buds, but a marked reduction in antero-posterior width, with apparent loss of both anterior and posterior tissue. Analysis of the total distal limb bud area of a sample population of \textit{Hd/Hd}, \textit{Hd/+}, and +/- limbs using NIH image analysis 5.8, shows that at 12.5 dpc there is a detectable reduction in the size of \textit{Hd/Hd} limbs when compared to \textit{Hd/+} and +/- limbs (Table 3.8); \textit{Hd/Hd} limb buds are now 40% smaller than the limb buds of their +/- or \textit{Hd/+} littermates. These results show that not only are the limb buds of \textit{Hd/Hd} embryos smaller than those of their +/- and \textit{Hd/+} littermates, but the relative increase in size from 11.5 to 12.5 dpc is reduced in \textit{Hd/Hd} embryos (Table 3.8). Between 11.5 and 12.5 dpc, the increase in size of \textit{Hd/Hd} limb buds is much less marked than of \textit{Hd/+} and +/- limb buds (Table 3.8), and at 12.5 dpc, the distal area of \textit{Hd/Hd} limbs buds is comparable to that of \textit{Hd/+} and +/- limb buds at 11.5 dpc. At 12.5 dpc, the apical ectodermal ridge on \textit{Hd/Hd} limb buds runs from anterior to posterior along the
distal tip of the buds, and this ridge is more pronounced than that seen on the paddle shaped buds of both $Hd/+ \text{ and } +/-$ embryos (Figure 3.12j, k, and l). In addition, a gap, sometimes filled with blood, is evident under the ridge and the ectoderm looks somewhat "baggy" around the distal tip of these pointed buds. Between 13.5, and 15.5 dpc, mutant limb buds maintain their pointed morphology, and by 16.5 dpc, when all of the limb components have been formed it is clearly evident that the pointed limbs will give rise to hands and feet with only one digit, as in the adult $Hd/Hd$ mutant (13 out of 74; Table 3.6; Figure 3.14c, f, I, and l).

Alterations in limb morphology of putative $Hd/+ \text{ embryos are much less dramatic and are detected at a later stage of development than those described for putative } Hd/Hd \text{ embryos. Slight changes in external morphology are first seen at 12.5 dpc, when a proportion of the embryos with paddle-shaped limb buds within a litter appear to show a difference in shape of the developing footplates (72 out of 256; Table 3.6). This is due to an apparent loss of tissue along the anterior margin, and embryos with such hindlimbs are assumed to be putative $Hd/+ \text{ embryos (Figure 3.12e, h, and k). The alteration in hindlimb morphology of putative } Hd/+ \text{ embryos becomes progressively more noticeable at later stages of development when the developing digits are more defined. From 13.5 dpc onwards, it is clearly evident that there is a loss of tissue along the anterior margin of the developing footplate where digit I usually develops (Figure 3.13b, e, h, and k).}
Figure 3.12

+/+, Hd/+, and Hd/Hd mice and limbs at 11.5 and 12.5 dpc. (a) Right hindlimb of a +/- or Hd/+ 11.5 dpc embryo showing the expected rounded morphology. Note, the two phenotypes can not be distinguished at this stage. (b) and (c) Right hindlimb of possible Hd/Hd 11.5 dpc embryos showing a more pointed morphology. (b) and (c) are from different embryos. (d) Paddle-shaped hindlimb bud of a 12.5 dpc +/- embryo. (e) Paddle-shaped hindlimb bud of a 12.5 dpc Hd/+ embryo. (f) Pointed hindlimb bud of a 12.5 dpc Hd/Hd embryo. (g) 12.5dpc +/- embryo. Anterior is to the left. (h) 12.5 dpc Hd/+ embryo, small arrow indicates a reduction in tissue along the anterior border of the hindlimb bud. Anterior is to the left. (i) 12.5 dpc Hd/Hd embryo, arrows indicate the pointed limb buds. Anterior is to the left. (j), (k), (l): Higher magnifications of limbs shown in (d), (e), (f) Arrow heads indicate reduction in tissue along the anterior border of the Hd/+ hindlimb buds. Except where otherwise indicated anterior is towards top.
Table 3.8
Total Area of +/+, Hd/+ and Hd/Hd Distal Limb Buds at 11.5 - 12.5 dpc

<table>
<thead>
<tr>
<th></th>
<th>+/+ and Hd/+</th>
<th>Hd/Hd</th>
<th>Relative size of Hd/Hd compared to +/+ and Hd/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.5 dpc</td>
<td>0.56 ±0.1</td>
<td>0.46 ± 0.07</td>
<td>Hd/Hd limb buds are 18% smaller than +/+ and Hd/+ limb buds at 11.5 dpc</td>
</tr>
<tr>
<td></td>
<td>(n = 66)</td>
<td>(n = 17)</td>
<td></td>
</tr>
<tr>
<td>12.5 dpc</td>
<td>0.91 ± 0.12</td>
<td>0.55 ± 0.04</td>
<td>Hd/Hd limb buds are 40% smaller than +/+ and Hd/+ limb buds at 12.5 dpc</td>
</tr>
<tr>
<td></td>
<td>(n = 24)</td>
<td>(n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

Relative increase in size from 11.5 to 12.5 dpc

| 12.5 dpc | 162.5% | 120% |

Table 3.8
Table showing the average area of distal limb buds of +/+ , Hd/+ , and Hd/Hd embryos at 11.5 and 12.5 dpc, the relative increase in limb buds size from 11.5 to 12.5 dpc, and the average difference in size of +/+ and Hd/+ limb buds in comparison to Hd/Hd limb buds. Measurements are in in arbitrary units as calculated by NIH image analysis 5.8. (n = ): represents number of limbs analysed.
Figure 3.13

+/+, Hd/+, and Hd/Hd mice between 13.5 and 16.5 dpc. Anterior is towards the top in all cases. (a) Right side of a 13.5 dpc +/+ embryo. (b) Right side of a 13.5 dpc Hd/+ embryo. Arrow indicates reduction in hindlimb digit I. (c) Left side of a 13.5 dpc Hd/Hd embryo. Note distinct reduction in size of developing hands and feet of this embryo resulting in a pointed morphology. (d) Left forelimb and hindlimb of a 14 dpc +/+ embryo. (e) Right forelimb and hindlimb of a 14 dpc Hd/+ embryo. Arrow indicates reduction in hindlimb digit I. (f) Right forelimb and hindlimb of a 14 dpc Hd/Hd embryo. The presence of one digit on both the forelimb and hindlimb is clearly evident, note central position of this digit, particularly in the forelimb. (g) Right forelimb and hindlimb of a 14.5 dpc +/+ embryo. (h) Right forelimb and hindlimb of a 14.5 dpc Hd/+ embryo. Arrow indicates reduction in hindlimb digit I. (i) Right forelimb and hindlimb of a 14.5 dpc Hd/Hd embryo. One digit is clearly evident on both the forelimb and hindlimb. Note central position of this digit on both forelimb and hindlimb. (j) Left hindlimb of a 16.5 dpc +/+ embryo. (k) Right hindlimb of a 16.5 dpc Hd/+ embryo. Arrow indicates reduced digit I. (l) Left forelimb and hindlimb of a 16.5 dpc Hd/Hd embryo. One digit is clearly evident on both forelimb and hindlimb. Note central position of this digit on both forelimb and hindlimb.
3.5.6.1 Difficulties experienced during analysis of limb polarising activity

In carrying out the experiments to analyse polarising activity described in sections 5.2 and 3.5.6, several difficulties were experienced. Mouse limb buds used in this study were very sensitive to the time spent in 2% trypsin; no longer than 30 minutes should be required if the buds are pricked with a needle prior to placing them into the trypsin. In addition, the trypsin must be kept ice-cold as when the trypsin warms up, this often leads to the limbs being over-trypsinised, making the mesenchymal tissue very sticky. The mesenchyme not only dissociates from the ectoderm, but the mesenchymal cells begin to dissociate from each other too. Sticky blocks of mesenchyme are unsuitable for grafting. Furthermore, on many occasions, mouse mesenchyme grafted into the host chick limb became very white and opaque. The appearance of the graft tissue was similar to that seen in \( H^d/H^d \) limb bud mesenchyme at 11.5 - 12.5 dpc (see section 4.3.1 in Chapter 4). This mesenchyme is thought to have an elevated level of mesenchymal cell death. In addition, a similar observation is also seen when grafts of chick mesenchyme die once they are grafted into the host chick wing. It is therefore possible that many of the heterospecific grafts from mouse to chick carried out in this study were not behaving as would be expected due to necrosis induced by the experimental procedure; mesenchyme of all three phenotypes behaved in a similar way, suggesting that this phenomenon is not specific to mutant mesenchyme. Mouse mesenchymal cells appear to be very sensitive to alterations in pH of the medium in which they are placed; micromass cultures of mouse mesenchymal cells are carried out in CMRL and not growth medium as for chick cells. During the experimental procedure to analyse polarising activity in \( H^d \) mutant limbs, limbs and individual blocks of mesenchyme were placed into growth medium before and after trypsinisation. It is therefore possible that the viability of the mouse mesenchymal cells was not maintained by this medium, leading to an increase in cell death/necrosis in the grafted tissue. Thus, results obtained in this study may not be representative of the actual situation \textit{in vivo}. 
3.5.6 Polarising activity is present in posterior mesenchyme of *Hd/Hd* limb buds

Blocks of posterior mesenchyme taken from *Hd/Hd*, *Hd/+*, and */+* limb buds at 10.5, 11.5, and 12.5 dpc were grafted to the anterior margin of a stage 20 chick wing bud underneath a loop in the apical ectodermal ridge. At 10.5 dpc, when the phenotypes of embryos were not distinct enough to visualise, 60% (15 out of 25) of grafts induced ectopic digits, 8 of which induced an extra 2 and/or 3, the other 7 induced an extra 4 (Table 3.9). A day later, at 11.5 dpc, 23% (3 out of 13) of grafts taken from *Hd/Hd* limb buds induced an extra 2 or 3, and 27% (3 out of 11) of grafts taken from *Hd/+* limb buds induced an extra 2, 3, or 4. Finally, at 12.5 dpc, 25% (2 out of 8) of grafts from *Hd/Hd* limbs induced an extra digit 2, and 28% (2 out of 7) of grafts from */+* or *Hd/+* limbs induced an extra 2, these duplications were caused by posterior mesenchyme taken from *Hd/+* hindlimbs. Mesenchyme taken from */+* hindlimbs, and */+* or *Hd/+* forelimbs did not induce extra digits. These results, although not conclusive, suggest that polarising activity is present in the limbs of *Hd* mutant mice, and this activity is reduced and present right up to 12.5 dpc, a stage when polarising activity is normally no longer detected in posterior limb bud mesenchyme of normal limb buds.
Table 3.9

<table>
<thead>
<tr>
<th>Age and phenotype embryo</th>
<th>10.5</th>
<th>11.5/12.0</th>
<th>12.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+, Hd/+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>++/Hd+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hd/Hd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+/+ or Hd/Hd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>and Hd/Hd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Final digit pattern*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>36%</td>
<td>73%</td>
<td>77%</td>
</tr>
<tr>
<td>Extra 2 and/or 3</td>
<td>32%</td>
<td>18%</td>
<td>23%</td>
</tr>
<tr>
<td>Extra 4</td>
<td>28%</td>
<td>9%</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>(7/25)</td>
<td>(1/11)</td>
<td>(0/13)</td>
</tr>
</tbody>
</table>

Table 3.9

Table summarising results obtained from grafting posterior mesenchyme from +/+,
Hd/+, and Hd/Hd limb buds to the anterior border of a normal stage 20 chick wing.
Figures in brackets represent number of cases. * Identity of most posterior additional
digit specified, in addition other, more anterior digits may also have been induced.
3.5.7 Gene expression in the limbs of +/+; Hd/+, and Hd/Hd embryos

3.5.7.1 Expression of Shh

Expression of Shh in limbs of mutant and normal mice was assessed between 10.5 and 12.5 dpc. At 10.5 dpc, no differences were detected in limb morphology or distribution of Shh expression in the limbs of 3 litters (Figure 3.14A and B). Assuming that at least a quarter of these mice may have been homozygous, Hd/Hd mice, this suggests that the initiation of Shh expression in mutant limbs is unaffected. Throughout subsequent developmental stages analysed, no differences in Shh expression were detected in limbs of Hd/+ embryos when compared with +/+ embryos.

At 11.5 dpc, Shh expression is detected in posterior mesenchyme of the forelimb and hindlimb buds; expression is restricted to a stripe of posterior distal mesenchyme along the posterior edge of the developing hand and footplates (Figure 3.14C), and is more extensive in hindlimb than in forelimb buds. Older embryos within a litter have little or no forelimb expression and a reduction in the size of the hindlimb expression domain. Shh expression in the limbs of Hd/Hd embryos is detected in a thick band of posterior mesenchyme along the distal posterior border of the developing hand and footplates (Figure 3.14D). Half a day later, at 12.0 dpc, Shh expression is not detected in the forelimbs of +/+ or putative Hd/+ and hindlimb expression is restricted to a narrow stripe of mesenchymal cells along the distal posterior border of the buds.

Expression in putative Hd/Hd limbs is still detected in a thick band of mesenchyme. By 12.5 dpc, Shh expression is not detectable in the limbs of +/+ or Hd/+ embryos, but continues to be expressed in the notochord and neural tube (Figure 3.14G and H). In contrast, the footplates of 12.5 dpc Hd/Hd embryos continue to express Shh in posterior mesenchyme (Figure 3.14I), and in some cases very weak expression in posterior mesenchyme of the forelimbs can be detected, although this expression is not much above background levels.
Table 3.10
Area of expression of Hoxd13 and Hoxd11 in developing limb buds of normal and mutant embryos at 11.5 and 12.5 dpc

<table>
<thead>
<tr>
<th></th>
<th>+/-</th>
<th></th>
<th>+/-</th>
<th></th>
<th>+/-</th>
<th></th>
<th>+/-</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>11.5 dpc</td>
<td>12.5 dpc</td>
<td>11.5 dpc</td>
<td>12.5 dpc</td>
<td>11.5 dpc</td>
<td>12.5 dpc</td>
<td>11.5 dpc</td>
<td>12.5 dpc</td>
</tr>
<tr>
<td>Total area of distal limb*</td>
<td>0.56±0.1</td>
<td>0.91±0.12</td>
<td>0.46±0.07</td>
<td>0.55±0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(66)</td>
<td>(24)</td>
<td>(17)</td>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area of Hoxd13 expression in distal limb</td>
<td>0.23±0.03</td>
<td>0.46±0.06</td>
<td>0.16±0.02</td>
<td>0.24±0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td>(12)</td>
<td>(9)</td>
<td>(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area of Hoxd13 expression as a % of the distal limb</td>
<td>43.42±4.49</td>
<td>53.01±4.55</td>
<td>35.88±5.65</td>
<td>45.86±1.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area of Hoxd11 expression in distal limb</td>
<td>0.32±0.07</td>
<td>0.54±0.14</td>
<td>0.35±0.06</td>
<td>0.17±0.21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(32)</td>
<td>(12)</td>
<td>(8)</td>
<td>(2)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area of Hoxd11 expression as a % of the distal limb</td>
<td>54.15±9.52</td>
<td>56.40±7.34</td>
<td>72.59±8.94</td>
<td>45.41±11.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Area expressed in arbitrary units and number of limbs analysed in brackets.
Note that the area of the distal limb increases much more in +/- than in Hd/Hd embryos.
3.5.7.2 Expression of *Fgf4*

At 11.5 dpc, *Fgf4* expression is detected in the posterior apical ectodermal ridges of *Hd/+ /+* forelimbs and hindlimbs (Figure 3.14E), and the expression patterns are as previously reported for normal mouse limbs (Niswander and Martin, 1992). At this stage, extensive *Fgf4* expression is also detected in the apical ectodermal ridges of *Hd/Hd* embryos, and this expression appears to encompass the anterior as well as posterior ridge (Figure 3.14F). By 12.5 dpc, transcripts are no longer detected in apical ectodermal ridges of *+/+ or Hd/+* forelimbs or hindlimbs (Figure 3.14J and K), in accordance with Niswander and Martin (1992). However, *Fgf4* transcripts are still detected in the apical ectodermal ridge of *Hd/Hd* hindlimbs. Expression is relatively low in the posterior but more extensive in the anterior of the apical ectodermal ridge (Figure 3.14L).

3.5.7.3 Expression of *Hoxd13*

Throughout developmental stages analysed here, *+/+* limb buds express *Hoxd13* transcripts in domains as previously described (Dolle et al., 1989; Dollé and Duboule, 1989; Duboule and Dollé, 1989; Oliver et al., 1989). At 11.5 dpc, limb buds of the mixed population of *Hd/+ and +/+* embryos all express *Hoxd13* in a crescent-shaped domain in the posterior distal mesenchyme (Figure 3.15A). This expression domain covers approximately 43% of the total distal limb area (Table 3.10). At this stage, the smaller *Hd/Hd* limb buds express *Hoxd13* at the distal tip of the limb buds (Figure 3.15B). This expression domain is not crescent-shaped and appears smaller than in the mixed population of *Hd/+ and +/+* embryos. However, when the *Hd/Hd Hoxd13* expression domain is described as a percentage of the total distal limb area, the *Hd/Hd* domain is only slightly reduced when compared to the *Hd/+ or +/+* domains (36% versus 43%; Table 3.10).

At 12.5 dpc, *+/+ and Hd/+* limb buds continue to express *Hoxd13* in distal mesenchyme (Figure 3.15C and D), covering approximately 53% of the total distal limb area (Table 3.10). The proximal border of the expression domain is indented in regions where lower levels of transcripts are detected, these areas are thought to correspond to where the digital condensations are developing (Figure 3.15C and D). *Hd/Hd* limbs continue to express *Hoxd13* at 12.5 dpc, however, expression is reduced when compared
to +/+ and Hd/+ limb buds and is restricted to posterior distal mesenchyme in a triangular-shaped domain (Figure 3.15E). Expression is not found at the posterior border of the limb, and the proximal limit of expression is well defined and smooth; no indentations are detectable and no digital condensation are evident. The Hd/Hd Hoxd13 domain covers approximately 46% of the total distal limb area (Table 3.10).

During the later stages of limb development, the expression patterns of Hoxd13 become more complex; forelimbs of +/+ and Hd/+ embryos have similar Hoxd13 expression patterns in cells adjacent to developing digital chondrogenic blastemas in digits I to V (Figure 3.15F and G). Expression at this stage is much lower than at earlier stages. +/+ and Hd/+ hindlimbs are clearly distinguishable from one another due to the reduction along the anterior border of the developing Hd/+ footplate. A slight alteration in the distribution patterns of Hoxd13 is also observed in Hd/+ embryos; both phenotypes show expression of Hoxd13 in cells adjacent to the digital chondrogenic blastemas, however, Hd/+ hindlimbs show additional expression of Hoxd13 at the distal tip of the reduced digit I (Figure 3.15G). This is not detected in +/+ hindlimbs (Figure 3.15F). These expression domains in both the forelimbs and hindlimbs become more distinct at 14.5 dpc. Hd/Hd embryos at 13.5 dpc express Hoxd13 in both hand and footplates, where it is clear that only one digit is developing. This single digit expresses Hoxd13 transcripts in cells adjacent to the developing chondrogenic blastema, as seen in +/+ digits. However, there is also expression in mesenchyme at the distal-most aspect of the developing hand and footplates (Figure 3.15H and I). By 14.5 dpc, the mesenchymal expression in the hand and footplates have ceased, and expression is only detected in cells adjacent to the single developing digit.

3.5.7.4 Expression of Hoxd11
At 11.5 dpc, limb buds of the mixed population of +/+ and Hd/+ embryos all express Hoxd11 in similar patterns, covering approximately 54% of the total distal limb area (Table 3.10). Forelimb expression is localised in two distinct stripes, one in the mesenchyme along the distal edge of the developing hand and footplates, the other positioned more proximally (Figure 3.16A). The distal domain is a thick uniform
crescent-shaped band of expression sweeping from along the posterior border of the hand and footplates, up along the distal edge and into the anterior mesenchyme. The proximal domain begins at the posterior limb-body wall junction, from where it progresses along the posterior border of the proximal limb towards the developing handplate, where it curves up anteriorly towards the anterior border of the limb (Figure 3.16a). A weak line of expression joins the anterior limit of the proximal domain to that of the previously described distal domain. Hindlimb expression of Hoxd11 is also localised in two domains (distal and proximal). These domains are less well separated from one another than in forelimb buds (Figure 3.16a). The distal domain encompasses the mesenchyme at the distal tip of the developing hindlimb bud. The proximal domain is located within the mesenchyme of the posterior region of the limb. Expression begins weakly at the level where the limb joins the body wall, and then gets stronger more distally. Transcripts are not found in the mesenchyme right along the posterior edge of the developing limb, but slightly anterior to it. As the expression in this proximal domain approaches the distal domain, the band of expression curves up anteriorly towards the centre of the limb (as seen in the forelimb). Weak, punctate expression is detectable in the region of cells between the distal and proximal domains, giving the appearance that the two domains are not yet distinct from one another.

Hoxd11 is detected in the limb buds of Hd/Hd embryos at 11.5 dpc (Figure 3.16B). However, expression is localised to a single domain encompassing the posterior mesenchyme at the distal tip of the bud and in posterior mesenchyme more proximally (Figure 3.16B). This expression domain covers approximately 73% of the total distal limb area (Table 3.1); thus there appears to be an increase in Hoxd11 expression in the limb buds of 11.5 dpc Hd/Hd embryos.

At 12.5 dpc, the limbs of +/- and Hd/ + embryos continue to express Hoxd11 in the mesenchyme (Figure 3.16C and D), and despite the morphological differences between +/- and putative Hd/+ hindlimb buds, no differences in the distribution of Hoxd11 are detected. Expression in the forelimbs continues to be located in two regions, one distally in mesenchyme of the distal handplate, the other more proximally, in the region of the wrist (Figure 3.16C and D). At this stage, there is visible evidence of the
developing forelimb digits; the digital rays of the future digits II, III, an IV can be seen. These digital rays are made more conspicuous by the fact that they lack *Hoxd11* expression, i.e. there is no purple staining (Figure 3.16C). In addition to the distal domain, there are two patches of expression, one anterior, the other posterior, in the region of the forearm and wrist (Figure 3.16C and D). The anterior patch is just proximal to the condensations of digit II and III in the region of the developing wrist. The posterior patch begins in the posterior part of the developing wrist, then curves round and extends proximally along the posterior border of the developing forearm and on into part of the developing stylopod. Hindlimb expression is similar to that detected in the forelimbs. A distal mesenchymal domain runs diagonally from the proximal posterior limit to the distal anterior limit of the footplate, and the proximal domain consists of two patches of expression, one posterior the other anterior, at the level of the ankle region. Taken together, these domains of *Hoxd11* expression in the limbs of *Hd/+* and +/+ embryos cover approximately 56% of the total distal limb area (Table 3.0).

