Congenital Limb Malformations
A study of Mice and Man

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Voor mijn ouders

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PREFACE

The development of a complete organism from a single fertilized oocyte is a complex process, which has been the subject of investigation in many morphological studies. In recent years, the rapid progress in molecular biology has allowed identification of some of the key genes that regulate embryonic development. This makes it possible to study embryonic development, and the regulating processes. It has also become clear that some of these genes are not exclusively involved in embryonic development, but that they can also play a significant role later in live.

For example, some of the signaling molecules that are involved in embryonic development also regulate processes such as cell proliferation, cell differentiation and programmed cell death in adult tissues. A defect in this signaling, can lead to deregulation of these processes and cancer growth (as reviewed in Pawson and Hunter, 1994). Therefore, studying genes that are involved in the precisely controlled cell growth during embryonic patterning, might contribute to the understanding of uncontrolled cell growth in cancer in adult live. Furthermore, developmental pathways can be re-activated in the regeneration of wounds. The newt, for example, is an amphibian that can regenerate its limbs after amputation in the adult stage. To achieve this, the newt uses the same developmental pathways, which normally pattern its limbs during embryonic development (Imokawa and Yoshizato, 1998). A similar principle can be seen in mammalian species. This is nicely illustrated by the finding that a range of developmental genes is activated in an attempt of the body to repair damage after a cardiac arrest (Molkentin *et al.*, 1998). The knowledge gained by studying developmental biology can have a practical use in medicine as well. A nice example is the clinical application of *bone morphogenetic proteins* to stimulate bone growth after fractures.

During the past thirty years vertebrate limb development has become a powerful model system for studying developmental mechanisms and pattern formation during embryogenesis. The developing limb is an easily accessible structure that makes observations and experimental handling practical. Limb development is by no means an uncomplicated model for embryonic development. It takes many complex embryological processes and interplay of a diverse set of genes to construct an anatomical correct limb from a group of undifferentiated cells.

In the quest for knowledge about limb development, two animal models, the chicken and the mouse, have proven to be invaluable. The chicken is a model system with an important advantage over other model systems: the egg makes a chicken embryo easily accessible and it can be used as a natural incubator to study the effects of experimental manipulations. Classical experiments in chicken embryos provided the first insights in the processes that control limb development. In recent years, mice have become the most important model system for studying molecular biology of limb development. Supported by ever increasing knowledge of the mouse, it is to some extent possible to extrapolate experimental insights from this model to the human situation. A large number of mutant limb phenotypes have been described in the mouse that can

be used to study limb development (MGD, 1998). Some of these mutants arose spontaneously, others are the result of induced mutagenization experiments or targeted null-mutations.

Hand malformations are the most common congenital malformations observed in newborns (Flatt, 1994). They can occur in an isolated form, or as part of a syndrome that includes anomalies of other body structures. Two well-known examples of such syndromes are the Holt-Oram syndrome (Terrett *et al.*, 1994), a combination of hand – and heart malformations, and the craniosynostosis syndromes (Mulliken and Warman, 1996), which can affect the limb and skull. Syndromic hand malformations are a clear indication that genes that are important in limb development also play a role in other structures of the developing embryo. Apparently, similar mechanisms are used in pattern formation of the limbs, the heart and the skull. The study of (abnormal) limb development will therefore surely contribute to the understanding of the pathology of other developmental disorders.

The aim of this study was to investigate the etiology and pathogenesis of two congenital limb malformations: pre-axial polydactyly (duplication of digits on the thumb side) and syndactyly (fusion of fingers). Studies were performed in families affected with pre-axial polydactyly and in mouse mutants that serve as a model for pre-axial polydactyly and syndactyly.

The initiation of this study dates back to the period of 1983 to 1991 when 11 children with a positive family history for pre-axial polydactyly were surgically treated at the Department of Plastic and Reconstructive Surgery of the Sophia's Children's Hospital in Rotterdam. On close inspection, the families of these children all originated from a small area in the South Western part of the Netherlands, and could be traced back to a common ancestor couple, that lived approximately 200 years ago. Affected individuals showed a large phenotypic variation, but the most characteristic phenotypical observation was a non-opposable thumb, with three phalanges instead of the normal two, or a triphalangeal thumb. The hand malformations segregated as an autosomal dominant disorder, and in 1994 it was shown that the abnormal phenotype in this family is genetically linked to chromosome 7q36 (Heutink *et al.*, 1994). In addition to the triphalangeal thumb two other hand malformations, polydactyly of the index finger and complex polysyndactyly (a combination of pre-axial polydactyly and syndactyly), have been linked to chromosome 7q36 (Tsukurov *et al.*, 1994; Hing *et al.*, 1995; Radhakrishna *et al.*, 1996).

A mutation in the "pre-axial polydactyly gene" on chromosome 7q36 is thought to disturb the antero-posterior (thumb to little finger) axis of the developing limb during embryogenesis. Because the underlying biochemical defect is unknown, a cloning approach based on chromosomal location is the only way to identify the disease gene.

Two spontaneous mouse mutants, the *Hemimelic extra toes* - and the *Hammertoe* mutant, are considered to be a model system for the hand malformations linked to human chromosome

7q36. Both mutations map to a region on mouse chromosome 5, syntenic to human chromosome 7q36 (MGD, 1998), and their mutant phenotype shows a striking resemblance with the human hand malformations linked to 7q36 (Knudsen and Kochhar, 1981; Green, 1989). Although the genetic defect underlying these mutant phenotypes is still unknown, they provide a great opportunity to study the genetics and pathogenesis of pre-axial polydactyly without knowing the exact genetic defect.

Isolated syndactyly is a common congenital hand malformation in man. It generally segregates as an autosomal dominant trait and is characterized by syndactyly of the soft tissues between the digits on the hands and feet. An exception to this general rule is a new form of syndactyly that has recently been described in a Turkish family. The syndactyly phenotype segregates as a recessive trait and is characterized by synostosis of the phalanges in digits three and four (Percin *et al.*, 1999).

Recently, a mouse mutant with a comparable form of syndactyly was observed in an independent line of mice transgenic for the human rhodopsin gene. The mutant phenotype segregates as a recessive trait and is characterized by a synostosis of phalanges in digits two and three of the hind limbs. The transgene integration in this mouse mutant is an important starting point in the identification of the responsible gene. Apparently, the transgene that has no role in limb development, has integrated in or near a gene that is important for limb development, and has disrupted or altered its function. Once the responsible gene has been identified it can be investigated whether the human and mouse limb phenotypes are caused by defects in homologous genes.

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CHAPTER 1

General Introduction

Adapted from

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1.1 GENETICS OF CONGENITAL HAND MALFORMATIONS

It is estimated that one in 626 newborns has a congenital malformation of an upper limb (Flatt, 1994). These malformations can occur in an isolated form or in combination with other hand and/or foot anomalies, or as part of a syndrome. Many of these syndromes show heart or craniofacial malformations, indicating that some of the genes that are involved in limb development are also essential for development of these structures.

The etiology of congenital malformations can be subdivided into environmental and genetic causes. A well known example of an environmental cause is the wave of thalidomide induced hand malformations that occurred in the 1960s. Malformations caused by a genetic defect can be subdivided into three categories: single gene disorders with Mendelian inheritance patterns, multiple gene disorders, and chromosomal abnormalities (Temtamy and McKusick, 1978). For most malformations no large chromosomal abnormalities like translocations or deletions, which can be visualized with cytogenetic techniques and that can point towards a chromosomal localization of the gene defect are found. Most of the congenital hand malformations are single gene disorders.

Until recently, little was known about the etiology and pathogenesis of congenital hand malformations, which is reflected in present classifications based on descriptions of morphology, or osseous anatomy. However, this era is coming to an end. A number of genes involved in the etiology of human hand malformations has been localized and/or identified (see table 1.1). Pre-axial polydactyly, and several hand malformations with similarities to the polydactylous phenotype, will be discussed here.

1.1.1 Polydactyly

Polydactyly, or the duplication of a finger, or part of a finger, is the most frequently observed hand malformation (Ivy, 1957). Polydactyly often occurs in combination with other congenital malformations as part of a syndrome. According to location, polydactyly can be divided in radial or pre-axial polydactyly (PPD), ulnar or post-axial polydactyly (PAP), and central polydactyly. It is not clear whether central polydactyly represents a separate entity. The prevalence of polydactyly, with or without an associated malformation, varies between 5 and 17 per 10,000 live births (Sesgin and Stark, 1961; EUROCAT-working-group, 1991; de Walle *et al.*, 1992). Temtamy and McKusick (Temtamy and McKusick, 1978) define two types of post-axial and four types of pre-axial polydactyly (see table 1.2).

PPD type I has further been divided in six subtypes, depending on the level of duplication of the bones (Wassel, 1969). This type of pre-axial polydactyly is usually sporadic, often unilateral, and less frequently associated with thenar hypoplasia than the other three types.

Disorder	Localization	Gene	OMIM Entry	References
Isolated limb malformations				
Brachydactyly type C Haws	12q24	?	113100	Haws (1963)
Brachydactyly type C Robins	20q11	*CDMP1*	113100	Polinkovsky *et al.* (1997)
Post-axial polydactyly type a1	7p13	*GLI3*	174200	Radhakrishna *et al.* (1997)
Pre-axial polydactyly type II / III	7q36	?	190605	Heutink *et al.* (1994)
Pre-axial polydactyly type IV	7q36	?	174700	Tsukurov *et al.* (1994)
Pre-axial polydactyly type IV	7p13	*GLI3*	174200	Radhakrishna *et al.* (1998)
Synpolydactyly	2q31	*HOXD13*	186100	Muragaki *et al.* (1996)
Split hand / split foot 1	7q21	?	183600	Scherer *et al.* (1994)
Split hand / split foot 2	Xq26	?	313350	Faiyaz *et al.* (1993)
Split hand / split foot 3	10q24	?	600095	Nunes *et al.* (1995)
Syndromes involving limbs and the craniofacial region				
Apert syndrome	10q26	*FGFR2*	101200	Wilkie *et al.* (1995)
Cleidocranial dysplasia	6p21	*CBFA1*	119600	Mundlos *et al.* (1997)
Cornelia de Lange syndrome	3q26.3	?	122470	Ireland *et al.* (1991)
Greig syndrome	7p13	*GLI3*	175700	Vortkamp *et al.* (1991)
Orofaciodigital syndrome	Xp22	?	311200	Feather *et al.* (1997)
Oculodentodigital dysplasia	6q22-q24	?	186100	Gladwin *et al.* (1997)
Saethre-Chotzen syndrome	7q21	*TWIST*	101400	Howard *et al.* (1997)
Shprintzen-Goldberg syndrome	15q21.1	*FBN1*	182212	Dietz *et al.* (1995)
Other syndromes involving the limbs				
Acromesomelic dysplasia	20q11	*CDMP1*	201250	Thomas *et al.* (1996)
Ellis- van Creveld syndrome	4p16	?	225500	Francomano *et al.* (1995)
Hand-foot-genital syndrome	7p15	*HOXA13*	140000	Mortlock and Innis (1997)
Holt-Oram syndrome	12q24	*TBX5*	142900	Basson *et al.* (1997)
Pallister-Hall syndrome	7p13	*GLI3*	146510	Kang *et al.* (1997)
Townes-Brocks syndrome	16q12.1	*SALL1*	107480	Kohlhase *et al.* (1998)
X-linked radial aplasia	Xq24-25	?	312190	Galjaard *et al.* (in prep.)

Table 1.1 A selection of genes that have been localized or identified responsible for human congenital malformations involving the limbs.

Post-axial polydactyly

Type A	A fully developed extra digit including bony structures
Type B	A rudimentary extra digit without bony structures

Pre-axial polydactyly

Type I	Duplication of skeletal components of a biphalangeal thumb
Type II	Polydactyly of a triphalangeal thumb
Type III	Polydactyly of an index finger
Type IV	Polysyndactyly, combination of pre-axial polydactyly and syndactyly

Table 1.2 Tentamy and McKusick classification for polydactyly

Type II, type III and type IV are usually inherited as autosomal dominant traits (Temtamy and McKusick, 1978).

In 1994, two independent studies reported linkage of two different phenotypes of pre-axial polydactyly (PPD) type II/III and type IV to chromosome 7q36 - namely the previously mentioned triphalangeal thumb and complex polysyndactyly (Heutink *et al.*, 1994; Tsukurov *et al.*, 1994). The phenotype in the family with triphalangeal thumbs type II/III varied between opposable and non-opposable triphalangeal thumbs, indicating a common genetic origin of these two phenotypic variants (Heutink *et al.*, 1994). Linkage of complex polysyndactyly or PPD type IV to the same chromosomal region suggested that PPD type II/III and type IV could be caused by different mutations in the same gene (allelic heterogeneity), or by mutations in two different, but closely linked genes (locus heterogeneity). Presence of different degrees of post-axial polydactyly in both phenotypes brought up the question whether this gene (these genes) could also be responsible for isolated post-axial polydactyly. However, studies of seven Dutch Caucasian kindreds with isolated post-axial polydactyly type A or B showed no linkage with the locus on chromosome 7q36 (Zguricas *et al.*, 1996), suggesting that pre - and post-axial polydactyly have a different genetic origin. As will be discussed later, some forms of PPD and post-axial polydactyly are caused by mutations in the *GLI3* gene on chromosome 7p13.

1.2 RELATED HAND MALFORMATIONS

1.2.1 Split Hand / Split Foot

Some individuals affected with complex polysyndactyly show a phenotype that resembles split hand/split foot (N. Akursu, personal communication). Split hand/split foot

malformation (SHSF), also termed ectrodactyly, is characterized by the absence of the central digital rays, deep median cleft and syndactyly of the remaining digits. Typical and atypical categories are recognized (Temtamy and McKusick, 1978). Atypical cases are usually sporadic. The majority of the familial cases are inherited in an autosomal dominant fashion, but autosomal recessive (Verma *et al.*, 1976), and X-linked (Faiyaz *et al.*, 1993) inheritance have been described as well. The most frequent syndromic association of SHSF is the EEC (ectrodactyly, ectodermal dysplasia and cleft lip / palate) syndrome (Temtamy and McKusick, 1978). Both the isolated and syndromic form of this disorder show great phenotypic variability. SHFM is genetically heterogeneous - until now three different loci have been reported to play a role in the etiology of this disorder. The first autosomal locus is termed SHFM1, and is localized at chromosome 7q21 (Scherer *et al.*, 1994). Various authors have reported ectrodactyly patients with chromosomal aberrations of the 7q21 region (Sharland *et al.*, 1991; Genuardi *et al.*, 1993; Cobben *et al.*, 1995). An X-linked locus termed SHFM2 has been mapped at Xq26 (Faiyaz *et al.*, 1993). A second autosomal locus termed SHFM3 has been mapped to chromosome 10q24-25 (Nunes *et al.*, 1995; Gurrieri *et al.*, 1996).

Several genes from the critical chromosomal region of the SHFM1 gene have been investigated as candidate genes (Crackower *et al.*, 1996). Recently, a gene named *DSS2*, has been identified from the SHFM1 candidate region (Lee *et al.*, 1997). The murine *Dss2* homologue is expressed during limb development. Although the gene is interrupted and deleted in SHFM1 patients (Sinasac *et al.*, 1998) more direct evidence for the the involvement in SHFM1 will have to come from mouse models that lack the *Dss2* gene.

1.2.2 Greig Cephalopolysyndactyly Syndrome and allelic Hand Malformations

Several congenital hand malformations are caused by mutations in the human *GLI3* gene. The *GLI3* gene was originally isolated by cross-hybridization to the zinc-finger gene *GLI*, a sequence-specific DNA binding transcription factor from the *GLI-Kruppel* family. The coding region of the *GLI3* gene is composed of 14 exons, including an exon of more than 2500 bp (Kang *et al.*, 1997). The defining features of the syndromes caused by mutations in the *GLI3* gene are overlapping but distinct, and include combinations of polydactyly and syndactyly. The phenotypic spectrum associated with mutations in the *GLI3* gene is of particular interest to the hand malformations linked to chromosome 7q36. It is possible that, in analogy with the *GLI3* gene, mutations or deletions of different domains in a single gene are responsible for the polydactylous and syndactylous phenotypes linked to chromosome 7q36 (see chapter 5 of this thesis).

The Greig cephalopolysyndactyly syndrome (GCPS) is an autosomal dominant disorder affecting limb and craniofacial development. Affected individuals are characterized by post-axial polysyndactyly of hands, pre-axial polysyndactyly of feet, macrocephaly, a broad base of the

nose with mild hypertelorism and a prominent forehead. Large deletions, translocations, and point-mutations resulting in haplo-insufficiency of the *GLI3* gene have been found in association with GCPS (see figure 1.1) (Brueton *et al.*, 1988; Pettigrew *et al.*, 1989; Vortkamp *et al.*, 1991).

PPD type IV is characterized by broadening and/or duplication of the big toes and, less prominently, the thumbs. Other associated features are polydactyly of the third and fourth finger and/or the fifth toes, and soft-tissue syndactyly of the third and fourth fingers and/or second and third toes (Temtamy and McKusick, 1978). PPD type IV is a genetic heterogeneous disorder. Different forms of PPD type IV are linked to chromosome 7q36 and chromosome 7p13. Recently a mutation in the *GLI3* gene on 7p13 was found in a family with PPD type IV (Radhakrishna *et al.*, 1998). The mutation is a one nucleotide insertion in codon 1216 of the GLI3 protein, and is predicted to lead to a truncated protein with a length of 1245 amino acids.

Pallister-Hall syndrome (PHS) is an autosomal dominant trait that includes post-axial and midaxial polydactyly, syndactyly, imperforate anus, hypothalamic hamartome (brain tumor) and a range of other malformations. The disease is caused by frameshift mutations between the 3' the zinc finger domain and the 5' post-zinc-finger-1 (Pzf1) domain of the *GLI3* gene (Kang *et al.*, 1997).

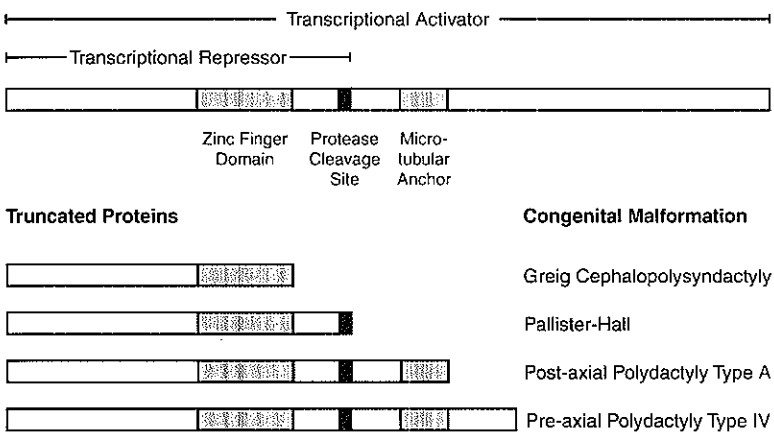

Figure 1.1 Protein truncations in the human *GLI3* gene and their associated congenital malformations. The *GLI3* transcription factor gene consists of seven domains, of which the DNA binding zinc finger -, the protease cleavage site -, and a microtubular anchor domain have been depicted here. Beneath the amino-terminal end of the *GLI3* protein are bars that represent proteins that are predicted to arise from truncating mutations in Greig cephalopolysyndactyly, Pallister-Hall syndrome, post-axial polydactyly type A, and PPD type IV. The truncated proteins may act to repress or activate transcription. This figure has been adapted from Biesecker (1997).

Post-axial polydactyly type A (PAP-A) is an autosomal dominant trait characterized by an extra digit in the ulnar and / or fibular side of the upper and / or lower extremities. The extra digit is usually functional, since it is well formed and articulates with the fifth or extra metacarpal / metatarsal. In a single family the gene responsible for PAP-A was mapped to the centromeric region of 7p. A mutation search in the *GLI3* gene demonstrated a mutation in codon 764 of the transcript (Radhakrishna *et al.*, 1997). The mutation occurred at the 3' end of the Pzf1 domain.

The vertebrate *GLI3* gene shows homology to the *Drosophila melagonaster* transcription factor *Cubitus interruptus* (*Ci*). The Ci protein is cleaved by a protease that is regulated by the Hedgehog protein, the *Drosophila* homologue of the vertebrate *Sonic hedgehog* (*Shh*) gene. The Ci amino-terminal fragment represses transcription of *Hedgehog* mRNA and some of its downstream targets. The intact Ci protein activates transcription, rather than repressing it. It is thought that the Hedgehog protein regulates transcription levels of its downstream targets by altering the balance of Ci repressor and activator forms (Aza-Blanc *et al.*, 1997). Like the Ci protein, it has been suggested that GLI3 protein function is regulated by proteolytic cleavage and that the transcription of *SHH* and downstream targets is repressed by the amino-terminal part of the GLI3 protein, while it is activated by the full length product (Biesecker, 1997). The combined transcriptional repressor and activator activities can explain that mutations and deletions of different domains in the *GLI3* gene cause a range of human congenital malformations, suggesting specific roles for the variant protein domains during development.

1.2.3 Holt-Oram Syndrome

Holt-Oram syndrome (HOS) is a developmental disorder affecting the heart and upper limbs. The characteristic findings in HOS are atrial or ventricular septal defects and thumb anomalies. The thumb may be a triphalangeal, non-opposable, finger-like digit and can be hypoplastic or even completely absent. The thumb metacarpal has both a proximal and distal epiphyseal ossification center. In most cases, the thumb defects are associated with hypoplastic thenar, or limited supination of the forearm. Limb defects are almost always bilateral, and asymmetrical. Cardiac defects are found in 95% of the familial cases, but patients having limb defects only may bear offspring showing the clinical phenotype of the complete syndrome (Temtamy and McKusick, 1978). The non-opposable, finger-like thumb that is associated with the HOS phenotype resembles that of PPD type III.

In a linkage analysis study in 7 HOS families, a gene responsible for HOS phenotype could be assigned to chromosome 12q, in five families. The two remaining families, that were phenotypically indistinguishable from the others, did not show linkage to 12q, thus demonstrating genetic heterogeneity. In the families linked to 12q it was shown that mutations in the *TBX5* gene are the basis of this disorder (Basson *et al.*, 1997).

The *TBX5* gene is a member of a family of transcription factors called the *Brachyury (T)* gene family, all sharing a T-box domain that binds DNA. A Glu-69-stop mutation in the *TBX5* gene was identified in affected members of a HOS family. The mutated gene is predicted to encode a truncated TBX5 protein that lacks most of the T-box residues, and is unable to bind DNA. In addition to heart and forelimb, the murine *Tbx5* gene is expressed in papilla, lung, pharynx, and thorax body wall. The observation that these tissues are not affected in HOS patients indicates that *TBX5* haplo-insufficiency on organ morphogenesis may differ between tissues. Alternatively, the human *TBX5* gene may be expressed in a more restricted pattern.

1.2.4 Synpolydactyly

Synpolydactyly is defined as syndactyly of the third and fourth fingers as well as syndactyly of fourth and fifth toes, associated with polydactyly of the same fingers and toes. It is usually inherited as an autosomal dominant trait (Temtamy and McKusick, 1978). Linkage studies in a large kindred with synpolydactyly (SPD), or syndactyly type II, (Akarsu *et al.*, 1995; Sayli *et al.*, 1995), mapped the SPD gene to a locus on chromosome 2q31 (Sarfarazi *et al.*, 1995) near the *HOXD* gene cluster. It was demonstrated that a mutation in the *HOXD13* gene is responsible for the SPD phenotype (Muragaki *et al.*, 1996). The mutation results in the expansion of a poly-alanine stretch in the amino-terminal part of the HOXD13 protein, a non-DNA binding part outside the homeodomain. It has been suggested that alanine stretches in homeoproteins play a critical role in modulating protein activity. Meanwhile it was reported that the SPD phenotype in two other families is caused by heterozygous and homozygous mutations in the *HOXD13* gene (Akarsu *et al.*, 1996; Muragaki *et al.*, 1996). The major features of the heterozygous phenotype are syndactyly and digit duplications. Branching of the metacarpals involved in digit duplications is also observed. The much more severe homozygous phenotype comprises of short hands and feet, complete cutaneous syndactyly of all four limbs, polydactyly, loss of normal tubular shape of the carpal, metacarpal and phalangeal bones resulting in polygonal structures, and bone fusions (Akarsu *et al.*, 1995) (Sayli *et al.*, 1995).

The phenotypes of these families correspond well with the phenotype of a mouse model with a targeted deficiency in the *HoxD* gene complex (Zakany and Duboule, 1996). The mouse *HoxD* genes are expressed in overlapping domains in the limb bud, which is suggestive of a role in the specification of the digit pattern (Izpisua-Belmonte *et al.*, 1991). *HoxD13* is the last gene to be activated during mouse limb development, and its expression is restricted to the most posterior region of the limb bud, which corresponds with the finding of poly- and syndactyly of post-axial rays in SPD phenotypes. It is not likely that the expansion of the alanine tract observed in the SPD patients causes a loss of function of the HOXD13 protein, but probably results in a protein with an altered function, DNA-binding specificity, or capabilities to repress or induce transcription (Akarsu *et al.*, 1996). As cooperative interactions between HOX proteins

are very important. a mutation in one member of the family is likely to influence the function of other members too.