At 12.5 dpc, *Hoxd11* expression in *Hd/Hd* forelimbs is focused on two different regions of the limb, one in the distal-most region of the developing limb, the handplates, and the other in a more proximal region, at the level of the wrist (Figure 3.16E). The larger and more distinct domain is the single focus of expression in the mesenchyme of the distal handplates. Here expression is specifically restricted to the posterior half of the developing handplates. No evidence of digital development in the distal handplates is yet detectable in the forelimbs of putative *Hd/Hd* mice. The proximal domain at level of the wrist, consists of two small patches of expression, and as in +/+ and putative *Hd/+* mice, these patches are positioned one anteriorly the other posteriorly. These two regions of expression are less well defined than the single distal domain (Figure 3.16E). Transcripts are more easily detectable in the *Hd/Hd* hindlimbs than in the forelimbs at this stage, and, as in the forelimbs, there are two regions of expression within the developing limb, one distal and the other more proximal. Distally, expression is restricted to mesenchyme of the posterior 2/3rds of the developing footplates. Expression in this domain appears not to be uniform throughout; centrally there is a region of cells that appear to have a lower level of expression than the surrounding cells (Figure 3.16E). These centrally positioned
cells co-localise to the region of the footplates where the single digit is thought to eventually develop. The proximal domain of expression within the *Hd/Hd* hindlimb is, as in the forelimb, found in the region of the ankle. In contrast to that seen in the forelimb, this domain of expression appears not to be totally separated from the distal domain, it extends proximally into the posterior half of the developing forearm as a thin line of expressing cells emanating from the posterior limit of the distal domain. Anterior to this posterior proximal domain is another small patch of expression that extends proximally from the anterior-most level of the distal expression domain. This more anteriorly positioned proximal domain localises to a central position within the ankle region. Taken together, these domains cover approximately 45% of the distal limb area (Table 3|0).

During the later stages of limb development, the expression patterns of *Hoxd11* become more complex. At 13.5 - 14.5 dpc, transcripts are detected in cells adjacent to the developing chondrogenic blastemas of digits II to V (Figure 3.16F, G, I, and J). Despite the obvious morphological differences between 13.5 dpc *Hd/Hd* and *+/+* limbs, *Hoxd11* expression in *Hd/Hd* limbs is somewhat similar to that observed in *+/+* limbs. In *Hd/Hd* limbs, *Hoxd11* transcripts are detected either side of the developing digital chondrogenic blastema (Figure 3.16H and K). The only slight difference observed is that in addition to expression in cells around the digital blastema, there are low levels of expression in the mesenchyme anterior and posterior to the single digit at the distal aspect of the developing hand and footplates. Weak expression is seen proximally in the region of the developing wrist. Expression of *Hoxd 11* in the hindlimb is still localised throughout the distal extent of the hand and footplates, and is noticeably stronger posteriorly than anteriorly. Half a day later, at 14.5 dpc, the mesenchyme around the developing digit in each of the *Hd/Hd* limbs has regressed, and only cells adjacent to the digital blastema express *Hoxd 11*(Figure 3.16K).
Figure 3.14
Wholemount *in situ* hybridisations of +/+, Hd/+, and Hd/Hd embryos between 10.5 and 12.5 dpc hybridised with species specific digoxygenin-labelled riboprobes to *Shh* and *Fgf4*. (A and B: 10.5 dpc; C to F: 11.5 dpc, and G to L: 12.5 dpc embryos; A to D, and G to I: *Shh*; E, F, and J to L; *Fgf4*). A and B: expression of *Shh* in the posterior limb bud mesenchyme of two different 10.5 dpc embryos. At this developmental stage, the three *Hd* phenotypes are not visibly distinguishable from one another, and in all embryos analysed, *Shh* expression was detected in posterior limb bud mesenchyme. It is therefore assumed that at this early stage, limb development and patterning in both *Hd/+* and +/- embryos is normal. C: expression of *Shh* in the posterior limb bud mesenchyme of an 11.5 dpc ++/+Hd+ embryo. Expression in the both the forelimb and hindlimb buds are similar, therefore, at this stage the forelimbs of *Hd/Hd* embryos have a stronger expression of *Shh* than the forelimbs of ++/+Hd+ embryos. G: expression of *Shh* in a 12.5 dpc ++/+Hd+ embryo; *Shh* transcripts are no longer detected in posterior limb bud mesenchyme, but are still strongly detected in the notochord (black arrow head) and neural tube (white arrow head). H: expression of *Shh* in a 12.5 dpc Hd/Hd embryo; as in +/- embryos at this stage, *Shh* transcripts are no longer detected in posterior limb bud mesenchyme, but are still strongly detected in the notochord (black arrow head) and neural tube (white arrow head). I: expression of *Shh* in a 12.5 dpc Hd/Hd embryo; *Shh* transcripts are detected in posterior mesenchyme of the hindlimb bud (white arrow), but not in the forelimbs. E: expression of *Fgf4* in an 11.5 dpc ++/+Hd+ embryo. *Fgf4* transcripts are detected in the posterior apical ectodermal ridge; expression in the hindlimb bud (black arrow) is detectably stronger than in the forelimb bud, which shows only very weak expression. F: expression of *Fgf4* in an 11.5 dpc Hd/Hd embryo. *Fgf4* transcripts are detected in the entire apical ectodermal ridge of both forelimb and hindlimb buds (black arrow heads). *Fgf4* expression in *Hd/Hd* embryos is stronger than in ++/+Hd+ embryos (see F). J: expression of *Fgf4* in an 12.5 dpc +/- embryo; no *Fgf4* transcripts are detected. K: expression of *Fgf4* in an 12.5 dpc Hd/+ embryo; no *Fgf4* transcripts are detected. L: J: expression of *Fgf4* in an 12.5 dpc Hd/Hd embryo; *Fgf4* transcripts are detected in the apical ectodermal ridge of the hindlimbs only. Expression is stronger anteriorly than posteriorly.

All embryos in this figure were bisected down the midline prior to *in situ* hybridisation, therefore the views are of half embryos. All except figures 3.14G and 3.14H are viewed from the dorsal side of the embryos which does not reveal the cut surface. Figures 3.14G and 3.14H are viewed from the ventral side of the embryos and therefore show the cut surface of the embryo such that the notochord and floorplate are visible.
Figure 3.15
Wholemount in situ hybridisations of +/+, Hd/+ and Hd/Hd embryos between 11.5 and 14.5 dpc hybridised with species specific digoxigenin-labelled riboprobes to Hoxd13 (A and B: 11.5 dpc; C to E: 12.5 dpc; F to I: 13.5 dpc; J to N: 14.5 dpc embryos/limbs).
A: expression of Hoxd13 in limb buds of an 11.5 dpc +/+ Hd/+ embryo which is localised to a large crescent-shaped domain in the distal limb bud mesenchyme; B: expression of Hoxd13 in limb buds of an 11.5 dpc Hd/Hd embryo which is focussed on the posterior distal mesenchyme of both fore- and hindlimb buds; expression domains are not crescent-shaped as in the limbs in A; C: expression of Hoxd13 in limb buds of a 12.5 dpc +/+ embryo which is localised to the distal limb bud mesenchyme. Regions of low expression within this distal domain are thought to correspond to where the digits will later develop; D: expression of Hoxd13 in limb buds of a 12.5 dpc Hd/+ embryo, which as in +/+ limbs, is localised to the distal limb bud mesenchyme. Regions of low expression within this distal domain are thought to correspond to where the digits will later develop; E: expression of Hoxd13 in limb buds of a 12.5 dpc Hd/Hd embryo which is localised to a triangular domain in the distal limb bud mesenchyme. No regions of lower expression are detected within this domain as in the limbs of +/+ and Hd/+ embryos; F: expression of Hoxd13 in cells adjacent to the developing digits in the hand and footplates of a 13.5 dpc +/+ embryo. Digital blastemas do not express Hoxd13.; G: expression of Hoxd13 in cells adjacent to the developing digits in the hand and footplates of a 13.5 dpc +/+ embryo. Note the reduction along the anterior border of the footplate corresponding to the region where digit I would normally develop (white arrow head). Digital blastemas do not express Hoxd13.; H: expression of Hoxd13 in cells adjacent to the developing digit in the handplate of a 13.5 dpc Hd/Hd embryo. The digital blastema does not express Hoxd13.; I: expression of Hoxd13 in the distal mesenchyme of a 13.5 dpc Hd/Hd hindlimb. There is no evidence of a developing digital blastema and Hoxd13 appears uniform across the entire distal antero-posterior extent of the footplate; J: expression of Hoxd13 in cells adjacent to the developing digits (white arrow) in the developing foot of a 14.5 dpc +/+ embryo; K: expression of Hoxd13 in cells adjacent to the developing digits (white arrow) in the developing hand of a 14.5 dpc +/+ embryo; L: expression of Hoxd13 in cells adjacent to the developing hand and footplates of a 14.5 dpc Hd/+ embryo, white arrow indicates region of strong Hoxd13 expression at the distal tip of the reduced hindlimb digit I; M: expression of Hoxd13 in cells adjacent to the developing digits (white arrow) in the developing hand of a 14.5 dpc Hd/+ embryo; N: expression of Hoxd13 in cells adjacent to the developing digits in the hand and foot of a 14.5 dpc Hd/Hd embryo.
Figure 3.16
Whole mount in situ hybridisations of +/+, Hd/+ and Hd/Hd embryos between 11.5 and 14.5 dpc hybridised with species specific digoxygenin-labelled riboprobes to Hoxd11 (A and B: 11.5 dpc; C to E: 12.5 dpc; F to I: 13.5 dpc; J to N: 14.5 dpc embryos/limbs).

A: expression of Hoxd11 in limb buds of an 11.5 dpc +/+Hd+ embryo. Hoxd11 transcripts are detected in two domains, one in the distal mesenchyme, the other more proximally.; B: expression of Hoxd11 in limb buds of an 11.5 dpc HdHd embryo. Hoxd11 transcripts are detected in almost all of the mesenchyme of the developing limb buds, being excluded from proximal anterior cells; C: expression of Hoxd11 in limb buds of a 12.5 dpc +/+ embryo; transcripts are localised in two regions, one distal the other proximal (black arrow; D: expression of Hoxd11 in limb buds of a 12.5 dpc Hd/+ embryo; as in +/+ limbs, transcripts are localised in two regions, one distal the other proximal (black arrow; E: expression of Hoxd11 in limb buds of a 12.5 dpc Hd/Hd embryo; transcripts are detected in the distal mesenchyme, but there is also weak expression in a more proximal domain (white arrow), similar to that detected in +/+ and Hd/+ limbs. Hindlimb expression is stronger than in the forelimb; F: expression of Hoxd11 in cells adjacent to the developing digits in the hand and footplates of a 13.5 dpc +/+ embryo; G: expression of Hoxd11 in cells adjacent to the developing digits in the hand and footplates of a 13.5 dpc Hd/+ embryo, arrow indicates reduction along the anterior border of the developing footplate in the region where digit I would normally form; H: expression of Hoxd11 in distal mesenchyme of the footplate and in cells adjacent to the developing digit in the hand of a 13.5 dpc Hd/Hd embryo; I: expression of Hoxd11 in cells adjacent to the developing digits in the hand and footplates (white arrow) of a 13.5 dpc +/+ embryo; J: expression of Hoxd11 in cells adjacent to the developing digits in the hand and footplates (white arrow) of a 13.5 dpc Hd/+ embryo, note the reduction along the anterior border of the developing footplate in the region where digit I would normally form ; K: expression of Hoxd11 in cells adjacent to the developing digits in the hand and foot of a 14.5 dpc Hd/Hd embryo.
3.6 Discussion and Conclusion

3.6.1 Summary of results

In this chapter, the external morphology of adult and embryonic $Hd/+$, and $Hd/Hd$ mice is described for the first time, and the skeletal morphology of adult mutants which was first reported by Hummel (1970), is confirmed. Limb defects in both $Hd/+\text{ and } Hd/Hd$ mice are specifically restricted to the distal regions of the limb, leaving more proximal limb elements unaffected. $Hd/+\text{ mice have a reduction of hindlimb digit I including an absent or malformed terminal claw, and soft tissue syndactyly between digits II and III.}$

The adult $Hd/+\text{ limb phenotype is variable, showing ranges of severity between animals, but also between left and right hindlimbs of the same mouse. } Hd/Hd\text{ mice have a single digit or digit-like structure on all four limbs. The single digit on each of the forelimbs is well formed and resembles the morphology of a normal forelimb digit. However, the single digit on each of the hindlimbs is less well specified and resembles a rudimentary digit-like structure. It is shown for the first time that morphological defects observed in adult } Hd/+\text{ and } Hd/Hd\text{ mice are associated with alterations in the size and shape of developing } Hd/+\text{ and } Hd/Hd\text{ limb buds, which are first detected between 11.5 and 12.5 dpc, as the hand and footplates are beginning to be specified.}$

The polarising region is involved in antero-posterior patterning in developing limbs, and loss of digits in hypodactyly suggested that there may be defects in this signalling region of the limb. However, it is reported here for the first time that loss of digits in $Hd/Hd\text{ limbs is not due to the lack of polarising activity, and mutant limb buds express both } Shh\text{ and } Fgf4\text{ transcripts in the posterior mesenchyme and apical ectodermal ridge respectively. Surprisingly expression of these two genes is slightly more prolonged than normal in the limb buds of } Hd/Hd\text{ embryos. } Hd\text{ is now known to be a mutation in } Hoxa13\text{ (Mortlock et al., 1996), and } Hoxa13\text{ and } Hoxd13\text{ are thought to function together in specification of the hands and feet (Fromental-Ramain et al., 1996). Expression of } Hoxd13\text{ in } Hd/Hd\text{ limbs is slightly reduced, and is initially associated with an increase, followed by a subsequent decrease in expression of another } Hoxd\text{ gene, } Hoxd11.\text{ However, the mutation does not appear to affect localisation of } Hoxd13\text{ and } Hoxd11.}$
expression around the developing digital blastemas during later stages of limb development.

3.6.2 Limb defects in *Hd/+* and *Hd/Hd* adult mice do not affect proximal structures and are more severe in the hindlimbs than in the forelimbs

Skeletal analysis of *Hd/+* and *Hd/Hd* adult mice shows that limb defects are specifically restricted to the distal-most regions of the limb; the long bones proximal to the wrist and ankle are unaffected. Defects in *Hd/+* mice are restricted to the feet where there is a variable reduction of the phalanges and terminal claw of digit I, whereas in *Hd/Hd* mice, defects are also detected more proximally in the wrist and ankle. It is evident that in both phenotypes, hindlimbs are more severely affected by the mutation than the forelimbs. In *Hd/+* mice, despite the presence of one *Hd* allele, normal forelimb development occurs, but specification of hindlimb digit I is incomplete. The presence of two *Hd* alleles in *Hd/Hd* mice increases the severity of limb defects; hands and feet of *Hd/Hd* mice have a severe reduction in the number of digits that develop. *Hd/Hd* limbs are no longer pentadactylous, and instead have a single digit, or digit-like structure, on each of the four limbs in addition to defects in the small bones of the wrist and ankle. These results imply that the *Hd* mutation affects a gene specifically required for the correct development of the hands and feet, but is not a prerequisite for development of more proximal limb structures, and a mutation in *Hoxa13* is now known to be the molecular basis of *Hd*.

*Hd/+* forelimbs are normal and their hindlimb defects are minimal compared to those of *Hd/Hd* mice. The mild nature of *Hd/+* limb defects suggest that the presence of HOXA13 protein transcribed from the wildtype *Hoxa13* allele in *Hd/+* mice, is almost sufficient to fully compensate for the presence of the *Hd* allele. This could imply that during normal limb development, *Hoxa13* functions in a partially redundant manner and other genes may be able to compensate for the loss of *Hoxa13*. Alternatively, there may normally be an excess to requirement of HOXA13 protein, thus permitting a certain amount of loss of protein to occur before severe defects are observed. This may allow the remaining wildtype HOXA13 protein present in *Hd/+* limbs to specify the forelimbs properly and almost fully specify the hindlimbs. Compensation may also occur by other
genes in the limb, such as the \textit{Hoxd} parologue of \textit{Hoxa13}, \textit{Hoxd13}, and is it possible that genes may function together to compensate for the loss of wildtype HOXA13 protein. \textit{Hd/Hd} mice are assumed to have a total lack of wildtype HOXA13 present in their developing limbs, and functional compensation by other genes present in the limbs may not be sufficient to compensate for the loss of this protein, thus accounting for the increased severity of the defects when compared to \textit{Hd/+} mice.

3.6.3 There is evidence to suggests that \textit{Hd} is not a null allele of \textit{Hoxa13}

Comparisons between the \textit{Hd} phenotypes and those of \textit{Hoxa13} knockout mice reveal that there are both similar and unique features. \textit{Hd/+} mice have normal forelimbs with a naturally truncated digit I and a variable reduction of hindlimb digit I which can be associated with soft tissue syndactyly between digits II and III, and an abnormal or absent terminal claw. \textit{Hoxa13}^{+/+} mice have a reduced digit I on both forelimbs and hindlimbs due to fusions of the phalanges and the presence of rounded terminal claws, in addition to soft tissue syndactyly between digits II and III in the hindlimbs (Fromental-Ramain et al., 1996). \textit{Hd/Hd} mice have a single, malformed digit/digit-like structure on all four limbs, in addition to absent or malformed carpal and tarsal bones in the wrists and ankles. In comparison, \textit{Hoxa13}^{+/-} mice lack digit I on both hands and feet, the second phalangeal element of the remaining digits is absent on the hindlimbs but not the forelimbs, they have soft tissue syndactyly between digits II and III, carpal and tarsal abnormalities, and exhibit an abnormal bending of the digits towards the preaxial side of the limb (Fromental-Ramain et al., 1996). Thus, it is evident that the two mutant phenotypes are not identical, and \textit{Hd/Hd} mice are more severely affected than \textit{Hoxa13}^{+/-} mice. The study of Fromental-Ramain et al. (1996) provides genetic evidence for the requirement of \textit{Hoxa13} during specification of the distal regions of the limb, since the lack of HOXA13 leads to the defects described above. However the situation in hypodactyly appears more complex suggesting that \textit{Hd} is not a null allele of \textit{Hoxa13}. In addition, the limb defects observed in \textit{Hoxa13}^{+/-} mutants are only slightly more severe than in \textit{Hoxa13}^{+/-} mice, whereas the defects of \textit{Hd/Hd} mutants are dramatically more severe than those of \textit{Hd/+} mice. These results suggest the possibility that \textit{Hd} is a gain of function mutation that not
only affects development of the hands and feet due to loss of wildtype HOXA13 function, but also due to an additional function of the putative product of the $Hd$ allele. However, it is important to note that production of a mutant protein by the $Hd$ allele has not yet been shown. Assuming that a mutant protein is translated, and that this protein has an additional function to that of wildtype HOXA13 protein, it is evident from analysis of $Hd/+\text{ and } Hd/Hd$ mice, that the amount of mutant protein may be an important contributing factor to the final phenotypic outcome of the $Hd$ mutation.

3.6.4 The hindlimbs are more susceptible to limb defects in both $Hd/+\text{ and } Hd/Hd$ mice

An interesting observation is that the hindlimbs of both $Hd/+\text{ and } Hd/Hd$ mice are more severely affected by the mutation than their respective forelimbs. Both developing forelimbs and hindlimbs express some of the same genes, for example $Hox$, $Bmps$, $Shh$, $Fgf4$, and so on, and this suggests that there are common factors involved in specification of the different limb types. However, there is evidence to suggest that different $Hox$ genes may function independently in different limbs. For example, $Hoxd12^{-/-}$ knockout mice have normal hindlimbs and mild defects of the forelimbs (Davis and Capecchi, 1996), whereas $Hoxd11^{-/-}$ and $Hoxd13^{-/-}$ knockout mice have defects of both forelimbs and hindlimbs (Davis and Capecchi, 1994; Doulé et al., 1993). Both $Hoxd11^{-/-}$ and $Hoxd13^{-/-}$ knockout mice have overlapping defects with $Hoxd12^{-/-}$ knockout mice, and it is possible that one or both of these genes could provide sufficient compensation to allow relatively normal limb development to occur in $Hoxd12^{-/-}$ mice, suggesting that some $Hox$ genes may function in a partially redundant manner (Davis and Capecchi, 1996). Evidence to support the idea that some $Hox$ genes expressed in the limbs may be functionally redundant comes from the finding that $Hoxa11^{-/-}$ and $Hoxd11^{-/-}$ knockout mice have relatively mild hindlimb abnormalities compared to those in the forelimbs, and this has been correlated with the presence of a $Hoxc$ paralogue, $Hoxc11$, which is expressed in developing hindlimbs but not forelimbs (Davis and Cappechi, 1994; Davis et al., 1995; Favier et al., 1995; Small and Potter, 1993). Thus $Hoxc11$ in the hindlimbs may compensate for the loss of $Hoxa11$ and $Hoxd11$ reducing the severity of the defects.
(Davis and Cappechi, 1994; Davis et al., 1995). In addition, studies in the chick show that
Hox expression domains have subtle differences in the developing wing versus leg
(Nelson et al., 1996), and, as in the mouse, different sets of Hoxc genes are expressed in
the wing and the leg (Nelson et al., 1996). If subtle differences in Hox gene expression
also exist in mouse forelimbs and hindlimbs, these differences could account for the
differential susceptibility of forelimbs and hindlimbs to defects caused by mutations in
different Hox genes.

It is not only Hox genes that are differentially expressed in developing forelimbs
and hindlimbs. For example, in the chick, Bmp7 is differentially expressed in the wing
and leg (Francis-West et al., 1995); in the wing, Bmp7 transcripts are localised to the
apical ectodermal ridge and posterior mesenchyme, whereas in the leg, they are expressed
in anterior and posterior mesenchyme and apical ectodermal ridge. Interestingly,
expression of Bmp7 in mice does not parallel that in the chick as transcripts are detected
in both anterior and posterior mesenchyme as well as the apical ectodermal ridge in both
forelimbs and hindlimbs (Hofmann et al., 1996). However, although the Bmp7
expression domains are similar, hindlimb expression is less intense than in the forelimb
(Hofmann et al., 1996). These differences in the levels of Bmp7 transcripts in hindlimbs
versus forelimbs of mice may in part account for the fact that limb defects in Bmp7−/−
mice preferentially affect the hindlimbs (Hofmann et al., 1996). Bmp7 is thought to be a
putative suppressor of growth, and 82% of Bmp7−/− mice develop an ectopic anterior
hindlimb digit compared to 12% in the forelimb (Hofmann et al., 1996). These findings
all suggest that although similar molecular mechanisms may be utilised during
development of forelimbs and hindlimbs, there may fundamental differences in the exact
mechanisms in order to allow development of different limb types. These limb-type
differences, as suggested by alterations in patterns of gene expression in forelimbs and
hindlimbs, may allow compensation for the presence of one Hd allele to occur, allowing
normal forelimb development in Hd/+ mice, and partially compensate for the presence of
two Hd alleles, allowing development of a well formed, single digit on Hd/Hd forelimbs.

The differences in severity of forelimb versus hindlimb in both Hd/+ and Hd/Hd
mice could be due to differences in the spatiotemporal expression patterns of Hoxa13 in
developing forelimbs and hindlimbs. A detailed documentation of \textit{Hoxa} expression domains in developing mouse limbs has only been carried out in the forelimb (Haack and Gruss, 1993), and it is therefore of vital importance to ascertain if forelimb and hindlimb expression patterns are the same. Haack and Gruss (1993) make a brief statement in their paper to say that although a detailed analysis of hindlimb expression has not been carried out, the few observations they have made suggest that expression is the same. However, even a subtle difference could account for the differences in defects seen in \textit{Hd} mutants. It is important to note that although \textit{Hoxa13} and \textit{Hd/Hd} are not equivalent, the hindlimb defects in \textit{Hoxa13} are more severe than in the forelimbs, supporting the idea that \textit{Hoxa13} may be differentially expressed in the developing forelimbs and hindlimbs.

However, it is worth noting that forelimb digit I is normally truncated, and the development of normal forelimbs in \textit{Hd/-} mice may reflect the fact that a full complement of wildtype HOXA13 is not required for development of this digit. It is therefore interesting to note that in this sense, the hindlimbs of \textit{Hd/+} mice are taking on a morphology similar to that of the forelimbs.

\textbf{3.6.5 The \textit{Hd/+} hindlimb phenotype is variable}

Severity of the \textit{Hd/+} hindlimb phenotype ranges from no external evidence of digit I including the terminal claw, to a slight reduction in length associated with an abnormal rounded terminal claw. In addition, varying levels of soft tissue syndactyly between digits II and III are also observed. Variations in severity are observed between different mice, but can also be present on the same mouse such that defects are asymmetrical, with one hindlimb being more affected than the other. Soft tissue syndactyly between digits II and III is the most variable component of the \textit{Hd/+} phenotype. The asymmetry of soft tissue syndactyly and terminal claw morphology do not appear to favour either left or right limbs, however digit reductions are often slightly more severe on the right than on the left foot.

Variability of \textit{Hd/+} hindlimb defects suggest that the \textit{Hd} mutation may have a variable expressivity; the \textit{Hd/+} genotype may be expressed to varying degrees in the phenotype. This could be due to influences from other genes or environmental factors.
which may affect the putative levels of genetic compensation that may occur in response to the presence of \(Hd\) alleles. Another possibility is that genetic compensation by other genes may not be under tight developmental control, thus resulting in less severe phenotypes when levels of compensation are high. It is unclear whether the \(Hd/Hd\) defects are variable between animals as only one adult \(Hd/Hd\) mouse has survived and been analysed here.

3.6.6 Defects induced by the \(Hd\) mutation are not restricted to the limbs

In previous reports (Hummel, 1970; Hummel and Chapman, 1963) the \(Hd\) mutation has been described as a semidominant lethal mutation which in addition to inducing limb defects, affects the viability and fertility of \(Hd/Hd\) mice. In this study, only 1 out of 612 live born offspring produced by the breeding colony showed the reported \(Hd/Hd\) phenotype. In addition to the limb defects discussed in the above sections, this \(Hd/Hd\) mutant appeared to have a genital defect; the genital area was red, distended and very slightly malformed, and the mouse was infertile. Female \(Hd/Hd\) mice are also reported to be infertile (Mortlock et al., 1996). The cause of infertility in \(Hd/Hd\) mice is not known, but the presence of abnormalities in the external genital area suggests that in addition to its role during limb development, \(Hoxa13\) is also required during development of the urogenital system. \(Hoxd\) genes are known to be expressed in the developing genitalia (Dolle et al., 1991b), but as yet, expression of \(Hoxa\) genes in this region have not been detected. It will be interesting to analyse \(Hox\) gene expression in this region of the embryo in order to ascertain whether they are involved in patterning and tissue specification. Recently, it has been shown that a mutation in \(HOXA13\) in humans causes hand-foot-genital syndrome (HFG) (Mortlock and Innis, 1997). The distinctive hand and foot abnormalities in HFG are similar to those of \(Hd/+\) mutants (Mortlock and Innis; 1997), and it has been proposed that both the HFG and \(Hd\) proteins may have an altered DNA-binding ability, and that the defects are caused by \(HOXA13\) haploinsufficiency in both \(Hd/+\) and humans affected by HFG (Mortlock and Innis, 1997). Genital defects in HFG are found internally, and the limb defects affect several different digits across the antero-posterior distal limb axis (Mortlock and Innis, 1997). This suggests that although
the two mutations are similar, *Hd* may be a model for HFG. In order to understand the nature and timing of the urogenital defects and infertility in *Hd/Hd* mice, analysis of the internal urogenital system of both male and female *Hd/Hd* and *Hd/+* mice will need to be carried out. As yet, genital defects have only been observed in *Hd/Hd* mice, whereas genital defects are found in humans affected with HFG in which there is only one copy of mutant *HOXA13* (Mortlock and Innis, 1997), suggesting that there are differences in the functioning of *HOXA13* in humans and *Hoxa13* in mice. Another explanation for the defects seen in humans affected with HFG and in mice affected with *Hd*, is that HFG and/or mutant Hd protein may have new and novel effects on development not normally induced by wild type protein (Mortlock and Innis, 1997).

### 3.6.7 *Hd/+* and *Hd/Hd* embryonic phenotypes

Adult *Hd* mutant phenotypes are associated with variable alterations in the size and shape of the developing embryonic hand- and footplates. Mutant mice, as detected by the presence of abnormal limb bud morphologies, are not seen until 11.5 dpc. This suggests that limb initiation and the very early stages of limb development during which the proximal elements of the limbs are specified, are unaffected in both *Hd/+* and *Hd/Hd* mutants. This is in agreement with the lack of proximal defects in adult phenotypes.

Severe loss of digits and malformations of the small bones of the hands and feet in *Hd/Hd* limbs are associated with smaller and more pointed limb buds, which are first detectable at 11.5 dpc, and are maintained and accentuated as development proceeds. The abnormal morphology initially appears to be due to a lack or loss of anterior limb bud mesenchyme, but later mesenchyme is also reduced along the posterior margin of the hand- and footplates. The timing of the defect is correlated with the time in development at which the hand- and footplates are being specified, and in the forelimb, the defects are detected nearly a day later than the activation of *Hoxa13* in the limb bud mesenchyme. This suggests that the lack of wildtype *Hoxa13* in the limbs of *Hd/Hd* mice has an effect on the amount of tissue available for digital development, which could account for the loss of digits. The finding that tissue is apparently lost from both anterior and posterior margins of the limb, implies that the single digit that develops may not be digit V as
suggested by Hummel (1970). The digit could be a more anteriorly located one, such as digit IV.

*Hd/+* embryos are first identified at 12.5 dpc due to alterations in shape of developing hindlimb buds; although paddle-shaped, they do not appear as angular along the anterior margin as would be expected. This lack of angularity appears to be due to a loss/lack of mesenchyme along the anterior margin of the hindlimb buds. As with the morphology of putative *Hd/Hd* developing limb buds, the abnormal hindlimb morphology of putative *Hd/+* embryos is maintained and accentuated as development proceeds.

Analysis of the external morphology of *Hd/+* and *Hd/Hd* developing limbs thus indicates that both mutant phenotypes are associated with a change in limb bud morphology during the time in development when the hands and feet are being specified, thus agreeing with the localisation of the limb defects in adult *Hd* mutants to the bones of the hands and feet. As previously mentioned, limb defects are often associated with alterations in size and shape of developing limb buds. Reducing the size of the developing limb bud limits the amount space available for digital development. The *os* mutant has a variable loss and fusion of digits preceded by a pre-axial mesenchymal deficiency that appears to be due to an abnormally large area of cell death in the pre-axial mesenchyme. In association with this, *os* has a reduction in the number and size of digital blastemas in the reduced distal mesenchyme (Milaire 1967 cited in Hinchliffe and Johnson, 1980 and Milaire, 1992). As in *os*, loss and reduction of digits in *Hd* mutant may therefore be due to the presence of a reduced number of cells available for digital development since digit loss is associated with a reduction in the size of the mutant limb buds. In addition, the reduction in size of *Hd* mutant limb buds is associated with an increase in mesenchymal cell death (see Chapter 4).

### 3.6.8 Polarising activity and expression of *Shh* and *Fgf4* in *Hd/+* and *Hd/Hd* embryonic limbs

The *Hd/Hd* limb phenotype is not associated with a total lack of polarising activity, however, polarising activity appears weaker than in +/- and *Hd/+* limb buds. *Shh* is
considered to be at least one of the important signals produced by the polarising region. *Shh* is assumed to be upstream of the *Hox* genes in the limb, but this has not been directly demonstrated for *Hoxa13*. The polarising activity of posterior mesenchymal cells is reduced after posterior apical ectodermal ridge removal, and can be maintained both *in vivo* and *in vitro* by FGF2 or FGF4. Reduction in polarising activity in *Hd/Hd* limb buds could be due to a lack of signalling between the ridge and cells of the polarising region and the relationship between these tissues seems to be disturbed in hypodactyly. This hypothesis could be tested by culturing *Hd/Hd* posterior limb mesenchymal cells in the presence of FGF4 or FGF2, then grafting them to the anterior of a normal chick wing and analysing the digit patterns that result to see if polarising activity can be rescued. If the lack of polarising activity is intrinsic to the *Hd/Hd* posterior mesenchymal cells, then one would not expect to see an increase in polarising activity after culturing these cells in the presence of FGF2 or FGF4.

Loss of digits in mice has previously been shown to be associated with a reduction or loss of *Shh* and *Fgf4* expression (Chiang et al., 1996; Haramis et al., 1995; Parr and McMahon, 1995). The limb deformity mutant (*ld*), exhibits limb defects including loss of digits, and this is associated with the premature termination of *Shh* expression in the developing limbs and lack of *Fgf4* expression in the apical ectodermal ridge (Haramis et al., 1995). Loss of digits observed in the *Wnt7a<sup>-/-</sup>* knockout mouse is correlated with a reduction of *Shh* expression in posterior limb bud mesenchyme (Parr and McMahon, 1995). Recent evidence obtained from the study of mice with a targeted disruption in *Shh* show that the limbs *Shh<sup>-/-</sup>* mice are severely malformed with distal structures being most affected (Chiang et al., 1996). However, since *Hoxa13* is probably downstream of *Shh* and *Fgf4* signalling, it is not surprising that, in hypodactyly, loss of digits is not associated with a reduction or loss of *Shh* and *Fgf4* expression.

Expression of *Shh* and *Fgf4* is slightly prolonged in the *Hd/Hd* mutant limbs, especially the hindlimbs. The presence and maintenance of expression suggests that the feedback loop that exists between these two genes is present. However, it is possible that the delay in termination of expression of *Shh* and *Fgf4* in *Hd/Hd* limbs may be due to defects in this feedback loop. Since the *Hd* mutation is known to involve *Hoxa13* which
is expressed in the mesenchymal component of the limb, any defects in the feedback loop are likely to stem from defects in the mesenchyme. This suggests that recombining mutant ectoderm with +/+ mesenchyme should restore the correct temporal distributions of Shh and Fgf4. Alternatively, Shh and Fgf4 may only be switched off once cells have committed to giving rise to digits. Therefore if digits are not specified, the expression of these genes may not be correctly terminated. Another possible explanation for the delay in termination of Shh and Fgf4 expression is that it may reflect heterochrony in Hd/Hd limbs. The finding that the relative increase in limb bud size of Hd/Hd embryos is less than for +/+ and Hd/+ limb buds from 11.5 to 12.5 dpc, also suggests that heterochrony may occur in the Hd mutation.

3.6.9 Hoxd gene expression in hypodactyly

Hoxd13 is still expressed in the limbs of Hd/Hd embryos, albeit in a slightly reduced expression domain. Interestingly, Hoxd11 was also expressed in the limb buds of Hd/Hd mutants where expression was initially more extensive, but was then reduced compared to normal buds. Thus although hypodactyly more closely resembles Hoxa13^+/Hoxd13^-/- mutant phenotype than the Hoxa13^-/- mutant phenotype, it seems unlikely that these slight changes in Hoxd gene transcription can account for the differences in morphology between hypodactyly and Hoxa13 deficient mice. Recent evidence from the study of a human disorder, synpolydactyly, caused by a mutation in the human HOXD13 gene (Muragaki et al., 1996) suggests that the mutant HOXD13 may block functioning of other Hox genes, as the human phenotype more closely resembles that of the Hoxd11^-/-, Hoxd12^-/-, and Hoxd13^-/- triple knockout mouse and not of the Hoxd13^-/- mice (Zakany and Duboule, 1996). A similar explanation could account for the severity of the digit reductions in hypodactyly; Hoxd13 is still transcribed in Hd/Hd limbs, but the mutant Hoxa13 product may interfere with the normal functioning of Hoxd13. This would explain why the severe phenotype of hypodactyly more closely resembles that of the Hoxa13^-/-/Hoxd13^-/- double mutant mouse. In this way, the hypodactyly mutation could be seen as a possible gain of function mutation, acting in opposition to wildtype HOXA13 protein, possibly as a negative regulator of growth and cell proliferation. It is
interesting to note that the *Hoxa13* gene dosage, and therefore amount of functional protein present is critically relevant to the phenotypic outcome; heterozygotes produce only half the amount of wildtype *HOXA13* protein which is unable to compensate for the 50% loss of wildtype protein and/or the presence of the mutant protein.

In the developing limb, the expression domains of *Hoxa11* and *Hoxa13* are mutually exclusive; *Hoxa11* is downregulated in distal mesenchymal cells when *Hoxa13* is being activated and expressed in these cells. This in some way indicates that *Hoxa13* may be directly regulating *Hoxa11* expression in a negative manner (Haack and Gruss, 1993). If the mutant HOXA13 protein in *Hd/Hd* embryos is unable to function in a wildtype manner, then it may no longer be able to regulate the expression of *Hoxa11* allowing it to be expressed in the distal limb. It is conceivable that this may play a part in the specification of the *Hd* limb phenotype. It is interesting therefore that the *Hoxd* paralogue of *Hoxa11* is initially overexpressed in *Hd/Hd* limbs. This suggests two possibilities. Either that *Hoxa13* also influences the expression of *Hoxd11*, or *Hoxa11* interacts with *Hoxd11* and influences it's expression. Analysis of *Hoxa11* expression in *Hd/Hd* embryos will help to clarify this matter.
Chapter 4

Analysis of cellular effects of the *Hd* mutation

4.1 Introduction

Analysis of adult and embryonic *Hd* mutant mice shows that limb development is disrupted such that morphological defects are restricted to the hands and feet, leaving more proximal limb structures unaffected. Recently, *Hd* has been shown to be a mutation in *Hoxa13* (Mortlock et al., 1996), although the exact mechanism by which this mutation affects normal development is not understood. Molecular signals known to be involved in limb patterning upstream of *Hoxa13*, such as *Shh* and *Fgf4*, are present in *Hd* limbs. Confirming that *Hoxa13* is downstream of both *Shh* and *Fgf4*. Analysis of transgenic mice in which *Hoxa13* and another *Hox* gene, *Hoxd13*, have been functionally inactivated, provides evidence to suggest that both *Hoxa13* and *Hoxd13* function together to specify the hands and feet (Fromental-Ramain et al., 1996). *Hoxd13* is transcribed in *Hd* limbs, but the *Hd/Hd* limb phenotype suggests that functioning of both *Hoxa13* and *Hoxd13* are affected by the *Hd* mutation. *Hox* genes are thought to function during limb patterning by influencing aspects of cell behaviour, and this chapter aims to analyse effects of the *Hd* mutation on cell behaviour during limb development in *Hd* mutants.

Loss of limb structures in *Hd* is associated with a reduction in limb bud shape and size (see Chapter 2), and mesenchymal cell death is thought to play a role in shaping the developing limb bud. Analysis of mesenchymal cell death in developing limbs of other mutants with limb defects shows that alterations in limb bud shape and size are often associated with alterations in mesenchymal cell death. Reduction in the shape and size of *Hd/Hd* limb buds appears to be due to an apparent loss of tissue anteriorly and posteriorly in the developing hand and footplates. This suggests that this loss of tissue may be due to an increase in mesenchymal cell death in the affected regions of the mutant limbs.

The limb skeleton arises from a cartilage model which is laid down in the limb bud mesenchyme in a proximal to distal direction as the limb develops. Defects in adult mutant mice suggest that the *Hd* mutation may affect this process in the distal limb when
the developing hand and footplates are being specified. Duplicated outgrowth and patterning
require a functional apical ectodermal ridge, which is dependent on maintenance signals
from the underlying mesenchyme. \textit{Hoxa13} expression is normally restricted to distal
limb bud mesenchyme, and defects in \textit{Hd} are therefore assumed to reside in the
mesenchymal component of the limb buds. However, due to interactions between limb
bud mesenchyme and the overlying ectoderm, it is possible that mesenchymal defects
caused by the \textit{Hd} mutation may induce secondary defects in the overlying apical
ectodermal ridge. These defects in the apical ectodermal ridge may in turn contribute to
the final phenotypic outcome of the \textit{Hd} mutation. Observations of \textit{Hd/Hd} mutant limb
buds made under the light microscope suggests that there may be alterations in the apical
ectodermal ridge morphology and its association with the underlying mesenchyme.

4.1.1 Programmed cell death

Programmed cell death is required during embryonic development, and has various
functions at many different sites in the developing embryo (reviewed Saunders, 1966;
Jacobson et al., 1997). During limb development, differential cell death is thought to be
involved in shaping the early limb buds in both the chick and the mouse (reviewed
Hinchliffe, 1981; Hinchliffe and Johnson, 1980), and later on it is essential for separation
of developing digits (reviewed in Hinchliffe, 1981; Saunders et al., 1962; van der
Hoeven et al., 1994; Zakeri and Ahuja, 1994).

During normal limb development, prior to digit specification, zones of
mesenchymal cell death are present at the anterior and posterior limits of the apical
ectodermal ridge, these regions are known as the anterior and posterior necrotic zones in
both mouse and chick (Milaire, 1992; Saunders et al., 1962). Hinchliffe and Ede (1967)
suggested that these necrotic zones act as ‘end stops’ to determine the extent of the apical
ectodermal ridge, possibly by limiting the production of factors such as apical ectodermal
ridge maintenance factor, which are required for ridge maintenance. Variations in patterns
of cell death in the limb are correlated with differences in limb bud size/shape and final
digit number. Comparative studies in different species show that differences in final limb
morphology are often preceded by alterations in patterns of cell death during limb
development (reviewed in Hinchliffe, 1981; Hinchliffe and Johnson, 1980). Morphology of the distal limb in chickens and mice are very different; the chicken wing and leg have 3 and 4 digits respectively, whereas mice have five digits on all four limbs. Morphological differences are first observed when the limb buds begin to assume a symmetrical shape, as the hand and footplates are being specified; chicken limbs develop from limb buds that remain narrow throughout development (Hamburger and Hamilton, 1951), in comparison, mouse limbs develop from limb buds that assume a broad and polygonal shape after the initial stages of outgrowth (Martin, 1990; Wanek et al., 1989). These differences in bud morphology have been correlated with the fact that cell death in the anterior and posterior necrotic zones is more extensive in the chick than in the mouse (Milaire, 1992). This finding strongly suggests that the anterior and posterior necrotic zones have roles in shaping the limb buds and influencing limb pattern, possibly by limiting the amount of mesenchyme available for digital development. Observations from the study of limb mutants also support this theory, as increases or decreases in cell death are correlated with increases or decreases in limb bud size and digit number respectively (Hinchliffe, 1974; Milaire, 1992).

4.1.2 Chondrogenesis during limb development

Analysis of limb defects in adult $Hd/Hd$ and $Hd/+ \,$ mice shows that the $Hd$ mutation affects specification of the bones of the hands/wrist and feet/ankles. These defects are associated with a reduction and alteration in the shape of developing mutant limb buds. Reductions in the size and shape of developing limb buds in other mutants are associated with digit reduction or fusion, possibly due to a reduction in the amount of cells and/or space available for digital development (Hinchliffe and Johnson, 1980; reviewed in Chapter 2 of Johnson, 1986).

Prior to the overt differentiation of the osseous limb skeleton, prechondrogenic mesenchymal condensations, often referred to as prechondrogenic blastemas, are laid down within the limb bud mesenchyme (Thorogood and Hinchliffe, 1975; Shubin and Alberch, 1986) the formation of which are an absolute requirement for chondrogenesis to occur. Prechondrogenic blastemas are thought to arise as a result of an increase in cell
density. There is debate as to how this increase in cell density is achieved, but there is evidence that it may involve changes in the surrounding extracellular matrix and cell-cell interactions (Janners and Searles, 1970; Thorogood and Hinchliffe, 1975). Fibronectin is proposed to have a role in the initial cell condensation step during blastema formation, as there is a marked increase in expression and accumulation of fibronectin at the sites of future skeletal elements (Kulyk et al., 1989; Downie and Newman, 1995), and an increase in the cell adhesion molecules NCAM and N-cadherin have also been reported during condensation (Oberlander and Tuan, 1994; Widelitz et al., 1993).

The exact molecular mechanisms involved in determining shape, size and position of the prechondrogenic blastemas in the limb are not fully understood. However, there is evidence that the mechanisms may involve the presence or absence of soluble secreted signalling molecules, as emphasised by characterisation of the mouse mutants short ear (se) and brachypodism (bp) (reviewed in Kingsley, 1994). These mutants have alterations in the size and shape of selected skeletal elements, which can be attributed to alterations in number, size and shape of prechondrogenic blastemas. The se and bp mutations result from inactivation of the genes for BMP5 (bone morphogenetic protein 5) and GDF5 (growth and differentiation factor 5), two members of the TGFβ superfamily (Kingsley et al., 1992; Storm et al., 1994). Once the prechondrogenic blastemas have been specified, cells within them undergo differentiation into chondrocytes and begin to secrete increasing amounts of extracellular matrix macromolecules such that a cartilage model of the future boney skeleton is formed.

The ability of undifferentiated limb mesenchymal cells to produce cartilage, i.e. the chondrogenic potential of cells, can be tested in vitro by disaggregating prechondrogenic limb mesenchymal cells and plating them at high density in micromass cultures. Over a 6 day period, cultures develop cell aggregates which undergo ultrastructural changes and subsequently differentiate into cartilage nodules (Ahrens et al., 1977; Royal and Goetinck, 1977). The extent of cartilage differentiation in cultures can be assessed by staining with alcian blue (pH 1.0), which binds to sulphated glycosaminoglycans in cartilage matrix (Ahrens et al., 1977; Lev and Spicer, 1964). The sequence of events leading to the differentiation of cartilage in culture is thought to be a
recapitulation of in vivo events; in culture, mesenchymal cells condense forming aggregates, cells within these aggregates competent to respond to appropriate signals in the environment then begin to express the cartilage phenotype and secrete cartilage-specific matrix molecules (Ahrens et al., 1977).

Members of the transforming growth factor β (TGFβ) superfamily are strongly implicated in aspects of skeletogenesis. Transcripts of members of this superfamily, such as genes encoding bone morphogenetic proteins (Bmps), growth differentiation factors (Gdfs) as well as transforming growth factors (Tgfs) themselves, are detected at sites where skeletogenesis takes place. Lyons et al. (1989) proposed that expression of different members of the TGFβ superfamily is required at distinct stages of differentiation during the process of chondrogenesis. There is also in vitro evidence supporting the possible role of members of the TGFβ superfamily in chondrogenesis; exogenous application of TGFβ1 or TGFβ2 to high density micromass cultures of limb mesenchyme stimulates the accumulation of type II collagen and cartilage-specific core proteoglycan mRNA (Kulyk et al., 1989), thus implying that they mediate levels of chondrogenesis. Similar results were obtained by Schofield and Wolpert (1990), who found that TGFβ1 increased chondrogenesis in chick limb micromass cultures in a dose dependent manner. In contrast, TGFβ1 can modulate chondrogenesis in micromass cultures of periotic mesenchyme by acting as either an activator or a suppressor of this process (Frenz et al., 1991), suggesting that the influence of members of the TGFβ superfamily on chondrogenesis, may depend on the region of the embryo analysed.

Another family of growth factors, fibroblast growth factors (FGFs) are thought to have putative roles in aspects of skeletal development. Application of 10 ng/ml of FGF2 to micromass cultures of stage 20-22 (Hamburger and Hamilton, 1951) progress zone mesenchyme leads to an inhibition of cartilage formation (Watanabe and Ide, 1993). In contrast, application of 0.1-10 ng/ml FGF2 to micromass cultures of stage 22-23 (Hamburger and Hamilton, 1951) distal limb mesenchyme results in an increase in the levels of chondrogenesis in culture (Schofield and Wolpert, 1990), suggesting that the response of mesenchymal cells to the action of FGF2 may be stage-specific. FGFs exert their effects on cells via low affinity and high affinity cell surface receptors (Klagsbrun
and further evidence for the involvement of FGFs in skeletal development comes from the findings that mutations in both murine (Deng et al., 1994; Yamaguchi et al., 1994; Deng et al., 1996; Colvin et al., 1996) and human FGFRs lead to skeletal defects (Muenke and Schell, 1995), implying that FGFs are an important part of the signalling mechanisms that lead to normal limb development.