SPD phenotypes show considerable overlap with polysyndactyly, or PPD type IV. Both disorders are characterized by the presence of poly- and syndactyly. However, an important difference between the synpolydactyly and PPD phenotypes is thumb involvement. No subjects from the reported kindreds affected with SPD phenotypes show thumb polydactyly. Genes responsible for pre- and post-axial polydactyly will be cloned in the near future and their functional analysis will provide explanations for the overlapping phenotypes.

1.2.5 Hand-Foot-Genital Syndrome

Hand-foot-genital (HFG) syndrome is characterized by small feet with small great toes, abnormal thumbs, and defects of the Mullerian ducts or its derivatives. Limb anomalies include short first metacarpals, small distal phalanges of the thumbs, short middle phalanges of the fifth fingers, and fusion or delayed ossification of wrist bones. In the feet, the great toe is shorter due to a short first metatarsal and distal phalanx (Stern *et al.*, 1970).

HFG syndrome is an autosomal dominant, fully penetrant disorder. Using linkage analysis the gene for HFG syndrome was mapped to chromosome 7p15-p14.2 near the *HOXA* gene cluster, and a nonsense mutation in the *HOXA13* gene was described (Mortlock and Innis, 1997). A stop codon was created by an A-to-G transition in a highly conserved tryptophan codon in the homeodomain, producing a truncated protein missing 20 C-terminal amino acids. The tryptophan is the only amino acid that is invariant in all known homeodomain proteins. This portion of the homeodomain normally folds into the last of the 3 alpha-helices, and is critical for DNA binding. The mutation likely eliminates, or greatly reduces, the ability of the HOXA13 protein to bind DNA.

The phenotype in the HFG families corresponds well to that of the *hypodactyly* (*Hd*) mouse mutant. *Hd* is a semidominant mutation that is caused by a 50 bp deletion in the first exon of the mouse *HoxA13* gene (Mortlock *et al.*, 1996). Heterozygous *Hd* animals have a shortened digit I on all four limbs, that varies from shortening of the nail to absence of one or two phalanges. These mouse mutants show alterations in the timing of ossification events, and fusions of carpals and tarsals. Homozygous *Hd* animals have a single digit on each limb, and loss of some of the carpal and tarsal elements. Homozygous *Hd* animals usually die in utero, those that are born survive to adulthood, but are infertile. Based on the expression pattern of the *HoxA13* gene in wild-type limbs, the digital arch defects in *hd* mice are consistent with a mutation in this gene. This is a good example of how mouse mutants can serve as a model system for a human congenital malformation.

1.3 LIMB DEVELOPMENT AND PATTERNING

The vertebrate limb has become an important model system for studying developmental mechanisms and pattern formation during embryogenesis. Pattern formation is the term used to describe the emergence of spatial biological organization during development. Mechanisms involved in the control of pattern formation include cell-cell communication, control of cell growth, tissue differentiation, and programmed cell death. Knowledge of normal pattern formation during embryonic limb development is important to understand the pathogenesis and underlying causes of hand malformations.

The upper limb bud in humans appears at 26-28 days after the fertilization, and the lower limb bud follows approximately two days later. The majority of the molecular events involved in limb patterning are the same for upper and lower extremities, which explains the often observed overlap in phenotypes of the affected hands and feet.

Digits in the upper limb become distinguishable at 41-43 days, and are fully separated approximately 10 days later. In the lower limb these events occur at 44-46 days and 54-56 days, respectively. During a period of 25 days, an interplay of genes and complex embryological processes have created a limb with the right amount of digits, the right appearances and functions at the right place along the proximo-distal, antero-posterior, and dorso-ventral axis (see figure 1.2).

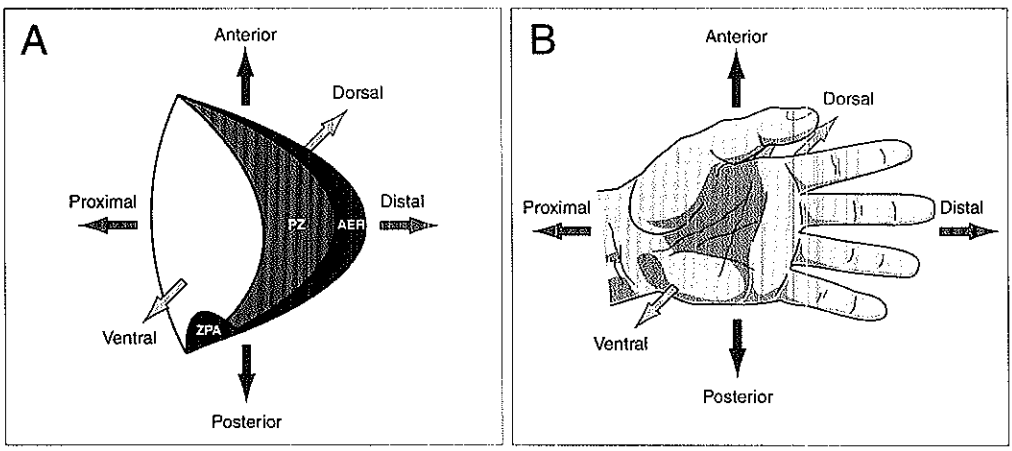

Figure 1.2 Schematic overview of the three axis in limb development. (A) A developing limb bud that will give rise to (B) a mature hand (depicted in the same orientation). ZPA; zone of polarizing activity, PZ; progress zone, AER; apical ectodermal ridge.

The general features of the animal body plan are laid out in broad strokes. At first, differential cell fates are specified along the rostral-caudal (head to tail) axis, a process

commonly referred to as regional specification. During the following development, further refinement of these broad fields occurs, leading to the formation of semi-autonomous regions. In these so-called secondary fields, the process of regional specification is repeated anew. The first clues to how the complex shape of a limb is achieved came from the work of experimental embryologists who identified the signaling centers, specific regions that are essential in directing growth and patterning in the developing limb bud. Studies of the developing limb, facilitated by its accessibility and large size, have resulted in now classical models for vertebrate pattern formation. Using powerful, complementary approaches, including surgical manipulations, ectopic expression studies in chick, and targeted gene disruption in mice, many of the processes involved in limb development have been elucidated.

Pattern formation starts with the definition of cells that make up the future limb, and is followed by the establishment of signaling centers that globally pattern the field. The positional information is recorded by the cells in the form of gene expression. With the help of this information and additional cues, the cells proliferate and differentiate to form a limb.

1.3.1 Definition of the Limb Field and Initial Outgrowth

The vertebrate limb bud develops from a dual contribution of lateral plate and somitic mesoderm (Chevallier *et al.*, 1977). The appearance of a limb bud starts with differential proliferation of lateral plate mesodermal cells at the appropriate level of the flank. Shortly thereafter, cells from the edges of nearby somites migrate into the limb. Limb muscle, nerve, and vasculature derive from these migratory cells, while all other limb tissues, including skeletogenic mesenchyme, cartilage, and tendons, derive from the lateral plate mesenchyme. It was found that members of the *Fibroblast Growth Factor* (*FGF*) gene family are capable of initiating limb bud formation. There are at least nine *FGF* gene family members (Miyamoto *et al.*, 1993). Beads soaked in FGF-1, FGF-2, FGF-4, or FGF-8 protein, that are placed in the inter-limb region in chick lateral plate mesoderm, form new ectopic limb buds. These limb buds develop into complete additional limbs, suggesting that local FGF protein production could be the required signal for limb bud initiation and subsequent patterning (Cohn *et al.*, 1995). The *FGF-1*, *FGF-2*, and *FGF-4* genes are not expressed at the right time or the right place to be the candidates for the real "limb inducer". The *FGF-8* gene expression pattern however, is consistent with the hypothesis that the FGF-8 protein might be responsible for the initiation of limb bud outgrowth (Vogel *et al.*, 1996). *FGF-8* gene expression is transiently localized to the intermediate mesoderm at forelimb and hind-limb levels just prior to limb outgrowth (Vogel *et al.*, 1996) (Crossley *et al.*, 1996). At this moment it is unclear whether *FGF-8* gene expression is essential/sufficient for limb bud initiation or that the endogenous signaling factor(s) include other as yet unidentified *FGF* gene family members, or other signaling molecules entirely. A problem with this model, is the rather long physical distance between the source of FGF-8

protein in the intermediate mesoderm and the target tissue, the forming limb bud. One model that would account for bridging this distance, is that FGF-8 protein induces a second signal in the lateral plate mesoderm, that in turn acts on the cells within the limb field. A strong candidate for such a secondary signal is *FGF-10* gene, which is expressed in the lateral plate mesoderm under the early limb bud and can be induced by ectopic application of FGF-8 protein (Ohuchi *et al.*, 1997).

1.3.2 Proximo-Distal Axis and the Apical Ectodermal Ridge

The rapidly dividing mesodermal cells of the limb bud are enveloped by an overlying ectodermal jacket whose distal tip forms a specialized epithelial structure called the Apical Ectodermal Ridge (AER) (see fig. 1.2). The AER is running along the antero-posterior axis at the interface of dorsal and ventral territories, is maintained by the underlying mesoderm, and is responsible for the outgrowth of the limb bud along the proximo-distal (shoulder to top of fingers) axis (Saunders, 1948; Saunders, 1977; Fallon and Kelley, 1977; Todt and Fallon, 1984). Experimental removal of the AER from a growing limb bud results in truncation of the limb. The proximo-distal level at which the truncation occurs is dependent on the time of AER removal (Niswander *et al.*, 1993b). This suggests that human transversal limb malformations could be caused by disturbances in differentiation along the proximo-distal axis.

The mesodermal cells located directly underneath the AER, the so-called "progress zone" (Summerbell *et al.*, 1973), show a high mitotic activity and maintain an undifferentiated state. As growth proceeds, cells located in the proximal parts of the progress zone, leave the progress zone. Experimental evidence suggests that, by this time, they have received their positional identities; in other words, patterning information is acquired in the progress zone. As the limb bud grows, differentiation becomes apparent, initially of the most proximal structures (primordia of the humerus), followed by the differentiation of the primordia of the more distal structures (radius, ulna, wrist bones and digits) (Niswander *et al.*, 1993b).

Once again, FGF proteins play an important role during this stage of limb development. At least three *FGF* gene family members are expressed in the limb ectoderm: the *FGF-2*, *FGF-4* and *FGF-8* genes (Vogel *et al.*, 1996). The *FGF-2* gene is expressed along the entire antero-posterior extent of the AER, and expression of the *FGF-4* gene is restricted to the posterior two-thirds (Niswander and Martin, 1992; Suzuki *et al.*, 1992; Savage *et al.*, 1993; Dono and Zeller, 1994). The *FGF-8* gene is expressed prior to AER formation in a broad stripe of cells along the distal tip of the limb bud. Once the ridge has been fully formed *FGF-8* gene expression is restricted to the AER (Heikinheimo *et al.*, 1994; Ohuchi *et al.*, 1994; Crossley *et al.*, 1996; Vogel *et al.*, 1996). It has been demonstrated that the signaling function of the AER can be replaced by the application of members of the *FGF* gene family. After experimental AER removal, local application of beads soaked in FGF-2, FGF-4 or FGF-8 protein leads to normal

proximo-distal outgrowth of the limb (Niswander *et al.*, 1993a) (Fallon *et al.*, 1992; Crossley *et al.*, 1996). Although these FGF proteins can substitute for normal AER function, the relative importance of individual *FGF* genes in maintaining proximo-distal outgrowth has not been clearly evaluated.

1.3.3 Apical Ectodermal Ridge positioning and the Dorso-Ventral Axis

The position of the AER is of crucial importance for proper pattern formation in the developing limb. The AER arises along the border of dorsal and ventral domains of the presumptive limb ectoderm (Michaud *et al.*, 1997). The homeobox containing transcription factor *engrailed-1* (*En-1*) and *Radical fringe* (*R-fng*) genes are expressed along this border prior to AER formation (see figure 1.3). *En1* gene expression is restricted to the ventral ectoderm, while the *R-fng* gene is solely expressed in the dorsal ectoderm. Both genes play a prominent role in AER positioning. The R-fng protein is believed to be a secreted molecule that modulates signaling through the *Notch* gene pathway. The interface between *R-fng* expressing and non-expressing cells is where the AER forms. Interruption of the normal *R-fng* expression boundary, either by ectopic expression or elimination of parts of the endogenous expression, results in formation of AERs at the new *R-fng* expression boundaries. The role of the En1 protein in AER positioning is to suppress *R-fng* gene expression in the ventral ectoderm, thereby creating the *R-fng* expression boundary. In support with this theory, mutant mice lacking En1 protein activity show a flattened AER that spreads into ectopic ventral locations (Loomis *et al.*, 1996).

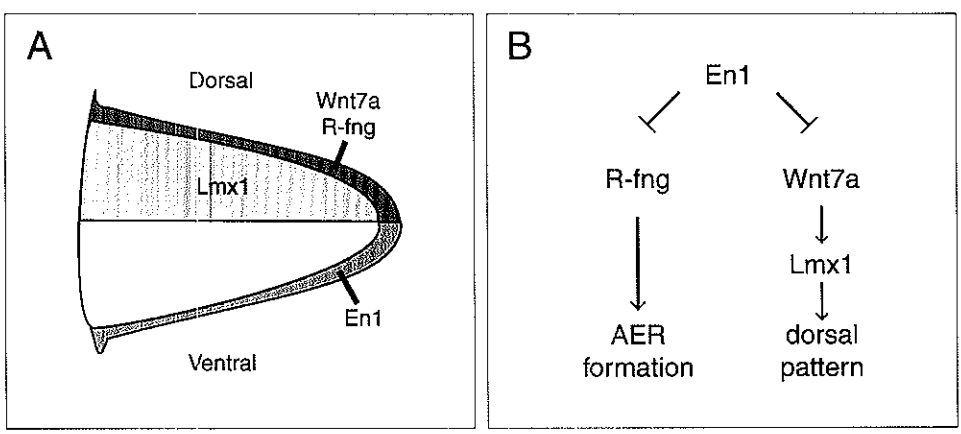

Figure 1.3 Mechanisms of AER positioning and dorso-ventral patterning. (A) gene expression along the dorso-ventral limb bud axis. The *Wnt7a* and *R-fng* genes are expressed in dorsal ectoderm. The *Lmx1* gene is expressed in the dorsal mesoderm. The *En1* gene is expressed in the ventral ectoderm. (B) Genetic interactions involved in AER formation and dorso-ventral patterning. *En1* expression in the ventral ectoderm restricts *Wnt7a* and *R-fng* gene expression to the dorsal ectoderm. The AER is positioned at the boundary of *R-fng* expressing and non-expressing cells. *Wnt7a* gene expression in the dorsal ectoderm instructs the underlying mesoderm to express the *Lmx1* gene and so acquire a dorsal fate.

Proper ectodermal signaling is important in dorso-ventral patterning. Classic experiments in which the ectoderm has been turned 180° around the dorso-ventral axis resulted in an inversion of dorso-ventral polarity of the limb mesoderm (MacCabe *et al.*, 1974; Patou, 1977). The *En1* gene is involved in dorso-ventral patterning (Loomis *et al.*, 1996; Laufer *et al.*, 1997; Rodriguez-Estaban *et al.*, 1997). *En1* gene expression is needed to restrict *Wnt7a* gene expression to the dorsal ectoderm (Dealy *et al.*, 1993; Parr *et al.*, 1993). The *Wnt7a* gene is a member of the *Wnt* gene family that encodes a group of secreted signaling molecules. Mutant mice lacking Wnt7a protein develop dorsal footpads, and flexor tendons on both, ventral and dorsal side of the digits, and accordingly abnormal "dorsal" flexion, indicating that the Wnt7a protein is a "dorsalizing" signal (Parr and McMahon, 1995).

The LIM-homeodomain containing transcription factor (the *Lmx1* gene) is believed to be a downstream target of the *Wnt7a* gene. Expression of the *Lmx1* gene is restricted to the dorsal mesoderm (Dealy *et al.*, 1993; Parr *et al.*, 1993; Riddle *et al.*, 1995; Vogel *et al.*, 1995). Ectopic expression of either the *Wnt7a* or *Lmx1* gene in the ventral ectoderm dorsalizes the ventral mesoderm (Riddle *et al.*, 1995; Vogel *et al.*, 1995).

Spatial restriction of the dorsalizing Wnt7a protein and the AER positioning R-fng protein to the dorsal ectoderm is maintained through the action of a single gene, *En-1*, that seems to co-ordinate both AER positioning and dorso-ventral patterning. However, the two processes are separable. Mice lacking Wnt7a protein activity have ventralized limbs, but do have defined AERs (Parr and McMahon, 1995), and ectopic expression of the *Wnt7a* gene in ventral ectoderm, does not influence the position of the AER (Riddle *et al.*, 1995; Vogel *et al.*, 1995).

1.3.4 The Antero-Posterior Axis and the Zone of Polarizing Activity

An important gain in the understanding of antero-posterior patterning came from grafting experiments in chicken (Saunders and Gasseling, 1968). When mesenchymal cells from the posterior border of a developing limb bud are transplanted to the anterior border of another limb bud, they will induce a symmetrical mirror image duplication of structures along the antero-posterior axis of a limb (see figure 1.4). When the grafted material was from quail origin, which is distinguishable from chicken tissues, it was clear that the duplicated structures were not derived from the graft itself, but from the surrounding host tissue that was induced to proliferate and change its antero-posterior identity. Because the graft reorganized antero-posterior order of structures within the limb, this region of posterior mesenchymal cells was called the Zone of Polarizing Activity (ZPA) (Saunders and Gasseling, 1968; Tabin, 1991; Tickle, 1991). These classic transplantation experiments produced animal phenotypes that mimic the human phenotype of the ulnar mirror hand.

It has been hypothesized that patterning along the AP axis is controlled by a signaling molecule, or a morphogen, that is released from the ZPA, and forms a gradient across the early

limb bud. A high concentration of the ZPA morphogen, which can be found along the posterior border of the limb bud, would give rise to digits with posterior character, and tissues exposed to the lower levels of the ZPA morphogen would develop into more anterior digits (Tickle *et al.*, 1975). The concept of a concentration dependent signal secreted by the ZPA was supported by the findings that the extent of digit duplications found in ZPA transplantation experiments is proportional to the number of transplanted ZPA cells (Tickle, 1981). The secreted Shh protein has been identified as the most likely ZPA morphogen.

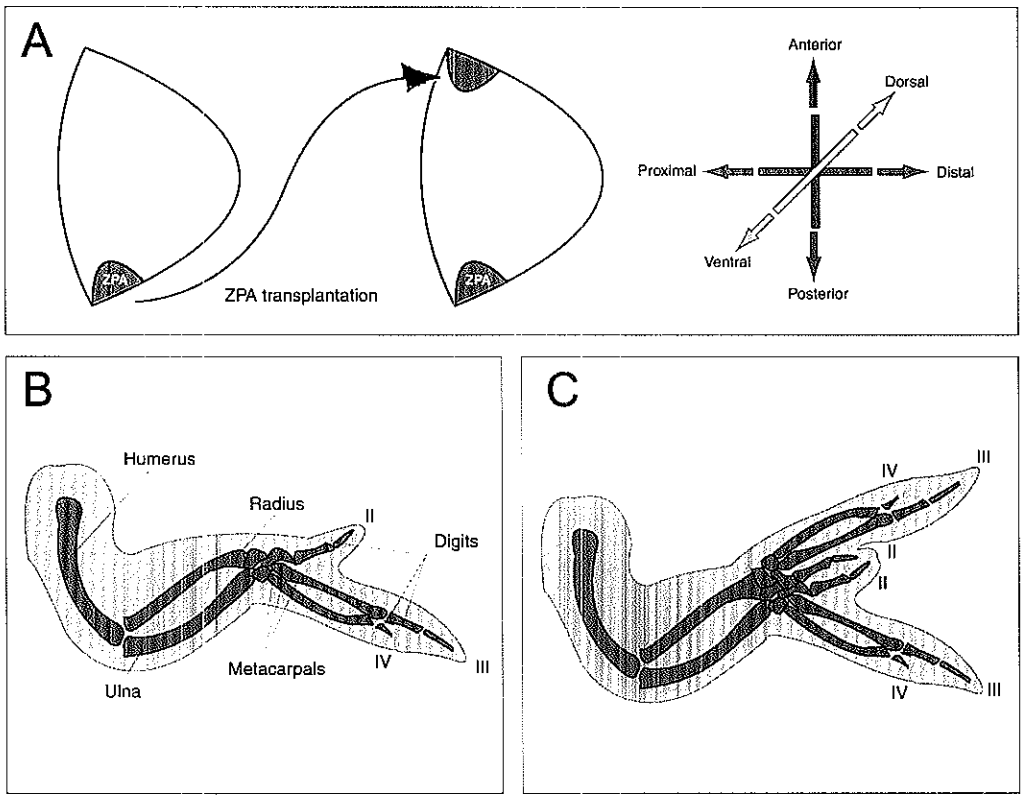

Figure 1.4 ZPA transplantation experiments in developing chicken wings. (A) Posterior mesenchymal cells are removed from a donor limb bud and transplanted to the anterior side of a host wing bud. (B) Normal chicken wing, with three morphologically distant digits which are designated from posterior to anterior IV, III and II. (C) Mirror image duplication after ZPA transplantation showing a IV, III, II, II, III, IV digit pattern.

The expression of the *Shh* gene co-localizes spatially and temporally with the ZPA (Riddle *et al.*, 1993). When *Shh* gene expressing cells, or beads soaked in Shh recombinant protein, are implanted along the anterior margin of a limb bud, a mirror image duplication just

like the one from the ZPA grafts, can be obtained (Riddle *et al.*, 1993; Chang *et al.*, 1994; Lopinez-Martinez *et al.*, 1995).

Careful ZPA deletion experiments, using *Shh* expression as a ZPA marker, have shown that ZPA removal in the early limb bud leads to truncated limb patterns along the antero-posterior and proximo-distal axes (Pagan *et al.*, 1996). Removal of *Shh* gene expression by genetic methods, confirmed these findings (Chiang *et al.*, 1996). So, Shh protein is required for polarizing activity and polarizing activity in turn, is required for correct antero-posterior and proximo-distal limb patterning.

Before the *Shh* gene was discovered, retinoic acid was thought to be the ZPA morphogen (Tickle *et al.*, 1985). Retinoic acid shows a "natural" gradient across the AP border of the limb bud: it is present in higher concentrations along the posterior border of the limb bud (Thaller and Eichele, 1987), and a bead soaked in appropriate concentrations of retinoic acid implanted in the anterior margin of the limb bud results in the mirror-image duplications observed with the ZPA grafts (Tickle *et al.*, 1982). However, evidence accumulated that retinoic acid does not fit into the "morphogen model". Retinoic acid acts by a mechanism dependent on absolute concentration, rather than on graded distribution. Polarizing activity in the limb fails to correlate with retinoic acid levels (Tabin, 1991). It appears that, rather than being the endogenous signal, retinoic acid can induce an ectopic ZPA (Wanek *et al.*, 1991). The relationship between *Shh* gene expression and retinoic acid is more clarified by the finding that retinoic acid has the ability to induce the expression of the *Shh* gene in the tissue of the anterior limb bud (Johnson *et al.*, 1994).

The question whether the Shh protein is a diffusible morphogen, or acts through local mechanisms remains unanswered. Within the limits of detection, Shh protein does not appear to diffuse from the site of synthesis (Lopinez-Martinez *et al.*, 1995; Marti *et al.*, 1995), and in insect and mammalian cell lines it was shown that both *Drosophila* hedgehog protein, and mouse Shh protein tightly bound to the cell surface (Chang *et al.*, 1994; Lee *et al.*, 1994; Bumcrot *et al.*, 1995). This strong adherence comes from a post-translational addition of a cholesterol moiety (Porter *et al.*, 1996a; Porter *et al.*, 1996b). The long range effect of Shh protein may be mediated by other signaling molecules like the Bmp2 protein. The *Bmp2* gene is expressed in the posterior mesenchyme in a broader domain than the *Shh* gene (Francis *et al.*, 1994). There is evidence that *Bmp2* expression can be induced by *Shh* expression and has weak polarizing activity itself (Laufer *et al.*, 1994; Duprez *et al.*, 1996). Questions regarding the range and mechanisms of Shh protein action have to be answered by definition of the Shh signaling pathway, and the identification of genes whose transcription is directly regulated by the *Shh* gene.

1.3.5 The Initiation of Antero-Posterior Positional Information

In embryos of the chicken mutant *limbless*, antero-posterior polarity is established in the form of posteriorly nested expression of *Hox* genes, without any detectable *Shh* gene expression, apical ectodermal ridge genes (the *FGF* gene family), or dorso-ventral asymmetry (Grieshammer *et al.*, 1996; Noramly *et al.*, 1996; Ros *et al.*, 1996). It has been suggested that gene expression in the emerging limb bud is established by axial influences on the limb field. This means that there is still a "missing piece", an yet unknown factor initiating antero-posterior polarity before the AER and ZPA appear.