During early stages of limb development, prior to the laying down of individual skeletal elements, outgrowth and patterning of the limb is dependent on the presence of the apical ectodermal ridge. This specialised epithelial structure at the distal tip of the bud expresses three FGF family members, \textit{Fgf2}, \textit{Fgf4}, and \textit{Fgf8}. Of these genes, \textit{Fgf4} is differentially expressed, being posteriorly restricted, whereas transcripts of both \textit{Fgf2} and \textit{Fgf8} are present throughout the entire ridge (see Chapter 1). \textit{Fgf4} is proposed to be one of the endogenous ridge signals required to maintain the polarising region, and both the ridge and the polarising region are required for limb outgrowth and patterning.

\subsection*{4.1.3 Role of the apical ectodermal ridge in shaping the limb bud}

In mice, the apical ectodermal ridge is first evident around 11.5 dpc as a thickening of the epithelium running antero-posteriorly along the distal edge of the buds (Carter, 1954; Wanek et al., 1989). Loss of limb structures in vertebrate mutants are sometimes associated with a premature loss or lack of the apical ectodermal ridge. Mutations in the limb deformity (\textit{ld}) gene disrupt differentiation of the apical ectodermal ridge and lead to the loss of distal limb structures (Zeller et al., 1989), and lack of an apical ectodermal ridge in the mouse mutant legless (\textit{lgf}), in association with an increase in mesenchymal cell death, leads to severe loss of hindlimb structures distal to the femur (Singh et al., 1991). Induction and maintenance of the apical ectodermal ridge requires reciprocal interactions between the ectoderm and underlying mesenchyme. Ridge defects may therefore arise due to intrinsic defects in the ectoderm, or as a secondary effect of defects in the underlying mesenchyme. The \textit{Hd} mutation affects \textit{Hoxa13}, which is usually expressed in the distal limb bud mesenchyme, and the presence of ridge defects in \textit{Hd/Hd} mutants implies that these defects may be a secondary effect of the \textit{Hd} mutation.
4.2 Aims of this Chapter

The first section of this chapter details the distribution of mesenchymal cell death in developing limbs of $Hd/Hd$, $Hd/+$, and $++$ embryos at the developmental stages when morphological alterations in limb bud size and shape are first observed. The alterations in limb bud size and shape suggests that the amount of tissue and/or space available for digital development is reduced in $Hd$ mutant limbs. The limb skeleton develops from mesenchymal condensations that form in the developing limb in a proximo-to-distal manner, such that proximal skeletal elements are specified prior to more distal ones. The $Hd/Hd$ adult phenotype suggests that specification of proximal skeletal elements of the limb occurs normally, but that the specification of more distal elements are affected by the mutation. The second section of this chapter analyses the appearance of cartilage rudiments in developing $Hd/Hd$, $Hd/+$ and $++$ limbs, and the results show that loss of digits is associated with lack of formation of mesenchymal condensations that give rise to digits. This suggests that the ability of $Hd/Hd$ undifferentiated mesenchymal cells to undergo chondrogenic differentiation is altered. In order to test this, the third section of this chapter analyses the ability of undifferentiated mesenchymal cells to produce cartilage in vitro culture. TGFβ, FGF2 and FGF4 are known to increase levels of chondrogenesis in culture, and the ability of $Hd/Hd$ mesenchymal cells to respond to these inductive signals were tested. Finally, observations made under the light microscope suggest that $Hd/Hd$ embryos may have an abnormal apical ectodermal ridge, and so the final section of this chapter discusses the results obtained from scanning electron microscopy and histological sections of $Hd/Hd$, $Hd/+$, and $++$ limbs. Results of this chapter are then discussed in relation to what they tell us about the nature of the $Hd$ mutation and the putative role of $Hox$ genes during normal vertebrate limb development.
4.3 Results

4.3.1 Mesenchymal cell death in the developing limbs of Hd mutants

The early phase of mesenchymal cell death normally observed in developing limbs that is thought to influence size and shape of the developing limb bud, was analysed in Hd/Hd, Hd/+, and +/+ limbs at 11.5 - 12.5 dpc by staining with vital dye Nile Blue Sulphate (NBS). At 11.5 dpc, both forelimbs and hindlimbs of Hd/Hd embryos (11 embryos analysed) are less paddle-shaped than their +/+/Hd/+ littermates (Figure 4.1A and B). In addition, these limb buds possess an opaque patch of cells at the distal tip that appears very white in comparison to the rest of the limb bud. This is visible under the light microscope (compare A and B in Figure 4.1). NBS staining of these Hd/Hd embryos reveals that both forelimbs and hindlimbs have a large area of strong staining in posterior mesenchyme at the distal tip (Figure 4.2C and D). This strong staining represents mesenchymal cell death, and correlates with the position of the opaque patch of cells. This massive distal mesenchymal cell death is not detected in either the forelimbs or hindlimbs of 11.5 dpc +/+/Hd/+ embryos (twenty-four embryos analysed; Figure 4.2 A and B).

At 12.5 dpc, Hd/Hd limbs are clearly not paddle-shaped, and have a more pointed morphology than their +/+ and Hd/+ littermates (Figure 4.1 C and D). In addition, the opaque patch of cells first observed at 11.5 dpc, is still present at the distal tip of the limbs buds (Figure 4.1 D). This is not present in +/+ or Hd/+ limb buds (Figure 4.1 C). Hd/Hd limb buds show punctate mesenchymal cell death was detected throughout the developing hand and footplates of Hd/Hd embryos (6 embryos analysed), with strong NBS staining in the anterior and posterior distal mesenchyme (Figure 4.2 G, H, and K). There is also strong staining in the apical ectodermal ridge (Figure 4.2 K). At this stage, developing limbs of Hd/+ and +/+ embryos exhibit two small regions of cell death in anterior and posterior mesenchyme of developing hand and footplates (Figure 4.2 E and F). However, in contrast to the pattern of cell death in +/+ footplates, the footplates of Hd/+ embryos which are reduced along the distal-anterior border (see chapter 3), have an increase in the anterior region of mesenchymal cell death (Figure 4.2 F). Cell death is detected in the apical ectodermal ridge of both +/+ and Hd/+ limbs at this stage (Figure
4.2 I and J) These regions of mesenchymal cell death in +/+ limbs have been previously identified as the anterior and posterior necrotic zones respectively (Milaire, 1992).

At 14.5 dpc when interdigital cell death is occurring, 

\textit{Hd/+} limbs have a strongly staining region of cell death along the anterior border of the developing feet in addition to interdigital cell death (Figure 4.2 L and M). Mice showing the 

\textit{Hd/Hd} limb phenotype at this stage have not been analysed.
Figure 4.1
Photographs of the hindlimbs of +/+Hd/+, +/+ and Hd/Hd embryos at 11.5 and 12.5 dpc showing the shape of the hindlimb buds and the opaque patch of cells in Hd/Hd limb buds that colocalise to regions where mesenchymal cell death is detected. (A): ventral view of a+/-+Hd/+ 11.5 dpc hindlimb bud; (B): ventral view of a Hd/Hd 11.5 dpc hindlimb bud indicating the region of cells that have a very white and opaque appearance (white arrows) in comparison to the rest of the bud and the appearance of +/+/Hd/+ buds; (C): dorsal view of a+/-+ 12.5 dpc hindlimb bud; (D): ventral view of a Hd/Hd 12.5 dpc hindlimb bud indicating the region of cells that have a very white and opaque appearance (white arrows) in comparison to the rest of the bud and the appearance of mesenchyme in +/- limb buds.

Figure 4.2
Photographs of +/-Hd/+, +/-, Hd/+ and Hd/Hd limbs at 11.5, 12.5 and 14.5 dpc showing regions of cell death as detected with Nile Blue Sulphate staining. (A): 11.5. dpc +/-Hd/+ hindlimb bud; (B): 11.5. dpc +/-/Hd/+ forelimb bud; (C): 11.5. dpc Hd/Hd hindlimb bud showing a region of cell death in the distal mesenchyme (arrow) not detected in +/-/Hd/+ buds; (D): 11.5. dpc Hd/Hd forelimb bud showing a region of cell death in the distal mesenchyme (arrow) not detected in +/-/Hd/+ buds; (E): lateral view of a 12.5 dpc +/- embryo showing regions of cell death in the anterior and posterior limb mesenchyme (black arrows), inset shows the ventral aspect of the left hindlimb bud indicating the anterior cell death (arrow); (F): lateral view of a 12.5 dpc Hd/+ embryo showing regions of cell death in the anterior and posterior limb mesenchyme (black arrows), inset shows the ventral aspect of the right hindlimb bud indicating the anterior cell death (arrow) which is more extensive than in +/- hindlimbs; (G): 12.5. dpc Hd/Hd forelimb bud showing regions of cell death in the distal mesenchyme (arrows) not detected in +/- or Hd/+ buds; (H): 12.5. dpc Hd/Hd hindlimb bud showing regions of cell death in the distal mesenchyme (arrows) not detected in +/- or Hd/+ buds; (I): 12.5 dpc +/- embryo showing cell death in the apical ectodermal ridge of the hindlimb buds (arrows); (J): 12.5 dpc Hd/+ embryo showing cell death in the apical ectodermal ridge of the hindlimb buds (arrows); (K): 12.5 dpc Hd/Hd embryo showing cell death in the apical ectodermal ridge of the hindlimb buds (white arrow head) and mesenchymal cell death in the hindlimb (black arrow) and forelimb (white arrow); (L): ventral aspect of a 14.5 12.5 dpc +/- hindlimb bud indicating interdigital cell death (arrows); (M): ventral aspect of a 14.5 12.5 dpc +/- hindlimb bud indicating interdigital cell death (arrows).

Note: In the procedure used above, Nile Blue Sulphate stains the entire embryo/limb blue. However, areas where staining is very dark blue and punctate represent regions of the embryo where cell death is taking place.
4.3.2 Development of skeletal elements of the limb

Patterns of cartilaginous rudiments of the limb skeleton were analysed by staining whole embryos and limbs with alcian green. At 11.5 dpc, there is one dense prechondrogenic mesenchymal condensation in the proximal portion of the pointed $Hd/Hd$ limb buds and two more distal condensations. These represent the developing humerus/femur and radius and ulna/tibia and fibula. At 12.5 dpc these proximal elements in $Hd/Hd$ embryos have developed into cartilage rudiments easily distinguishable from surrounding non-chondrogenic mesenchyme (Figure 4.3 c and f). Histological sections show that there are signs of a fourth prechondrogenic mesenchymal condensation beginning to develop in the posterior half of the developing hand also detected in histological sections (see later in this chapter). In comparison, the limbs of $+/+$ and $Hd/+\$ embryos are more advanced and at 12.5 dpc show clear evidence of digit formation in the hand and footplates (Figure 4.3 a, b, d, and e). Between 13.5 and 14.5 dpc, the condensations in $Hd/Hd$ limb buds become more prominent, and the distal condensation is more advanced in the forelimb than in the hindlimb (Figure 4.3 i and j; Figure 4.4 e and f). It is assumed that this condensation gives rise to the single digit that develops. In comparison, the limbs of $+/+$ and $Hd/+\$ embryos are well formed and there are five digits developing in each of the hands and feet (Figure 4.3 g and h). At 13.5 and 14.5 dpc the reduction in $Hd/+\$ hindlimb digit I is evident (Figure 4.3 h; Figure 4.4 e). By 15.5 dpc, staining with alcian blue and alizarin red s shows that skeletal elements are fully specified, and some of them are beginning to ossify; the $+/+$ and $Hd/+\$ but not the $Hd/Hd$ femur shows signs of ossification (Figure 4.5 A, C, and E), whereas all of the long bones in the forelimbs of all three phenotypes shows signs of ossification (Figure 4.5 B, D, and F). The reduction in the $Hd/+\$ hindlimb digit I is clearly shown in Figure 4.5 C, and the presence of a single digit in the forelimbs and hindlimbs of $Hd/Hd$ mice are shown in Figure 4.5 E and F. In the forelimb (Figure 4.5 F) this digit is made up of two small bones - one resembling a metacarpal the other a phalange. There is no sign of joint formation in the hindlimb digit until 16.5 dpc, when a small distal cartilage element is detected at the distal tip of the single hindlimb digit (Figure 4.6 A). At this stage the long bones of the hindlimbs have begun to ossify as in $+/+$ embryos of the same stage (Figure 4.6 B).
Figure 4.3

Alcian green stained limbs showing developing cartilage elements of +/+, Hd/+ , and Hd/Hd embryos at 12.5 and 13.5 dpc. Anterior of the limb is towards the top. (a) 12.5 dpc +/+ left hindlimb bud; (b) 12.5 dpc Hd/+ right hindlimb bud; (c) 12.5 dpc Hd/Hd right hindlimb bud; (d) 12.5 dpc +/+ right forelimb bud; (e) 12.5 dpc Hd/+ right forelimb bud. (f) 12.5 dpc Hd/Hd left forelimb bud; (g) 13.5 dpc +/+ left hindlimb bud. (h) 13.5 dpc Hd/+ right hindlimb bud. Arrow indicates reduced digit I; (i) 13.5 dpc Hd/Hd right hindlimb bud. Arrow indicates beginning of digital condensation; (j) 13.5 dpc Hd/Hd right forelimb bud. Arrow indicates developing digital condensation.
Figure 4.4

Alcian green stained limbs showing developing cartilage elements of +/+, Hd/+ and Hd/Hd embryos at 14.5 dpc. (a) 14.5 dpc +/+ hindlimb; (b) 14.5 dpc +/+ forelimb; (c) 14.5 dpc Hd/+ hindlimb showing reduced digit I; (d) 14.5 dpc Hd/+ forelimb; (e) 14.5 dpc Hd/Hd hindlimb; (f) 14.5 dpc Hd/Hd forelimb.
Figure 4.5

Alcian blue and Alizarin red S stained limbs of 15.5 dpc +/+, Hd/+, and Hd/Hd embryos. (A): +/+ hindlimb with five well formed digits distally (I - V), digit I is anterior and V is posterior. Ossification is present in the femur (fm) but not in the tibia (t), fibula (f) or other skeletal elements; (B): +/+ forelimb with five well formed digits distally (I - V), digit I is anterior and V is posterior. Ossification (staining) is present in the scapula (s), humerus (h), radius (r), and ulna (u); (C): Hd/+ hindlimb with a reduced anterior digit I (arrow), in this photograph, digit V is curled round under the rest of the hand and is therefore not fully visible; (D): Hd/+ forelimb with five well formed digits distally (I - V), digit I is anterior and V is posterior. Ossification (red staining) is present in the scapula (s), humerus (h), radius (r), and ulna (u); (E): Hd/Hd hindlimb with evidence of only one digit developing distally (arrow). The tibia (t), fibula (f) and femur (fm) are well formed but ossification has not yet occurred; (F): Hd/Hd forelimb with a single distal digit (arrow) located fairly centrally in the hand. Carpals are present proximal to this digit, and the radius (r), ulna (u), humerus (h), and scapula (s) are well formed and are beginning to ossify (red staining). Blue: cartilage; red: bone; t: tibia; f: fibula; fm: femur; r: radius; u: ulna; h: humerus; s: scapula.
Figure 4.6
Alcian blue and Alizarin red S stained \( H^d/H^d \) and +/- embryos at 16.5 dpc. (A): 16.5 dpc \( H^d/H^d \) embryo showing skeletal defects restricted to the distal region of the limbs where only one digit develops (arrows); (B): 16.5 dpc +/- embryo showing skeletal structures. Blue: cartilage; red: bone.
4.3.3 *In vitro* chondrogenesis

Chondrogenic differentiation, as demonstrated by alcian blue staining, was monitored in high density micromass cultures of mesenchymal cells taken from distal limb buds of +/+, Hd/+, and Hd/Hd embryos at 11.5 and 12.5 dpc. Results from different experimental runs were pooled and the mean number of cartilage nodules produced in culture by mesenchyme from individual +/+, Hd/+, and Hd/Hd limbs calculated.

Little overall difference was detected between the ability of ++/Hd+ and Hd/Hd limb mesenchyme to produce cartilage in culture. Figure 4.7 shows that there is a similar mean nodule number in micromass cultures of mesenchyme from 11.5 dpc rounded ++/Hd+ limb buds and narrow Hd/Hd limb buds. Although the mean number of nodules from ++ /Hd/+ and Hd/Hd limbs is similar, there was a large variation of nodule number in cultures assessed, as shown by the large standard deviation. However, it is important to note that mesenchymal cells from the limbs of Hd/Hd embryos from four out of five litters produced fewer cartilage nodules in culture than their ++/Hd+ littermates, suggesting that there may indeed be a difference between ++/Hd+ mesenchyme at 11.5 dpc. With cells taken from limb buds of 12.5 dpc embryos, there is a dramatic difference in the extent of chondrogenesis in cultures of Hd/Hd mesenchyme compared with that in cultures of +/- and Hd/+ mesenchyme. In +/- and Hd/+ cultures, many densely packed cartilage nodules are present whereas Hd/Hd cultures show a marked reduction or even a total loss of cartilage nodules as demonstrated by alcian blue staining (Figure 4.9). Figure 4.8 shows the average nodule number from 12.5 dpc +/-, Hd/+ and Hd/Hd embryos. Embryos were taken from five different litters. No marked difference between +/- and Hd/+ is observed. Although cultures of limb mesenchyme from 12.5 dpc Hd/Hd mutant mouse embryos show much less chondrogenesis, muscle still differentiates to around the same level as in Hd/+ and +/- cultures (Figure 4.9 c-f).
Figure 4.7
Histogram showing the average number of nodules in micromass cultures set up at normal density with mesenchyme from 11.5 dpc +/+/Hd/+ and Hd/Hd limbs.
Figure 4.8
Histogram showing the average number of cartilage nodules in micromass cultures set up with mesenchyme from 12.5 d.p.c. limbs including the standard deviation.
Figure 4.9
Cartilage nodules in 6 day micromass cultures of 12.5 dpc +/- and *Hd/Hd* limb bud mesenchyme stained with Alcian blue for cartilage. (A): 6 day micromass culture of 12.5 dpc +/- limb bud mesenchyme stained with Alcian blue for cartilage, note multiple cartilage nodules have developed; (B): 6 day micromass culture of 12.5 dpc *Hd/Hd* limb bud mesenchyme stained with Alcian blue for cartilage showing a reduction in the amount of cartilage produced by the mutant mesenchymal cells; (C): medium power phase contrast photograph of cartilage nodules (arrows) in a 6 day micromass culture of 12.5 dpc +/- limb bud mesenchyme; (D): medium power phase contrast photograph of cartilage nodules (arrows) in a 6 day micromass culture of 12.5 dpc *Hd/Hd* limb bud mesenchyme. Well developed cartilage nodules are not detected; (E): high power phase contrast photograph of a cartilage nodule and muscle fibres in a 12.5 dpc +/- micromass; (F): high power phase contrast photograph of a 12.5 dpc *Hd/Hd* micromass culture. Arrow heads indicate muscle fibres.
The reduction in chondrogenesis in cultures of \(Hd/Hd\) mesenchymal cells may be due to the production of a factor or factors that inhibit chondrogenesis, or because of the lack of production of a factor required for chondrogenesis. To begin to test these hypotheses, a series of cultures from 12.5 dpc \(+/+\) and \(Hd/Hd\) limbs were set up.

Cultures were fed daily either with a 50:50 or 25:75 mixture of fresh medium:conditioned medium. Feeding 12.5 dpc \(Hd/Hd\) cultures with conditioned medium obtained from \(+/+\) cultures at the same stage has little effect on levels of chondrogenesis in mutant cultures and at best only a very few cartilage nodules were formed (Figure 4.10). The \(+/+\) cultures fed with \(Hd/Hd\) conditioned medium showed no inhibition of chondrogenesis but instead showed a slight increase. In a second experiment, cultures were set up with a 1:1 mixture of cells from 12.5 dpc \(Hd/Hd\) and \(+/+\) limb buds. Results show that, in the mixed cultures, more cartilage nodules developed than in the 100% \(Hd/Hd\) derived cultures, but the number of nodules produced is less than in the 100% \(+/+\) derived cultures (Figure 4.11). In addition, increasing the cell density of \(Hd/Hd\) cultures has no effect on cartilage differentiation (Figure 4.12).

A second experiment was carried out to test the action of growth factors on cartilage differentiation in culture. Micromass cultures of 12.5 dpc ++, \(Hd/+\), and \(Hd/Hd\) limb mesenchymal cells were set up, cultured for six days and fed daily with fresh medium or medium containing 10 ng/ml of TGFβ1, FGF2, or FGF4. Preliminary results suggest that none of the growth factors rescued the ability of \(Hd/Hd\) mesenchymal cells to give rise to cartilage in culture to a level comparable to \(+/+\) or \(Hd/+\) mesenchymal cells. TGFβ1 and FGF2 both induced a decrease in the number of cartilage nodules in cultures of \(Hd/Hd\) limb mesenchymal cells, whereas FGF4 caused a slight increase in nodule number (Figure 4.13). When added to cultures of \(+/+\) or \(Hd/+\) mesenchymal cells, TGFβ1 appeared to cause a decrease and both FGF2 and FGF4 appeared to cause an increase in the number of cartilage nodules in culture (Figure 4.13).
Figure 4.10
Histogram showing average nodule number in cultures of +/+, Hd/+,
and Hd/Hd mesenchymal cells from 12.5 dpc embryos fed with
conditioned medium.
Figure 4.11
Histogram showing the number of cartilage nodules in micromass cultures set up with mesenchyme from 12.5 dpc +/+ and Hd/Hd limb bud mesenchyme, and in cultures of a 50:50 mixture of +/+ and Hd/Hd mesenchymal cells.
Figure 4.12
Histogram showing the average number of nodules in micromass cultures set up at double density with mesenchyme from 11.5 dpc $+/+Hd/+ \text{ and } Hd/Hd$ limbs.
Phenotype of mesenchymal cells in culture

Figure 4.13
Histogram showing average nodule number in cultures of mesenchymal cells taken from limbs of +/+ and Hd/Hd 12.5 dpc embryos fed with different growth factors.
4.3.4 The *Hd* mutation also affects the morphology of the apical ectodermal ridge

Observations made under the light microscope suggested that *Hd/Hd* limb buds have apical ectodermal ridge defects. Scanning electron microscopy (SEM) allowed high resolution visual analysis of *Hd* mutant limbs, and the results confirmed that prior to 11.5 dpc, no alterations in limb bud morphology are observed in litters produced by heterozygous crosses. At 9.5 and 10.5 dpc, limb buds of all embryos analysed look symmetrical in shape (see Figure 3.11 in Chapter 3) and are similar in size, with forelimb buds being more advanced than hindlimb buds. At 11.5 dpc, limb buds of *Hd/Hd* embryos are narrower and more pointed than those of+/+/*Hd/+ littermates (Figure 4.14 and 4.15). SEM reveals that these narrower limb buds have an apical ectodermal ridge that appears thicker and more pronounced than that of the rounded+/+/Hd/+ limb buds (Figure 4.14, and 4.15). At 12.5 dpc, normal limbs have well developed polygonal hand and footplates and the apical ectodermal ridge is less pronounced (Figure 4.16 and 4.19). At this stage, *Hd/Hd* limb buds are noticeably narrower due to an apparent reduction in width along the antero-posterior axis, and the limb buds are pointed rather than polygonal in shape (Figure 4.18). A prominent elevation running across the dorsal surface of the developing hands and feet is visible; this elevation is likely to correspond to the site of digit formation. In striking contrast to the normal buds, the apical ectodermal ridge of these pointed buds remains prominent (Figure 4.18 and 4.19). 24 hours later, at 13.5 dpc, the apical ectodermal ridge is not visible on +/+ and *Hd/+* hand and footplates, whereas the limbs *Hd/Hd* embryos still have a visible apical ectodermal ridge (Figure 4.20). By 14.5 dpc, individual digits are seen on each of the four limbs of *Hd/+* and +/+ embryos, whereas only a single digit is present on each of the four limbs of the *Hd/Hd* embryos. Even at this stage, long after the apical ectodermal ridge has totally regressed on both *Hd/+* and +/+ limbs, a prominent ridge-like structure persists along the whole distal outline of the single digit on the *Hd/Hd* limbs.
Figure 4.14

Scanning electron micrographs (SEM) of the limb buds of an 11.5 dpc $Hd/Hd$ embryo. (A): SEM of the left forelimb and hindlimb buds of an 11.5 dpc $Hd/Hd$ embryo; (B): higher power SEM of the forelimb bud in (A) indicating the prominent apical ectodermal ridge (arrows).
Figure 4.15
Scanning electron micrographs (SEM) of the limb buds of an 11.5 dpc +/+Hd/+ embryo. (A): SEM of the left forelimb and hindlimb buds of an 11.5 dpc +/+Hd/+ embryo; (B): higher power SEM of the hindlimb bud in (A); (C): higher power SEM of the paddle-shaped forelimb bud in (A).
Figure 4.16
Scanning electron micrographs (SEM) of the limb buds of a 12.5 dpc +/+ embryo. (A): SEM of the right forelimb and hindlimb buds of a 12.5 dpc +/+ embryo. Anterior is to the right; (B): higher power SEM of the hindlimb bud in (A), anterior is to the top; (C): higher power SEM of the paddle-shaped forelimb bud in (A), anterior is to the left.
Figure 4.17
Scanning electron micrographs (SEM) of the limb buds of a 12.5 dpc *Hd/+* embryo.