Positional information along the antero-posterior axis is already present in the lateral plate mesoderm prior to limb bud formation (Harrison, 1918; Harrison, 1921; Hamburger, 1938). Pre-limb bud flank mesoderm has polarizing activity even though at these stages the *Shh* gene is not expressed in the flank (Hornbruch and Wolpert, 1991). When that same tissue is transplanted to a permissive environment below the anterior AER, *Shh* gene expression is activated in the transplanted flank cells (Yonei *et al.*, 1995).

These results suggest that the region of cells competent of expressing the *Shh* gene is larger than that which will actually form the ZPA, and that proper initiation of *Shh* gene expression requires a signal from the AER. The localization of the ZPA to discrete limb bud cells may come from interfering with a Shh protein repressor, activating *Shh* gene expression, or giving the limb bud cells competence to respond to other positive regulators of *Shh* gene expression.

One of the candidates for a factor that stimulates *Shh* gene expression or Shh protein function is the *HoxB8* gene. The *HoxB* gene family members have known roles in antero-posterior patterning of the main body axis. The *HoxB8* gene is expressed in the lateral plate mesoderm of the flank, in a position that correlates with the antero-posterior domains present in the flank cells. When the *HoxB8* gene is ectopically expressed to include the anterior domain of pre-limb bud flank cells, an ectopic ZPA will form (Charité *et al.*, 1994). Because the endogenous *HoxB8* expression domain in the developing limb bud exceeds that of the *Shh* gene, *HoxB8* expression alone is not sufficient to induce *Shh* expression. Inhibitors of retinoid synthesis or activity placed in the flank of the embryo prevent initial *Shh* and *HoxB8* expression (Helms *et al.*, 1996; Stratford *et al.*, 1996; Lu *et al.*, 1997). Retinoic acid could play a role in regulating expression of *Hox* genes and influence the future domain of *Shh* expression.

Part of the restriction of *Shh* expression to the posterior mesenchyme appears to be due to an active suppression mechanism operating in the anterior limb. Several polydactylous mouse mutants show ectopic expression of the *Shh* gene in the anterior mesenchyme during limb development. These mutants include *Extra toes* (*Xt*), *Strong's luxoid* (*Lst*), *luxate*, *X-linked polydactyly*, *Rim4*, and the mouse model for human PPD linked to chromosome 7q36, the

Hemimelic extra toes (*Hx*) mutant. (Büscher and Rüther, 1988; Chan *et al.*, 1995; Masuya *et al.*, 1995; Masuya *et al.*, 1997; chapter 5 of this thesis). The disturbances in antero-posterior patterning seen in the developing limb buds of these mutants are thought to cause the polydactyly phenotypes. While the molecular defect in most polydactylous mutants is unknown they have been elucidated for the *Lst* and *Xt* mutants. The *Lst* phenotype is due to a loss of function of the paired-type homeodomain transcription factor gene *Aristaless-like-4* (*Alx4*) (Qu *et al.*, 1998). The *Xt* mutant phenotype is caused by a loss-of-function of the zinc-finger transcription factor *Gli3*, a gene that is also known to cause polydactyly in humans (Schimmang *et al.*, 1992; Vortkamp *et al.*, 1992; Hui and Joyner, 1993). Both *Alx4* and *Gli3* genes are normally expressed in the anterior mesenchyme of the developing mouse limb bud, and are proposed to be components of a regulatory program that restricts ZPA formation to the posterior limb bud mesenchyme (Qu *et al.*, 1997). Alternatively, some of the mutant polydactylous genes may normally function indirectly by inducing programmed cell death (PCD) of cells that would otherwise express the *Shh* gene at the wrong position in the developing limb bud. There are a number of regions that undergo PCD during limb development. The anterior necrotic zone at the anterior margin of the developing limb bud is one of the largest (Saunders and Fallon, 1966), and could be made up of cells that would otherwise activate *Shh* gene expression. Later on, PCD in the interdigital regions serves to free the digits of the mature limb. Mutations affecting PCD in both the anterior necrotic zone and in the later interdigital necrotic zone could explain the regular presence of polydactyly and syndactyly in the same syndromes.

1.3.6 Integration of Axial Patterning Mechanisms

All three axes (proximo-distal, antero-posterior and dorso-ventral) are closely linked during limb patterning and outgrowth (Yang and Niswander, 1995). For the maintenance of a functional ZPA, an intact AER is required (Vogel and Tickle, 1993). FGF-4 protein, which is produced by the AER, is supporting a functional ZPA by maintaining the expression of the *Sonic Hedgehog* gene (Vogel and Tickle, 1993; Niswander *et al.*, 1994). In addition, Shh protein produced by the ZPA, can induce *FGF-4* expression within the AER by a positive feedback loop (Laufer *et al.*, 1994). The feedback loop suggests a mechanism by which outgrowth and patterning along the proximo-distal and antero-posterior axes of the limb can be coordinately regulated, and explains why ZPA removal influences the antero-posterior and proximo-distal axes. The Wnt7a protein, the dorso-ventral patterning molecule, also plays a role in antero-posterior patterning (Parr and McMahon, 1995). Besides a disturbance in dorso-ventral patterning *Wnt7a* mutant mice also lack posterior digits. The signals from the dorsal ectoderm, where the *Wnt-7a* gene is expressed, are necessary to maintain *Shh* expression and thereby formation of the posterior skeletal elements.

1.3.7 The Homeobox Gene Family and Limb Development

The homeobox gene family is named after the "homeotic mutation" - a mutation which causes a body part to be replaced with a structure normally found elsewhere on the body. The first homeotic mutation was described in 1894 by W. Bateson (Krumlauf, 1994). In 1948, it was discovered by Lewis that homeotic transformation can be caused by a mutation in a single gene, and assumed that a mutation affected one of the "master genes" that control function of many other "subordinate" genes. In 1984, the first *Drosophila* gene with a homeobox motif was discovered (McGinnis *et al.*, 1984). The homeobox motif encodes a stretch of 60 amino acids that is conserved in proteins of different species. The homeobox domain regulates "subordinated" genes by binding to their promoters to activate or repress transcription. The proteins encoded by *Hox* genes can differ greatly from one another, except at the highly conserved homeodomain. It is now clear that homeotic genes play an important role in vertebrate development.

All vertebrates have four clusters of homeobox genes, called the *HoxA*, *HoxB*, *HoxC* and *HoxD* complexes, that contain 38 genes in total. The order of the genes in these cluster corresponds directly to position and timing of expression in developing embryos. The *Hox* gene complexes probably arose by duplication and divergence from a common ancestral cluster in invertebrates (Nelson *et al.*, 1996). Each complex contains different subsets of paralogous genes, indicating that some members of a cluster have not been duplicated during the evolutionary events that led to the formation of multiple complexes (Krumlauf, 1992). *Hox* motifs are also found in a number of other developmentally important genes located outside of the clusters. It is not yet clear how many homeobox containing genes are present in the human genome.

Activation of the *Hox* genes during limb morphogenesis follows distinct spatial and temporal patterns. Analysis of these patterns suggests that the expression of each of the *HoxA* and *HoxD* genes is regulated in three independent phases. Each of these phases is associated with the specification and patterning of one of the proximo-distal segments of the limb (upper arm, lower arm and hand) (Yokouchi *et al.*, 1991; Nelson *et al.*, 1996). Various models suggest that the genes from the *HoxD* complex regulate the positional identity along the AP axis of the limb bud, and *HoxA* genes are involved in patterning of the proximo-distal axis. However, *Hox* expression patterns are not always strictly correlated with antero-posterior or proximo-distal axes formation (Morgan and Tabin, 1994). It has been proposed that *Hox* paralogues function together in regions defined by their activation order and domains of expression to specify the patterns of growth of pre-chondrogenic condensations. Targeted *Hox* gene mutants exhibit alterations in the maturation of individual skeletal elements, suggesting that *Hox* genes influence multiple events during limb bud outgrowth and skeletal development. From combinations of targeted *Hox* gene mutants, a rule of posterior prevalence was suggested (Duboule, 1995). When two ore more *Hox* genes are co-expressed in the same cell, the more 5' gene(s) exerts a dominant effect. So, as the

dynamic patterns of *Hox* gene expression unfolds, different *Hox* genes play dominant roles in different limb bud regions. Because the different members of the *Hox* gene clusters have distinct effects on proliferation and differentiation, this leads to differential growth of limb elements.

Considerably less is known about the expression and regulation of the *HoxB* and *HoxC* genes even though it appears that expression of some members of these complexes is restricted to either fore or hind limb bud (Nelson *et al.*, 1996). It is possible that morphological differences between the upper and lower extremity can be correlated with these expression patterns.

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CHAPTER 2

Introduction to the Experimental Work

2.1 POSITIONAL CLONING APPROACHES

For some genetic disorders, chromosomal abnormalities, such as large re-arrangements and deletions, give a direct indication of the chromosomal region involved in a disease. In most cases, as in PPD, a disease causing gene has to be mapped to a chromosomal location using linkage analysis with polymorphic markers.

Linkage analysis makes use of the fact that all affected individuals in a family are expected to share the mutated gene and some of the surrounding DNA. By testing polymorphic markers around the chromosomal location, the smallest region of DNA shared by all affected individuals, that is not shared by non-affected individuals of the family can be defined. This candidate – or critical region must contain the disease gene. For a review on linkage analysis with polymorphic markers see White *et al.* (1989).

In 1994 it was shown that PPD type II/III is genetically linked to chromosome 7q36 (Heutink *et al.*, 1994). The generation of a physical map, an artificial reconstruction of the candidate region in overlapping genomic clones, was the next step in a positional cloning approach. This physical map was used to develop new polymorphic markers allowing further refinement of the candidate region by recombination analysis, and to identify candidate genes from the candidate region.

2.1.1 Identification of Candidate Disease Genes

There are several methods to identify genes in a candidate region. In this study, a combination of techniques such as computer-based homology – and gene-mapping searches , direct cDNA selection and exon trapping was used. There was an overlap in the genes that were found by these different methods, but some genes were identified only by a single method. Therefore a combination of methods is the best way to increase the chance of finding new genes.

The Human Genome Project is a worldwide initiative to identify all the estimated 80.000 genes in human DNA, and to determine the sequence of the 3 billion bases of the complete human genome. It is expected that a major part of the human genome consists of non-coding and repetitive DNA, containing little relevant biological information. To obtain biologically relevant sequences before the sequence of the whole genome is finished, a large number of cDNA clones are sequenced and the information is deposited in the Expressed Sequence Tag (EST) database. A first step to identify candidate genes is to see which genes have already been mapped to a certain chromosomal region. The Human Transcript Map is an example of a database that holds information on the chromosomal position of 30.000 genes and ESTs (Deloukas *et al.*, 1998). In the near feature the "positional candidate approach" will become very powerful as even more data on gene position accumulates rapidly.

A generally used method to identify candidate genes is screening copy DNA (cDNA) libraries with genomic clones from the critical region. This method has been used in many positional cloning projects, for example in cloning the genes responsible for Fragile-X syndrome (Verkerk *et al.*, 1991) and tuberous sclerosis complex 2 (European TSC consortium, 1993). This method can be very labor intensive for two main reasons. Firstly, only a small genomic region can be investigated in a single experiment. Secondly, in order to identify a transcript it will have to be expressed in the tissue that was used to generate the cDNA library. If the expression of a disease gene is expected to be low then multiple cDNA libraries prepared from different tissues will have to be screened.

A more efficient method that is also based on hybridization, but that is less labor-intensive, is direct cDNA selection (Lovett *et al.*, 1991; Parimoo *et al.*, 1991). In the direct cDNA selection method a pool of cDNA libraries is hybridized to immobilized genomic DNA (see figure 2.1). The different direct cDNA selection protocols that have been described differ mainly in the type and the preparation of the genomic DNA, and whether the hybridization is performed on genomic DNA immobilized on a membrane, or genomic DNA coupled to biotin.

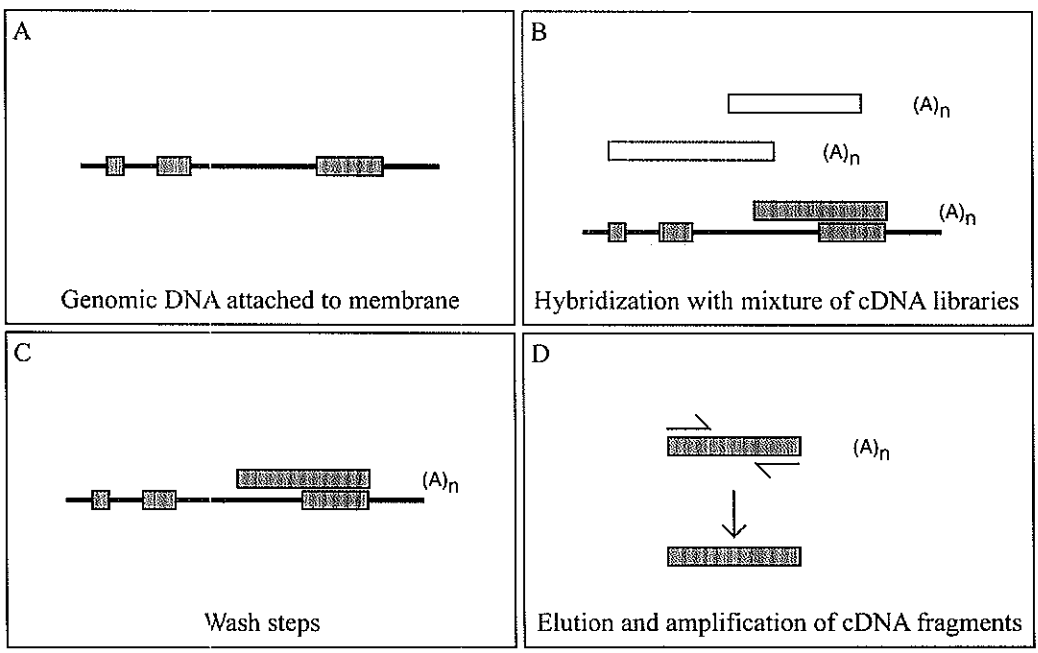

Figure 2.1 **cDNA selection method. (A)** Genomic DNA from the candidate region is attached to a nylon membrane. **(B)** The membrane is hybridized with a mixture of cDNA libraries. **(C)** cDNA clones that do not hybridize to the genomic DNA on the membrane are washed away. **(D)** The remaining cDNA clones are removed from the genomic DNA under stringent conditions and amplified by polymerase chain reaction.

A major advantage of this method is that it allows to screen large genomic regions with several cDNA libraries in a single experiment. Again, the success of the method relies on the number and type of cDNA libraries that are used in the hybridization step. Direct cDNA selection is, like all techniques that are based on hybridization, prone to false-positive findings that can result from genomic contamination of cDNA libraries, strong sequence homology with other genes, or the presence of pseudogenes in the region.

Exon trapping is a method based on the detection of coding sequences in genomic DNA by the splicing machinery of a cell (see figure 2.2) (Duyk *et al.*, 1990; Buckler *et al.*, 1991). The coding parts of genes, also called exons, are flanked by specific DNA sequences, the splice-acceptor and splice-donor sites. These splice sites are used by the cells splicing machinery to distinguish between exons, and the non-coding parts of a gene, the introns.

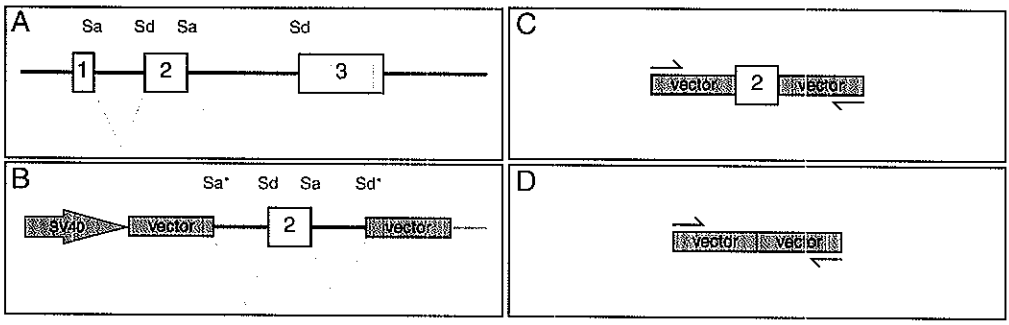

Figure 2.2 **Schematic overview of the exon trapping method (A)** Genomic DNA that contains a theoretical gene of three exons, named exon 1, 2, and 3. The second exon is flanked by a splice-acceptor (Sa) and a splice- donor (Sd) site that are used by the splicing machinery to distinguish between coding and non-coding sequences. Note that the first and last exon have only a single splice-acceptor or splice-donor site. **(B)** The genomic DNA is randomly cut with restriction enzymes and cloned into a special exon trapping vector that contains artificial splice-acceptor (Sa*) and splice-donor (Sd*) sites flanking the genomic insert. This "shotgun-cloned" DNA is transfected into a mammalian cell line, where, under the influence of the SV40 promoter also present in the vector, it is transcribed into RNA. **(C)** If the genomic insert contains exon 2 with a bona fide splice-acceptor and splice-donor site, the splicing machinery of the cell line will recognize this and join the splice sites of the vector with that of the genomic insert, thereby removing the non-coding DNA. To obtain the trapped exons, RNA is isolated from the cell line and reverse transcribed into cDNA using vector specific primers (half arrows). After PCR amplification, the exons are cloned into a vector and subjected to further analysis. **(D)** If the genomic insert in step B did not contain an exon with bona fide splice-acceptor and splice-donor sites, the artificial sites of the exon trapping vector are joined, and an empty PCR product of known size results.

A major advantage of exon trapping compared to direct cDNA selection is that the first step in the protocol is independent of expression. It is, only after the putative exons have been obtained, that cDNA libraries and sequence databases will have to be screened for extended transcripts. At this point several disadvantages of the method begin to play a role.

Cryptic splice sequences, that resemble bona fide splice-acceptor - and splice-donor sites, can result in the identification of putative exons that are in fact non-coding genomic sequence. If extended cDNA clones for a putative exon can not be identified, it either means that the cDNA libraries do not contain the gene of interest, or that the putative exon is in fact intronic sequence that resulted from a cryptic splicing event.

A second disadvantage of the exon trapping method is that it traps large exons in a very inefficient way. The average length of a putative exon is 100 nucleotides, making them rather small hybridization probes that complicate the cDNA library screening procedure. There are several reasons why the trapped exons are so small. Firstly, the protocol includes several Polymerase Chain Reaction (PCR) amplification steps that tend to amplify small fragments more efficiently than large fragments. Secondly, the exon trapping method relies on the presence of both a splice-acceptor and a splice-donor site. Therefore it cannot detect the first - and the last exon of a gene, which are usually the largest exons.

This automatically results in a third disadvantage of the exon trapping method. Most ESTs are sequenced from the ends of a cDNA clone, which are encoded by the first – or last exon. Therefore, the chance of finding an EST that matches a putative exon sequence is relatively small.

2.1.2 Gene Identification from Genomic Sequence Information

As the Human Genome Project advances, large-scale sequencing of whole disease candidate regions is becoming a widely used method in positional cloning. An example of a disease gene that was recently cloned with the help of large-scale sequencing is the tuberous sclerosis complex 1 (*TSC1*) gene on chromosome 9 (Slegtenhorst *et al.*, 1997). At this moment, the sequencing of the entire PPD candidate region on chromosome 7q36, is underway.

To analyze the vast amount of data that comes from the complete human genome sequence, new tools will have to be developed, and existing tools will have to be improved. Coding sequences are taking up only a small part of the human genome. Therefore, the challenge of finding genes in genomic sequence is to discriminate between the coding and non-coding parts. A good first step towards this goal, can be masking the repetitive DNA sequences. Repeated sequences can often disturb analyses, especially database searches. Programs to match repeat libraries to a query sequence, as well as repeat databases from several organisms are available on the World Wide Web (WWW). An excellent tool to find repeated sequences is the REPEATMASKER program (Smith *et al.*, 1996).

Searching for sequence similarity to other genes is a widely applicable method that relies on evolutionary relatedness. A direct advantage of finding homology with other proteins in a genomic sequence is that it gives a direct indication of the location of coding sequences. Furthermore, some of the biology of the gene might already have been elucidated. Nucleotide

sequence databases can be searched with programs like the Basic Local Alignment Search Tool (BLAST) (Karlin and Altschul, 1993) or the Fast Alignment Search Tool (FAST) (Pearson and Lipman, 1988; Pearson, 1990). This can help find conserved regulatory regions and identify known genes and ESTs. Another, very informative, strategy is to use an adaptation of the BLAST routine called BLASTX, that translates a nucleotide sequence to protein in all six possible reading frames, and uses the result as a query against databases of amino acid sequences and functional motifs (Gish and States, 1993). BLASTX allows identifying homologies on the amino acid level that are not directly clear from the comparison of nucleotide sequences.

When no sequence similarities to other genes are found, one depends on computational methods to identify the coding sequences in genomic DNA. Most of these methods recognize regularities in protein coding regions, which have a bias in their codon usage when compared to non-coding regions. All gene identification programs make use of a table that summarizes the regularities in coding regions. These tables can be calculated with the help of a so-called "coding measure"; a rule that defines how to search for regularities in coding regions. Coding measures are extracted from sets of "training" sequence data, from which the position of the protein coding regions are exactly known. The most elementary example of a coding measure is simply to determine the frequency in which a certain triplet occurs in a coding sequence versus the frequency of this same triplet in a non-coding sequence. Other examples of coding measures are: counting successive triplet pair occurrence, counting the multiple occurrences of the same nucleotide at regular intervals, counting simple sequences (such as long homopolymer runs) versus complex nucleotide sequences, and the occurrence of open reading frames.

Many coding region detection programs, like the well-known GRAIL program (Lopez *et al.*, 1994), combine the results from several coding measures to make the predicted outcome more accurate.

The pure mathematical approach of recognizing a coding sequence by codon bias has nothing in common with the way the transcription machinery in a cell recognizes coding regions in genomic DNA. Therefore, the accuracy of predicting coding regions might be increased by the recognition of signals like transcription-factor-binding sites and intron-exon junctions, places where the gene expression machinery interacts with the DNA. Signal sites can be recognized by searching for consensus sequences that describe the most common base at each position in a signal. Unfortunately, programs that make use of signal consensus sequences are not very good in discriminating true signal sites from pseudosites. More sophisticated search algorithms make use of a Position Weight Matrix (PWM) (Stormo, 1990; Hippel, 1994), in which a score is assigned to each nucleotide in the consensus sequence that tells how important the base is. The individual nucleotide scores are then added up to give a total score to the potential site. One example of a signal recognition program is PromoterScan that applies a PWM for the TATAA box, and takes into account the consensus sequence binding sites of a large number of general

and tissue-specific transcription factors. PromoterScan can identify 70% of all known primate promoters (Prestridge, 1995).

The tools described above mainly consider the isolated aspects of genes. Taking into account the overall consistency of putative gene features would significantly increase prediction accuracy. A putative exon is likely to be a real hit when, for example, it is predicted by a combination of a codon bias, an ORF, and flanking splice signals. Lately, there is a trend towards the development of integrated gene-finding programs. These programs mask repeated sequences, do homology searches, perform coding region analysis, and search for signal sequences. The results of all these analyses are presented in a single output. The programs attempt to define exons, and propose a tentative gene structure that seems most consistent with the available data.

2.1.3 Evaluation of Candidate Disease Genes

A disease gene is expected to be expressed in tissues that are affected in patients. For example, the PPD gene is likely to be expressed during embryonic limb development. The expression profile of a gene can be investigated by Northern blot analysis and whole mount *in situ* hybridization.

Northern analysis can provide information on the approximate length of a mRNA, and show tissue-specific expression of a transcript. The availability of human tissues to make Northern blots often poses a large problem. Some tissues, like the developing limbs of a human embryo, are very difficult to obtain. In those cases, more accurate data on the expression profile of a candidate gene can be obtained by whole-mount *in situ* hybridization on mouse embryos. A problem that accompanies whole-mount *in situ* hybridization of human candidate genes in mouse embryos is the *in situ* probe. To ensure a complete fit of the probe to the target mRNA in the embryos, the mouse homologues of the human candidate genes have to be identified first. When expression of a candidate gene is not detectable it could either mean that the gene is not expressed in the tissues that were examined, or that the expression level of the gene is below the detection limit of the method.

The evidence that a candidate gene is involved in a disease comes from the identification of changes in the nucleotide sequence that are specific for affected individuals. These nucleotide changes must segregate with the disease phenotype, must be rare or absent in the general population, and must predict to influence protein function. If the genomic structure of a gene is known, it is possible to screen for mutations in the coding region, intron-exon boundaries, and regulatory elements like promoters and enhancers. Otherwise, mutation analysis has to be done on the coding region only. This is usually performed on cDNA obtained from the mRNA that the gene produces.