(A): SEM of the right forelimb and hindlimb buds of a 12.5 dpc *Hd/+* embryo. Anterior is to the left; (B): higher power SEM of the hindlimb bud in (A), anterior is to the left.
Figure 4.18
Scanning electron micrographs (SEM) of the limb buds of a 12.5 dpc Hd/Hd embryo. (A): SEM of the left forelimb and hindlimb buds of a 12.5 dpc Hd/+ embryo. Anterior is to the left; (B): higher power SEM of the hindlimb bud in (A) indicating the prominent apical ectodermal ridge (arrows), anterior is to the left. fl: forelimb bud; hl: hindlimb bud.
Figure 4.19

Scanning electron micrographs (SEM) of the limb buds of 12.5 dpc +/+ and Hd/Hd embryos indicating the apical ectodermal ridge. (A): SEM of the distal tip of a 12.5 dpc +/+ forelimb bud. The apical ectodermal ridge (arrows) is not as pronounced as in (B); (B): SEM of the distal tip of a 12.5 dpc Hd/Hd hindlimb bud indicating the prominent apical ectodermal ridge (arrows). Anterior is to top; (C): higher power SEM of the apical ectodermal ridge in (A); (D): higher power SEM of the apical ectodermal ridge in (B) indicating the prominent apical ectodermal ridge (arrows).
Figure 4.20
Scanning electron micrographs (SEM) of +/+, Hd/+ and Hd/Hd embryos at 13.5 dpc clearly indicating the differences in limb bud morphology between the three phenotypes. 
(A): 13.5 dpc +/+ embryo with well developed polygonal forelimbs and hindlimbs; (B): 13.5 dpc +Hd+ embryo with well developed polygonal forelimbs and hindlimbs with a reduction along the anterior margin of the developing foot; (C): 13.5 dpc Hd/Hd embryo with narrow and pointed forelimbs and hindlimbs.
Semi-thin sections of +/-, Hd/+, and Hd/Hd limbs between 11.5 and 12.5 dpc were cut from the tip to the base, or from anterior to posterior of the bud. Sections show that at 11.5 dpc, ++/Hd+ limb ectoderm consists of a basal ectodermal layer of cuboidal epithelial cells, and a more superficial layer of flattened cells, the periderm (Figure 4.21). The apical ectodermal ridge consists of tightly packed, pseudostratified epithelial cells, that are continuous with the basal epithelial cells and covered with periderm (Figure 4.21). In contrast, the basal layer of Hd/Hd limb ectoderm is not a simple cuboidal epithelium (Figure 4.21). It appears multicellular in places and the apical ectodermal ridge is very prominent, consisting of many cells, bulging at the basal surface and protruding into the underlying limb mesenchyme. At 12.5 dpc, the ectoderm of Hd/+ limbs was similar in appearance to that of +/- limbs, however, the apical ectodermal ridge was slightly thicker anteriorly than in +/- limbs. At this stage, sections of Hd/Hd limbs showed that the apical ectodermal ridge is very prominent, and a large gap exists between the ridge and the underlying mesenchyme of the limb.

There are visible differences in the appearance of Hd/Hd limb mesenchyme when compared to that of Hd/+ and +/- limb buds. At 12.5 dpc, mesenchymal cells of both +/- and Hd/+ limb buds are densely packed and digital condensations are visible in which cells are tightly packed together (Figure 4.21), these condensation will later develop into the digits. The marginal venous sinus is visible in the subapical mesenchyme which is in close association with the ridge. In contrast, mesenchyme of Hd/Hd limb buds appear somewhat less organised, mesenchymal cells are not densely packed, many macrophages are detected, and digital condensations are not present in Hd/Hd limbs analysed here. The marginal venous sinus is expanded, and surrounded by large extracellular spaces, resulting in a large gap between the underlying mesenchyme, marginal venous sinus and apical ectodermal ridge.
Figure 4.21

Semi-thin araldite sections of 11.5 and 12.5 +/+ and \textit{Hd/Hd} limbs showing the morphology of the apical ectodermal ridge. \textbf{(A)}: semi-thin section of an 11.5 dpc +/+ forelimb showing the apical ectodermal ridge (black arrow), the marginal venous sinus (white arrow head) in the subapical mesenchyme, and a central mesenchymal condensation (white arrow); \textbf{(B)}: semi-thin section of an 11.5 dpc \textit{Hd/Hd} forelimb showing the apical ectodermal ridge, the enlarged marginal venous sinus and gap under the apical ectodermal ridge (black arrow), and the presence of macrophages indicating regions of mesenchymal cell death (white arrows); \textbf{(C)}: section of a 12.5 dpc +/+ forelimb bud indicating mesenchymal condensations (white arrow) that will give rise to digits. Interdigital mesenchyme is loosely packed (black arrow), the marginal venous sinus is present in the subapical mesenchyme (white arrow head), and the apical ectodermal ridge (black arrow head) is flattened and less prominent than at 11.5 dpc; \textbf{(D)}: section of a 12.5 dpc \textit{Hd/Hd} forelimb bud indicating a single mesenchymal condensation (white arrow) in the posterior half of the bud which will give rise to digit. The remainder of the limb bud mesenchyme is loosely packed (black arrow). The marginal venous sinus is present in the subapical mesenchyme. Anterior is at the bottom left of the photograph; \textbf{(E)}: high power of the apical ectodermal ridge (black arrow) of a 12.5 dpc \textit{Hd/Hd} hindlimb bud. There is a gap between the apical ectodermal ridge and the underlying mesenchyme (white arrow head), and the marginal venous sinus is extended (white arrow).
4.4 Discussion and Conclusion

4.4.1 Summary of results
In this chapter it is shown that the limb defects in *Hd* mutants are associated with alterations in mesenchymal cell behaviour and in apical ectodermal ridge morphology. Limbs of *Hd* mutants exhibit an increase in mesenchymal cell death that corresponds to the reported alterations in limb bud morphology. Loss of digits in *Hd/Hd* mice is preceded by a reduction in the number of digital blastemas that develop in the mutant hand and footplates, and *Hd/Hd* mesenchymal cells appear to have an autonomous inability to undergo cartilage differentiation, as analysed in vitro. Furthermore, there is evidence to suggest that the alterations in mesenchymal cell behaviour induce a secondary defect in the apical ectodermal ridge.

4.4.2 Mesenchymal cell death is affected by the *Hd* mutation
The reduction or loss of limb structures in *Hd* mutants is associated with reductions in the size and shape of developing mutant limb buds (Chapter 3). Previous studies have shown that mesenchymal cell death plays a putative role in determining the size and shape of the developing limb bud, and in doing so influences limb patterning (reviewed Hinchliffe and Johnson, 1980; Hinchliffe, 1981; Saunders et al. 1962; Zakeri and Ahuja, 1994). The results reported here show that alterations in limb bud morphology of *Hd* mutants are associated with changes in the normal patterns of mesenchymal cell death in developing hand- and footplates. An increase in mesenchymal cell death is first observed in the limbs of *Hd/Hd* embryos at 11.5 dpc, which then becomes more pronounced 12.5 dpc; cell death is extensive throughout most of the distal mesenchyme of both hand- and footplates. In contrast, alterations in the normal patterns of cell death are only detected in the footplates of *Hd/+* embryos; there is an increased area of mesenchymal cell death at the anterior margin. This is first observed at 12.5 dpc, when morphological differences are first detected between *Hd/+* and +/+ footplates, and becomes more pronounced at 13.5 dpc.

Increases in mesenchymal cell death are observed in *Hd/+* and *Hd/Hd* limbs at the same time as reductions in size and shape of the limbs buds are first detected. These
reductions in size and shape suggest that there may be a decrease in the numbers of cells present in the mutant limb buds. As in other developing systems, the number of cells in developing limb buds may be controlled by the levels of cell proliferation and cell death. Homeostasis of the number of cells is maintained by a critical balance of cell proliferation and cell death, and alterations in this balance may lead to an increase or decrease in the numbers of cells available for further development. Little is known about the exact mechanisms involved in regulation of cell proliferation and cell death in the limb. However, genetic studies provide evidence that, as in the rest of the embryo, cell death in the limb may be under genetic control (Hinchliffe and Ede, 1967; Hinchliffe and Thorogood, 1974; van der Hoeven et al., 1994; Zakeri et al., 1994). One could therefore predict that the $Hd$ mutation in some way affects cell death control mechanisms in the limb leading to an increase in mesenchymal cell death in $Hd$ limbs reducing the number of viable mesenchymal cells available for further development of the limb. Timing and localisation of the increase in mesenchymal cell death may be responsible in part, for the alterations in size and shape of mutant limb buds. It is not evident how the observed changes in cell death in $Hd$ limbs occur. It is possible that they could be due to direct alterations in cell death regulation, or to the withdrawal of cell maintenance/survival factors, or inability of cells to respond appropriately to these factors due to the lack of the required receptors. Irrespective of how cell death is modulated by the $Hd$ mutation, results described here provide circumstantial evidence to support the importance of cell death during limb development, and its putative role in limb patterning as suggested by Zakeri and Ahuja (1994).

The molecular basis of $Hd$ is a deletion in $Hoxa13$ (Mortlock et al., 1996), which is normally expressed in the distal mesenchyme of developing limbs (Haack and Gruss, 1992). Recent evidence from analysis of transgenic mice in which $Hoxa13$ has been functionally inactivated, suggest that this gene is required for specification of the hands and feet (Fromental-Ramain et al., 1996). Other $Hox$ genes of the $Hoxa$ and also of the $Hoxd$ clusters are expressed in developing limbs. They are involved in limb patterning, although the precise mechanisms by which they influence limb pattern is not fully understood. However, there is evidence to suggest that $Hox$ genes are important in
controlling the amount of tissue available for skeletal development (Davis and Cappechi, 1994, 1996; Dolle et al., 1993; Favier et al., 1995, 1996; Small and Potter, 1993; Davis et al., 1995; Fromental-Ramain et al., 1996). The amount of tissue could be controlled by influencing the levels of cell proliferation and/or mesenchymal cell death. Hd/Hd limbs exhibit an increase in mesenchymal cell death which accompanies a reduction in the size of the developing limb buds. It is therefore possible that one of the normal functions of Hoxa13 could be to prevent or control mesenchymal cell death in the limb, thus allowing them to contribute to the limb skeleton. Hox genes encode proteins that have the ability to bind to specific DNA sequences in target genes influencing transcription of these genes. Target genes of HOXA13 have not yet been identified, but the observed increase in cell death in Hd/Hd limbs which are thought to lack functional wildtype HOXA13, tentatively suggests that these, as yet unidentified target genes, could include cell survival factors and/or their receptors, or even genes known to mediate and/or modulate cell death in other areas of the embryo, for example cmyc, bcl2, bax, or Nedd2 (reviewed in Korsmeyer, 1995; Meredith and Schwartz, 1997; Williams and Smith, 1993).

\textit{cmyc} is an important element in control of cell proliferation, but its presence under conditions of growth arrest, such as growth factor deprivation, can induce apoptosis (Evan et al., 1992). The cellular decision between cell proliferation and cell death may be influenced by other signals, in the distal region of the limb, one of these signals could be HOXA13. Alternatively, HOXA13 could influence the production of a growth factor or other survival stimuli, which when absent would lead to cell death. \textit{Bcl2} encodes a protein which forms part of a multicomponent protein complex that includes Bax (Bcl2 associated protein). Bcl2 has been found to oppose cell death whilst Bax promotes it, and Bcl2 heterodimerises with Bax, counteracting its death promoting qualities (reviewed in Korsmeyer, 1995). It has been proposed that the Bcl2:Bax ratio within a cell represents a cell-autonomous mechanism that predetermines a cell’s life or death response to apoptotic stimuli (reviewed in Korsmeyer, 1995). There is no evidence to suggests that either \textit{bcl2} or \textit{bax} are targets of HOXA13. Nevertheless, one could envisage that the increased cell death in \textit{Hd} mutants could be due to an inappropriate response to signals that normally control cell death in the limbs, due to alterations in the
Bcl2:Bax ratio in mutant mesenchymal cells. However, since functional inactivation of Hoxa13 results in a less severe phenotype than that of Hd/Hd mice, this suggests that the altered function of Hd may be complex. If a mutant HOXA13 protein is transcribed, and is stable in cells, it is possible that the more severe phenotype in Hd/Hd mice could be due to a gain-of-function of this protein. Therefore, it may be that in the normal limb, HOXA13 does not interact with genes known to mediate and/or modulate cell death, but in Hd, the mutant HOXA13 gains the ability to influence these genes and/or other genes involved in cell survival.

There is evidence to suggest that Hoxa13 may be involved in the regulation of homophilic cell-to-cell adhesiveness (Yokouchi et al., 1995), and it has been suggested that cell-to-cell adhesions may in some way modulate cell survival (reviewed in Meridith and Schwartz, 1997). In addition, survival of many cell types is thought to involve integrin-mediated adhesion of cells to extracellular matrix. In the absence of the appropriate contacts, cells may die (reviewed in Meridith and Schwartz, 1997), and there is evidence to suggest that signals from growth factor receptors can be modulated by cell adhesion, such that events downstream of growth factor binding are affected. It is possible therefore that Hoxa13 may also influence cell-to-matrix as well as cell-to-cell adhesiveness, in such a way that lack of functional HOXA13 alters cell interactions which either directly or indirectly lead to cell death.

4.4.3 Specification of mesenchymal condensations in developing hand and footplates are affected by the Hd mutation

Proximal skeletal elements of Hd mutant limbs are unaffected by the mutation, but elements distal to the radius/ulna and tibia/fibula are abnormal or missing. During normal limb development, skeletal elements are specified as the buds grow out. Within the mesenchymal core of the limb bud, condensations, also known as blastemas appear in a fixed order in a given limb with proximal blastemas being laid down earlier than more distal ones. These blastemas undergo chondrogenic differentiation giving rise to a cartilagenous model of the future skeletal elements. In the mouse, the digital blastemas are specified in a specific order such that digit IV is specified first, followed by III, II and
then finally digits I and V appear around the same time. In *Hd/Hd* limbs, specification of proximal skeletal elements is unaffected, but the number of digital condensations are reduced from five to one, and these single condensations appear almost a day later than the digital condensation in *+/+* and *Hd/+* limbs. Therefore, loss of distal limb structures in *Hd/Hd* limbs is not due to regression of digital condensations as only one condensation is ever specified in the hand and footplates. It appears that the *Hd* mutation in some way affects the ability of mutant mesenchymal cells to form digital condensation, but not of more proximal ones. Hindlimb defects observed in adult *Hd/+* mutants are also reflected in embryonic limbs; proximal skeletal elements are laid down as normal, but the phalanges of hindlimb digit I *Hd/+* are reduced or absent. Reduction in size or absence of these structures reflect the reduction in the size of *Hd/+* footplates. If the amount of cells or space available for development of the distal region of the digits is reduced, this may inhibit specification of the phalanges. Phalanges are thought to arise due to segmentation of the initial digital condensation, it has been suggested that this condensation may have to reach a critical mass before segmentation will occur, therefore, by reducing it's size further segmentation events may be prevented.

The exact mechanisms that control mesenchymal condensation in the limb are not fully understood, but they are thought to involve an increase in cell density brought about by changes in cell interactions. These interactions are thought to increase the levels of intercellular contact, drawing cells closer together and reducing intercellular spaces between them, thus increasing cell density within the forming condensations (Thorogood and Hinchliffe, 1975). Once blastemas are specified, there is evidence that a threshold number of cells are required for a condensation to become chondrogenic (Wolpert et al., 1979), and that cell-cell interactions are required for cartilage differentiation (Solursh and Reiter, 1980). Deviation from normal cell surface properties which appear to be genetically controlled may thus have dramatic effects on the emerging chondrogenic pattern. The development of defects in *Hd* mutants tentatively suggest that the mutation may alter an early step during specification of the distal limb skeleton, as once cells form a condensation they have the ability to undergo chondrogenic differentiation and subsequent ossification. The mutation may affect the ability of cells to interact with one
another and with the surrounding extracellular environment. This could not only account for the increase in mesenchymal cell death, but also for the inability of Hd/Hd mutant mesenchyme to condense and form digital condensation. If highly specific cell-cell or cell-matrix interactions are required during the specification of the hands and feet, then it is conceivable that defects affecting these interactions will affect development of these regions of the limb. The putative loss of wildtype HOXA13 in Hd/Hd limbs, may alter the ability of mutant mesenchymal cells which would normally express Hoxa13 to interact with surrounding cells and the extracellular environment, which could lead to an increase in mesenchymal cell death and a decrease in chondrogenesis. This suggests that Hoxa13 may be involved in regulation of cell behaviour in the developing hand and footplates, possibly by mediating cell surface properties, as suggested by Yokouchi et al. (1995). It is therefore possible that Hox genes have multiple functions in the developing limb; they could influence the amount of cells available for limb development by mediating cell death and proliferation, and then later, they may influence mesenchymal cell behaviour such cells interact with each other and the extracellular environment, giving rise to mesenchymal condensations, which go on to develop into a cartilage model of the future boney skeleton. Evidence from analysis of other Hox mutants, suggests that different Hox genes could carry out these functions in different regions of the limb, such that Hox 13 paralogues are required for distal development, whereas group 12 and 11 paralogues are required for specification of more proximal limb elements. This may account for the fact that in both Hd/+ and Hd/Hd mutants, proximal limb elements are unaffected by the mutation. The differences in severity of the Hd/+ and Hd/Hd phenotypes and the reported differences in severity of forelimbs versus hindlimbs in both Hd/+ and Hd/Hd mice may be due to compensation of the remaining wildtype protein in Hd/+ mice, and possible limb-type-specific differences in expression patterns of Hoxa13 and compensation by other genes (see Chapter 3).
4.4.4 Chondrogenic potential of undifferentiated mesenchymal cells *in vitro* is affected by the *Hd* mutation

Undifferentiated limb bud mesenchyme has the ability to produce cartilage when cultured at high density for five to six days. Changes in skeletal pattern of *Hd/Hd* limbs compared to +/+ limbs are associated with a reduction in the ability of undifferentiated *Hd/Hd* mesenchymal cells to produce cartilage in culture; dissociated mesenchymal cells from the limbs of *Hd/Hd* mutants do not produce normal levels of cartilage in culture. This decrease in chondrogenesis appears to be a cell autonomous attribute as increasing cell density or mixing *Hd/Hd* cells with +/+ cells in culture does not rescue chondrogenesis. It is becoming increasingly evident that secreted growth factors of the TGFβ superfamily are important in skeletal development (reviewed Kingsley, 1994; Vortkamp, 1997), and mutations in these genes can cause skeletal dysmorphology. The mouse mutant brachypodism (*bp*) exhibits decreases in the length of long bones in the limbs, and changes the number of phalanges that develop on each of the digits (Gruneberg and Lee, 1973). It has been shown that the *bp* mutation also alters early skeletal condensation, and acts in a cell non-autonomous manner both *in vitro* and *in vivo* (Elmer and Selleck, 1975; Gruneberg and Lee, 1973; Owens and Solursh, 1982), which suggested that the mutation might prevent formation of a secreted signalling molecule required for normal skeletal development. *bp* is now known to be caused by mutations in a BMP-like protein known as growth/differentiation factor 5 (*Gdf5*). In contrast to the *bp* mutation, *Hd* does not appear to affect the production of a secreted signalling molecule, as culturing *Hd/Hd* mesenchymal cells in the presence of conditioned medium obtained from +/+ cultures cannot rescue chondrogenesis in mutant cultures. This suggests that in contrast to the *bp* mutation, the defect in *Hd* is cell autonomous. It does not however disprove the possibility that mutant mesenchymal cells have an intrinsic inability to respond to secreted signalling factors due to lack of the appropriate receptors. Another possibility is that *Hd/Hd* mesenchymal cells secrete a factor into the extracellular environment that inhibits chondrogenesis, however, culturing +/+ cells in the presence of conditioned medium obtained from *Hd/Hd* cultures does not inhibit the production of cartilage by the +/+
derived mesenchymal cells. This suggests that the reduction in chondrogenic potential of 
\textit{Hd/Hd} mesenchymal cells is not due to secretion of an inhibitory factor.

It therefore appears that the \textit{Hd} mutation causes a change in mesenchymal cell 
behaviour in culture, and it is possible that these changes prevent mutant mesenchymal 
cells from aggregating. The formation of cell aggregates in culture is a prerequisite for 
cartilage nodule formation (Solursh and Reiter, 1980), and therefore, inhibition of this 
process may result in a decrease in nodule number. It is conceivable that the inability of 
\textit{Hd/Hd} mesenchymal cells to produce cartilage in culture may be due to changes in cell 
surface properties of mutant mesenchymal cells, providing further circumstantial 
evidence to suggest that \textit{Hoxa13} may normally influence cell behaviour by mediating 
cellular interactions. Chondrogenesis requires cell adhesion, and it is thought that one of 
the properties of dead/dying cells is that adhesion is reduced, thus the increase in 
mesenchymal cells death in the limbs of \textit{Hd/Hd} embryos may also have an affect on 
levels of chondrogenesis.