There are several classes of mutations that can occur in a gene. Sequence changes leading to a premature stop of protein translation, like frame shifts, deletions, insertions and non-sense mutations, are usually regarded as disease causing mutations because they can lead to a (partial) loss of protein function. Mutations in intron-exon boundaries, or regulatory elements can influence splicing efficiency and transcription levels. Missense mutations could change amino acids that are known, or expected, to be crucial for the protein function. Because the effect of a mutation is not always obvious, it has to be demarcated from a polymorphism, by testing a number of control chromosomes for the same sequence change or by doing functional studies on the effect of the mutation on mRNA and protein stability and function.

2.1.4 The function of a disease gene

After a disease gene has been identified, one would like to understand the function of the gene and its role in the pathogenesis of the disease. A disease gene must have a biological function that can explain the disease phenotype. Investigating this function is not always straight forward. This is nicely illustrated by the investigation of the fragile X and Huntington's disease genes, which is still ongoing many years after these gene have been identified and where many questions on the function of the genes remain.

An important clue to what the function a disease gene could be, can come from homology with previously characterized genes. To identify protein relationships the protein sequence (predicted from the mRNA sequence) is compared to all protein sequences in the publicly available databases, and the presence of protein motifs is investigated. A protein motif is a highly similar region in an alignment of protein sequences that most likely share a common ancestry. Protein motifs are widely used to identify functional and structural regions of proteins. For example, the presence of a zinc finger - or a homeobox motif indicates that a protein can bind DNA and is most likely a transcription factor. A wide range of protein motifs can be searched in databases at the Baylor College of Medicine (BCM) web site (http://dot. imgen.bcm. tmc.edu:9331/ seq-search/ protein-search.html). Additional information about protein function can be derived from the presence of signal sequences that route proteins to different cell organelles. It is, for example, expected that a transcription factor contains signal sequences that direct it to the nucleus.

Another way to predict protein function is by investigating protein relationships. Protein relationships have evolved over long periods of time by speciation (orthologous relationships) and duplications fixed in genomes (paralogous relationships). Orthologs are collections of genes that share a common ancestry, and typically perform the same role in different organisms. An example of orthologous proteins are the *Drosophila hedgehog* and the vertebrate *Sonic hedgehog*, which share sequence homology and are both involved in similar processes during embryonic patterning (Johnson *et al.*, 1994). Paralogs are multiple proteins that arose from the

same ancestral gene within an organism. A classical example of paralogous genes are the alfa-globin, beta-globin, and myoglobin genes, that most likely arose from duplications of an ancestral globin gene in the vertebrate lineage (Ingham, 1963).

PSORT (Nakai and Kanehisa, 1992) is a computer program that predicts the sub-cellular localization of proteins. It requires the amino acid sequence and a source of origin, e.g. mammalian, as input data. PSORT analyzes the amino acid sequence by applying rules for various sequence features of known protein sorting signals. The program can recognize signal sequences in the N-terminus of a protein, mitochondrial proteins, nuclear proteins, peroxisomal proteins, endoplasmatic reticulum proteins, proteins in vesicular pathways, lysosomal and vacuolar proteins, lipid anchors in proteins, coiled-coil domains in proteins, and transmembrane segments. If transmembrane segments are detected, PSORT will also make an attempt to predict the membrane topology of the protein.

2.2 MOUSE MODELS FOR LIMB DEVELOPMENT

Over the years mice have become an important model system for studying human congenital malformations. There are several benefits to working with mouse models. The majority of developmental processes have remained largely unchanged during the course of evolution from mice and man, making it possible to extrapolate experimental insights from these models to the human situation. For a mammalian species, mice have a short generation time, making them ideal for genetic experiments. By using mouse inbred strains, it is possible to rule out influences of differences in genetic background, something that is virtually impossible to do in human populations. The practical advances of the mouse as a model system, and the experimental techniques that are available, have led to an enormous increase in our understanding of (abnormal) embryonic development (as has been discussed in chapter 1.3)

2.2.1 Mouse Models for Human Pre-Axial Polydactyly and Complex Polysyndactyly

In the Mouse Genome Database there are 25 entries that display the word polydactyly as one of the characteristics of a mutant phenotype (MGD, 1998). One of these polydactylous mutants is the *Hemimelic extra toes* (*Hx*) mutant. The *Hx* mutation is mapped on mouse chromosome 5, in a region that is syntenic to human chromosome 7q36 (MGD, 1998). The phenotype of the mouse *Hx* mutant resembles that of the human PPD phenotype linked to human 7q36 (Knudsen and Kochhar, 1981). A second mouse mutant, the *Hammertoe* (*Hm*) mutant, has been mapped very close to the *Hx* mutation on mouse chromosome 5 (MGD, 1998). The *Hm* mutant is characterized by webbing of the digits (Green, 1989), and this phenotype is comparable to the complex polysyndactyly phenotype linked to human 7q36. Because of the

comparable phenotypes and the syntenic chromosomal location, we assume that the *Hx* and *Hm* mutant phenotypes are caused by mutations in genes that are homologues to the genes responsible for the hand malformations on human chromosome 7q36.

2.2.2 A mouse model for syndactyly

In a single line of transgenic mice for the human rhodopsin gene (Li *et al.*, 1996), synostosis of the phalanges of digits two and three on the hind limbs was observed. The human rhodopsin gene has no role during limb development. Apparently in this line the transgene has integrated in, or near, a gene that is involved in limb development and disrupted or altered its function. The syndactyly phenotype is inherited as a recessive trait. Homozygous mutants show a complete syndactyly and synostosis of digits two and three of the hind limbs, while heterozygous mice do not show any limb malformations.

Recently, a human congenital hand malformation was reported that shows a remarkable similarity to the syndactylous mouse phenotype (Percin *et al.*, 1998). The severe, presumably homozygotic, human phenotype is characterized by complete osseous syndactyly of the third and fourth fingers and complete or partial soft tissue syndactyly of the toes.

2.2.3 Experimental Techniques in the Mouse

Several very powerful experimental techniques have been developed that can be used to study mice. It is possible to introduce (altered) genetic material into the germline of mice. This technique allows studying the effect of mutations in a gene, or can be used to express a gene in an ectopic location. Genes can be made inactive by removing (part of) the gene from the genetic material, these null-mutations, or "knock-out" mice, can be used to gain more insight into the function of a gene, and serve as a model for many human diseases. Theoretical and technical issues regarding these techniques are reviewed in (Glover and Hames, 1995).

Studying the expression pattern of a gene in tissues and/or different time-points in development can give insight into the function of a gene. The expression pattern of a gene can be visualized on the mRNA level by whole mount *in situ* hybridization, as reviewed in (Rosen and Beddington, 1993). This technique allows detailed examination of the tissue, or even the cell type, where a gene is expressed, and can be readily applied to mouse embryos in the various stages of development. The position of the protein in the cell can be an important clue to what its function might be. This can be investigated with antibodies that are directed against the protein.

Similar to the Human Genome Project, the goal of the Mouse Genome Project is to sequence the entire mouse genome. Availability of sequence information will facilitate extensive comparative studies between mouse and human genes, and will hopefully increase our knowledge about (human) biology, and the etiology of congenital malformations. Already large

amounts of information on mouse genetic markers, molecular segments, phenotypes, comparative mapping data, and experimental mapping data is accumulating in databases like the Mouse Genome Database (MGD, 1998).

During the long history of the mouse as an animal model, numerous abnormal phenotypes that arose as a result of induced mutagenization experiments, spontaneous mutations, or transgenic - and knock-out methods have been described and collected. Among the mouse mutants there are many that have congenital malformations of the limbs.

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CHAPTER 3

Clinical and genetic studies on 12 preaxial polydactyly families and refinement of the localisation of the gene responsible to a 1.9 cM region on chromosome 7q36

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Clinical and genetic studies on 12 preaxial polydactyly families and refinement of the localisation of the gene responsible to a 1.9 cM region on chromosome 7q36

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Abstract

Polydactyly is the most frequently observed congenital hand malformation with a prevalence between 5 and 19 per 10 000 live births. It can occur as an isolated disorder, in association with other hand/foot malformations, or as a part of a syndrome, and is usually inherited as an autosomal dominant trait. According to its anatomical location, polydactyly can be generally subdivided into pre- and postaxial forms. Recently, a gene responsible for preaxial polydactyly types II and III, as well as complex polysyndactyly, has been localised to chromosome 7q36.

In order to facilitate the search for the underlying genetic defect, we ascertained 12 additional families of different ethnic origin affected with preaxial polydactyly. Eleven of the kindreds investigated could be linked to chromosome 7q36, enabling us to refine the critical region for the preaxial polydactyly gene to a region of 1.9 cM. Our findings also indicate that radial and tibial dysplasia/aplasia can be associated with preaxial polydactyly on chromosome 7q36.

Combining our results with other studies suggests that all non-syndromic preaxial polydactylies associated with triphalangism of the thumb are caused by a single genetic locus, but that there is genetic heterogeneity for preaxial polydactyly associated with duplications of biphalangeal thumbs. Comparison of the phenotypic and genetic findings of different forms of preaxial polydactyly is an important step in analysing and understanding the actiology and pathogenesis of these limb malformations.
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Keywords: preaxial polydactyly; chromosome 7q36; localisation

The human limb bud starts to develop late in the fourth week of intrauterine life. Approximately four weeks later, an interplay of genes and molecular factors results in the development of a complete set of limbs with a well defined appearance, function, and a specific number of digits. Proper positional signalling within the three dimensional structure of the developing limb is of crucial importance for the future cell fate during embryogenesis. Disturbances in these signalling pathways can result in a large number of congenital limb deformities.

Limb malformations occur as isolated malformations of the hands or feet or as a part of a syndrome. In The Netherlands, approximately 16 per 10 000 children are born with congenital hand malformations.[1] Of all congenital hand malformations, polydactyly is the most frequently observed and it is even one of the most frequent congenital disorders in general.[2] The estimated prevalence of polydactyly, with or without an associated malformation, varies between 5 and 19 per 10 000 live births.[1 2 4 5] Sporadic occurrence has been described, but the majority of cases show an autosomal dominant mode of inheritance. The large variety of recorded prevalences in different studies can partly be explained by the fact that clusters of affected families often inhabit specific geographical areas.

Our present knowledge on limb development and congenital limb disorders has mainly been gathered in two ways. Firstly, because of its accessibility and size, the vertebrate limb has become a model system for studying developmental mechanisms and pattern formation during embryogenesis of vertebrate embryos, and this has resulted in models that are now classical for vertebrate pattern formation.[3] Secondly, in recent years, congenital limb malformations have attracted enormous research attention. Finding the genes responsible and correlations of phenotypes with genotypes have made a large contribution to the understanding of human development.[3] However, the majority of genes involved in the actiology of human limb malformations remain to be identified and in view of this most of the present classifications of congenital hand malformations are descriptive. The most frequently used are the ones by Swanson[7] and Wassel[8] based on anatomical findings, the classification by Temtamy and McKusick[9] widely used among geneticists, and the classification proposed by Winter and Tickle[10] based on embryological findings. However, none of these classification systems has succeeded in clarifying the considerable overlap in phenotype between the defined subgroups.

Polydactyly can be defined as the duplication of a finger or a part of it. Isolated (non-

syndromic) polydactyly can be generally subdivided into pre- and postaxial polydactyly. Preaxial polydactyly refers to an excess of parts on the radial side of the limb and it describes the so called duplicated thumbs, as well as the various forms of triphalangeal thumbs and index finger duplications. Temtamy and McKusick[17] define the following four types of preaxial polydactyly: type I (PPD-I) or thumb polydactyly is the duplication of one or more of the skeletal components of a biphalangeal thumb; type II (PPD-II) or polydactyly of a triphalangeal thumb; type III (PPD-III) or polydactyly of an index finger; type IV (PPD-IV) or polysyndactyly. Both preaxial polydactyly and syndactyly are cardinal features of this phenotype, but syndactyly never occurs without polydactyly.

PPD-I, or thumb polydactyly, has further been subdivided into six subtypes by Wassel[18] according to the level of duplication in bone anatomy. This type of preaxial polydactyly is usually sporadic, often unilateral, and less frequently associated with thenar hypoplasia than the other three types mentioned above.

In 1994, two independent studies reported linkage of two different phenotypes of preaxial polydactyly to chromosome 7q36. Two kindreds were affected with PPD-II/III[12,13] and the other kindred was affected with complex polysyndactyly.[14] Subsequently in two other studies, linkage of two families with PPD-II/III to the same chromosomal locus was reported.[15,16]

Our group is presently working on the identification of the gene responsible for PPD-II/III on chromosome 7q36. As part of these efforts, we collected a large number of families affected with different forms of poly- and syndactyly. In this paper we will address the question of which forms of polydactyly are linked to chromosome 7q36. We report the phenotypic and genetic findings of 12 families with different ethnic backgrounds that are affected with various forms of preaxial polydactyly.

All families with PPD-II/III could be linked to chromosome 7q36. Within the linked families a large variation in the phenotypes was observed, including PPD-I, postaxial polydactyly, and associated radial and tibial dysplasia/aplasia. Linkage to chromosome 7q36 of a family with PPD-I but not PPD-II/III could be excluded, indicating that there is genetic heterogeneity for different forms of preaxial polydactyly. A comparison of all phenotypes that have been linked to the genetic locus on chromosome 7q36 is an important step in analysing their aetiology and understanding their overlapping phenotypes.

Subjects and methods

THE DUTCH FAMILIES

After our initial studies, the two Dutch families originally described in 1994[12,13] were expanded by another seven families originating from the same small geographical region in The Netherlands. Clinical and genealogical investigation was performed and peripheral blood samples were obtained for DNA analysis. A genealogical search for the common ancestor of all affected families from this region was performed by using the population and census records and civil registration in the municipal archives. All participating family members were personally examined by one of the authors with consent.

In addition, through a review of medical records of the Department of Plastic and Reconstructive Surgery of the Sophia Children's Hospital in Rotterdam, we ascertained a proband with PPD-I of the hands and feet. The proband had a positive family history for the same disorder. This family was not related to the above mentioned kindreds. Clinical investigation and peripheral blood sampling was performed by one of the authors with consent.

THE BRITISH FAMILY

Through a review of medical records of the Department of Plastic, Hand and Reconstructive Surgery of St James's University Hospital in Leeds, 10 probands with preaxial polydactyly were ascertained which were referred to the department for primary or secondary surgical correction of their hands. Each of these probands had a positive family history for the same limb malformation. Most of the probands and their relatives originated from the same small village in the Lake District in the United Kingdom. Clinical and genealogical investigation and peripheral blood sampling was performed by one of the authors with consent.

THE TURKISH FAMILY

Through a review of medical records of the Department of Orthopaedic and Hand Surgery of the Social Security Hospital in Ankara, Turkey, four probands with PPD-II were ascertained, all of whom had been referred for hand surgery. All four patients were members of the same family in which PPD-II was inherited as an autosomal dominant trait with complete penetrance. Clinical investigation and peripheral blood sampling was performed by two of the authors with consent.

THE CUBAN FAMILIES

A case of a girl with bilateral tibial aplasia and preaxial polydactyly born into a family in which PPD-II was inherited as an autosomal dominant trait has previously been reported by one of us.[17] This family will be referred to as Cuban family A. In addition to the already performed clinical and genealogical examination, peripheral blood samples were obtained for DNA analysis from all informative family members with each subject's consent.

Through a review of medical records of the Department of Clinical Genetics of the National Center for Medical Genetics in Havana, Cuba, another family affected with PPD-II/III was ascertained. This family will be referred to as Cuban family B. Clinical and genealogical examination and sampling of the peripheral blood was performed by one of the authors with consent.

DNA STUDIES

Genomic DNA was isolated from peripheral blood as described by Miller *et al.*[18] Microsatel-

Table 1 Pairwise lod scores between chromosome 7q36 markers and PPD-II/III families at various recombination fractions

	Recombination fractions (θ)						
Family	*0.00*	*0.01*	*0.50*	*0.10*	*0.20*	*0.30*	*0.40*
Turkey 1							
D7S550	3.357	3.295	3.041	2.713	2.021	1.288	0.550
D7S2465	3.110	3.051	2.809	2.493	1.815	1.086	0.376
D7S559	1.288	1.259	1.143	0.994	0.683	0.378	0.133
D7S2423	1.926	1.957	1.972	1.873	1.500	1.002	0.453
UK							
D7S550	4.749	4.667	4.333	3.900	2.979	1.978	0.904
D7S2465	4.269	4.194	3.890	3.494	2.650	1.734	0.766
D7S559	1.308	1.290	1.211	1.103	0.854	0.571	0.276
D7S2423	−0.297	1.182	1.642	1.643	1.305	0.799	0.265
Cuba A							
D7S550	0.183	0.237	0.363	0.422	0.406	0.308	0.168
D7S2465	1.764	1.734	1.610	1.449	1.104	0.734	0.356
D7S559	1.764	1.734	1.610	1.449	1.104	0.734	0.356
D7S2423	−4.823	−0.397	0.199	0.367	0.386	0.272	0.125
Cuba B							
D7S550	5.612	5.499	5.040	4.457	3.266	2.071	0.944
D7S2465	7.052	6.922	6.393	5.708	4.268	2.759	1.262
D7S559	6.895	6.772	6.274	5.631	4.277	2.852	1.395
D7S2423	2.676	2.622	2.402	2.120	1.545	0.975	0.453

lite markers were tested in multiplex reactions essentially as described by Weber and May[19] using a Perkin-Elmer-Cetus 9600 Thermocycler. Initial denaturation was 10 minutes at 94°C followed by 25 cycles of 30 seconds denaturation at 94°C, 30 seconds annealing at 55°C, and 90 seconds extension at 72°C. After 25 cycles a final extension time of five minutes at 72°C was used. Gel electrophoresis on polyacrylamide gels was performed as described by Weber and May.[19] Information for microsatellite markers D7S550, D7S559, D7S2423, and D7S2465 was obtained from the Genome DataBase (GDB).[20] Marker order was also obtained from GDB. A microsatellite marker within the Sonic hedgehog gene (Shh) was described by Marigo *et al.*[21]

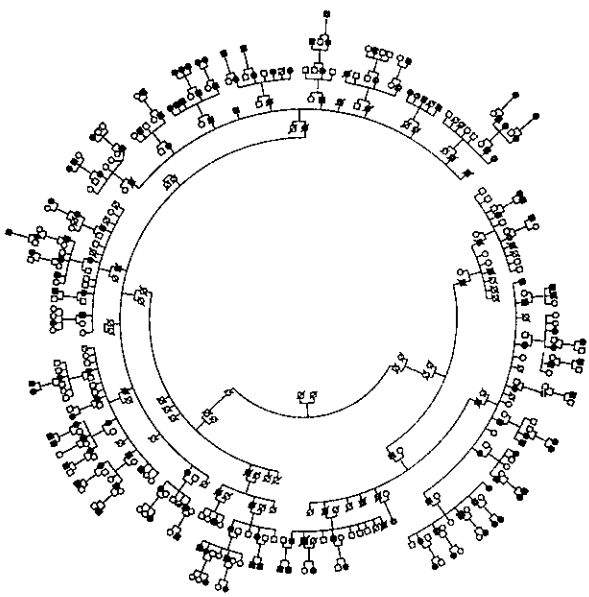

Figure 1 Pedigree of the Dutch kindreds affected with PPD-II/III according to Temtamy and McKusick,[6] and their common ancestral couple. Filled symbols represent affected subjects.

LINKAGE ANALYSIS

Pairwise lod scores were calculated for each family using the MLINK program of the LINKAGE package (version 5.1)[22] assuming polydactyly to be an autosomal dominant disease with a gene frequency of 0.001 and a conservative penetrance estimate of 95% (table 1). Mutation rate was set at zero and equal recombination rates between males and females were assumed. Marker allele frequencies were kept equal because population frequencies were not available and the families were too small to calculate reliable allele frequencies from people marrying into the polydactyly kindreds.

Results

THE DUTCH PPD-II/III PHENOTYPE

Seven investigated families were affected with a similar phenotype of PPD-II/III according to Temtamy and McKusick.[6] A detailed description of the phenotype has been provided elsewhere.[11] We had the opportunity to examine 298 subjects of whom 138 were affected, 94 were unaffected sibs, and 66 were healthy partners of affected subjects. By means of genealogical investigation, all affected families could be linked to a single common ancestral couple who lived approximately 200 years ago (fig 1).

Given its size and structure, lod scores were not calculated for the complete family since strong evidence for linkage in this family has already been reported.[12] Instead we performed haplotype analysis on the complete pedigree in order to detect recombination between the disease phenotype and genetic markers that would allow us to reduce the critical region for the genetic defect. In our original study we reported recombinations with marker D7S550 on the centromeric side of the critical region and with marker D7S794 on the telomeric side. In addition to what was reported in our original study, we tested four chromosome 7q36 markers (D7S550, D7S2465, D7S559, and D7S2423) on all available family members. All affected subjects share a common haplotype for the markers D7S2465 and D7S559 but several recombinants were observed with D7S550 on the centromeric side and with D7S2423 on the telomeric side, reducing the candidate region to a 1.9 cM interval between D7S550 and D7S2423 (table 2). The Sonic hedgehog (Shh) gene is localised within this 1.9 cM region close to D7S550. Shh is known to play an important role in limb development and specifically in the determination of the anterior-posterior pattern of the limb. Therefore, the gene can be considered as a good candidate for PPD. We tested a polymorphic marker within the Shh gene in the branch of the family that shows a recombination with D7S550. We detected a recombination between one affected subject and the Shh polymorphism, excluding Shh as a candidate gene for PPD (data not shown).

The "disease" haplotype was not found in unaffected subjects except for two brothers

Table 2 Haplotype sharing between affected subjects in several branches of the Dutch PPD-II/III family

	Branch					
Markers	*2515*	*2537*	*2776*	*2997*	*3026*	*7640*
D7S550	−	+	+	+	+	+
D7S2465	+	+	+	+	+	+
D7S559	+	+	+	+	+	+
D7S2423	−	+	+	+	−	+

+ = haplotype shared between all patients. − = haplotype not shared by all patients.

who shared the "disease" haplotype between D7S550 and D7S2423. These two brothers showed no (preaxial) polydactyly at first sight. On more detailed examination, one had a rudimentary postaxial polydactyly on his left hand in the form of a wart with a diameter of 3 mm on the lateral border of his middle phalanx.[3] No other abnormalities were found. This subject had two children and one of them inherited the "disease" haplotype and showed full blown preaxial polydactyly. The second brother had no abnormal findings even on detailed examination. He had one unaffected child who did not inherit the "disease" haplotype. In total, 140 members of this family shared a common haplotype for D7S2465 and D7S559. Of these 140 subjects, 139 showed a polydactyly phenotype. The calculated penetrance is therefore almost complete.

THE BRITISH PHENOTYPE

Ten probands were originally ascertained and appeared to be members of the same family. According to family history, 28 out of 81 subjects were affected. Twenty-five family members were personally examined. Fifteen of them were affected, five were unaffected relatives, and five were healthy partners of affected subjects who had affected children. The pattern of inheritance was autosomal dominant with complete penetrance and variable expression. All affected family members had hexadactyly of all four extremities, with opposable or non-opposable thumbs.

In the "opposable thumb phenotype" (fig 2), a fully developed thumb is placed in a normal "thumb" plane with an angle of 90° to the digital rays. The thenar muscles are well developed and opposition function is not significantly impaired. On *x* ray, the thumb shows either a biphalangeal or triphalangeal appearance, but without noticeable increase in length. The supernumerary digits are always localised between the thumb and index finger, partly rotated towards the thumb plane.

In the "non-opposable phenotype" (fig 3), the thumbs are small and hypoplastic. The "second" digital rays have the appearance of a fully developed index finger. All digits are placed in the same plane. The thenar musculature is hypoplastic and the thumbs are only capable of "pseudo-opposition".

All affected subjects showed hexadactyly of both feet. The supernumerary ray is localised between the big and the second toe. On *x* ray, the supernumerary toe is biphalangeal and usually has the appearance of a somewhat hypoplastic (duplicated) hallux. The phenotype of this family can be classified as PPD-II/III.

All affected persons in this family shared a common haplotype for all four markers. However, this haplotype was different from the one in the other families in this study (fig 4A).

THE TURKISH PHENOTYPE

According to the family history, 21 out of 51 subjects were affected. Fifteen affected sub-

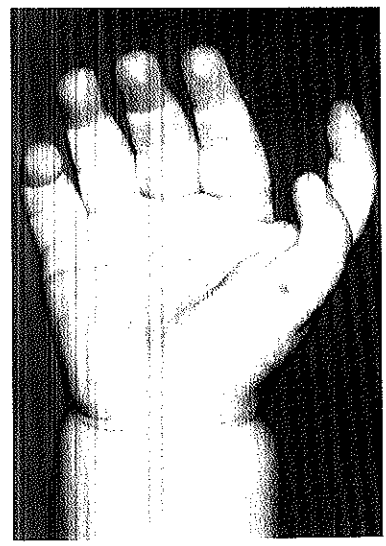

Figure 2 The "opposable" phenotype in the British kindred.