4.4.5 Apical ectodermal ridge morphology is affected by the \textit{Hd} mutation

Limbs of \textit{Hd/Hd} embryos have a persistent and abnormal apical ectodermal ridge, the 
association of which with the underlying mesenchyme is also abnormal. These 
abnormalities are first detected between 11.5 and 12.5 dpc, when the \textit{Hd} mutant 
phenotypes are first observed. The ridge defect is likely to be a secondary affect of the \textit{Hd} 
mutation, as expression of the gene affected in \textit{Hd} is normally restricted to the distal limb 
mesenchyme. Maintenance of the apical ectodermal ridge requires intricate epithelial-
mesenchymal interactions, and it is therefore possible that mesenchymal defects in \textit{Hd} 
mutant induce defects in the overlying ectoderm. Lack of intimate association of the 
\textit{Hd/Hd} ridge and the underlying mesenchyme, may account for persistence of the ridge. 
During early limb development, the apical ectodermal ridge directs outgrowth and 
patterning of the limb, and it is thought that the ridge gives a "stay" signal to the 
underlying mesenchyme maintaining it's lability and proliferation. There is evidence that 
all cells of the progress zone will undergo cartilage differentiation when removed from 
the influence of the ectoderm (Cottrill et al., 1987). Thus, patterning of the cartilage
elements in the limb may essentially involve inhibition of cartilage formation. If this inhibition is the result of an interaction between the ridge and the underlying mesenchyme, then prolonging the presence of the ridge along with its functionality may result in a reduction in the amount of cartilage produced. Therefore, the presence of an abnormal apical ectodermal ridge in \textit{Hd/Hd} limbs suggests that if the ridge does influence cartilage differentiation in the limb, the mutant ridge may also influence the final phenotypic outcome of the \textit{Hd} mutation.
Chapter 5
Distribution of polarising activity and expression of Fgf4, Shh, and Bmps in the developing limbs of the polydactyous chicken mutant Talpid3

5.1 Introduction

Talpid3 (ta3) is a recessive lethal mutation found in chickens. Homozygous (ta3/ta3) embryos rarely survive past 14 days of development and have multiple developmental defects affecting the craniofacial region, vertebral column, cardiovascular system, and limbs (Ede and Kelly, 1964a, b). ta3/ta3 embryos have polydactyous limbs with up to 8 - 10 morphologically similar digits, many of which are partially fused forming a pattern that lacks any obvious antero-posterior polarity (Hinchliffe and Ede, 1967; reviewed by Hinchliffe and Johnson, 1980).

Limb abnormalities in ta3/ta3 limbs are first evident towards the end of the third day of development (Hinchliffe and Ede, 1967); the base of the forelimb buds is elongated along the antero-posterior axis, and proximo-distal outgrowth is reduced (Figure 5.1A. Towards the end of the fourth day, the limb buds begin to take on a characteristic mushroom-like shape (Figure 5.1B) which is capped by an extensive apical ectodermal ridge. As development proceeds, there is a significant lack of programmed cell death within the mesenchyme of ta3/ta3 limb buds (Hinchliffe and Ede, 1967). Mesenchymal condensations begin to form in the normal proximal-to-distal sequence around the sixth day. Separation of the developing condensations however into distinct longitudinal elements does not occur. This is thought to be due to alterations in mesenchymal cell adhesiveness (Ede and Flint, 1975). By the eleventh day of development, polydactyly is clearly evident and there are up to 10 digits present on all four limb rudiments. Limb defects in ta3/ta3 embryos are not restricted to the digits, more proximal skeletal elements are also affected. The long bones of the limbs maybe shortened and fused, and metacarpals and carpals form a single block of cartilage proximal to the digits (Hinchliffe and Ede, 1967; Hinchliffe and Thorogood, 1974).
Figure 5.1
Photographs of a stage 20 and a stage 25 ta\(^3\)/ha\(^3\) embryos *in ovo*. (A) Stage 20 ta\(^3\)/ha\(^3\) embryo showing the abnormal bending of the embryo and the presence of "corckscrew" blood vessels (small arrows). At this stage, the right wing bud is only slightly broader than in normal embryos (large arrow). (B) Stage 25 ta\(^3\)/ha\(^3\) embryo showing the characteristic "mushroom-shaped" wing bud (arrow).
Antero-posterior limb pattern is governed by signalling interactions between the polarising region, apical ectodermal ridge and underlying mesenchyme, involving signals such as Shh and Fgf4 (see Chapter 1). The molecular basis of the ta^ mutation is not yet known, but the lack of antero-posterior polarity in digit pattern in ta^/ta^ limbs suggests that the mutation affects mechanisms involved in patterning. The spatiotemporal distribution of Hoxd genes in the developing limb is suggestive of them playing a role in antero-posterior patterning, and Hoxd genes are downstream targets of signalling from the polarising region and apical ectodermal ridge (see Chapter 1 and 3). Further evidence to suggest that the ta^ mutation affects mechanisms involved in limb patterning comes from the finding that 5' Hoxd genes are ectopically expressed in ta^/ta^ limbs (Izpisúa-Belmonte et al., 1992b). Instead of being expressed in nested domains, mesenchymal expression of Hoxd9, Hoxd11, and Hoxd13 is uniformly distributed across the entire antero-posterior axis of ta^/ta^ buds (Izpisúa-Belmonte et al., 1992). Association between the lack of antero-posterior patterning and mis-expression of Hoxd genes in ta^/ta^ limbs provides further evidence for the importance of these genes in limb patterning, and suggests that the absence of discrete patterns of Hoxd gene expression may contribute to the ta^/ta^ limb phenotype. It is not known how this altered pattern of Hoxd expression arises (Izpisúa-Belmonte et al., 1992), but there are two possibilities; it could be due to a change in the signalling pathways in ta^/ta^ limb buds that normally set up these patterns of gene expression, or it could be due to an inappropriate response of ta^/ta^ cells to patterning signals in the limb.

5.1.1 Aims of this chapter

This chapter addresses the question of how the alterations in Hoxd gene expression patterns and lack of antero-posterior patterning in ta^/ta^ limbs arise. The possibility that they could be due to a change in the distribution of the polarising signal within the mutant limb is assessed by mapping the polarising region in ta^/ta^ limbs. In order to determine whether the mutation affects the response of ta^ limb cells to patterning signals in the limb, the ability of ta^ limb mesenchymal cells to respond to polarising signals are analysed. In addition, the distribution of Fgf4 transcripts is documented, and in
collaboration with Dr. P. Francis-West and Dr. J-C. Izpisúa-Belmonte, the distribution of $Shh$, $Bmp2$, $Bmp4$, and $Bmp7$ transcripts within $ta^3ta^3$ limbs is also determined. The results give insights not only into the basis of defective antero-posterior patterning in $ta^3ta^3$ limbs, but also into the roles of the $Shh$ signal and BMPs during normal limb morphogenesis.

5.2 Materials and Methods

5.2.1 Chick embryos
Fertilised wildtype chicken eggs (White Leghorn) were obtained from Poyndon Farm, Waltham Cross, Hertfordshire, U.K. and fertilised Talpid$^3$ ($ta^3$) eggs were obtained from Dr. D. Burt at the Roslin Institute (BBSRC), Roslin, U.K. Eggs were incubated at 38 ± 1 °C. Wildtype embryos were staged according to Hamburger and Hamilton (1951) and $ta^3ta^3$ embryos were staged according to Hinchcliffe and Ede (1967).

5.2.2 Identification of homozygous $ta^3ta^3$ mutants
Homozygous $ta^3ta^3$ mutants are identified by the presence of corkscrew blood vessels, altered head carriage, abnormal limb morphology and craniofacial defects (Ede and Kelly, 1964a, b; Hinchcliffe and Ede, 1967; see Figure 5.1). In order to confirm the homozygous mutant status of stage 18-20 $ta^3ta^3$ embryos used in the grafting experiments, a single wing or leg bud was removed from the putative $ta^3ta^3$ embryo using sharpened tungsten needles. The isolated bud was then placed carefully into a normal host egg in which the embryo had been prepared by removing the right wing bud in ovo, and the donor bud was grafted onto the host wing stump and pinned into place with pins made from platinum wire (0.025mm, Goodfellow) (Figure 5.2). The egg was resealed, and the manipulated embryo allowed to develop for a further 6 days at 38 ±1 °C, after which the embryo was fixed and stained for skeletal analysis (see below). Skeletal morphology of the grafted limb was compared to that of the contralateral host limb to confirm the $ta^3$ status of the original donor embryo (Ede and Kelly, 1964a; Hinchcliffe and Ede, 1967). At 10 days, grafted $ta^3ta^3$ limbs are broad and have a skeletal pattern with up to 10 digits.
Figure 5.2
Diagram showing how the identification of $ta^3/ta^3$ embryos is confirmed. A single wing or leg bud is removed from a putative $ta^3/ta^3$ embryo and grafted onto the stump of a normal host embryo, which is prepared by removing the right wing bud in ovo. The isolated $ta^3/ta^3$ bud is carefully placed into the host egg and pinned onto the stump with pins made of platinum wire (0.025mm, Goodfellow).
Stage 20/23

Donor wing/leg bud

Discard host wing tip

Stage 21/22

Normal Host Right Wing

— line of incision

Tip of host wing removed to provide a stump on which to graft the $\alpha^3/\alpha^3$ donor wing/leg bud

$\alpha^3/\alpha^3$ donor wing/leg bud removed from embryo using fine forceps

$\alpha^3/\alpha^3$ donor wing/leg bud pinned onto wing stump of normal host embryo and allowed to develop further

Pin

Figure 5.2

Diagram showing how the identification of $\alpha^3/\alpha^3$ embryos is confirmed. A single wing or leg bud is removed from a putative $\alpha^3/\alpha^3$ embryo and grafted onto the stump of a normal host embryo, which is prepared by removing the right wing bud in ovo. The isolated $\alpha^3/\alpha^3$ bud is carefully placed into the host egg and pinned onto the stump with pins made of platinum wire (0.025 mm, Goodfellow).
5.2.3 Mapping polarising activity in developing limb buds of \( ta^3/ta^3 \) embryos

In order to determine the distribution of polarising activity in \( ta^3/ta^3 \) limb buds, blocks of mesenchyme from various locations along the antero-posterior axis of normal and \( ta^3/ta^3 \) limb buds were grafted to the anterior margin of a host wing bud and the subsequent limb pattern was analysed.

Host embryos were prepared by windowing normal, stage 20 chick embryos. A hole in the extra-embryonic membranes surrounding the embryo was made around the area of the right limb bud with fine forceps to allow access to this bud. The future implantation site was prepared by cutting along the anterior margin of the right wing bud close to the base of the apical ectodermal ridge using a sharpened tungsten needle. The apical ectodermal ridge was pulled away from the underlying mesenchyme to create loop in the apical ectodermal ridge (Figure 5.3) under which blocks of mesenchyme were to be grafted.

Donor tissue was prepared from stage 20-24 normal and \( ta^3/ta^3 \) embryos. Embryos were removed from their eggs and placed into sterile growth medium (Appendix 2), their necks were severed and limb buds removed from the body using sharpened tungsten needles. The isolated buds were marked with a small cut along the anterior margin and pricked several times before being placed into 2\% trypsin on ice at 4°C for 30 - 60 minutes in order to remove the ectoderm. After trypsinisation, buds were washed twice in ice-cold growth medium, and the ectoderms were carefully removed by teasing them away from the underlying mesenchyme with the aid of tungsten needles and fine forceps. The mesenchymal cores were placed into fresh medium, and blocks of mesenchyme were cut out from four different levels (anterior, distal tip, mid-posterior or posterior; Figure 5.4) along the antero-posterior axis along both \( ta^3/ta^3 \) and wildtype embryos. Blocks of mesenchyme were stored in sterile medium on ice prior to grafting.

In order to graft the donor mesenchyme into the host wing buds, the isolated blocks of mesenchyme were gently pipetted into the host egg with a little sterile medium, being careful not to pipette too vigorously and lose the piece of mesenchyme. The aim was to place the block of mesenchyme onto the body of the embryo just proximal to the right wing. The donor tissue was then eased into position under the prepared loop in the
apical ectodermal ridge along the anterior margin of the host's right wing bud. The egg was resealed, and the manipulated embryo allowed to develop for a further 6 days at 38 ±1 °C.
Loop cut in the apical ectodermal ridge along the anterior margin of a host wing bud

Apical ectodermal ridge

Limb bud mesenchyme

Figure 5.3
Schematic representation of a stage 20 chick wing bud showing a loop in the anterior apical ectodermal ridge under which block of mesenchyme were grafted
**A**

Anterior (A)

Apical ectodermal ridge

Tip (T)

Mid-posterior (MP)

Posterior (P)

*ta3ta3 wing/leg bud*

**B**

Loop in apical ectodermal ridge

Block of donor *ta3ta3 wing/leg bud mesenchyme*

*Stage 20 host wing bud*
Figure 5.4
Diagram showing how polarising activity was mapped in $ta^3/ta^3$ limb buds. (A): Blocks of mesenchyme were taken from various positions of a $ta^3/ta^3$ wing/leg bud. A, anterior; T, tip; MP, mid-posterior; P, posterior. (B): Blocks of mesenchyme were then grafted under the apical ectodermal ridge at the anterior margin of a stage 20/21 normal right wing bud.
5.2.4 Grafting a normal polarising region to the anterior margin of a stage 20/21 \(ta^3/ta^3\) limb bud

In order to determine if \(ta^3/ta^3\) limb bud mesenchyme has the ability to respond to a normal polarising signal, a block of mesenchyme from the posterior region of a normal limb (stage 20/21) was grafted into the anterior margin of a stage 20/21 \(ta^3/ta^3\) limb.

Host and donor embryos were prepared as described above. However, the majority of \(ta^3/ta^3\) embryos die on day 6 or 7 of incubation before the limb skeletal pattern can be determined. Therefore manipulated \(ta^3/ta^3\) limb buds were removed 3 hours after grafting and were pinned on to the stump of a wing bud in a normal host (as described in section 5.2.2). The contralateral un-operated \(ta^3/ta^3\) limb was also grafted to the stump of a normal host wing bud to allow direct comparison of manipulated and un-manipulated limbs. Manipulated embryos were allowed to develop for a further 6 days at 38 ±1 °C, then fixed and stained for skeletal analysis (see below).

5.2.5 Fixation and staining of manipulated embryos

After a further 6 days incubation, manipulated embryos were removed from the eggs and culled by decapitation. Embryos were washed in phosphate buffered saline (PBS; Appendix 1) and lower limbs and guts removed prior to fixing overnight at room temperature in a solution of 5 % trichloroacetic acid (TCA) in H\(2O\). Specimens were then stained in a solution of 1% alcian green in acid alcohol (70% ethanol, 1% hydrochloric acid) overnight at room temperature, after which they were placed in acid alcohol overnight to remove stain differentially from non-cartilagenous structures. Prior to clearing, the embryos were dehydrated twice in 100 % alcohol then cleared in methyl salicylate allowing visualisation of cartilaginous elements of the developing skeletal system. Skeletal pattern was analysed and photographed with the aid of a dissection microscope and attached camera.

5.2.6 Retinoic acid treatment of \(ta^3/ta^3\) limb buds

AG1-X2 beads (from Bio-Rad, in formate form) of 200 μm diameter were picked out manually under a dissecting microscope using a calibrated eye piece graticule. The beads
were soaked in 100 µl of all-trans-retinoic acid (0.05 mg/ml, 0.1 mg/ml or 10 mg/ml) in dimethylsulfoxide (DMSO). After a 20 minute soak at room temperature, the beads were rinsed in 100µl drops of culture medium (minimum essential medium plus 10% foetal calf serum) and left to wash in 1.0 ml of culture medium for 20 minutes at 38 ±1°C. Stage 20/21 ta^3/ta^3 wing buds were prepared by creating a loop in the apical ectodermal ridge at the anterior margin of the buds (as described in section 5.2.3). Retinoic acid-soaked beads were then implanted under the apical ectodermal ridge at the anterior margin, the eggs were resealed and the embryos incubated at 38 ±1°C for a further 30 hours.

After further incubation, manipulated embryos were removed from the eggs and placed into ice-cold PBS, before being fixed in 4% (w/v) paraformaldehyde in PBS for in situ hybridisation. In order to analyse skeletal morphology as a result of retinoic acid treatment, manipulated ta^3/ta^3 wing buds were grafted the isolated wing onto the stump of a normal host (as described above in section 5.2.2). The grafted limbs were allowed to develop for a further 5 days to assess skeletal pattern. An un-operated contralateral limb bud of each of the ta^3/ta^3 mutants was also grafted onto a limb stump of a normal host and allowed to develop to confirm the embryos mutant status (as described above in section 5.2.2) and to allow for comparison of digit number and morphology of un-manipulated and retinoic acid treated ta^3/ta^3 limbs.

5.3 General molecular biology methods

5.3.1 Chicken Fgf4 plasmid
This plasmid clone (pLN20) was provided by L. Niswander (Niswander et al., 1994). It contains ~ 600 base pairs of chicken Fgf4 cDNA derived from a clone pLN19 inserted into a blueskript vector (2959 base pairs). The vector-insert junctions are at the EcoRV site (T7 end) which is not preserved, and the EcoRI site (T3 end) which is preserved. An antisense riboprobe for in situ analysis was produced by linearising the plasmid with EcoRI restriction enzyme and transcribing with T7 RNA polymerase.
5.3.2 Chicken Shh plasmid

This plasmid was provided by Dr. J-C Ispizúa-Belmonte, and contains 500 base pairs of chicken Shh cDNA covering the 3’ end of the gene inserted into Blueskript (pBS). The vector/insert junctions are the XbaI and EcoRI sites. An antisense riboprobe for in situ analysis was produced by linearising the plasmid with SmaI restriction enzyme and transcribing with T7 RNA polymerase.

5.3.3 Chicken Hoxd13 plasmid

This plasmid was provided by Dr. D. Duboule and contains ~ 600 base pairs of chicken Hoxd13 cDNA inserted into Blueskript (pKS). The vector/insert junctions are the XhoI and PstI sites. An antisense riboprobe for in situ analysis was produced by linearising the plasmid with EcoRV restriction enzyme and transcribing with T3 RNA polymerase as described by Ispizúa-Belmonte et al. (1991).

5.3.4 Chicken Bmp2 plasmid

This plasmid (p5.1) was provided by Dr. P. H. Francis-West and contains ~ 797 base pairs of chicken Bmp2 cDNA inserted into Blueskript (pSK+). Antisense riboprobes specific for chicken Bmp2 transcripts were synthesised by linearising the plasmid DNA with HindIII restriction enzyme, and transcribing with T3 RNA polymerase, as described by Francis et al. (1994).

5.3.5 Chicken Bmp4 plasmid

This plasmid (p6.1) was provided by Dr. P. H. Francis-West and contains ~ 953 base pairs of chicken Bmp4 cDNA inserted into Blueskript (pSK+). Antisense riboprobes specific for chicken Bmp4 transcripts were synthesised by linearising the plasmid DNA with BamHI restriction enzyme, and transcribing with T3 RNA polymerase, as described by Francis et al. (1994).
5.3.6 Chicken Bmp7 plasmid

This plasmid (pBH2) was sent to Dr. P. H. Francis-West by Dr. B. Houston and contains ~ 752 base pairs of chicken Bmp7 cDNA inserted into Blueskript (pSK II+). Antisense riboprobes specific for chicken Bmp7 transcripts were synthesised by linearising the plasmid DNA with BamHI restriction enzyme and transcribing with T7 RNA polymerase, as described in Houston et al. (1994).

5.3.7 Preparation of plasmid DNA

Elution of plasmid DNA from Whatman filter paper, transformation of competent cells, small and large-scale preparation of plasmid DNA, and purification of the plasmid DNA were carried out as described in Chapter 2.

5.4 Analysis of gene expression in limb bud of ta^3/ta^3 embryos

5.4.1 35S in situ hybridisation

In situ hybridisation to tissue sections using 35S-labelled Hoxd13 and Shh riboprobes was carried out by J-C. Izpisúa-Belmonte as described by Francis et al. (1994) on specimens prepared by K. E. Robertson.

5.4.2 Non-radioactive, wholemount in situ hybridisation

Wholemount in situ hybridisation using digoxigenin-labelled Bmp2, Bmp4, and Bmp7-specific riboprobes were carried out by P. Francis-West as described by Francis et al. (1994), except prior to the proteinase K treatment, embryos were bleached in 6% H2O2 for 1 hour and were then washed three times with PBS containing 0.1% Tween-20 (PBT) for a total of 15 minutes. Wholemount in situ hybridisations using digoxigenin-labelled Shh and Hoxd13-specific riboprobes were carried out by J-C. Izpisúa-Belmonte as described in Izpisúa-Belmonte et al. (1993). Wholemount in situ hybridisations with the Fgf4-specific riboprobe were performed according to the protocol of Niswander et al. (1994), see below.
Fixation and prehybridisation treatment:
Embryos were fixed overnight at 4°C in 4% paraformaldehyde (w/v) and dehydrated on ice through PBS, 25% methanol in PBS, 50% methanol in PBS, 75% methanol in PBS, and 100% methanol. Dehydrated embryos were stored in 100% methanol at -20°C. Prior to hybridisation, embryos were rehydrated on ice by taking them through a series of graded methanol in PBS containing 0.1% Tween 20 (PBT) (75%, 50%, 25% methanol in PBT), and washed three times for 5 minutes in fresh PBT with gentle rocking. Rehydrated embryos were incubated in a solution of 10μg/ml proteinase K in PBS for 5 minutes at 37°C then washed twice at room temperature in PBT before refixing in 4% paraformaldehyde for 20 minutes at room temperature. After refixation, embryos were rinsed in freshly prepared hybridisation solution and then prehybridised for an hour at 60°C. The hybridisation solution was replaced with fresh solution, and the embryos prehybridised for a further 2 hours at 60°C.

Hybridisation and posthybridisation washes:
Hybridisation was carried out over night at 60°C in hybridisation solution (Appendix 1) containing 1μg/ml of chicken Fgf4-specific riboprobe. Post-hybridisation washes of increasing stringency of 2X SSC (Appendix 1) in prehybridisation solution and DEPC treated water (75% prehybridisation solution, 25% 2X SSC; 50% prehybridisation solution, 25% 2X SSC, 25% DEPC H2O; 25% prehybridisation solution, 25% 2X SSC, 50% DEPC H2O) were carried out at 55°C with gentle rocking for 5 minutes. Embryos were then washed twice for 30 minutes in 2X SSC containing 0.1% CHAPS, then in 0.2X SSC containing 0.1% CHAPS for 5 minutes, and finally twice for 30 minutes the same. Embryos were allowed to cool to room temperature and washed twice in PBS, then once in PBS containing 0.1% Triton X100. Embryos were then blocked at 4°C for 3 hours in a blocking solution of 15% heat inactivated sheep serum in PBS containing 0.1% Triton X100.
Preabsorption of the antibody and antibody binding:
In order to preabsorb the sheep anti-digoxigenin antibody, 5 mg of chick embryo powder (Appendix 1) was resuspended in 1ml of TBST (Appendix 1) and heat inactivated at 65-70°C for 30 minutes then quenched on ice. The antibody was diluted 1:200; 5µl of antibody was preabsorbed for an hour with 1ml of heat inactivated embryo powder in TBST and 50µl of 20% heat inactivated sheep serum, at 4°C with gentle rocking. The mixture was spun in a microfuge at 4°C and the resulting supernatant diluted 1:10 in 15% heat inactivated sheep serum in PBS containing 0.1% Triton X100, to a final concentration of 1:2000. The blocking solution was replaced with this preabsorbed antibody solution, and the embryos incubated overnight at 4°C with gentle rocking.

Post antibody washes and colour detection:
After overnight antibody incubation, embryos were washed four times for 1 hour at room temperature in PBS containing 0.1% Triton X100 with gentle rocking, then twice for 30 minutes in the same. Prior to colour detection, embryos were washed twice for 15 minutes at room temperature in alkaline phosphatase buffer (see Appendix 1). Colour detection was carried out at room temperature in the dark in a solution of alkaline phosphatase buffer containing 4.5µl/ml of stock nitroblue tetrazolium salt (NBT; Appendix 1) and 3.5µl/ml of stock 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Appendix 1). Embryos were left for at least 30 minutes, then periodically checked for signal. The colour reaction was stopped by washing several times in PBS containing 0.1% Tween 20 then refixing in 4% PFA in PBT. Embryos were viewed and photographed using a dissecting microscope and attached camera containing Kodak T64 colour reversal film.

5.5 RESULTS
5.5.1 Distribution of polarising activity in ta^3/ta^3 limb buds
Polarising activity in ta^3/ta^3 limb buds was mapped by assessing the ability of mesenchyme from different positions along the antero-posterior axis of ta^3/ta^3 leg or wing buds to induce additional digits when grafted to the anterior margin of normal wing
buds (Figure 5.4). In normal limb buds, only tissue taken from the posterior margin of the limb had polarising activity and an extra digit 4 was invariably induced (see also MacCabe et al. 1973; Tickle et al. 1975; Honig and Summerbell, 1985). Tissue taken from the anterior of normal chick limb buds never specified additional digits or ectopic cartilage (4 of 4 cases; see also MacCabe et al. 1973). In contrast, in \( ta^3/ta^3 \) limb buds between stages 19 and 23, polarising activity was found to be graded along the distal antero-posterior axis (Table 5.1). Mesenchyme from the posterior tip of a \( ta^3/ta^3 \) limb bud often induced digit patterns with a digit 4 found next to the graft (55%; 5 out of 9 cases; Figure 5.5; Table 5.1). However, there was also weak polarising activity at the anterior margin of the \( ta^3/ta^3 \) limb bud. The majority of anterior grafts induced the formation of either an additional digit 2 (33%; 3 out of 9 cases; Table 5.1) and or a blip of extra cartilage (44%; 4 out of 9 cases; Fig. 5.5; Table 5.1). Cells taken from positions between the two margins of \( ta^3/ta^3 \) limb buds gave intermediate degrees of duplication (Figure 5.5). Cells from the mid-posterior tip and mid-distal tip induced an extra digit 4 in 40% (2 out of 5) and 14% (1 out of 8) of the cases, respectively. Typically these grafts induced digits with a more anterior identity with the majority of grafts from distal and mid-posterior inducing digits 3 and 2 respectively.

Mesenchyme from younger \( ta^3/ta^3 \) limb buds had higher polarising activity than mesenchyme from older limb buds. In normal limbs there is no significant diminution in polarising activity between stages 19 and 24 (MacCabe et al. 1973; Honig and Summerbell, 1985). When mesenchyme was taken from stage 19-23 \( ta^3/ta^3 \) limb buds, 97% (31 out of 32) of the grafts resulted in the induction of ectopic cartilage or well defined additional digits. At stages 23/24, polarising activity was much weaker and only 36% (4 out of 11) of the grafts induced extra structures with only the most anterior digit, digit 2 being duplicated (Table 5.1). Table 5.1 pools data obtained from both leg and wing buds. Separate analysis of the data shows that, as in normal leg and wing buds (Hinchcliffe and Sansom, 1985), there is a similar distribution of polarising activity in both leg and wing buds of \( ta^3/ta^3 \) embryos.
Table 5.1

<table>
<thead>
<tr>
<th>Origin of graft from</th>
<th>Normal wing</th>
<th>Normal Blip</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>ta&lt;sup&gt;3&lt;/sup&gt;/ta&lt;sup&gt;3&lt;/sup&gt; wing or leg bud</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stages 19-23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>1</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Tip</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Mid-posterior</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Posterior</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Stages 23-24</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tip</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mid-posterior</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Posterior</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1

Table showing number of mesenchymal grafts from different regions of stage 19 - 23 and stage 23 - 24 ta<sup>3</sup>/ta<sup>3</sup> wing and leg buds that were grafted to the anterior border of normal chick wings, and the identity of the most anterior additional digit formed. *In addition, other more anterior digits may also have been induced.
Figure 5.5
Skeletal patterns of 10-day chick wings that resulted from mesenchyme grafts from a stage 19/20 ta<sup>3</sup>/ta<sup>3</sup> limb bud into the anterior margin of a normal stage 20/21 host. Embryos were fixed and stained with Alcian green to show the skeletal pattern.

Mesenchyme was taken from (A) the posterior region of a ta<sup>3</sup>/ta<sup>3</sup> wing; (B) the distal tip of a ta<sup>3</sup>/ta<sup>3</sup> leg; (C) the anterior region of a ta<sup>3</sup>/ta<sup>3</sup> leg. A and B have a digit pattern 4, 3, 2, 3, 4 and blip, 3, 3, 3, 4 respectively. Limb C only has an additional blip of cartilage at the anterior margin (open arrow), and the coracoid is thickened (solid arrow). In all three limbs, the radius is abnormal in shape and the distal part of the humerus is thickened. H: humerus; R: radius.
In addition to digit duplications, \( ta^3/ta^3 \) grafts induced other skeletal abnormalities (27 out of 32 cases; Table 5.2). In 3 of these cases, mesenchyme from stage 19-23 \( ta^3/ta^3 \) limbs, induced the formation of additional small unarticulated cartilage elements between the distal ends of the digits. Other effects of grafts of stage 19-23 \( ta^3/ta^3 \) limb mesenchyme included malformation of the coracoid (Figure 5.5), thickening of the distal part of the humerus (Figure 5.5) and thickening (Figure 5.5) or deletion of the radius. Mesenchyme from stage 23/24 \( ta^3/ta^3 \) limb buds affected only development of the radius, resulting in the thickening (6 out of 9) or deletion (2 of 9) of the radius. These abnormalities are not induced by grafts of mesenchyme from limb buds of normal embryos.
### Table 5.2

Additional skeletal defects induced by grafts of *Talpid*³ mesenchymal cells to the anterior margin of a stage 20 normal chick wing bud.

<table>
<thead>
<tr>
<th>Antero-posterior origin of graft</th>
<th>Duplicated humerus</th>
<th>Thick Radius</th>
<th>Thin/absent Radius</th>
<th>Coracoid mis-shapen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>3/7</td>
<td>4/7</td>
<td>1/7</td>
<td>2/7</td>
</tr>
<tr>
<td>Tip</td>
<td>2/6</td>
<td>3/6</td>
<td>2/6</td>
<td>-</td>
</tr>
<tr>
<td>Midposterior</td>
<td>1/4</td>
<td>2/4</td>
<td>1/4</td>
<td>-</td>
</tr>
<tr>
<td>Posterior</td>
<td>-</td>
<td>2/7</td>
<td>1/7</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.2

Table showing the number of cases where skeletal alterations additional to the induction of additional digits were induced by grafted *Talpid*³ mesenchyme.
5.5.2 Response of \(ta^3/ta^3\) limb mesenchyme to polarising region grafts and retinoic acid

In a normal wing bud, placing a polarising region graft or a bead soaked in retinoic acid at the anterior of the limb bud induces digit duplications in the host limb. To test whether \(ta^3/ta^3\) mesenchyme is able to respond to a polarising signal, a polarising region from a normal limb bud was grafted to the anterior margin of a \(ta^3/ta^3\) limb bud. Manipulated \(ta^3/ta^3\) limb buds were then grafted to a stump of a right wing bud of a normal chick embryo to allow continued development. The digit patterns that resulted were compared with those of un-manipulated \(ta^3/ta^3\) limb buds grafted to wing bud stumps of normal embryos. Unlike normal limb buds, there was no increase in the number of digits formed when a polarising region was grafted into the anterior margin of a \(ta^3/ta^3\) limb bud (n=4). The most marked effect was that the digits appeared to be more distinct rather than being fused along the antero-posterior axis and were bifurcated at the tip (Figure 5.6). However, they were still abnormal in shape and individual digits could not be classified.

When retinoic acid was applied anteriorly to \(ta^3/ta^3\) limb buds, the number of digits that developed was reduced in all cases (5 out of 5). High levels of retinoic acid (10 mg/ml) inhibited outgrowth of the limb bud (3 out of 5) and prevented the formation of any digits whereas with lower doses (0.1 and 0.05 mg/ml) a few digits developed (2 out of 5; average of 1.5 digits compared with average of 5 in un-manipulated limbs). As with polarising region grafts, these digits could not be identified as particular digits but were more distinct and separated from each other than those in un-manipulated limbs.
Figure 5.6
Skeletal pattern of a 10-day (A) control ta^3/ta^3 wing or (B) contralateral ta^3/ta^3 wing after a polarising region graft from a stage 20 normal wing had been grafted to the anterior margin at stage 20. Both limb buds were grafted onto the stump of the right wing bud of a normal host and allowed to develop to day 10. The limbs were stained with Alcian green to show the skeletal pattern. In the limb (A) the cartilage elements are fused across the antero-posterior axis and the digits are indistinguishable. In the manipulated limb (B) the cartilage elements are more distinct and there are 4 digits, 3 of which are bifurcated distally.
5.5.3 Expression of Shh and Hoxd13 in ta<sup>3</sup>/ta<sup>3</sup> limb buds

Expression of Shh and Hoxd13 was mapped by in situ hybridisation to wholemounts and tissue sections (performed by J-C. Izpisúa-Belmonte). In normal limb buds, Shh is expressed in a small region of mesenchyme in the posterior part of the limb bud, corresponding to the polarising region (Riddle et al., 1993). In ta<sup>3</sup>/ta<sup>3</sup> limbs, Shh was also expressed in a discrete domain at the posterior margin of the limb and was associated with the region found to have highest polarising activity, i.e. posterior and mid-posterior mesenchyme (Figure 5.7A). Although there are low levels of polarising activity in more anterior regions of the ta<sup>3</sup>/ta<sup>3</sup> limb bud, Shh transcripts were not detectable in these regions even by in situ hybridisation to tissue sections (Figure 5.7B). In contrast, Hoxd13 transcripts were shown to be present in anterior mesenchyme (Figure 5.7C).

In normal limb buds, expression of Shh at the anterior margin of the limb can be induced by application of retinoic acid. To determine whether expression of Shh can be induced in anterior mesenchyme of a ta<sup>3</sup>/ta<sup>3</sup> limb bud, a bead soaked in retinoic acid was applied to the anterior margin of a limb bud. Within 30 hours, Shh expression at the anterior margin of the limb bud was activated (2 out of 2 cases). In one case, Shh transcripts were found in the mesenchyme both anterior and posterior to the bead (Figure 5.7D). In the other case, a small ectopic domain of Shh was induced just distal to the bead (Figure 5.7E). These results show that expression of Shh can be induced in anterior ta<sup>3</sup>/ta<sup>3</sup> mesenchyme by a polarising signal.

5.5.4 Expression of Fgf4 in ta<sup>3</sup>/ta<sup>3</sup> limb buds

In normal limb buds, Fgf4 is expressed in the posterior part of the apical ectodermal ridge (Niswander et al., 1994b; Figure 5.8A). FGF4 can maintain the expression of several genes, including Shh and the Hoxd13 in the posterior mesenchyme in the absence of the apical ectodermal ridge (Niswander et al., 1993, 1994). In ta<sup>3</sup>/ta<sup>3</sup> limbs buds, Fgf4 is expressed throughout the apical ectodermal ridge (Figure 5.8B).
Figure 5.7

(A) and (B) show the expression of Shh in a stage 22 ta<sup>3</sup>/ta<sup>3</sup> limb as detected by non-radioactive wholemount <i>in situ</i> hybridisation and radioactive <i>in situ</i> hybridisation to tissue sections. (C) shows the expression of Hoxd13 in a stage 22 ta<sup>3</sup>/ta<sup>3</sup> limb as detected by radioactive <i>in situ</i> hybridisation to tissue sections. (D) and (E) show the expression of Shh in ta<sup>3</sup>/ta<sup>3</sup> limbs, 30 hours after a bead soaked in 0.1 mg/ml retinoic acid was placed at the anterior margin. In the control limbs (A and B), expression of Shh is confined to a small region at the posterior margin of the limbs, whereas in the manipulated limbs (D, E) there is an additional domain of Shh expression at the anterior margin of the bud (marked with arrows). The open arrow shows the position of the bead. A, D, and E are <i>in situ</i> hybridisations to whole embryos, and regions in which Shh transcripts are present are stained dark blue/purple. B and C are <i>in situ</i> hybridisations to tissue sections; signal is shown by white grains under dark field illumination. The anterior part of the limb is at the top of the pictures.
Figure 5.8
Expression of Fgf4 in (A) a stage 22 normal leg bud (side view) and (B) a stage 22 ta^3/ta^3 leg bud (ventral view). In the normal limb, Fgf4 transcripts are restricted to the posterior part of the apical ectodermal ridge, whereas in the ta^3/ta^3 leg bud, Fgf4 transcripts are present throughout the extended apical ectodermal ridge. Regions in which Fgf4 transcripts are present are stained dark blue/purple. The anterior part of the limb is at the top of the pictures.
5.5.5 Expression of Bmp genes in ta<sup>3</sup>/ta<sup>3</sup> limb buds

At least three members of the Bmp gene family, Bmp2, Bmp4 and Bmp7, are expressed in limb bud mesenchyme. *Bmp4* is expressed in anterior and posterior mesenchyme (Francis et al., 1994; Figure 5.9B) and *Bmp2* is expressed in the polarising region of the normal early limb buds (Francis et al., 1994; Figure 5.9A). In the normal wing bud, *Bmp7* transcripts are predominantly confined to the polarising region (Figure 5.9C) whereas in the normal leg bud, the domain of *Bmp7* expression is similar to that of *Bmp4*, transcripts being located at the anterior and posterior margins of the limb bud (data not shown). All three genes were expressed in the apical ectodermal ridge (Francis et al., 1994; Figure 5.9A, B, and C). Expression of *Bmp2* and *Bmp7* in anterior mesenchyme can be induced by application of retinoic acid or by grafts of polarising region cells placed at the anterior margin of developing wing buds (Francis et al., 1994).

Wholemount *in situ* hybridisation was used to determine expression patterns of these genes in ta<sup>3</sup>/ta<sup>3</sup> limbs. In both developing leg and wing buds, between stages 20 and 25, all three genes were expressed around the rim of the bud (Figure 5.9D, E, and F). This uniform pattern across the antero-posterior axis correlates with the uniform expression of *Hoxd* genes and *Fgf4* and contrasts with restriction of *Shh* expression to posterior mesenchyme in ta<sup>3</sup>/ta<sup>3</sup> limb buds.
Figure 5.9

Normal (A-C) and \( ta^3/ta^3 \) (D-F) wing buds were probed by non-radioactive wholemount \textit{in situ} hybridisation for expression of \( Bmp2 \) (A and D; stage 25), \( Bmp4 \) (B and E; stage 20 and stage 22, respectively), and \( Bmp7 \) (C and F; stage 26 and stage 25, respectively). Regions in which \( Bmp \) transcripts are present are stained dark blue/purple. Note that in normal limb buds, the expression domains of \( Bmp2 \) and \( Bmp7 \) are predominantly at the posterior margin, while \( Bmp4 \) is expressed at the anterior and posterior margins of the limb bud, with lower levels of expression in the progress zone under the apical ectodermal ridge. In contrast, in \( ta^3/ta^3 \) limbs, expression of all three genes is symmetrical across the antero-posterior axis (D-F). In \( ta^3/ta^3 \) limbs, the extended apical ectodermal ridge expresses transcripts of all three Bmp genes, illustrated for \( Bmp7 \) in (F). In each photograph, the anterior part of the limb bud is at the top. Scale bars = 250 \( \mu \)m.
5.6 Discussion and conclusion

$ta^3/ta^3$ limbs have many digits which lack characteristic antero-posterior pattern, are morphologically similar and develop from abnormally broad buds (Ede and Kelly, 1964; Hinchliffe and Ede, 1967). In normal limb buds, antero-posterior patterning is controlled by a signal from the posterior mesenchyme. The results described in this chapter show that in $ta^3/ta^3$ mutants, polarising activity is graded across the entire antero-posterior axis of the limb, with highest levels of polarising activity at the posterior margin and weak polarising activity anteriorly. Polarising activity at the posterior margin is lower than in normal buds and is not maintained as the bud grows out. Application of retinoic acid anteriorly does not induce digits with antero-posterior pattern suggesting that $ta^3/ta^3$ mesenchyme does not respond normally to polarising signals. Expression of $Shh$ is associated with the region of the limb bud mesenchyme which has highest polarising activity and is thus confined to the posterior margin as in normal limb buds. In contrast, $Bmp2$, $Bmp4$ and $Bmp7$, are expressed uniformly around the margin and distal tip of $ta^3/ta^3$ limb buds, in association with extended ectodermal expression of $Fgf4$ throughout the entire apical ectodermal ridge.

This study shows that there is a functional polarising signal but it is abnormally distributed in $ta^3/ta^3$ mutants and $ta^3/ta^3$ mesenchyme is unable to respond to a polarising signal as in normal limb buds. However, the abnormal distribution of polarising activity in $ta^3/ta^3$ limbs should not abolish antero-posterior patterning. A series of identical digits would only be expected if polarising activity was uniformly distributed across a limb bud, whereas in $ta^3/ta^3$ limbs, polarising activity is graded. In addition to abnormal distribution of polarising activity, response of $ta^3/ta^3$ mesenchyme to a polarising signal also appears to have changed. In $ta^3/ta^3$ limbs, $Hoxd13$ expression is not restricted to regions of the limb that have high polarising activity, as in normal limb buds where $Hoxd13$ expression is activated locally by cells with high polarising activity (Izpisúa-Belmonte et al., 1992c). In $ta^3/ta^3$ limbs, $Hoxd13$ expression is also associated with regions of the limb that have weak polarising activity. It is unclear whether expression of the $Hoxd$ gene complex anteriorly is a consequence of the low polarising activity, but preliminary experiments have shown that the $Hoxd$ gene complex cannot be
activated in anterior mesenchyme cells of normal limb buds by this low polarising activity (P. Francis-West, personal observation).

The ta^3/ta^3 limb phenotype resembles the phenotype of another mutant, Talpid^2 (ta^2) (MacCabe and Abbott, 1974). Like ta^3/ta^3 mutants, ta^2/ta^2 mutants have polydactylous limbs with digits lacking obvious antero-posterior patterning, associated with mis-expression of Hoxd genes across the antero-posterior axis and a reduction of Msx2 gene expression in limb bud mesenchyme (Rodriguez et al., 1996; Coelho et al., 1992). In addition, strength of the polarising signal at the posterior margin of ta^3/ta^3 and ta^2/ta^2 limbs is weaker than the polarising signal in normal limb buds, and both mutants respond partially to polarising signals, showing increased separation of digits (Dvorak and Fallon, 1992). However, there are slight differences in their antero-posterior signalling pathways. In contrast to ta^3/ta^3 embryos, ta^2/ta^2 embryos do not exhibit polarising activity in anterior limb bud mesenchyme (Dvorak and Fallon, 1992). Furthermore, in ta^2/ta^2 limbs, some of the cartilage elements such as the radius and ulna are distinguishable and, following a polarising region graft into the anterior mesenchyme of a ta^2/ta^2 limb bud, some degree of re-patterning is evident as the radius is respecified to form the ulna (Dvorak and Fallon, 1992). In ta^2/ta^2 mutants, both grafts of polarising region and application of retinoic acid result in reduction in digit number, whereas in ta^3/ta^3 mutants, a reduction in the number of digits was only observed with retinoic acid. The basis of the talpid phenotype in both mutants, therefore appears to be similar with changes in both polarising signal and response although the ta^2/ta^2 mutant has a less severe phenotype than the ta^3/ta^3 mutant.

Examination of the signalling molecules expressed in ta^3/ta^3 limb bud gives insights into their normal function during limb patterning. In ta^3/ta^3 limb buds, expression of Shh transcripts is confined to the posterior margin as in normal limb buds and is only associated with cells with high polarising activity i.e. the ability to specify the most posterior digits. Anterior cells do not express Shh but possess weak polarising activity. It seems likely from work on the homologous gene in Drosophila that SHH protein acts locally, inducing long-range effects via induction of other signalling molecules (Basel and Struhl, 1994), and diffusion of SHH protein into anterior
mesenchyme of ta\textsuperscript{3}/ta\textsuperscript{3} limb buds seems unlikely. Therefore, our data suggest that factors, other than Shh, are able to confer polarising activity. In addition, the data show that the defect in ta\textsuperscript{3}/ta\textsuperscript{3} limbs does not involve control of Shh expression. Not only are Shh transcripts confined to the posterior margin of the limb bud, but they can be activated in anterior cells by retinoic acid as in normal limb buds.

Genes that are activated as a consequence of polarising signal include Bmp-2, Bmp7, and genes of the Hoxd complex (Izpisúa-Belmonte et al. 1991; 1992c; Francis et al., 1994). In the developing wing bud, Bmp2 and Bmp7 have similar domains of expression and it is possible that Bmp7 is involved in determining patterning across the antero-posterior axis as has been suggested for Bmp2 (Francis et al., 1994). In the leg, Bmp7 expression, unlike that of Bmp2, is equally intense in both anterior and posterior mesenchyme. The significance of the different domains of Bmp7 expression in the leg and the wing buds is unclear. However, the TGFβ family act as dimers which may be homodimers or heterodimers with distinct activities (Hsueh et al., 1987). Heterodimers of BMP2/BMP7 have been isolated from bovine bone extracts (Sampath et al., 1990) raising the possibility that BMP2 and BMP7 function as heterodimers in the polarising pathway.

In ta\textsuperscript{3}/ta\textsuperscript{3} limb buds Bmp2 and Bmp7 are expressed uniformly across the antero-posterior axis at the distal tip of the ta\textsuperscript{3}/ta\textsuperscript{3} limb bud. It is at present unclear whether their expression across the antero-posterior axis is responsible for the low polarising activity or a consequence of it. However, when anterior cells are respecified to form posterior structures by the action of polarising region grafts or retinoic acid, ectopic Bmp2 expression and Hoxd13 are induced in similar regions of the anterior mesenchyme (Izpisúa-Belmonte et al., 1991; Francis et al., 1994; Laufer et al., 1994), and ectopic expression of Fgf4 is activated in the anterior apical ectodermal ridge (Laufer et al., 1994; Niswander et al., 1994). Ectopic expression of Hoxd11 and Hoxd13 in anterior mesenchyme, and Fgf4 in anterior apical ectodermal ridge, can be activated by BMP2 expressing cells (Duprez et al., 1996a), suggesting that BMP2 is involved in regulating Hoxd gene and Fgf4 expression in the normal limb bud. In ta\textsuperscript{3}/ta\textsuperscript{3} limb buds, mis-expression of Bmp2 corresponds to mis-expression of Hoxd13 in the limb mesenchyme.
and $Fgf4$ in the apical ectodermal ridge, providing further evidence that BMP2 may function to control the expression of these genes.

Other features of the $ta^3/ta^3$ phenotype, such as formation of additional small un-articulated cartilage elements, and thickening of skeletal structures, could be related to the abnormal expression of $Bmp$ genes. Evidence supporting this idea is the fact that in addition to their putative signalling roles during early limb patterning, BMPs along with other TGFβ superfamily members, have been implicated in cartilage formation and in determining the size and shape of cartilage elements in the limb and at other sites throughout the skeleton (Duprez et al., 1996b; Francis et al., 1994; Kingsley et al., 1994; Lyons et al., 1989; Vortkamp, 1997). In $ta^3/ta^3$ limbs, cartilage elements are fused, chondrocytes are disorganised and the perichondrium, surrounding the cartilage element, fails to form (Hinchliffe and Ede, 1967). All of these features are seen in normal limbs following over expression of BMP2 and BMP4 using a retrovirus (Duprez et al., 1996b).