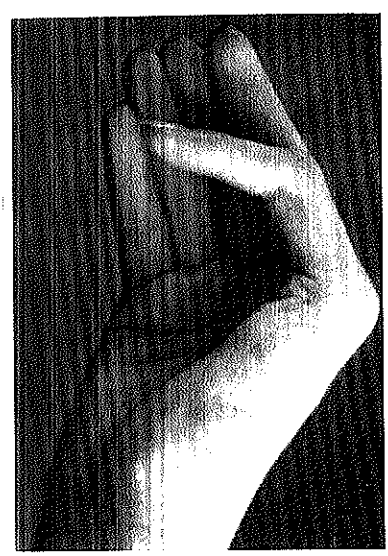

Figure 3 The "non-opposable" phenotype in the British kindred. The photograph was taken postoperatively. Only amputation of the preaxial extra ray was performed. No further reconstructive surgery was performed.

jects, 12 unaffected relatives, and five healthy partners of affected subjects who had affected children were examined.

All affected family members had a strikingly similar phenotype of hexadactyly of both hands and feet (fig 5). The first digital ray was too long for a thumb and was rotated in the "thumb plane". On x ray, the thumb was triphalangeal, with a fully developed rectangular extra phalanx and a long, slender metacar-

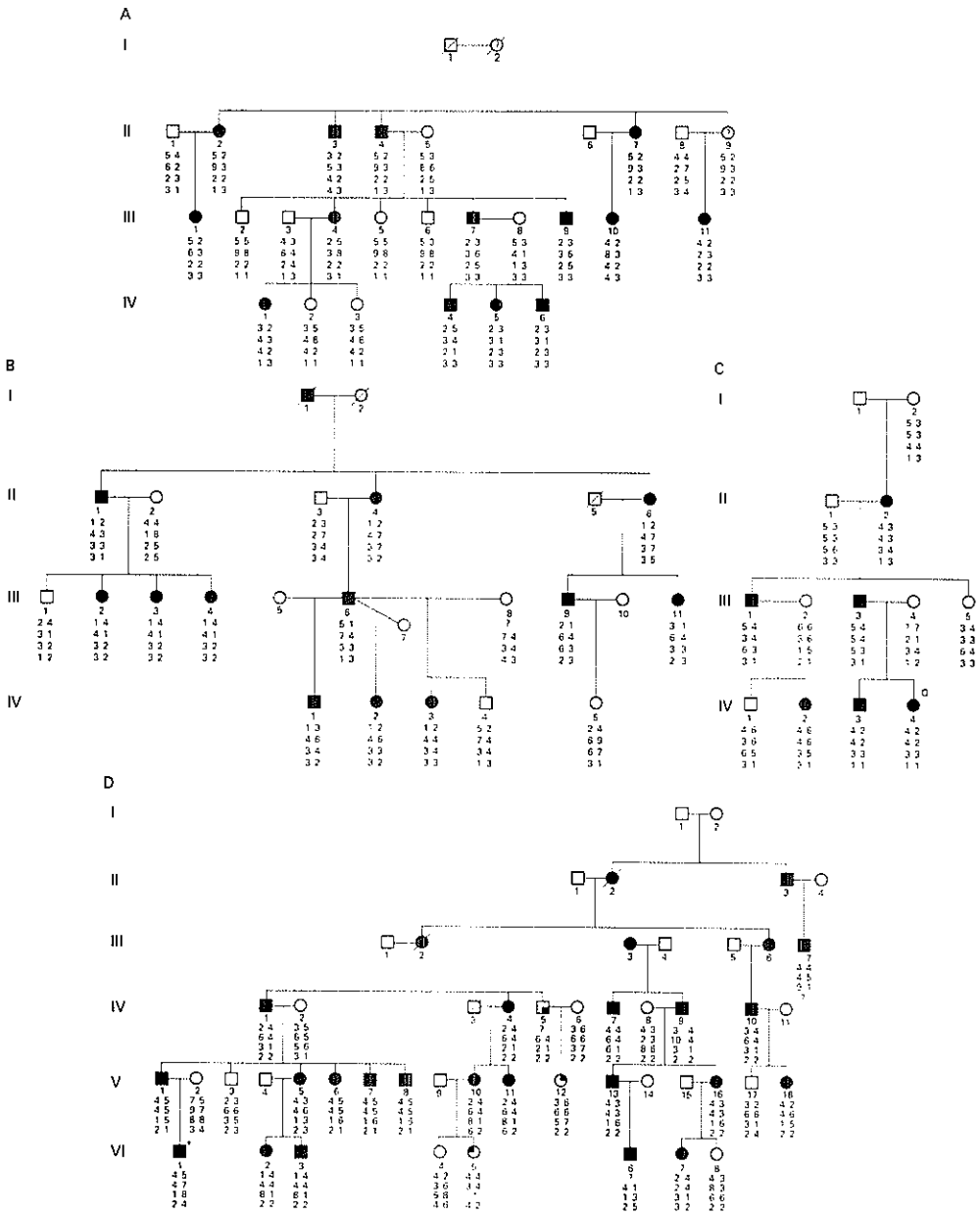

Figure 4 Pedigrees of PPD-IIIII families with haplotype for markers D7S550, D7S2465, D7S559, and D7S2423 (from top to bottom). (A) UK family. (B) Turkish family. (C) Cuban family A. (D) Cuban family B. Filled symbols represent affected subjects. The 0 next to IV.4 in (C) represents the additional radius and tibia dysplasia/aplasia. The + next to VI.1 in (D) represents an additional phenotype of scaphocephaly. The partly filled symbols in (D) with the top left quarter filled represent duplication of the thumbs, the top right quarter represents brachydactyly, and the bottom right quarter represents duplication of the big toes.

Figure 5 The "Turkish phenotype".

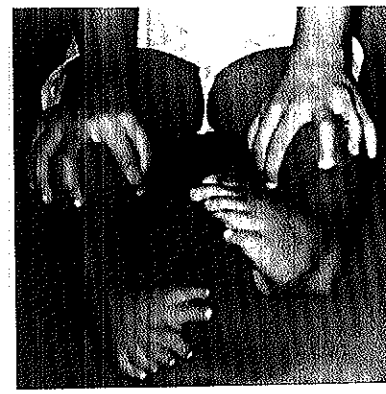

Figure 6 Proband from Cuban family A. Note hexadactyly of the left hand and a non-opposable triphalangeal thumb on the pentadactylous right hand; note also the severe bowing of the lower extremities with bilateral absence of the tibia and preaxial polydactyly of both feet.

pal bone. There were no sesamoid bones, which corresponded with the clinical finding of a moderately developed thenar eminence and moderate opposition impairment. The second digital ray had the appearance of a fully developed index finger. It was difficult to distinguish whether the duplicated digit had the "identity" of an index or a middle finger.

On *x* ray of the feet, the supernumerary digit was located between the big and the second toe. Again, the identity of the duplicated ray caused difficulties in view of the fact that in some subjects it contained two and in other affected subjects three phalanges.

All affected subjects were symmetrically affected, with little intra- and interpersonal variation in phenotype. The phenotype of this family can be classified as PPD-II/III. Patients shared a common haplotype for all markers, but not with any of the other families in this study (fig 4B).

THE CUBAN PHENOTYPES

Family A

Six out of 11 members of this family were affected with preaxial polydactyly of the hands and feet. All affected persons showed a very similar phenotype of bilateral, non-opposable, triphalangeal thumbs and preaxial polydactyly of both feet. No other malformations were recorded. The common phenotype of this family can be classified as PPD-II/III.

The previously described proband showed a much more severe phenotype.[17] Both upper extremities had a mild degree of radial dysplasia, associated with hexadactyly of the left hand, and a non-opposable triphalangeal thumb on the pentadactylous right hand (fig 6, IV.4 in fig 4C). All digital rays on the upper extremities had three phalanges and were placed in the same plane. Thenar hypoplasia was severe on both hands. In addition, there was cutaneous syndactyly between the first, second, and third digital ray of the left hand. On *x* ray, the fourth metacarpal of the right hand showed a partial duplication in the form of a bifurcation.

Both lower extremities were short, with bilateral absence of the tibia, severe bowing of the lower legs, and preaxial polydactyly of both feet. The right foot had six digital rays, which corresponded with six metatarsal bones on *x* ray. The left foot had seven digital rays on *x* ray accompanied by only five metatarsal bones. The two "floating" supernumerary digits were located between the big and the second toes.

This family is too small to obtain a significant lod score of more than 3 by itself. However, all affected family members share a common haplotype, and none of the unaffected subjects share this haplotype resulting in positive lod scores for all four markers, indicating that this family is very likely to be linked to 7q36. IV.2 shows a recombination with marker D7S2423. The severely affected proband showed the same disease haplotype as all other affected family members (fig 4C). The "disease" haplotype was different from that of the other families investigated.

Family B

We had the opportunity to examine 52 subjects of whom 32 were affected, 14 were unaffected sibs, and six were healthy partners of the affected subjects. The phenotype consisted of the opposable digit-like triphalangeal thumb in association with a preaxial extra ray which usually resembled a hypoplastic thumb. The common phenotype of this family can be classified as PPD-II/III.

Subject VI.1 showed the above described PPD phenotype on his left hand and foot. The right hand showed six well developed metacarpals with hypoplasia of the middle and distal phalanges. On the right foot, only the big and little toes were well developed, while the middle toes consisted of only the metatarsal bones and hypoplastic proximal phalanges. In addition, this subject also had mild scaphocephaly. No other relatives had craniosynostoses. Subject V.12 had brachydactyly type C of both feet and no polydactyly. Subject VI.5 had complete duplication of both thumbs or PPD-I (fig 7),

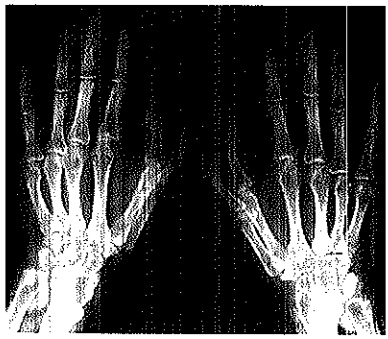

Figure 7 X ray of V.15 affected with PPD type I from Cuban family B.

but there was no triphalangism. The ulnar thumb showed a long, fully developed metacarpal with a well developed proximal and distal phalanx. The radial thumb showed a rather thumb-like, shorter metacarpal, with slightly hypoplastic proximal and distal phalanges. Subject IV.5 had PPD type I of his feet or duplication of the big toes. All subjects affected with polydactyly also had hexadactyly of their feet. On *x* ray, the extra ray was localised between the big and the second toe and contained two phalanges.

All affected subjects shared a common haplotype, different from the other families in this study, except V.12 affected with brachydactyly (fig 4D). The reason for the occurrence of additional congenital malformations in this family apart from polydactyly remained unclear.

DUTCH PPD-I FAMILY

Because of the finding of PPD-I in the Cuban family B, we included another family in which PPD-I occurred in our study. The Dutch proband with preaxial polydactyly type I came from a family affected with pre- and postaxial polydactyly. Twenty-three family members were personally examined. Sixteen subjects were unaffected relatives, two were healthy partners of affected subjects who had affected children, and five showed a polydactyly phenotype. The proband showed broad big toes, which on *x* ray appeared to be caused by complete duplication of the osseous elements. On both hands, this subject showed broad thumbs with ulnar clinodactyly in the interphalangeal joints. On *x* ray no duplication was seen, but there were broad, trapezoidal proximal and broad distal phalanges. The proband's father and brother showed only duplications of their big toes. Two subjects from the pedigree showed only unilateral postaxial polydactyly type B of their right hands. No other malformations were found. This family was too small to obtain significant evidence for linkage, but we tested the chromosome 7q36 markers on the family branch affected with the PPD type I, excluding the subjects with the postaxial polydactyly, and found no segregation of the marker alleles with the disease (data not shown), indicating that PPD-I can be caused by more than one genetic locus.

Discussion

In this study, we report clinical as well as genetic findings in 12 families with preaxial polydactyly. In addition to the two Dutch families described in our original linkage study,[12] we examined another seven families originating from the same geographical region. The phenotype of the affected subjects from these kindreds was very similar to that of those in our original study and could be classified as PPD-II/III according to Temtamy and McKusick.[10] Extensive genealogical studies showed a single common ancestor couple for all nine families. This genetic relationship was confirmed by the finding that all affected subjects in the nine families shared a common haplotype for markers D7S559 and D7S2465. This haplotype was not observed in unaffected subjects apart from one, so the trait shows almost complete penetrance.

Subsequent haplotype analysis on all available family members showed recombination events with markers D7S550 and D7S2423 reducing the critical region for the PPD-II/III gene to a region of 1.9 cM. The British, Turkish, and Cuban PPD-II/III kindreds described in this study also showed strong evidence for linkage to chromosome 7q36. A recombination event in the Cuban family A confirmed D7S2423 as the flanking telomeric marker for the critical region.

Recent studies by Hing *et al*[13] and Radhakrishna *et al*[14] described linkage of two families with PPD-II/III to chromosome 7q36; one family was of North American origin and the other of Indian origin, respectively. Until now, all families affected with PPD-II/III that have been investigated are linked to the same locus. Some families come from a completely different ethnic background and therefore the question arises whether this disorder is caused by a single, ancient mutation that has spread throughout the world population, or by several independent mutations in a single gene? We were not able to find a commonly shared allele for any of the tested markers between the Dutch, British, Turkish, and Cuban kindreds reported in the present study, suggesting that independent mutational events have occurred in the gene at chromosome 7q36.

Since one member of the Cuban family B showed a phenotype of PPD-I, and one subject from the kindred reported by Hing *et al*[13] showed a similar PPD-I phenotype, we wondered whether PPD-I is part of the phenotypic spectrum of the locus on chromosome 7q36 or if it can be caused by other genetic mutations. We ascertained a small family in which three subjects had a PPD-I phenotype and tested chromosome 7q36 markers. This Dutch kindred did not show linkage with the 7q36 locus, indicating that there is genetic heterogeneity for PPD-I. Considering the fact that in the Dutch PPD-I patients two have postaxial polydactyly type B (PAP-B), there is the possibility that the above described Dutch PPD-I kindred is affected with a distinct form of polydactyly

affecting the preaxial as well as the postaxial borders of the upper and lower extremities. However, the classification of Temtamy and McKusick[10] does not accomodate combined pre- and postaxial polydactyly as found in this family. Therefore the diagnosis in this family might be better described as crossed polydactyly as reviewed by Goldstein *et al*,[23] although the fact that the proband showed preaxial polydactyly on both hands and feet would fit best with PPD-I. In each case, linkage analysis in more families with PAP-B and PPD-I phenotypes are needed to study the molecular aetiology of these conditions.

In 1994, at the same time that we reported our original linkage findings, an independent study by Tsukurov *et al*[14] reported linkage of a kindred with complex polysyndactyly to chromosome 7q36. The family that participated in this study was of Dutch origin. It has been suggested that this family, as well as the kindred reported in our study,[12] originated from the same common ancestor from Belgium.[15 24] However, an extensive genealogical search, which identified the common ancestral couple of the Dutch PPD-II/III families in our study, excluded common ancestry for the complex polysyndactyly family in the period of the past 200 years. This finding suggested that the two phenotypes are either caused by different mutations in the same gene (allelic heterogeneity) or by mutations in different, but closely linked genes (locus heterogeneity).

Three patients from the kindreds reported here deserve particular attention because of their phenotype. Patient V.12 from Cuban family B is affected with brachydactyly. The patient does not share the "disease" haplotype that is found in other patients in this family, suggesting that the brachydactyly in this patient represents a separate finding unrelated to the gene defect on chromosome 7q36. Patient VI.1 from the same family has a more extensive limb phenotype than most other patients and mild scaphocephaly. This patient shares the "disease" haplotype with the other patients from the family. It is not clear whether the scaphocephaly phenotype may be connected with the gene defect on chromosome 7q36 or represents a separate entity.

The proband from Cuban family A showed a phenotype of bilateral tibial aplasia with polydactyly (MIM 188770) which has not previously been associated with the locus on chromosome 7q36.[21] In addition, this patient had a mild degree of radial dysplasia on both upper extremities. Interestingly, in the kindred reported by Hing *et al*,[15] one subject is also described with long biphalangeal thumbs and radiographical evidence of distal radial hypoplasia. This suggests that radial and tibial dysplasia/aplasia can be variant expressions of the PPD-II/III phenotype. Radial aplasia or hypoplasia usually occurs sporadically, which limits the possibilities for genetic research. In contrast, tibial aplasia or hypoplasia without associated duplication of the fibula, but with preaxial polydactyly of the toes and fingers, is known as a rare autosomal dominant trait with variable penetrance and expression.[25] It would be of interest to test these families for linkage to chromosome 7q36.

More evidence for the suggestion that radial and tibial dysplasia/aplasia are variant expressions of the PPD-II/III phenotype comes from two mouse mutants strains, *Hemimelic extratoes (Hx)* and *Hammertoe (Hm)*.[25 26] It has already been suggested that these mouse strains are excellent models for studying the PPD-II/III and complex polysyndactyly phenotypes linked to 7q36, respectively.[12 14] Both mutations are localised very close together on mouse chromosome 5, in a region that is syntenic to human chromosome 7q36. The *Hx* mouse shows tibial aplasia and various degrees of radial dysplasia and extra metacarpals, metatarsals, and digits, all of which are located on the preaxial side. The striking resemblance of the *Hx* mouse phenotype to the phenotype of the proband of Cuban family A strongly suggests that radial dysplasia and tibial hypoplasia are probably part of the phenotypic spectrum of the 7q36 locus.

Could the radial and tibial dysplasia/aplasia be the result of homozygosity for a mutation in the gene on chromosome 7q36, or is the variation the result of a modifier gene or an environmental factor? The possibility that the patients have a homozygous phenotype seems unlikely because both patients that have been described with radial/tibial dysplasia/aplasia in addition to PPD are heterozygous for the "disease" haplotype in the respective families. Furthermore, in both cases only one of the parents has the disease phenotype (fig 4C).[15] The answer to the question whether the variation in phenotype is caused by a modifier gene or by environmental factors could come from studies on the mouse model.

The *Hx* mutation arose in a non-inbred strain[27] and shows great phenotypic variability, similar to that seen in the human families. The variation in phenotype is likely to have a genetic background since environmental factors for mice are strictly controlled. By breeding the *Hx* mutation mice until the strain is completely isocongenic, the phenotype should become stable. These experiments are currently being performed by our group.

Several genes have been localised to chromosome 7q36 within the critical region. Two genes seem to be excellent candidates for the poly-/complex polysyndactyly phenotypes; the Sonic Hedgehog (Shh) gene and a homeobox gene HB9.[27 28] The mouse homologue of Shh is known to be expressed in the developing limb and is an important signalling molecule for the anterior-posterior patterning of the early limb bud. In humans, mutations in this gene can cause holoprosencephaly. Marigo *et al*[21] cloned the human homologue of the gene and performed recombination analysis in the family with complex polysyndactyly. In an unaffected subject from this family the authors reported a recombination event, making it unlikely that the Shh gene is responsible for the complex polysyndactyly phenotype. We performed haplotype analysis with the same polymorphic marker in the branch of the Dutch pedigree that showed the recombination with D7S550.

We detected a recombination event in an affected subject, thereby clearly excluding Shh as a candidate gene for PPD II/III. Additional evidence comes from experiments performed in the mouse mutant where several recombination events were observed between the *Hx* locus and *Shh*.[11]

The homeobox gene HB9 is distantly related to the *Drosophila melanogaster proboscipedia* gene.[28] The gene is mainly expressed in pancreas, small intestine, and colon. Whether the gene is expressed in the developing limb bud and is responsible for the polydactyly and complex polysyndactyly phenotypes is currently under investigation by our group.

The identification of the PPD gene(s) and the subsequent functional analysis will show how this gene is involved in the molecular pathways controlling the patterning of the preaxial portion of the limb, and in particular of the thumb.

The authors wish to thank all the participating families. Their contribution has been crucial for this work. We also wish to thank Professor Galiaard for his continuous support.

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CHAPTER 4

A physical and transcriptional map of the pre-axial polydactyly locus on chromosome 7q36

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ABSTRACT

Preaxial polydactyly is a congenital hand malformation that includes duplicated thumbs, various forms of triphalangeal thumbs and duplications of the index finger. A locus for preaxial polydactyly has been mapped to a region of 1.9 cM on chromosome 7q36 between polymorphic markers D7S550 and D7S2423. We constructed a detailed physical map of the preaxial polydactyly candidate region. With a combination of methods we identified and positioned 11 transcripts within this map. By recombination analysis on families with preaxial polydactyly, using newly developed polymorphic markers, we were able to reduce the candidate region to approximately 450 kb. The homeobox gene *HLXB9*, a putative receptor C7orf2, and two transcripts of unknown function, C7orf3 and C7orf4, map in the refined candidate region and have been subjected to mutation analysis in individuals with preaxial polydactyly.

INTRODUCTION

Human limb malformations are relatively common and are mostly associated with syndromes that display other congenital malformations as well. Of all congenital hand malformations, preaxial polydactyly (PPD) is most frequently observed, and includes duplicated thumbs as well as various forms of triphalangeal thumbs and index finger duplications (OMIM 190605). Sporadic cases of isolated PPD have been described, but most cases show an autosomal dominant mode of inheritance. By linkage analysis a PPD locus has been mapped near polymorphic marker D7S559 on human chromosome 7q36 (Heutink *et al.*, 1994). The critical region is flanked by polymorphic markers D7S550 and D7S2423 and spans approximately 1.9 cM (Zguricas *et al.*, 1999). The penetrance of the PPD phenotype is almost complete; 139 out of 140 related individuals, sharing the disease associated haplotype for D7S2423 and D7S559, showed a polydactyly phenotype (>99%)(Zguricas *et al.*, 1999).

In the same region the gene responsible for complex polysyndactyly (CPS) has been mapped (Tsukurov *et al.*, 1994). CPS is a congenital hand malformation characterized by pre- and postaxial limb anomalies combined with webbing of the fingers. Sacral agenesis (OMIM 176450), an autosomal dominant condition that includes a presacral mass, and urogenital and anorectal anomalies, has also been mapped close to polymorphic marker D7S559 on chromosome 7q36 (Lynch *et al.*, 1995).

A high-resolution map of a genomic area is essential for the positional cloning of disease genes. These maps are useful for the various transcript identification methods, they allow the development of new polymorphic markers and can serve as a template for large-scale genomic sequencing. The holoprosencephaly type 3 (HPE3) region is adjacent to the PPD candidate region. And previously, a contig of genomic YAC clones has been constructed to allow positional cloning approaches on the PPD - and HPE3 genes on chromosome 7q36 (Scherer *et al.*, 1992; Kunz *et al.*, 1994; Belloni *et al.*, 1996). This YAC contig was used as a starting point in our effort to identify the PPD gene.

To make the candidate region more suitable for experimental analysis we constructed a detailed contig of genomic P1, PAC and cosmid clones. Using a combination of exon trapping, cDNA selection, and EST mapping methods, 11 transcripts were identified, and precisely positioned in the genomic map. We developed 11 new polymorphic markers and used these for detailed recombination analysis on PPD families. This enabled us to localize the gene responsible for the PPD phenotype to a region of approximately 450 kb.

Four transcripts map to this refined PPD candidate region. These are the homeobox containing transcription factor *HLXB9*, the putative receptor C7orf2, and two transcripts with an unknown function, called C7orf3 and C7orf4. All four transcripts have been analyzed and sequenced in PPD patients, but no pathogenic mutations were identified. Large scale genomic

sequencing of the entire PPD candidate region will hopefully lead to the identification of the PPD gene.

MATERIALS AND METHODS

Construction of a high resolution genomic contig

Genomic library screening The ICRF cosmid library (113-L4/FS7), PAC library (RPCI1) constructed by P.J. de Jong (Ioannou *et al.*, 1994), and a P1 library (Shepherd *et al.*, 1994) were hybridized with long-range-inter-ALU products (Expand Long Template PCR System, Boehringer Mannheim) derived from YAC clones HSC7E445, HSC7E158, and HSC7E70. These YAC clones were obtained from "The Hospital for Sick children chromosome 7-specific library" and previously used for the construction of a physical map of the HPE3 critical region (Belloni *et al.*, 1996). STS assays from the ends of each P1 clone were used to identify overlapping clones. All resulting clones were mapped back to the contig of YAC clones by hybridization, using standard techniques as described by Sambrook *et al.* (1989). The position of individual clones is depicted in figure 1.

STS name	Size (bp)	Forward primer 5'-3'	Reverse Primer 5'-3'
HSC7E445(L)	150	gtttcctccatctgacttcacc	catggtgactgagtgaaaaagc
381E2(R)	132	aaagttcttcttctgcctaacaga	cgctctttaactgacgggac
99E12(T7)	110	gctggaggaggagcgtcatgaac	gacaccaaaaactctgccctgaa
HSC7E70(L)	100	gagagtctctgccagcctgt	aagagtagaattcttgctagtggtca
34E3(SP6)	85	gcttctgtgctgaagggg	gtctctagggtacacaaactgcg
K0922(T7)	163	tgcctttttacctgattcgtg	attatatgttgttgcttttc
B1120(SP6)	84	ttttcctctaaagaaaacgtattgc	tcttcagacttcaaacaagctcc
106C9(T7)	108	agtctgtgtcatttctgcctc	gaggacggtgacataacggt
1123(T7)	183	tgagcactaagcatcctg	aaacaaaaacccaacata
HSC7E739(R)	109	agatcccacaaatgagcgag	gttaatcacgagaataaacggacc
HSC7E158(L)	119	cgcagggtccataaatagaa	gaagaggggaagaagatccg
H0153(SP6)	150	aattacctattggaaccctacccatc	aacataatgcacaagatgatgcaa
HSC7E445(R)	123	accettgacagcagcagt	ttagcatagagctgtgtcactatgg
N2113(T7)	84	aaccccatctctacaaaa	tgcctcagcctcccaagt

Table 1 Sequence tags sites positioned in the genomic contig from centromere to telomere.

YAC specific cosmid library construction DNA of YAC clones HSC7E445 and HSC7E158 was obtained by growing single yeast colonies in Achilles heel cleavage (AHC) medium at 30°C for 48 hours. Total yeast DNA was prepared in 100 µL Seaplaque (FMC, Rockland, ME) agarose plugs. Agarose plugs were incubated overnight in MboI restriction buffer containing 0.1 U MboI restriction enzyme at 4°C. Restriction of the DNA was performed at 37°C for 20 minutes and was stopped by adding EDTA to a final concentration of 50 mM. The presence of 30-35 kb restriction fragments was checked by size separation on a pulse field agarose gel as has been described by van Ommen (1986). The remaining agarose plugs were treated with agarase (Boehringer Mannheim) according to the manufacturer's protocol. DNA fragments were cleaned by phenol extraction, precipitated with ethanol, and ligated into the BamHI site of the sCOGH2 cosmid vector (Datsun *et al.*, 1996) using standard techniques as described by Sambrook *et al.* (1989). The resulting cosmid library was packaged into phage particles using the GigapackII XL extract (Stratagene) according to the manufacturer's protocol. The cosmid

library was plated, transferred to nylon filters and hybridized with total human genomic DNA to identify the clones that contain a human genomic DNA insert. We picked, isolated DNA from, and fingerprinted 68 cosmid clones that contained a human DNA insert using standard techniques (Sambrook *et al.*, 1989). Cosmid clones have been designated FRS followed by a number. The position of the individual clones is depicted in figure 1.

Map integration Sequence tagged sites (STSs) were generated from vector-insert junction sequence from YAC, P1 - and cosmid clones using the ligation mediated PCR technique as described in Mueller and Wold (1989). Table 1 lists the primer sequences for the STSs.

Sequencing All sequencing in this study was done on a ABI-377 automated fluorescence dye sequencer using Bigdye chemistry (Perkin Elmer) or Dye terminators (Amersham).

Identification of Polymorphic Markers Genomic clones were partially restricted with Sau3A, shotgun cloned into BamHI linearized pBluescript vector (Stratagene) and plated. Colonies were transferred to nylon filters, and hybridized with the following set of di-, tri- and tetra nucleotide oligos: $(CA)_{15}$, $(CTG)_{10}$,$(AATT)_8$, $(AAAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(AGAT)_8$, and $(ACAT)_8$. Plasmid inserts from positive colonies were sequenced and PCR amplification primers flanking the repeated regions were designed. Each marker was tested on several unrelated individuals to test whether it was polymorphic. In this way 11 new polymorphic markers were integrated into the map (see table 2). The position of individual markers is depicted in figure 1. Existing markers, D7S559 and D7S2465, were also integrated into the map: (Green *et al.*, 1991). Positions of the polymorphic markers in the map are indicated in figure 1. Additional markers that were used for recombination analysis are: D7S550, D7S3037, D7S3036, D7S104, and D7S2423 (Green *et al.*, 1991; Belloni *et al.*, 1996).

Marker Name	Parent Clone	Size (bp)	Forward Primer 5' – 3'	Reverse Primer 5' – 3'
CGR13	264E9	154	cctctatggcaatctcctgc	cttgggtggcatacagtgtg
CGR12	140E19	212	ccaggagaactgtgttttcc	tggccacagttgaactttca
CGR6	K0922	112	aaaagacagtgtttccaaagca	gtggccattcattgagcc
HING1	B1120	169	gtaggctgacaaagaagaataac	actagatactaatatctaactac
CGR5	G2238	295	ggaggcagaggtttcagtga	acttcagtagtttctaggttt
CGR17	255D6	168	cactccagcctgggtgac	accttctgccccaagaattc
CGR16	N1914	132	actccactacctcaattttaata	ttggattgtattgttatttaaatg
CGR3	1280C3	300	gccaagcatgttggctta	cagaaactcctggcttgag
CGR8	I1338	194	tgacaagagcaaaactctgtca	ttcctgcctttcatcagcct
CGR10	FRS11	109	ctctgcctctctctgtagcca	tgcatgggctggttttaaat
CGR2	P1317a1	100	gcataggcccacacaacac	gtaaaaactcagcactgaaggc

Table 2 New polymorphic markers in the genomic contig from centromere to telomere

Amplification of polymorphic markers Amplification of polymorphic markers was performed in a 15 µL reaction containing 20 mM Tris HCl pH8.4, 1.5 mM $MgCl_2$, 50 mM KCl, 200 µM dNTPs, 0.5 U Taq DNA polymerase (Gibco BRL), and 10 pmol forward and reverse primers. Cycling conditions were 10 min at 94°C, 30 cycles of 30 s at 94°C; 30 s at 55°C; 90 s at 72°C, and a final step of 5 min at 72°C.

cDNA selection DNA from genomic clones was transferred to a nylon membrane. The membrane was hybridized with a cDNA mixture made of poly A+ RNA from fetal brain, fetal liver, fetal kidney, placenta, adult testis and adult brain (frontal cortex). To block repetitive sequences cDNA made from total RNA isolated from the Caco-2 (ATTC HTB37) cell line and sonicated human placental DNA was added to the cDNA mixture. Hybridization was carried out in Church hybridization solution at 60°C (Church and Gilbert, 1984). The final stringency for washing was 0.2xSSC / 0.1% SDS at 60°C. After two consecutive cycles of hybridization cDNA fragments were eluted from the filters and amplified by PCR. PCR products were cloned into the pBluescript vector (Stratagene), sequenced and subjected to further analysis.

Exon trapping analysis Exon trapping was done as described by Buckler *et al.* (1991). After the secondary amplification step, PCR products of the putative exons were cloned into the pAMP10 vector (Gibco BRL) and plated. Plasmid inserts were amplified by PCR directly from individual colonies using dUSD2 / dUSA4 primers. Insert size was checked by size separation on a 2% agarose gel. Putative exons were sequenced and sequences were screened for repeats using REPEATMASKER (Smith *et al.*, 1996). A non-redundant set of putative exons was mapped back to genomic clones from the candidate region by hybridization. Exon trapping was performed on the hatched clones depicted in figure 1.

EST mapping Based on the localization of ESTs to the radiation hybrid bins of the Human Transcript Mapping Project (http://www.ncbi.nlm.nih.gov/science96/) and the sequence nucleotide polymorphisms (SNPs) map (Wang *et al.* 1998), 16 primer pairs of transcripts that potentially map between markers D7S550 and D7S2423 were identified. These primer pairs were tested by PCR on DNA from a selection of genomic clones covering the region between polymorphic markers CGR2 and D7S2465. Total genomic DNA served as control.

cDNA library screening For each library $1x10^6$ plaques were plated, transferred to nylon filters, and hybridized with the probes of interest using standard procedures (Sambrook *et al.*, 1989). The following cDNA libraries were used: Human fetal brain (Clontech, 5'-stretch library HL1149x), human placenta (Clontech, 5'-stretch library HL1144x), human testis (Clontech, HL1010b).

5'RACE All 5'RACE experiments were done with the human fetal brain Marathon-Ready cDNA kit (Clontech) as recommended by the manufacturer.

Northern blot analysis Multiple-tissue Northern blots were purchased from Clontech. Hybridization in ExpressHyb solution (Clontech) and washes were done as recommended by the manufacturer. The Northern contains RNA from the following tissues: heart, brain, placenta, lung, liver, muscle, kidney, and pancreas. Hybridization probes were prepared by PCR, and the nucleotide sequence was confirmed. The following probes were used: a 558 nucleotide probe from C7orf3(1), a 431 nucleotide probe from C7orf2(3), a 149 nucleotide probe from C7orf4(1). See table 3 for the primer sequences of these PCR products.

Mutation analysis The families used in this study have been described before (Heutink *et al.*, 1994; Zguricas *et al.*, 1999) and are unrelated. Affected individuals do not share a common haplotype across the candidate region. PCR products from either genomic DNA or reverse transcribed (RT) products were amplified (see table 3 for primer sequences), cleaned with the QIAquick PCR purification kit (Qiagen) and sequenced. Mutation analysis for the *HLXB9* and C7orf4 transcripts was performed on genomic

DNA. *HLXB9* primers were designed to amplify all three exons of the *HLXB9* gene including their intron-exon boundaries. Exon 1 has been amplified with 286 bp upstream and 166 bp downstream sequences. Exons 2 and 3 have been amplified including the 598 bp intron that separates them. The amplification of these exons included 58 bp of sequence upstream of exon 2 and 89 bp of sequence downstream of exon 3. Mutation analysis on the *HLXB9* and C7orf4 transcripts was carried out on an affected individual from each of the following four families; Dutch, Cuban A, UK, and Turkish families (Heutink *et al.*, 1994; Zguricas *et al.*, 1999). C7orf4 primer pairs were designed to amplify the known transcript sequence. Control sequence was obtained from non-affected individuals from the above mentioned families and YAC clone HSC7E445. Mutation analysis on the partly known C7orf2 and C7orf3 transcripts was carried out on three affected persons from the Dutch and Cuban A and B families (Heutink *et al.*, 1994; Zguricas *et al.*, 1999). Transcripts were amplified from RT-PCR products made from Epstein Barr Virus transformed lymphocyte culture RNA. Control sequence was obtained from non-affected persons. Primer pairs for the C7orf2 and C7orf3 transcripts were designed to cover all known sequence of the transcripts.

PCR Product	**Forward primer 5'-3'**	**Reverse primer 5'-3'**
HLXB9(1)	cagcccgggctgcttaggac	atttgccaataatcaaagtcgc
HLXB9(2)	agggctgcagcgcaaagaa	ctctgcgcagaggcggctc
HLXB9(3)	aaccaccataacaataccgagc	ctgcaggctgccgctagtc
HLXB9(4)	gagccgcctctgcgcagag	gaagcccggcttgggcag
HLXB9(5)	tgcgcgccgagagcccgt	cagggtgcagccccagcgc
HLXB9(6)	ccaccaccacgcgcatc	gccactggtccagctggaag
HLXB9(7)	acgcgcatccgggcgcagc	cggggtgcgcgccttgca
HLXB9(8)	ggcgctctcctactcgtaccc	ataattcgctgcccgtctcatg
HLXB9(9)	tgtagtggtacaagccagcaacgg	gcaaaggtaacagtgtcccctg
HLXB9(10)	gggacttgagggacagtgac	ggaggccgcggccgaatcc
HLXB9(11)	ctccggggacaggaagc	ctcgtcctccgaggagcagt
HLXB9(12)	agagcaggctcgaccgcc	ccagcagtttgaacgctcg
HLXB9(13)	cttgaaaccgcctctggag	gactccgattttcaccctcagc
HLXB9(14)	gcccaagcgcttcgaggtg	gggacttttctacccgcgcc
HLXB9(15)	ggccgcattcgagaaattttgttc	ttcatccgccggttctggaa
C7orf3(1)	agctggagcgttgcctcgg	cactcaacctgttaagtagacc
C7orf3(2)	attcctcggaggacggtgac	ggagagactgtaccacgtgg
C7orf3(3)	tggtgaggaagttcgatgcc	gacccgtctgctccgcac
C7orf3(4)	tgcagagagctttggtccg	ggcaagttttccaagtcccg
C7orf3(5)	cctggctagcaaattctgtg	ttacagtcaaagggccacc
C7orf3(6)	ccttgcaacgaagtgtctgc	tgcctcagccacctcagtagc
C7orf2(1)	tgctgaggcgacggcggt	aaggcaaagggcatcaatac
C7orf2(2)	ctcagaactactatattcag	acgagaaaggccaactggtgtac
C7orf2(3)	gagttctatctaccctattt	gttccttttggcattgctg
C7orf2(4)	atctcggtcctcttggtg	gccaagttcttcgtagatta
C7orf2(5)	cgtcttctgccaccaagag	tacaagcagttgccattgc
C7orf2(6)	ctttattcagaatgtagaagc	cctataaggtaaaaatccaag
C7orf2(7)	gaagtatcatgtaatcacagt	cagtggacttaaagccaag
C7orf4(1)	cttctatcctttcccaactctcctccac	cgctattaaataccatgcaggaaattggg

Table 3 Primer sequences used in mutation analysis of the candidate genes

RESULTS

Construction of a Genomic Contig

YAC clones HSC7E445, HSC7E158, and HSC7E70 span a large part of the region between markers D7S550 and D7S2423, and have been used as a starting point to construct a detailed genomic contig. In general, two strategies were applied. Firstly, we screened cosmid, PAC and P1 libraries with long range inter-ALU products of these YAC clones. Secondly, we constructed a YAC-specific cosmid library from YAC clones HSC7E445 and HSC7E158 and positioned 67 additional cosmid clones in the map. End sequencing of several clones allowed the development of sequence tagged sites (STSs) (see table 1) that were used to rescreen the P1 library for overlapping clones. The 20 ICRF cosmids, 21 P1 clones, and 13 PAC clones we identified were positioned in the map and form a contig with a total size of approximately 1200 kb. This contig is continuous, except for a gap between the YAC derived cosmid FRS7 and the P1 clone 181C4. Based on the size and overlap of YAC clones HSC7E445 and HSC7E158, the gap is estimated to be less then 100 kb. Clones covering the gap were not present in any of the genomic cosmid, PAC and P1 libraries screened. A schematic overview of the high-resolution genomic contig and the positions of the STSs is presented in figure 1.

Transcript Identification and Characterization

To identify transcripts within the genomic contig we used a combination of cDNA selection, exon trapping analysis and public database searching. Several transcripts were identified by more than one method. Table 4 gives an overview of the identified transcripts, and figure 1 indicates their precise location in the genomic contig.

cDNA Selection

cDNA selection was performed on clones HSC7E158, HSC7E445, and P1317A1 that span almost the entire genomic contig. This yielded 18 unique cDNA fragments that were positioned in the contig and sequenced. By searching publicly available databases with these sequences 9 fragments obtained from P1317A1 were observed to encode fragments of phogrin, a tyrosin phosphatase like protein that is expressed in brain and pancreas (Kawasaki *et al.*, 1996). Five other fragments are part of KIAA0010 (Nomura *et al.*, 1994). This 5160 nucleotide transcript encodes a protein that contains a ubiquitin-ligase domain. Northern analysis of KIAA0010 showed bands of 5.1 kb in all tissues, with an additional band of 3.2 kb in skeletal muscle (data not shown). A single cDNA selection fragment that maps to N2113 showed homology with ubiquitin. Fragment 2AD21 (AF107458) maps to cosmid clone N2113, and

shows homology with several mouse ESTs of unknown function (AA516622, AA516843, and AA522017). Fragments GT725 (AF107459) and GT727 (AF107460), both map to cosmid clone N1042 and do not show homology with any known sequences. Screening of cDNA libraries yielded no clones that would extend these two cDNA selection fragments.

Name	GB ID	Method	Proposed Function	Expression	Reference
Phogrin	U66702	cs	Tyrosin phosphatase like	brain, pancreas	(Kawasaki *et al.*, 1996)
P40	Y11395	db	G protein coupled receptor	Predominantly heart, brain	(Mayer *et al.*, 1998)
Ubiquitin	X63237	cs	Ubiquitin	Not done	
KIAA0010	D13635	cs et db	Ubiquitin ligase	All tissues MTN	(Nomura *et al.*, 1994)
HLXB9	AF107457	db	Homeobox transcription factor	lymphoid tissues, pancreas	(Harrison *et al.*, 1994)
C7orf3	AF107455	et	Unknown function	All tissues MTN	
C7orf2	AF107454	cs et db	Transmembrane receptor	All tissues MTN	
C7orf4	AF107456	db	Unknown function	No signal	
2AD21	AF107458	cs	Unknown function	No signal	
GT725	AF107559	cs	Unknown function	Not done	
GT727	AF107460	cs	Unknown function	Not done	

Table 4 Overview of identified transcripts between markers CGR2 and D7S2465. The methods that have been used for identification of transcripts are cs cDNA selection; et exon trapping; db database searching.

The C7orf2 transcript (AF107454) was identified by a separate direct cDNA selection experiment on genomic clones from chromosome 7 performed by one of the authors (unpublished results). Northern analysis of the C7orf2 transcript showed bands of 1.9 and 4.8 kb in all tested tissues, with highest expression in heart and pancreas (see figure 2). By using 5' RACE and screening of cDNA libraries a sequence of 4849 nucleotides was obtained. When the Northern blot was exposed for 7 days, an additional faint band of 5.2 kb was visible in brain, indicating a possible third transcript, predicting an additional 400 nucleotides of sequence that was not found in clones we isolated from cDNA libraries. The C7orf2 transcript sequence encodes a 492 amino acid open reading frame (ORF), that starts with a methionine residue. The ORF is preceded by a 171 nucleotide 5'-UTR that contains a stop codon positioned three triplets before the start of the ORF. The ORF is followed by a relatively long 3202 nucleotide 3'-UTR, that does not contain any extensive ORFs. The C7orf2 ORF predicts a protein that shows homology to proteins in *Fugu Rubripes* (AF056116), and *Caenorhabditis elegans* (P35535). Both are hypothetical proteins and of unknown function. Because the C7orf2 protein contains nine transmembrane domains, a coiled-coil domain, and is probably located in the plasma membrane (Nakai and Kanehisa, 1992) it might encode a receptor protein.

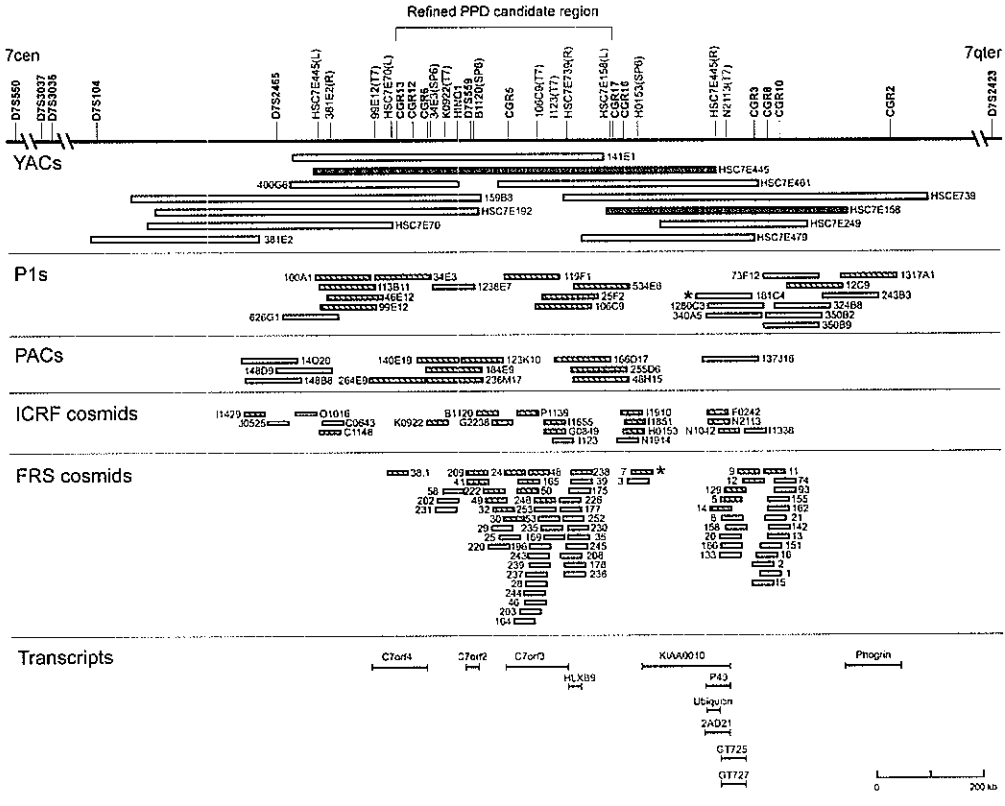

Figure 1 **Schematic representation of the detailed physical map around the PPD candidate region.** The position of STSs and polymorphic markers is indicated at the top line. For markers D7S2423, D7S104, D7S3036, D7S3037, and D7S550 the relative position is indicated. Genomic YAC clones HSC7E445 and HSC7E158, that were used to make the FRS cosmid library, are depicted in black. Clones FRS7 and 181C4 flank a small gap in the contig and have been marked with an asterisk. The hatched P1 - , PAC -, and cosmid clones were used for exon trapping. Additional information on the YAC clones can be found at http://www.genet.sickkids.on.ca/chromosome7/.

Exon Trapping Analysis

Exon trapping of 33 cosmid, 11 P1, and 8 PAC clones, covering the entire genomic contig, yielded 68 unique putative exons. Several exons identified a transcript that included the EST C7orf3. A total of 45 putative exons could not be assigned to any previously identified

transcript, and we were unable to find corresponding cDNA clones in several libraries. The sequences of these putative exons have been submitted to Genbank (AF107407-AF107451).

The C7orf3 transcript (AF107455) is highly expressed in heart and skeletal muscle with two major bands of 1.4 and 2.4 kb, and a number of minor bands up to 7.3 kb in size (see figure 2). After 5'RACE experiments, and screening human fetal brain, fetal limb, testis and placenta cDNA libraries we obtained a sequence of 2754 nucleotides. This sequence codes for a 738 amino acid ORF that is followed by a 540 nucleotide 3'-UTR containing a possible poly-adenylation signal. The ORF starts at the first nucleotide of the known sequence, and is not complete, but we were unable to find additional cDNA clones or ESTs that extend the transcript further 5'. The partial C7orf3 protein shows homology to proteins of unknown function from *Schizosaccharomyces pombe* (Z98601) and *Saccharomyces cerevisiae* (S51341).

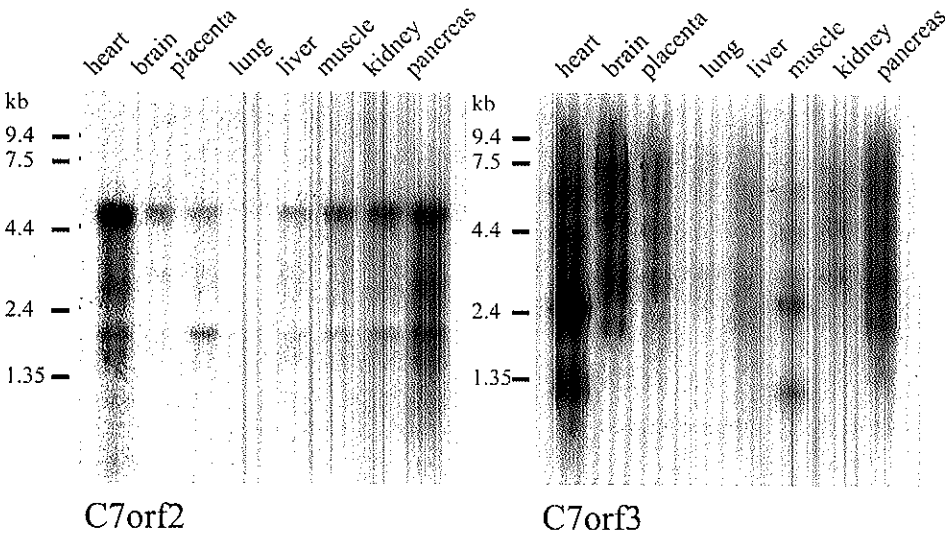

Figure 2 **Northern blot analysis.** (A) C7orf2 transcript after one day of exposure showing 1.9 and 4.8 kb bands. (B) C7orf3 transcript after three days of exposure.

EST Mapping

As a third strategy to identify transcripts we searched several public databases for ESTs that potentially map within the PPD critical region, and mapped three transcripts in the region. A 4544 nucleotide transcript called P40 encodes a 399 amino acid seven-transmembrane domain protein, which is predicted to be a putative G-protein coupled receptor. P40 was initially identified by isolating a 40 kDa integral membrane protein from human erythrocytes. Northern blot analysis of P40 shows a major 4.8 kb transcript that is predominantly expressed in heart and brain (Mayer *et al.*, 1998).