Recombination of normal mesenchyme and $ta^3/ta^3$ ectoderm result in the formation of normal limbs whereas reciprocal experiments result in formation of limbs with the $ta^3/ta^3$ phenotype (Ede, 1980). This shows that the defect in $ta^3/ta^3$ is due to a change in mesenchymal signalling. In normal limbs buds, $Fgf4$ is expressed in the posterior part of the ridge and FGF4 protein can maintain outgrowth (Niswander et al., 1993). As outlined in chapter 1, FGF4 maintains the expression of several genes in the posterior mesenchyme, can maintain polarising activity of the limb bud (Niswander et al., 1993, 1994; Vogel and Tickle, 1993) and $Fgf4$ and $Shh$ expression act in a loop with each able to maintain the expression of the other (Niswander et al., 1994). However, the intermediate signals have not yet been identified. In $ta^3/ta^3$ limb buds, whilst $Shh$ expression is restricted to the posterior mesenchyme, $Fgf4$ transcripts are found throughout the apical ectodermal ridge again suggesting $Shh$ expression is disconnected from the expression of other genes in the polarising pathway. This would indicate that there is at least one intermediate signal between $Shh$ expression and the activation of $Fgf4$, and the finding that BMP2 can induce expression of $Fgf4$ (Duprez et al., 1996), and that $Bmp2$ is expressed uniformly across the antero-posterior axis of $ta^3/ta^3$ limb buds, suggests that this factor may be BMP2. In $ta^3/ta^3$ limb buds, $Fgf4$ is expressed
throughout the apical ectodermal ridge, and in the normal limb bud, Fgf4 maintains polarising activity in posterior mesenchyme (Anderson et al., 1993; Vogel and Tickle, 1993), therefore Fgf4 expression could be involved in maintaining the low polarising activity of anterior ta^3/ta3 cells.

In both ta3/ta3 and ta2/ta2 mutants, expression of Shh is restricted to the posterior mesenchyme and the polarising signal is functional, yet Fgf4 and Hoxd genes, and Bmp2 are expressed around the margins of the limb bud across the entire antero-posterior axis (Rodriguez et al., 1996). The Talpid2 and Talpid3 mutations have similar phenotypes, with that of Talpid3 is more severe than that of Talpid2 (Dvorak and Fallon, 1992). However, although the presence of similar patterns of mis-expression of Fgf4, Hoxd genes, and Bmp2, is associated with normal expression of Shh in both mutants suggests that the ta2 and ta3 mutations affect the same molecular pathway, there are differences between the two mutants; ta3/ta3 limbs have weak anterior polarising activity which is not detected in the limbs of ta2/ta2 mutants (MacCabe and Abbott, 1974; Dvorak and Fallon, 1992). These differences may reflect subtle differences in the molecular basis of each of the mutations. In both mutants, Bmp2 is overexpressed across the entire antero-posterior limb axis, and is a good candidate for a gene that might be affected by the ta mutation and is responsible for the observed morphological defects. However, Bmp2 is also overexpressed in a similar manner in the polydactylous mutants Diplopodia1 and Diplopodia4 (Rodriguez et al., 1996), yet the phenotypes are quite different from the Talpid phenotypes. Diplopodia mutants have polydactylous limbs that have a preaxial extra-growth of the limbs that, unlike talpid limbs, is patterned (Rodriguez et al., 1996). It has been proposed that the differences in patterning could be due to a graded difference in protein distribution (Rodriguez et al., 1996). Alternatively, factors other than Shh, in association with Bmp2, could act together to pattern the limb (Rodriguez et al., 1996). Similar differences in protein distribution could account for differences between the Talpid3 and Talpid2 phenotypes.

Thus, the series of morphologically similar digits of ta3/ta3 limbs correlates with uniform expression of Bmp2, Bmp4 and Bmp7 and members of the Hoxd complex. In this sense, the polarising pathway could be viewed as a simple cascade of signals.
starting with the polarising signal. If one member of this pathway is expressed across the entire antero-posterior axis, this would then activate all other following members of the polarising cascade uniformly across the axis. In our data suggest a defect in the signalling pathway downstream of Shh signalling, but upstream of all other genes analysed. It has been proposed that Ptc and vSmo, a vertebrate homologue of the Drosophila gene smo (Stone et al., 1996), are involved in transduction and/or modulation of Shh signalling. In the developing chick wing between stage 17 and 24, Ptc is strongly expressed in posterior limb mesenchyme in a region slightly larger than that of Shh (Marigo et al., 1996). This region of high Ptc expression in posterior limb mesenchyme is approximately the same size as that expressing Bmp2 in response to Shh (Marigo et al., 1996). At stage 24, the Ptc expression domain splits into two regions, one proximal that remains posterior, and another more distal domain that spreads anteriorly. By stage 29 expression in these domains fades (Marigo et al., 1996), but a second phase of expression is activated in cells around the developing skeletal elements (Marigo et al., 1996).

Drosophila ptc encodes a HH receptor, which may constitutively repress HH target genes (reviewed in Hammerschmidt et al., 1997), and repression is only relieved by HH binding to PTC. Drosophila smo encodes a transmembrane protein, SMO, with structural similarities to G-protein-coupled receptors (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). Vertebrate smo homologues have been identified in the rat (rSmo) and human (hSmo) (Stone et al., 1996), but chicken smo homologue has yet to be identified. In the mouse, rSmo is expressed in SHH-responsive tissues, such as the early neural folds, neural tube, presomitic mesoderm and somites, and developing limb bud, gut and eye, showing an overlap with expression of rPTC (Stone et al., 1996 and references therein). There is direct biochemical evidence that in vertebrates, PTC binds both SHH and SMO, whereas SMO has no SHH-binding activity (Marigo et al., 1996; Stone et al., 1996), and it is thought that PTC might be the binding component and SMO the intracellular signalling component of a receptor complex for SHH involved in transduction of Shh signalling (reviewed in Hammerschmidt et al., 1997).

In the developing chick embryo, Ptc is expressed adjacent to all tissues where Shh or other members of the hedgehog family are expressed, such as the neural tube,
notochord, regions of the developing brain, gastrointestinal tract, developing lung, feather germs and developing limbs (Marigo et al., 1996), and it is possible that a chicken homologue of smo is similarly expressed in ta^3/ta^3 mutants have multiple developmental abnormalities; defects are observed in neurally derived tissues such as the eyes, pituitary body and diencephalic and mesencephalic regions of the brain, and in midline structures such as the upper beak, and vertebral column. The viscera protrude through the ventral body wall, there is incomplete feather development, and limbs are short and polydactyly (Ede and Kelly, 1964a, 1964b). It is therefore interesting that Shh, Ptc, and vSmo (in mice) are expressed in many of these tissues, suggesting that the multiple developmental defects in ta^3/ta^3 mutants may have a similar aetiology. In limbs of ta^3/ta^3 mutants, we have observed wildtype expression of Shh, implying that the defects is downstream of this gene, although Shh expression in other regions of ta^3/ta^3 mutants has not been analysed. It is therefore possible that alterations in expression or functionality of Ptc in ta^3/ta^3 mutants could be responsible for the defects observed. For example, in the developing limb, PTC may in some way constitutively repress activation of Bmp2. It is thought that PTC repression of SHH target genes is removed by SHH binding to PTC, thus, binding of SHH to PTC would permit activation of Bmp2. There is evidence to show that Shh can activate Bmp2 expression (Laufer et al., 1994). Alternatively, PTC could in some way activate expression of an as yet unidentified gene expressed in anterior mesenchyme that inhibits activation of Bmp2 in anterior mesenchyme, and localise the polarising signal to the posterior limb. In either case, removal or inappropriate expression of PTC, or for that matter of vSmo (as yet unidentified in chickens) could possibly lead to overexpression of Bmp2 in anterior mesenchyme, which would then activate Hoxd genes and Fgf4, and in some way permit expansion of polarising activity into anterior mesenchyme. Thus, the molecular basis of ta^3 could involve PTC or vSmo.

Observations made in the polydactyly mouse mutant extra-toes (Xt) suggest another possibility. Xt is caused by a mutations in a gene known as Gli3, a zinc-finger gene of the GLI-Krüppel family which can function as sequence-specific DNA-binding proteins (Hui and Joyner, 1993). Xt mutations affect craniofacial and limb development,
and Xr is a mouse model for the human condition known as Grieg cephalopolysyndactyly syndrome (GCPS) which includes craniofacial and limb defects, and is caused by mutations in the human GLI3 gene (Vortkamp et al., 1991). In the mouse, Gli3 is strongly expressed in the developing brain, and craniofacial and limb bud mesenchyme (Hui and Joyner, 1993). Later on in limb development, Gli3 is detected in the interdigital mesenchyme and interzonal mesenchymal regions which will give rise to the joints, but not in cartilage condensations (Hui and Joyner, 1993). Thus, Gli3 expression is associated with regions of the developing limb where mesenchymal cell death takes place, suggesting that Gli3 may be involved in the control of this process which is important during digital separation and joint formation. Further genetic evidence for this idea comes from the finding that the polydactylous mouse mutant polydactyly Nagoya (Pdn) has a decrease in the levels of cell death in the limbs, and Pdn defines a novel allele of Gli3 (Schimmang and Rüther, 1994). It is therefore evident that a deficiency in Gli3 can lead to incomplete cell death and polydactyly.

$ta^3/ta^3$ and $ta^2/ta^2$ embryos have defects of the craniofacial region and the limbs (which exhibit polydactyly associated with a decrease in mesenchymal cell death). It is therefore possible that the $ta^3$ and $ta^2$ mutations may affect the chicken homologue of Gli3. The phenotypic differences between the two talpid mutations could be due to subtle differences in the mutation of Gli3, as seen in the mouse mutants Xt, Pdn and another mutant, anterior digit pattern deformity (add). Xt, Pdn, and add are all slightly different alleles of Gli3, and all three mutants have different phenotypes (Hui and Joyner, 1993; Schimmang and Rüther, 1994; van den Hoeven et al., 1993). However, a contradictory finding related to Xt and the talpid mutants is that although polydactyly is associated with ectopic expression of Fgf4, Xt mutant limbs also exhibit ectopic anterior expression of Shh (Masuya et al., 1995). This has led to the suggestion that Gli3 may be involved in downregulating the activity of the polarising region, specifically at the anterior margin of the limb (Masuya et al., 1995). The presence of ectopic Shh in the limbs of Xt mutants therefore argues against the $ta^3$ mutation involving the chicken homologue of Gli3, unless Gli3 functions both upstream and downstream of Shh signalling in the limb. It is unknown whether Bmps, Hox genes, PTC and vSmo are misexpressed in Xt limbs, but
this information, along with how the link between Shh expression and expression of downstream genes, such as Bmps, Fgf4 and Hoxd genes has been broken in ta3/ta3 limbs will give us further insights into these mutations and the mechanisms that control antero-posterior patterning during normal limb development.
Chapter 6
General Discussion

6.1 The aim of this thesis was to analyse limb development in two vertebrate limb mutants, hypodactyly (Hd) in the mouse and talpid<sup>3</sup> (ta<sup>3</sup>) in the chick. My main findings about the hypodactyly mutation are that limb defects induced by the mutation are specifically restricted to distal-most regions of the limbs with a single digit developing on all four limbs of Hd/Hd mice, and a reduction of hindlimb digit I in Hd/+ mice. Loss of digits in Hd/Hd mutants is associated with a reduction in size and alteration in shape of the distal region of developing forelimb and hindlimb buds; in Hd/+ embryos the hindlimb buds only are slightly reduced along the distal anterior margin of the developing footplates (Chapter 3). Polarising activity can be detected in Hd/Hd limb buds, and Shh and Fgf4 are expressed (Chapter 3). Expression of Hoxd13 in Hd/Hd limbs is only slightly reduced compared to normal, and expression of Hoxd11 is transiently increased. Cellular analysis of Hd/Hd mutants shows an increase in mesenchymal cell death, a decrease in chondrogenesis and thickening of the apical ectodermal ridge (Chapter 4).

In the polydactylous chicken mutant talpid<sup>3</sup>, polarising activity is distributed in an anterior to posterior gradient within ta<sup>3</sup>/ta<sup>3</sup> limb buds, with weak polarising activity in anterior mesenchyme and the highest polarising activity localised posteriorly (Chapter 5). Shh transcripts are posteriorly restricted and associated with the region of highest polarising activity. In direct contrast, Fgf4 is ectopically expressed throughout the entire apical ectodermal ridge, and Bmp2, Bmp4, and Bmp7 are also ectopically expressed across the entire antero-posterior axis. Finally, retinoic acid and polarising region grafts do not induce digit duplications in ta<sup>3</sup>/ta<sup>3</sup> limbs, instead they lead to separation of the digital elements (Chapter 5).

6.2 General conclusions

Limb defects observed in Hd and ta<sup>3</sup> mutants are very different, but both are associated with alterations in limb bud size and shape; Hd/Hd limbs are narrow and ta<sup>3</sup>/ta<sup>3</sup> limb buds are wide distally (Chapter 3; Ede and Kelly, 1964; Hinchliffe and Ede, 1967). Localisation and timing of limb bud abnormalities in Hd and ta<sup>3</sup> mutants reflects the
phenotypic outcome of the individual mutations. *Hd* mutants have defects that are specifically restricted to the hands and feet, and limb bud abnormalities are detected at the time when the hand and footplates are being specified. *Hd/+* hindlimb bud abnormalities are detected a day later than *Hd/Hd* limb bud abnormalities, thus reflecting the less severe and more distally restricted defects in *Hd/+* hindlimbs. In addition, the *Hd/Hd* adult mouse analysed here appears to have abnormalities in the gential area. In contrast, *ta^3/ta^3* embryos have multiple defects affecting many different systems, and the limb defects involve all skeletal elements of the limbs; long bones are shortened and can be fused, metacarpals and carpals form a single block of cartilage, and up to 10 morphologically similar digits develop which may be fused (Ede and Kelly, 1964). Shortening of long bones is associated a reduction in limb bud length along the proximo-distal axis, and polydactyly is associated with a widening of the distal limb bud along the antero-posterior axis (Ede and Kelly, 1964). Abnormalities in limb bud morphology are detected early.

Outgrowth and patterning of the limb requires the production of SHH and FGF4 by the polarising region and apical ectodermal ridge respectively, and *Hox* genes act downstream of these signals in limb patterning (see Chapter 1). Polarising activity is posteriorly restricted and possibly slightly reduced in *Hd* mutant limb buds, whereas *ta^3/ta^3* limb buds exhibit ectopic polarising activity across the entire antero-posterior axis of the distal bud. Interestingly, both mutants show a normal distribution pattern of *Shh* transcripts in posterior limb mesenchyme, but slightly altered expression of both *Fgf4* and *Hox* genes. In *Hd/Hd* limb buds, a reduction in expression of *Hoxd13* is observed, whereas in *ta^3/ta^3* mutant limb buds, expression of this gene is extended across the entire antero-posterior axis of the distal limb. These results strongly suggest that factors other than and downstream of *Shh* in the signalling cascade, are required and/or have the ability to pattern the limb. In *ta^3/ta^3* mutant limb buds, *Bmp2* is a good candidate as it is overexpressed across the entire distal antero-posterior limb axis, and BMP2 is known to induce digit duplications and expression of *Fgf4* and *Hoxd* genes (Duprèz et al., 1996). If *Hox* genes control the number of cells available for skeletal development as discussed in Chapter 4, one would predict that overexpression would lead to an increase, and reduced or lack of expression of *Hox* genes would lead to a decrease in the number of potentially
skeletal cells available, resulting in gain and loss of skeletal structures respectively. In
*Hd/Hd, Hoxa13* is assumed not to function in a wildtype manner, whereas in *ta3/ta3*
limbs, *Hoxd13* is overexpressed. Therefore, results from analysis of both mutants
supports the idea that *Hox* genes may pattern the limb by controlling the amount of tissue
available for development of the digits.

Limb defects in *Hd* and *ta3* mutants are associated with alterations in the
behaviour of mesenchymal cells and the morphology of the apical ectodermal ridge. The
defect in *ta3* is known to be restricted to the mesenchymal component of the limb, and in
*Hd* it is assumed that this is also the case, as *Hoxa13* expression is normally restricted to
the mesenchyme. The *ta3/ta3* phenotype is associated with a lack of mesenchymal cell
death and an increased adhesiveness of mesenchymal cells, whereas *Hd* is associated
with an increase in mesenchymal cell death and a reduction in chondrogenesis. It is
possible in both cases, that changes in mesenchymal cell behaviour results from
alterations in *Hox* gene function. The mesenchymal alterations in the apical ectodermal
ridge in both mutants further emphasises the inter-relationship between the apical
ectodermal ridge and underlying mesenchyme during limb development, such that
mesenchymal defects can induce secondary defects in the overlying ectoderm.

### 6.3 Future work

Work on the *Hd* mutation described in this thesis provides information about how the
observed defects in the adult mutant arise. They support the idea that during limb
development, *Hox* genes can influence mesenchymal cell behaviour, and are intimately
involved in limb patterning by controlling the amount of tissue available for skeletal
development. The findings also support the idea that *Hox* genes of cognate group 13
function downstream of the polarising region and *Shh* in specification of the distal regions
of the limb, and that *Hox* genes in the same linkage group or across groups may interact
during limb development, as suggested by Davis et al. (1995). Further analysis of the
expression patterns of other *Hox* genes in the limbs of *Hd/+* and *Hd/Hd* mutants will
help to determine if and how this takes place. It will be important to analyse cellular
interactions and proliferation rates of *Hd/Hd* mesenchymal cells. For example, do mutant
mesenchymal cells segregate from normal cells when mixed together, and are proliferation rates of mutant mesenchymal cells reduced? In addition, analysis of the expression of cell adhesion molecules by mutant mesenchymal cells will help to determine how Hox genes influence mesenchymal cell behaviour in the limb. It will be interesting to analyse the expression of other genes such as Hoxa, Bmp, Msx and Evx genes in the limbs of Hd mutants in order to look for downstream targets which will enhance our understanding of the steps in the molecular pathways that lead to patterning of the limb.

Hypodactyly is the first naturally occurring Hox gene mutation to be analysed, and in order to understand fully the roles of Hox genes in the developing limb, it will be vital to analyse the embryology of transgenic mice with targeted disruptions in other Hox genes. For example, do mice with targeted disruptions in Hox genes have alterations in developing limb bud morphology and mesenchymal cell behaviour? Furthermore, by crossing Hd with these mutants and analysing the results in terms of embryology, cell behaviour and final phenotypic outcome, it may be possible to gain new information on how the Hox genes of different clusters and cognate groups function during limb development.

Work on the ta^3 mutation described in this thesis has helped to gain a better understanding of how the mutation leads to the limb defects observed by altering the molecular signalling pathways that usually pattern the limb. The studies have shown that there must be intermediate signals between Shh and Fgf4, Hoxd, and Bmp genes in the limb, and support the idea that factors other than Shh are required to pattern the limb. Misexpression of Hoxd and Bmp genes in ta^3/ta^3 limb buds also supports the roles of these genes during development of the limb skeleton. Hoxd genes are misexpressed in ta^3/ta^3 limb buds and associated with development of an abnormal number of digits. However, other more proximal skeletal defects are also observed in the limbs of ta^3/ta^3 embryos, which suggests that it might be interesting to analyse expression of genes of the Hoxa cluster. In addition, it will be interesting to analyse expression of genes such as Gli3, Ptc and the chicken homologue of smo, in addition to other members of the hedgehog family in order to determine if their expression is affected. The molecular basis
of the \( ta^3 \) mutation is not yet known and in order to fully understand the mutation, we need to identify the gene involved. This work is currently being carried out by Dr. D. Burt at the Roslin Institute in Edinburgh. Unlike \( Hd \), \( ta^3 \) affects many different systems suggesting that it involves a gene(s) required for many different aspects of development. Alternatively, it could affect a gene(s) utilised as a signalling molecule at different sites in the embryo, suggesting conservation of signalling mechanisms throughout the embryo.

Recently it has been shown that beads soaked in certain members of the FGF family can induce extra limbs when implanted into the lateral plate mesoderm of the lateral flank (Cohn et al., 1995). It will be interesting to analyse whether the flank of \( ta^3/ta^3 \) embryos behaves in a similar manner, and if additional limbs are induced, whether they have a normal or \( ta^3/ta^3 \) phenotype.

Over the last 10 years, great advances have been made in understanding the molecular mechanisms of limb development, and a good deal of this information has been gained from the study of both mouse and chick mutants. Similarities between limb development in mice and humans has been implicated by the cloning of human homologues of genes known to be important in mouse limb development. Although this does not necessarily imply that these genes have the same expression patterns and functions in humans as they do in mice, there is recent evidence to suggest that similarities must exist. Mutations in \( GLI3 \) (Vortkamp et al., 1991), \( HOXD13 \) (Muragaki et al., 1996) and \( HOXA13 \) (Innis et al., 1997; reviewed in Scott, 1997) in humans are associated with different types of developmental abnormalities, all of which affect limb development. In each of these cases, mutations in the mouse homologue of the affected gene causes a similar or related defect (Hui and Joyner, 1993; Mortlock et al., 1996; Zakany and Duboule, 1996). Further analysis of mutations such as hypodactyly and talpid\(^3\) will therefore continue to add to our existing knowledge of how the vertebrate limb is patterned and help us to understand how defects arise in different vertebrate species.
Appendix 1

Solutions:

Sterile Growth Medium: Minimum Essential Medium (MEM) containing 10% foetal calf serum, 100 units ml\(^{-1}\) penicillin, 100 \(\mu\)gml\(^{-1}\) streptomycin, 0.25 \(\mu\)gml\(^{-1}\) fungizone, and 2 mM L-Glutamine

Solution I: 50 Mm glucose, 25 mM Tris. Cl (pH 8.0), 10 mM EDTA (pH 8.0). Autoclave for 15 minutes at 10 lb/sq., and store on ice.

Solution II: 0.2 N NaOH (freshly diluted from a 10 N stock), 1% SDS. Freshly prepared.

Solution III: 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid, 28.5 ml of H\(_2\)O.

Phosphate buffered saline: prepared using Dulbecco "A" tablets (Oxoid) (PBS)

Paraformaldehyde in PBS: dissolve 4 g of paraformaldehyde solid in 90 ml of H\(_2\)O and two drops of NaOH at 65 °C. Filter, cool, then add 10 ml of 10X PBS.

TE buffer: 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0

Nieto et al. prehybridisation: 50% ultrapure, deionised formamide, 5X SSC, 2% blocking powder (Boehringer, Cat. No. 1096176; dissolve directly in this mix), 0.1% Triton X-100, 0.1% CHAPS (Sigma), 0.1 mg/ml tRNA, 5 mM EDTA, 50 \(\mu\)g/ml heparin. Dissolve at 65 °C.

20X SSC stock: 3 M NaCl, 0.3 M sodium citrate, pH 7.0

KTBT: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, 0.1% Triton X-100.

NTMT: 100 mM Tris-HCl, pH 9.5, 50 mM MgCl\(_2\), 100 mM NaCl, 0.1% Triton X-100.
NBT stock: 75 mg/ml in 70% dimethylformamide.

BCIP, X-phosphate stock: 50 mg/ml in dimethyl formamide.

Tyrodes: for 1 litre; 8 g NaCl, 0.2 g KCl, 0.2 g CaCl, 0.1g MgCl$_2$$\cdot$6H$_2$O, 0.05 g NaH$_2$PO$_4$$\cdot$H$_2$O, 1.0 g glucose, and 1.0 g NaHCO$_3$. Dissolve and make up to 1 litre.

Karnovsky’s: 1.0 g paraformaldehyde, 25 ml distilled H$_2$O, 2 drops of NaOH, 5 ml of glutaraldehyde, and 18 ml of freshly made cocodylate buffer.

Cocodylate buffer: Stock A; 0.2 M sodium cocodylate (4.28 g of sodium cocodylate in 100 ml H$_2$O.
Stock B; 1.7 ml of HCl in H$_2$O.

Embryo powder: homogenise stage 20-23 embryos in a minimum volume of ice-cold PBS. Add 4 vol ice-cold acetone, mixm incubate on ice for 30 minutes. Centrifuge at 10 000g for 10 minutes, remove the supernatant, and then wash the pellet out and grind it into a fine powder on a sheet of filter paper. Air dry and store at -20°C.


265


270


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