The homeobox motif containing *HLXB9* gene was originally mapped to chromosome 1q41 (Harrison *et al.*, 1994), but was reported in the Human Transcript Map to map to chromosome 7q36 (Deloukas *et al.*, 1998). We could confirm this localization with fluorescence *in situ* hybridization (data not shown). The *HLXB9* gene consists of three exons spread over 6 kb of genomic DNA, and encodes a 2.2 kb transcript that is expressed in lymphoid and pancreatic tissues (Harrison *et al.*, 1994). The homeodomain of the *HLXB9* protein is distantly related to that of the *Drosophila* gene *proboscipedia* (*pb*). The most closely related human homeodomain is that of *HOXB2* (Deguchi and Kehrl, 1991; Harrison *et al.*, 1994).

The STS sTSG8150, that contains the SNP WIAF2185, has been mapped on chromosome 7q36 in-between polymorphic markers D7S550 and D7S2423. sTSG8150 identified a contig of four overlapping cDNA clones in dbEST, with the 630 nucleotide ZI25H08 as the most extended cDNA clone. All four ESTs came from the same Soares fetal liver / spleen cDNA library (NFLS S1), suggesting a tissue-specific expression of the transcript. This transcript has been called C7orf4 and showed no signals on Northern blot, and cDNA library screening and 5'RACE experiments did not result in clones that extended the transcript any further. The known sequence encodes a 108 amino acid ORF, followed by a 304 nucleotide 3'-UTR containing a putative poly-adenylation site. No homologies to other known proteins were identified with the partial C7orf4 protein sequence.

The following ESTs could be excluded from the PPD refined candidate region (see below) by hybridization and PCR: cda0ba09, SGC34464, A002Q20, WI-6320, StSg2621, bda72a07, and a008429.

Refinement of the Candidate Region

During the construction of a detailed physical map we also attempted to reduce the size of the candidate region. Several new polymorphic markers within the candidate region were developed (see table 2) and additional markers became available. The order of polymorphic markers in the region starting from the centromeric side is D7S550- D7S3037- D7S3036 - D7S104- D7S2465- CGR13- CGR12- CGR6- HING1- D7S559- CGR5- CGR17- CGR16- CGR3- CGR8- CGR10- CGR2- D7S2423. To detect possible recombination events between the disease phenotype and these genetic markers we performed haplotype analysis on individuals from Cuban and Dutch PPD families. Previously, we reported recombinations in these families with D7S550 on the centromeric side and with D7S2423 on the telomeric side, an interval of 1.9 cM (Zguricas *et al.*, 1999). By looking for recombination events in these affected persons we were able to further refine the PPD candidate region between CGR17 on the telomeric site and D7S3037 on the centromeric site, an interval of 1 cM that excludes *Sonic Hedgehog* as a candidate gene (see table 5). In addition we identified two recombination events on the centromeric site in non-affected persons with markers D7S104 and CGR13. Because penetrance

of PPD is almost complete (>99%) we have focused our attention to a region of approximately 450 kb in-between markers CGR17 and CGR13.

Markers	Cuba-A Affected	Dutch Affected	Dutch Not Affected	Dutch Not Affected
D7S550	■	○	■	■
D7S3037	.	○	■	■
D7S3036	.	I	■	■
D7S104	I	■	■	■
D7S2465	■	■	■	I
CGR13	■	■	■	○
CGR12	.	■	I	.
CGR6	.	■	○	○
D7S559	■	■	I	○
CGR5	■	■	○	.
CGR17	○	■	○	.
CGR16	○	■	○	.
CGR3	I	I	○	I
CGR10	I	■	○	.
CGR2	○	■	○	○
D7S2423	○	■	○	○

○ Non affected allele ■ Affected allele
I Not informative . Not done

Table 5 Haplotype analysis of individuals in Cuban and Dutch PPD families. The polymorphic markers that were not informative in all four individuals have been left out of this table for reasons of clarity.

Mutation Analysis on Candidate Genes

After reduction of the PPD candidate region four transcripts remained as potential candidate genes: *HLXB9*, C7orf2, C7orf3, and C7orf4. We performed mutation analysis on these transcripts, but no sequence alterations specific for affected individuals were found. Several heterozygous sequence alterations were detected for the *HLXB9* gene and the C7orf2 and C7orf3 transcripts but these alterations were also found in healthy family members and non-related controls and should therefore be regarded as non-pathogenic polymorphisms. None of the

sequence alterations influenced amino acid coding potential of the *HLXB9* gene and the C7orf2 and C7orf3 transcripts.

The *HLXB9* sequence we obtained from human genomic DNA, and the YAC clone HSC7E445, differs somewhat from the published genomic sequence, and the corrected sequence has been submitted to Genbank (AF107452, AF107453, AF107457).

DISCUSSION

Generation of high-resolution genomic maps of the human genome is the first step towards characterization of the genes within a region. We focused our attention on the region between polymorphic markers CGR2 and D7S2465 on chromosome 7q36. This region contains the gene responsible for PPD, and is directly adjacent to that described for HPE3 (Belloni *et al.*, 1996). Using an available contig of YAC clones, a high resolution contig of P1, PAC and cosmid clones was constructed. This contig is continuous except for a gap that could not be covered by any of the genomic cosmid, PAC and P1 libraries used. The gap is bridged by the KIAA0010 transcript, which maps on clones flanking the gap. Since the genomic libraries were constructed from independent DNA sources we assume that this gap contains sequences that are difficult to clone, such as highly repeated, or extremely GC rich sequences.

The assembly of an ordered high-resolution contig of this region has enabled the precise location of several genes. A combination of cDNA selection, exon trapping and database searching was employed to make the transcription map as complete as possible. The 11 independent transcripts that were identified are a tyrosin-phosphatase like protein called phogrin, an ubiquitin ligase KIAA0010, a transmembrane receptor C7orf2, a G-protein coupled receptor P40, the homeobox containing transcription factor *HLXB9*, 5 transcripts of unknown function, and a fragment that shows homology with ubiquitin. These transcripts are candidate genes for congenital malformations such as PPD, CPS, and sacral agenesis that have been mapped within this region.

Using clones from the genomic contig, new polymorphic markers were developed that allowed us to refine recombination events in PPD families. The first polymorphic marker that recombined with the PPD phenotype of an affected individual on the centromeric side is D7S3037. A second recombination event on the centromeric side was identified with polymorphic marker CGR13. This recombination event was found in a non-affected individual and could be a case of non-penetrance of the PPD phenotype. However, we previously reported that penetrance of the PPD phenotype is almost complete (Zguricas *et al.*, 1999), and further detailed clinical examination by one of us with no prior knowledge of the genetic status of this individual showed no abnormalities of hands and feet. Therefore the possibility that this person is a case of non-penetrance is small. In addition, we found a third recombination event on the

centromeric side, also in a non-affected person, with polymorphic marker D7S104 that lies somewhat centromeric from CGR13. The recombination event that defines the PPD candidate region on the telomeric side is with polymorphic marker CGR17 and was identified in an affected individual. In this way the PPD candidate region was reduced to approximately 450 kb between markers CGR13 and CGR17. In the refined candidate region of 450 kb, four genes remained as candidates for PPD.

HLXB9 is the most plausible candidate gene in the region. Homeobox containing transcription factors play an important role in regulatory processes during embryonic development and are shown to be involved in a number of other congenital hand malformations. Examples are HOXD13 in synpolydactyly (Muragaki *et al.*, 1996) and HOXA13 in hand-foot-genital syndrome (Mortlock and Innis, 1997). Recently, it was found that mutations in the *HLXB9* gene are involved in the pathogenesis of sacral agenesis (Ross *et al.*, 1998). The C7orf2 transcript encodes a protein that might function as a receptor located in the plasma membrane. Receptors play an important role in signaling cascades that function during embryonic patterning and are therefore potential candidates for congenital malformations like PPD. The remaining two transcripts in the refined PPD candidate region are C7orf3 and C7orf4. At this moment it is not known what the function of these transcripts is, and whether they are biologically plausible candidate genes. It is also unknown whether any of the candidate genes is expressed during embryonic limb development, something that is expected for the gene responsible for the PPD phenotype.

Although we tested PPD patients for mutations in these four candidate genes, no pathogenic sequence alterations were found. The heterozygous sequence alterations that have been identified in the *HLXB9* gene, and the C7orf2 and C7orf3 transcripts were also found in non-affected individuals and rule out deletions of whole genes. Comparable peak heights at the variable positions in the C7orf2 and C7orf3 transcripts indicated that both forms of the transcripts are expressed at similar levels, ruling out instabilities of these transcripts as a cause of the PPD phenotype. There is still a possibility that candidate genes are mutated in parts of the genes that we were not able to investigate; C7orf4 and C7orf3 transcript sequences are not complete. The transcript size of the full length C7orf4 transcript is unknown and from Northern analysis we estimate that the C7orf3 transcript has an additional 4.5 kb of sequence that has not been investigated. Furthermore, it is possible that the PPD phenotype is caused by mutations at intron-exon boundaries or in regulatory elements, such as promoter regions or enhancer sequences. Mutation analysis for the C7orf2 and C7orf3 transcripts were done on reverse transcribed mRNA. We will not be able to investigate mutations at intron-exon boundaries or in regulatory elements until the genomic organization of the candidate genes is known. Finally, based on the exon trapping results there is a possibility that there are additional genes present in the PPD candidate region.

The high-resolution contig between CGR2 and D7S2465 has been submitted to a large scale-sequencing center where it will be sequenced as a part of the human genome project. The data will help identify possible additional transcripts, complete the sequence of known transcripts, and resolve the complete genomic organization of the candidate genes. Hopefully this will lead to the identification and characterization of the gene responsible for PPD, and encourage the identification of the gene responsible for CPS. Ultimately, we hope that identification of the PPD gene will give us more insight in embryonic limb development, and the underlying processes leading to congenital malformations of the hand.

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CHAPTER 5

The Mouse Mutations *Hammer toe* and *Hemimelic extra toes* interact

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ABSTRACT

Mouse mutants are often used as model systems to study human congenital malformations. Two mouse mutants have been described that can be regarded as animal models for complex polysyndactyly and pre-axial polydactyly linked to human chromosome 7q36. These are the *Hammer toe* mutant, characterized by syndactyly, and the *Hemimelic extra toes* mutant, characterized by pre-axial polydactyly and shortening of the tibia. Both mutations lie very close together and have been mapped to mouse chromosome 5 in a region that is syntenic to human chromosome 7q36. The original *Hemimelic extra toes* mutant shows a wide range of phenotypic variation and it has been suggested that the homozygous condition is embryo-lethal. In this study we show that the *Hemimelic extra toes* mutant phenotype stabilizes in an inbred genetic background and that the homozygous condition is fully viable and fertile. Genetic and phenotypic characterization of double mutant mice, heterozygous for both *Hammer toe* and *Hemimelic extra toes* mutations, provide evidence that the mutations show a genetic interaction. To investigate possible common mechanisms in the pathogenesis of both mutant phenotypes, we examined antero-posterior patterning and apical ectodermal ridge formation during limb development of both mutants.

INTRODUCTION

During the past several years mouse mutants have been described as model systems for human dysmorphic syndromes. Examples are the craniosynostoses syndromes caused by mutations in Fibroblast Growth Factor Receptors (*FGFRs*) (Webster and Donoghue, 1997) and the finding that the *Gli3*, *HoxA13* and *HoxD13* genes are involved in mouse and human limb malformations (Vortkamp *et al.*, 1991; Muragaki *et al.*, 1996; Mortlock and Innis, 1997).

Limb malformations are relatively common, and are mostly associated with syndromes that display other congenital malformations as well. Previously, we have investigated families with an isolated form of pre-axial polydactyly and mapped a locus to human chromosome 7q36 using linkage analysis (Heutink *et al.*, 1994; Zguricas *et al.*, 1994). In the same region a gene responsible for complex polysyndactyly has been localized (Tsukurov *et al.*, 1994). Interestingly, two mouse strains with similar limb phenotypes have been described. The mouse mutant *Hammer toe* (*Hm*) is characterized by syndactyly (Green, 1989), and the mouse mutant *Hemimelic extra toes* (*Hx*) is characterized by pre-axial polydactyly and shortening of the tibia (Green, 1989). Both mutations have been mapped to the same region of chromosome 5, that is syntenic to human 7q36 (Green, 1989; Mouse Genome Database, 1998). The *Hm* and *Hx* mutations lie very close together. One recombination event between the two mutations in 3664 offspring was observed (Sweet, 1982). Because of the striking similarity between the mouse and the human phenotypes and the synteny of the chromosomal localisation, we consider *Hm* and *Hx* likely to be the mouse equivalent of the human mutations linked to chromosome 7q36, and a good model system to study these congenital hand malformations.

The *Hm* mutant (C3HeB/FeJLe-a/a-Ca^{J} *Sl Hm*) shows webbing between digits 2, 3, 4 and 5 on all four paws, while digits 1 and 2 are separated normally. The hind feet are always more severely affected than the forefeet. The *Hm* mutant displays a semi-dominant phenotype. In the hind feet of homozygous *Hm* mutant mice there is complete webbing between digits 2, 3, 4 and 5 extending to the top of the distal phalanges. The forefeet show incomplete webbing that is gradually decreasing towards the pre-axial side. In heterozygous *Hm* mutant mice the forefeet have a similar phenotype as the homozygous mutant mice, but the hind feet show only webbing between digit 2, 3 and 4, extending to the base of the distal phalanges, while the webbing between digit 4 and 5 is complete (Green, 1989).

The *Hx* mutation has arisen in outbred strain B10.D2/oSn. Heterozygous mutant mice have pre-axial polydactyly on all four paws, and the hind limbs are always more severely affected than the fore limbs. The *Hx* phenotype shows a variable expression that might be caused by the outbred genetic background. The phenotype typically includes shortening of the radius, tibia, and talus with extra pre-axial metacarpals, metatarsals, and digits. The fibula and ulna are normal in size but often bowed. The humerus, femur, and limb girdles are normal and no other

skeletal defects are present (Green, 1989). It has been suggested that the homozygous *Hx* condition is embryo-lethal at an early stage of development (Knudsen and Kochhar, 1981).

The vertebrate limb develops from a group of undifferentiated cells into a structure that differentiates along three body axes: the proximo-distal, the antero-posterior and the dorso-ventral axis. The pattern formation along these axes is controlled by so called 'signaling centers'. The proximo-distal axis is regulated by signals from the apical ectodermal ridge (AER), an epithelial structure overlying the mesoderm that secretes *Fibroblast growth factors* (*Fgf*). These molecules promote proliferation of the underlying mesoderm. *In vivo* removal of the AER leads to truncation of the limb, and the extent of truncation depends on the time of AER removal. Truncation can be rescued by application of beads soaked in *Fgf* (Niswander *et al.*, 1993; Fallon *et al.*, 1994). The zone of polarizing activity (ZPA) is a group of posterior mesodermal cells controlling pattern formation along the antero-posterior axis. Grafting of ZPA cells, as well as application of *Sonic Hedgehog* (*Shh*) protein, to the anterior side of a chick limb induces additional digits in a mirror-image sequence along the antero-posterior axis (Saunders and Gasseling, 1968; Tickle *et al.*, 1975; Tickle, 1981). Signaling centers such as the AER and ZPA need to be precisely located in space and time to function properly.

The first morphological abnormalities in the *Hx* mutant can be detected at 11.5 days after conception (dpc) when a mesenchymal protrusion has been formed at the anterior side of the developing limb bud. At the same time ectopic *Shh* expression is seen in the anterior mesenchyme of the developing limb bud (Masuya *et al.*, 1995). Similar to the *Hx* mutant, there are other mouse mutants with pre-axial polydactyly that show ectopic *Shh* expression. These mutants include *Extra toes* (*Xt*), *Strong's luxoid* (*lst*), *luxate*, *X-linked polydactyly* and *Rim4* (Chan *et al.*, 1995; Masuya *et al.*, 1995; Masuya *et al.*, 1997; Büscher and Rüther, 1988). While the molecular defect in most polydactylous mutants is unknown they have been elucidated for the *Lst* and *Xt* mutant. The *Lst* phenotype is due to loss of function of the *Alx4* gene that codes for a paired-type homeodomain protein (Qu *et al.*, 1998). The *Xt* mutant phenotype is caused by an inactivation of the *Gli3* zinc-finger DNA binding protein (Schimmang *et al.*, 1992; Vortkamp *et al.*, 1992; Hui and Joyner, 1993). Both genes are expressed in the anterior mesenchyme of the developing limb bud and loss of function of the protein causes ectopic ZPA formation.

In this study we obtained a stable *Hx* phenotype after breeding the *Hx* mutation into an inbred background. In this inbred background, homozygous *Hx* mutant mice are fully viable and fertile and show a more severe phenotype than heterozygous mutant mice. We then generated a *Hm / wt ; Hx / wt* double mutant. The phenotype of this compound heterozygote mutant provides evidence that the *Hm* and *Hx* mutations show a genetic interaction with each other. In order to investigate whether a common mechanism in the pathogenesis of the *Hm* and *Hx* mutant phenotypes exists, we investigated antero-posterior patterning and AER formation in both mutants.

EXPERIMENTAL PROCEDURES

Polymorphic marker testing Offspring from crosses *Hx / wt* x *wt / wt* and *Hx / wt* x *Hx / wt* was tailed at weaning. PCR amplification on tail DNA and D5mit387 primer design were done as recommended (Mouse Genome Database, 1998).

Skeletal preparations Mice were killed, skinned and eviscerated. Skeletons were fixed in 100% ethanol for 1 day followed by staining overnight with alcian blue and alizarin red (150 mg/l alcian blue, 50 mg/l alizarin red in 80% ethanol / 20% acetic acid). Skeletons were cleared in 3% KOH and stored in glycerol.

Embryos Mice were caged together and checked for a vaginal plug the following day (plug = day 0). After cervical dislocation the embryos were collected, and staged after removal of extra-embryonic membranes. Unless stated differently, histology and whole mount *in situ* experiments were done on homozygous *Hm* embryos and heterozygous *Hx* embryos.

Whole-mount in situ hybridization Whole-mount *in situ* hybridization experiments for *Shh*, *Fgf4*, *Fgf8*, *HoxD11*, and *HoxD13* (Dolle *et al.*, 1991; Izpisua-Belmonte *et al.*, 1991; Niswander and Martin, 1992; Echelhard *et al.*, 1993; Crossley and Martin, 1995) were performed essentially as described (Sasaki and Hogan, 1993). Probes were digoxigenin labeled and visualization was by alkaline phosphatase reaction.

Histology Morphology and programmed cell death were examined. Embryos were cultured in HEPES medium at 37°C. Viable embryos were injected into a heart ventricle with approximately 3 l Annexin V biotin (APOPTEST-BIOTIN, product B500, NeXins Research B.V., Hoeven, The Netherlands). Annexin V binds to the phospholipid phosphatidylserine (Heerde *et al.*, 1995). Phosphatidylserine is exposed on the outside of the cell membrane of apoptotic cells early in the process of apoptosis, whereas it resides on the cytoplasmic side of the plasma membrane of viable cells (Diaz and Schroit, 1996). In this way apoptotic cells are detected before any nuclear changes (van den Eijnde *et al.*, 1997a; Fadok *et al.*, 1992). After 30 minutes of incubation at 37°C, embryos were fixed and processed for light microscopy as described (van den Eijnde *et al.*, 1997b).

RESULTS

Stabilization of *Hx* phenotype

To see whether the variable phenotype of the heterozygous *Hx* mutant stabilizes in a constant genetic background, and to test whether the *Hm* and *Hx* phenotypes are variant expressions of the same genetic defect, we crossed the *Hx* mutation into the C3HeB/FeJLe-a/a-Ca^J genetic background, the same background as the *Hm* mutant. After ten generations the phenotype was stable and all mice show pre-axial polydactyly and normal tibia. This finding indicates that the variation in the phenotype of the original *Hx* mutant must be caused by the presence of genetic modifiers elsewhere on the genome that influence the expression of the *Hx* mutant phenotype. Because the *Hx* phenotype is still present in the *Hm* background, the *Hm* and

Hx phenotypes are not the result of variant expression of a single mutation caused by differences in genetic background, but represent two distinct mutations.

We tested whether homozygous *Hx* mice are viable in the C3HeB/FeJLe-a/a-Ca^J genetic background. We used polymorphic marker D5mit387 to distinguish the mutant *Hx* from wildtype (*wt*) alleles. This marker lies in the proximity of the *Hx* / *Hm* locus (Mouse Genome Database, 1998) and in a *Hx* mutant backcross we observed 3 recombinations in 161 offspring. This correlates with a genetic distance between D5mit387 and *Hx* of 1.8 cM.

Genotype	**Number of *wt* offspring**	**Number of affected offspring**
1/1	25	0
1/2	1	40
2/2	0	28

Table 1 D5mit387 Genotype Phenotype Correlation in Offspring from Double Heterozygous *Hx* Mutant Cross

Offspring from heterozygous *Hx* crosses was tested for the presence of homozygosity for the D5mit387 allele associated with the *Hx* mutation. In 93 offspring we found 28 mice homozygous for this allele (see table 1). This finding is in agreement with Mendelian inheritance, providing that the homozygous *Hx* condition is viable ($P<0.05$). As was expected, the entire offspring from a backcross of homozygous *Hx* mutants with *wt* mates showed the *Hx* mutant phenotype. No significant differences in litter size and embryo resorption rates were found (see table 2).

Genotype	**Litters**	**Embryos**	**Resorbed**	**Frequency**
wt / *wt*	39	343	38	11.1%
Hm / *Hm*	49	416	39	9.4%
Hx / *wt*	42	378	42	11.1%

Table 2 Embryo Resorption in *Wt*, *Hm* and *Hx* Mutant Mice

Homozygous *Hx* mutant mice could phenotypically be distinguished from heterozygous *Hx* mutant mice. All four paws showed polydactyly with 6 to 8 digits, but unlike the heterozygous *Hx* mutant mice, the bones of the extra pre-axial digits tended to be fused. All homozygous *Hx* mutant mice showed shortening of the tibia, which was not seen in the heterozygous *Hx* mutant. In a few cases shortening of the radius was observed. Bone staining of

neonates and adult animals revealed no other skeletal defects. Crossing of homozygous *Hx* mice yielded fully viable and fertile offspring, that was born with normal litter sizes (see table 3).

Parent 1	Parent 2	Number of litters	Number of newborn	Mean Litter-size
wt / wt	*wt / wt*	19	140	7.4
Hm / Hm	*Hm / Hm*	26	181	7.0
Hx / wt	*wt / wt*	23	170	7.4
Hx / wt	*Hx / wt*	14	99	7.1
Hx / Hx	*wt / wt*	11	76	6.9
Hx / Hx	*Hx / Hx*	6	44	7.3

Table 3 Litter-size in *Wt*, *Hm* and *Hx* Mutant Mice

Hx and *Hm* mutations show a genetic interaction

Availability of homozygous *Hx* mutants, in the same genetic background as the *Hm* mutant, made it possible to test whether the *Hx* and *Hm* mutant mice show a genetic interaction. Because the *Hx* and *Hm* mutations lie very close together, the offspring from a homozygous *Hx* with homozygous *Hm* cross will carry one copy of both the *Hx* and *Hm* mutations. If the *Hm* and *Hx* phenotypes are caused by two independent mutations, the double mutant is expected to show an additive combination of both heterozygous phenotypes. We carefully examined 16 offspring from three independent double mutant crossings.

The *Hm / wt ; Hx / wt* double mutant mice showed pre-axial polydactyly with normal tibia, combined with webbing between all hind feet digits that extended to the top of the distal phalanges (Fig. 1). The polydactyly phenotype of the compound heterozygote mutant was indeed what was expected from the presence of a single mutant *Hx* allele, but the syndactyly phenotype was more severe than was expected from the presence of a single mutant *Hm* allele. The phenotype seemed to be a combination of a heterozygous *Hx* phenotype and a homozygous *Hm* phenotype. This clearly shows that the *Hm* and *Hx* mutations show a genetic interaction. Because a common mechanism in the pathogenesis of both mutants might exist, we examined developmental mechanisms that could explain both the *Hm* and *Hx* mutant phenotypes.

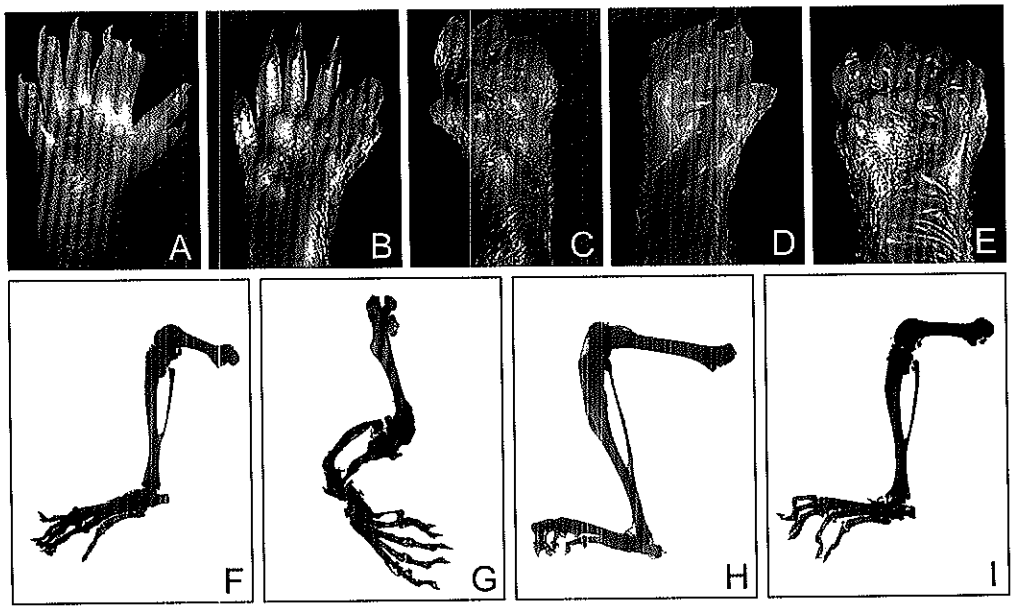

Figure 1 **Macroscopy of ventral hind limbs** (A-E) and skeletal preparations of hind limbs (D-I). *Hx / wt* (A, F) show pre-axial polydactyly with normal tibia; *Hx / Hx* (B, G) show pre-axial polydactyly with shortening of the tibia; *Hm / wt* (C) show partial webbing; *Hm / Hm* (D, H) show complete webbing with normal tibia.; *Hm / wt* ; *Hx / wt* (E, I) show pre-axial polydactyly, complete webbing and normal tibia.

Common mechanisms in the pathogenesis of *Hm* and *Hx* phenotypes

The *Hm* and *Hx* mutations could disturb antero-posterior patterning. Ectopic *Shh* expression in the anterior mesenchyme of 11.5 days post coitum (dpc) *Hx* mutant embryos was reported before (Masuya *et al.*, 1995). This suggests that disturbances in antero-posterior patterning and the formation of an ectopic ZPA are the events that lead to the *Hx* mutant phenotype. The *Hm* mutant phenotype could be explained by a disturbance in antero-posterior patterning that affects interdigital cell fates and leads to a lack of interdigital cell death (Zakeri and Ahuja, 1994a; Zakeri *et al.*, 1994b ; Ahuja *et al.*, 1997; Zakeri and Ahuja, 1997).

We used *Shh* and *Fgf4* as markers for ZPA activity. In *wt* embryos there is polarized expression of *Shh* in the posterior mesenchyme and *Fgf4* in the posterior two-third of the AER. Because of these non-overlapping expression patterns we tested both genes in a single whole-mount *in situ* hybridization. Expression patterns were investigated in *Hm* and *Hx* mutant embryos from 9 to 13 dpc and the results were compared to those in *wt* embryos.

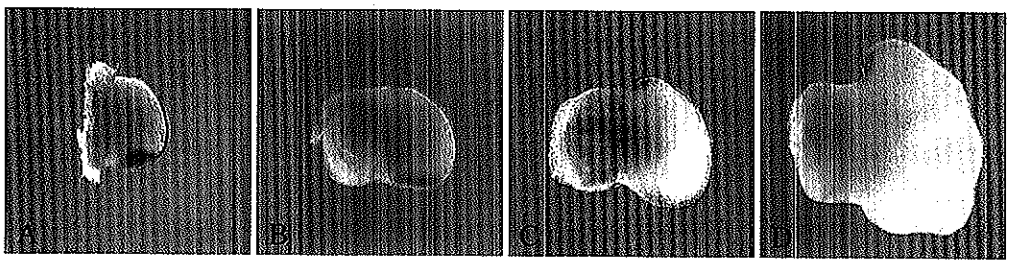

Figure 2 Whole mount *in situ* hybridization of ventral hind limbs of *Hx / wt* embryos with *Fgf4* and *Shh*. Anterior side facing upwards. (A) 10 dpc with normal posteriorly restricted expression of both genes, (B) 10.5 dpc with ectopic *Fgf4* expression in the anterior AER, (C) 11.5 dpc with ectopic expression of both *Fgf4* and *Shh* on the anterior side of the limb bud, (D) 12.5 dpc with continued ectopic expression of *Fgf4* and *Shh*. At this stage posterior expression has already faded away.

The first abnormality in *Hx* mutant limb buds was seen at 10.5 dpc when *Fgf4* is ectopically expressed in the most anterior part of the AER (Fig. 2B). One day later *Shh* was ectopically expressed in the anterior mesenchyme directly underneath the ectopic *Fgf4* expression domain (Fig. 2C). This ectopic *Fgf4 / Shh* expression continued until 13 dpc, long after normal expression on the posterior side faded away (Fig. 2D). No abnormalities in *Shh / Fgf4* expression were observed in *Hm* mutant embryos. Because the chick mutant *limbless* shows polarized *HoxD* expression in the limb mesenchyme without the presence of *Shh* expression, it has been suggested that *HoxD* genes play a role in antero-posterior patterning (Noramly *et al.*, 1996; Ros *et al.*, 1996). We therefore compared *HoxD11* and *HoxD13* expression in 9 to 11 dpc *Hm* and *Hx* mutant embryos to *wt* embryos and found normal posteriorly restricted expression patterns. We did however observe strong anterior *HoxD11* and *HoxD13* expression in *Hx* mutant limb buds after 11.5 dpc, another indication that in the *Hx* mutant polydactyly is associated with disturbances in antero-posterior patterning and ectopic ZPA formation (data not shown). These results clearly indicate a clear antero-posterior disturbance in *Hx* mutant limb buds, which was not observed in *Hm* mutant embryos.

In *Hx* mutant embryos, *Fgf4* expression in the anterior AER is the first observed defect. The ZPA and AER are interdependent (Niswander *et al.*, 1994), so ZPA duplication in the *Hx* mutant could be a secondary effect of a primary AER disturbance. If this is the case, the mutations causing the *Hm* and *Hx* phenotypes could disturb the initiation, positioning or maintenance of the AER.

A way to explain the *Hm* mutant phenotype with this model is that abnormal initiation or maintenance of the AER could lead to aberrant signaling from the AER to the underlying mesenchyme, and result in lack of interdigital cell death. We used *Fgf8* as a marker to study if the AER is initiated and maintained normally in *Hm* and *Hx* mutant embryos from 9 to 11.5 dpc. In both mutants we observed normal *Fgf8* expression in the ectoderm overlying the future limb

at 9 dpc, the ventral ectoderm of pre-AER limb buds at 9.5 dpc and in the established AER at 10 dpc. In agreement with the normal *Fgf8* expression, histological sections of 10.5 dpc *Hm* and *Hx* embryonic limbs showed a normally sized AER containing apoptotic cells. After ectopic ZPA formation, *Hx* mutant embryos showed high *Fgf8* expression in the pre-axial AER and histological sections showed a thick pre-axial AER containing few apoptotic cells. This was not observed in *wt* or *Hm* embryos, and is likely to be a secondary effect of ectopic ZPA activity. In 13 dpc *Hm* mutant embryos, at the stage where reduced interdigital cell death was observed, there was normal morphology and cell death of the AER overlying the interdigital mesenchyme (data not shown). This suggests normal AER initiation, positioning and maintenance in both mutants, with AER abnormalities in the *Hx* mutant only on the anterior side, after the ZPA duplication has taken place.

DISCUSSION

The mutant *Hx* phenotype in a stable genetic background

The original *Hx* mutant arose in a non-inbred strain, and shows a wide range of phenotypic variation. By breeding the *Hx* mutation into the stable C3HeB/FeJLe-a/a-Ca^{J} background we showed that the phenotype stabilizes. Therefore the phenotypic variation in the original strain must be caused by additional factors elsewhere on the genome. Identification of genetic modifiers in the original *Hx* mutant outbred strain, by linkage analysis and positional cloning, may reveal genes in similar or parallel pathways to the *Hm* / *Hx* gene. Obvious candidates for genetic modifiers are genes known to cause polydactyly in other mutants.

Our data showed that the homozygous *Hx* condition was fully viable and fertile in a stable genetic background. Furthermore we found no evidence for increased embryolethality, or reduced litter sizes at weaning as has been suggested in earlier studies (Knudsen and Kochhar, 1981).

There are two possible explanations for the phenotypic differences between heterozygous and homozygous *Hx* mutant mice. Firstly, increased penetration of the *Hx* phenotype in the homozygous *Hx* mutant mice could result in an ectopic ZPA that is established at an earlier developmental age, and therefore affects more proximal skeletal elements. Secondly, in homozygous *Hx* mutant embryos the ectopic ZPA may be spread more widely. The range of ectopic ZPA activity could be extended to include the presumptive tibial chondroblast region. Normal separation of tibial and fibular pre-cartilage rudiments is established by a region of programmed cell death within the zeugopodal condensation in the limb bud, the so-called 'opaque patch'. Ectopic ZPA signaling could set the positional value of the presumptive tibial chondroblasts to that of the opaque patch. Interestingly, extension of the opaque patch to the

anterior side has been observed in combination with tibial reduction in the original outbred *Hx* mutant strain (Knudsen and Kochhar, 1981).

Hm and *Hx* mutations show a genetic interaction

Because the phenotype of the *Hx* mutant is still present in the genetic background of the *Hm* mutant, it is clear that *Hm* and *Hx* do not represent a single mutation and their differences are not caused by the genetic background. The compound heterozygote mutant, carrying one copy of both mutations, showed a heterozygous *Hx* - combined with a homozygous *Hm* mutant phenotype. An explanation for this phenotype, could be that *Hm* and *Hx* represent two mutations in a single gene. It is tempting to think that the *Hx* mutation would result in a loss-of-function and the *Hm* mutation in a gain-of-function of the protein. Based on a recombination event between *Hm* and *Hx* Sweet *et al.* (1982) concluded that the mutations are not allelic. However, because of the extremely low recombination frequency there is still a possibility that the observed recombination is intragenic. Alternatively, the *Hm* and *Hx* phenotypes may be caused by mutations in two adjoining genes that act in the same genetic pathway, or by mutations in a shared regulatory element. The *Shh* gene is positioned on mouse chromosome 5, close to the *Hm* and *Hx* mutations, but is not allelic with the mutations (Marigo *et al.*, 1995). The same situation applies to the *SHH* gene and the pre-axial polydactyly / complex polysyndactyly locus on human chromosome 7q36 (Zguricas *et al.*, in press). Because of the changes in *Shh* expression pattern, and the short distance between the *Shh* gene and the *Hm* / *Hx* mutations, Masuya *et al.* (1995) suggested that the *Hx* phenotype might be caused by a long range cis-acting effect on the expression of the *Shh* gene. Although this option cannot be excluded until the mutations underlying the *Hm* and *Hx* phenotypes have been elucidated, we think our findings make this hypothesis less likely. We have shown that ectopic *Shh* expression in the *Hx* mutant is preceded by *Fgf4* expression in the anterior AER, so it seems that ectopic *Shh* expression is a secondary effect. If we assume that *Hm* and *Hx* are allelic, one would also expect a change in the *Shh* expression pattern of the *Hm* mutant, but this was not observed.

Common mechanisms in the pathogenesis of *Hm* and *Hx* phenotypes

If the *Hm* and *Hx* mutations show a genetic interaction there is the possibility of a common mechanism in the pathogenesis of both mutants. A clear disturbance in antero-posterior pattern formation was present in the *Hx* mutant, starting with abnormal *Fgf4* expression in the anterior AER, followed by abnormal *Shh*, *HoxD11* and *HoxD13* expression in the anterior mesenchyme. The different signaling centers in the limbs are interdependent and can mutually reinforce each other (Niswander *et al.*, 1994). An initial problem in the AER, like the abnormal anterior *Fgf4* expression, could stimulate the anterior mesenchyme to express *Shh*, and establish

a reinforcing loop between *Shh* and *Fgf4* expression that leads to ZPA duplication. We found normal *Shh* and *Fgf4* expression in *Hm* mutant limb buds, making it unlikely that disturbance of antero-posterior patterning is a common mechanism in the pathogenesis of both phenotypes.

In early limb development, normal AER initiation and positioning was found in both mutants using *Fgf8* expression as a marker, and no changes in morphology of the AER were detected. After ectopic ZPA formation in *Hx* mutant embryos, the anterior AER was thickened and highly expressed high levels of *Fgf8*. However, in *Hm* mutant embryos the morphology of the AER was normal through all stages, as was *Fgf8* expression. This indicates that initiation or positioning of the AER is not a common mechanism in the pathogenesis of both phenotypes. Aberrant signaling between the AER and the underlying mesenchyme may go undetected by just looking at *Fgf8* expression. Therefore, miscommunication between AER and mesenchyme remains a possible mechanism in the pathogenesis of both phenotypes.

In the *Hx* mutant *Fgf4* expression in the anterior AER is detected at 10.5 dpc, one day before anterior *Shh* in the mesenchyme. Interestingly, in the polydactylous *Alx4* null mutant ectopic expression of *Shh* is also preceded by ectopic *Fgf4* expression in the anterior AER (Qu *et al.*, 1997). At this moment it is not clear what the function of the *Alx4* gene is, or how loss of function of the *Alx4* protein causes *Fgf4* expression in the anterior AER. We examined *Alx4* expression in heterozygous and homozygous *Hx* mutant limb buds from 9 to 11 dpc and observed normal expression in the anterior mesenchyme (unpublished results). This indicates that the *Hx* mutation does not influence *Alx4* expression and that *Hx* and *Alx4* most likely act in a separate biochemical pathway.

Mutations and deletions of the different functional domains in the human *GLI3* protein cause a range of congenital malformations that include distinct combinations of polydactyly and syndactyly (Vortkamp *et al.*, 1991; Kang *et al.*, 1997; Radhakrishna *et al.*, 1997). It has been reported that after ectopic ZPA formation *Gli3* expression profiles in the *Hx* mutant embryos are changed (Büscher and Rüther, 1988). Whether this is an effect of a direct or indirect interaction of the *Hm / Hx* gene with *Gli3* remains to be determined. But, in analogy with the *GLI3* gene, we suggest that mutations or deletions of different domains in a single gene could be responsible for the differences between the *Hm* and *Hx* mutant mice and the human PPD and CPS phenotypes. Miscommunication between AER and mesenchyme could be the common mechanism in the pathogenesis of both phenotypes.

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CHAPTER 6

The *transgene induced phalange synostosis* mutant: a new mouse model for syndactyly

INTRODUCTION

Isolated syndactyly is a common congenital hand malformation affecting hands, feet, or both. Several attempts have been made to classify the different forms of syndactyly, but none of them could account for all forms of syndactyly that have been reported. Tentamy and McKusick (1978) specified five different forms of isolated syndactyly that are all inherited in an autosomal dominant fashion showing complete penetrance with variable expression.

- Type I is the most common form of syndactyly, characterized by complete or partial webbing between the third and fourth fingers, or the second and third toes, or both.
- Type II is also called synpolydactyly and is characterized by various degrees of duplication of fingers along with syndactyly of the third and fourth fingers, and fourth and fifth toes. This form of synpolydactyly can be caused by mutations in the *HOXD13* gene on chromosome 2q31 (Akarsu *et al.*, 1996; Muragaki *et al.*, 1996).
- Type III shows complete or partial webbing between digits four and five.
- Type IV is characterized by complete syndactyly of all fingers.
- Type V is associated with metacarpal and metatarsal synostosis, and normally affects the fourth and fifth fingers and toes, with an occasional involvement of the third and fourth fingers and toes.

Over the years, mice have become a powerful model system for studying embryonic limb development. Because the majority of developmental processes have remained largely unchanged during evolution in species such as mice and man, it is possible to extrapolate experimental insights from the mouse to the human situation. Several mouse mutant limb phenotypes with syndactyly have been described that can be used to study the genes and developmental processes involved in the pathogenesis of syndactyly (MGD, 1998).

Here we describe a new mouse model for syndactyly that was found in an independent line of mice transgenic for the human rhodopsin gene. The main characteristic of the mutant phenotype is a synostosis of the phalanges in the third and fourth digits of the hind limbs, therefore it is named the *transgene induced phalange synostosis* (*tipsy*) mutant.

The mouse mutant phenotype does not fit the classifications for human syndactyly that have been reported so far, but recently a human syndactylous phenotype was reported that shows a remarkable similarity to that of the mouse mutant. One of the characteristic findings in this human phenotype is a phalange synostosis in the third and fourth fingers (Percin *et al.*, 1998).

The transgene integration in this mutant provides an excellent starting point to identify the responsible gene. We localized the position of the transgene integration in the mouse genome, and characterized the integration site. This will lead to the identification of the responsible gene and provide more insight in the pathogenesis of this form of syndactyly.

MATERIAL AND METHODS

Skeletal preparations Mice were killed, skinned and eviscerated. Skeletons were fixed in 100% ethanol for 1 day followed by staining overnight with alcian blue and alizarin red (150 mg/l alcian blue, 50 mg/l alizarin red in 80% ethanol / 20% acetic acid). Skeletons were cleared in 3% KOH and stored in glycerol.

Fluorescent in situ hybridization Preparation of metaphase chromosomes and fluorescent *in situ* hybridization was done as has been previously described by Mulder *et al.* (1995).

Construction of a genomic library Mouse genomic DNA was partially digested with the Sau3A1 enzyme. The presence of restriction fragments sized between 11 and 23 kb was checked on a 0.4% agarose gel by electrophoresis. The complete restriction mixture was ligated in Lambdagem-11 phage vector (Promega, catalog number B1880) and packaged into phage particles using GigaPack Gold and GigaPack XL (Clontech) packaging extracts. Packaged phages were used to infect *E.coli* cells (strain KW251, Promega).

Genomic library screening For each library $1x10^6$ plaques were plated, transferred to nylon filters, and hybridized with a 263 nucleotide PCR product located in the first exon of the human rhodopsin gene using standard procedures (Sambrook *et al.*, 1989). The PCR product of the human rhodopsin gene (depicted black in figure 2) was amplified with the following primers forward (5'-3') ggcacagaaggccctaact and reverse gctggtgaagccacctag.

Mapping of genomic phage clones Phage clones were digested with SalI restriction enzyme. The resulting DNA fragments were separated by electrophoresis on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with individual SalI restriction fragments using standard procedures (Sambrook *et al.*, 1989).

Sequencing All sequencing was done on a ABI-377 automated fluorescence dye sequencer using Bigdye chemistry (Perkin Elmer) or Dye terminators (Amersham).

RESULTS

Phenotypic Description of the *transgene induced phalange synostosis* Mutant

Both heterozygous and homozygous *tipsy* mutants are viable and fertile. Heterozygous mutants show no abnormalities, indicating that the phenotype segregates as a recessive trait. The homozygous mutant phenotype is characterized by a synostosis of the phalanges in digits two and three of the hind limbs (see figure 1a). Complete fusion can occur between all three phalanges in the digits, but sometimes the distal ends of the most proximal phalanges are only partially fused. The mutants show a normal separation of the metatarsal bones in the hind limbs and a normal pattern of digits and metacarpals in the forelimbs.

In addition to syndactyly, several other bone anomalies were observed. In the hind limbs there is an amorphous bone attached to the calcaneum, that faces upwards alongside the heel (see

figure 1b). The long bones of the fore – and hind limbs, the scapula - and pelvic girdle bones are normal in overall size and shape, but appear very thin and fragile. The sacral vertebrae are abnormal in shape and the distal ends of the sacral ribs are fused. Occasionally, a twist at the end of the tail is observed. No other malformations of the axial skeleton, or the skull, could be detected.

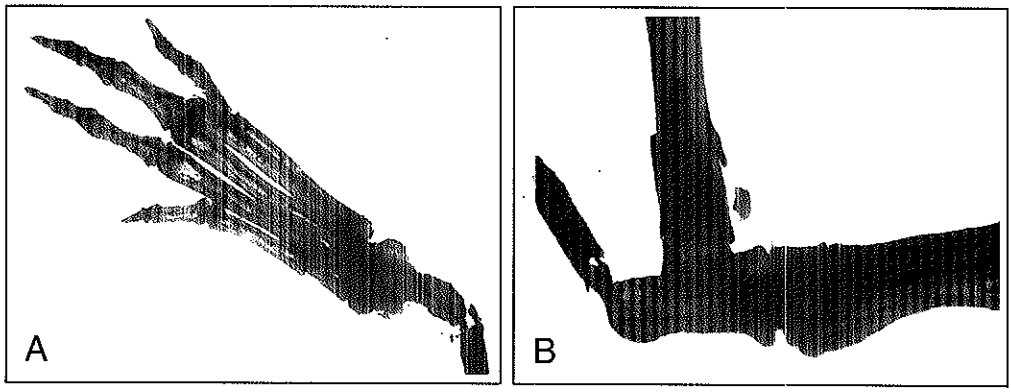

Figure 1 **Bone staining from the hind limb of a homozygous *tipsy* mutant mouse.** a) Dorsal view of the right hind limbs that shows complete phalange synostosis of digits two and three. Notice the normal appearance of the metatarsals. b) Lateral view of the shapeless bone attached to the calcaneum.

Characterization of the Transgene Integration Site

The syndactyly phenotype was observed in an independent line of mice transgenic for the human rhodopsin gene. The construct that was used to make the transgenic mice contains 13.3 kb of genomic DNA, that covers the entire transcriptional unit of the human rhodopsin gene including 3.4 kb upstream - and 4.8 kb downstream sequence (Li *et al.*, 1996). The transgene itself, has no role in limb development, but must have integrated in the mouse genome in or near a gene that is involved in limb development. As a result of this, the function of this gene has been disrupted or altered. If the transgene integration disrupts a gene that is needed for normal separation of the phalanges, then the *tipsy* mutant can be used to identify the interrupted gene.

To characterize the transgene integration site, a genomic phage library of homozygous mutant DNA was constructed, and screened with a 263 nucleotide probe that is located in the first exon of the human rhodopsin gene (see figure 2). Rhodopsin-positive phage clones were grown and sequenced from the 5' – and 3' sides. The orientation of phage clones was determined by aligning these end sequences to the sequence of the human rhodopsin gene (Genbank accession number AC000380).

Three out of ten rhodopsin-positive phage clones that have been sequenced contain part of the rhodopsin transgene and flanking mouse DNA located 5' of the transgene insertion (see figure 2). The order of the phage clones has been determined by comparison of SalI restriction sites and hybridization of individual SalI restriction fragments to the phage clones. A probe positioned at the other side of the human rhodopsin gene will be used to identify phage clones that contain mouse DNA located 3' of the transgene insertion.

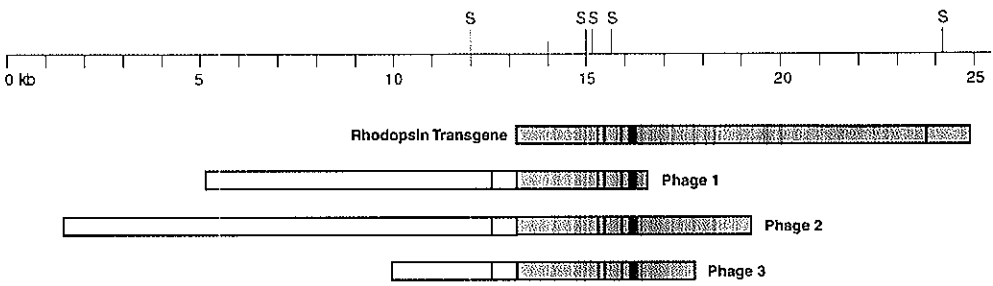

Figure 2 Schematic representation of the 5' part of the transgene integration site in the *tipsy* mouse mutant. The Gray box represents the 13.3 kb human rhodopsin gene. White boxes represent flanking mouse genomic DNA. The Black box represents the PCR product that was used to identify the phage clones. Vertical lines indicate SacI restriction sites that were used to order the DNA fragments (designated as S).

Sequence analysis of the 3 kb SalI restriction fragment on the 5' side of the rhodopsin transgene indicates that the integration has taken place in non-coding sequence. This indicates that the transgene integration does not disrupt an exon of a gene.

Chromosomal Localization of the transgene integration

In order to determine the chromosomal location of the transgene integration we performed fluorescent *in situ* hybridization (FISH) on mouse metaphase chromosomes, using the human rhodopsin transgene as a probe. While no signal was observed in *wildtype* mouse chromosomes, there was a clear fluorescent signal observed on mouse chromosome 18qE1 when mutant chromosomes were examined (see figure 3).

The localization of the transgene integration site was confirmed by co-hybridization with the 92/A probe, located at 18qE1 as well (personal communication R. Delwel and N. G. Copeland). Two other syndactylous mutants, the *shaker-with-syndactylism* (*sy*) - and *fused phalanges* (*fp*) mutant, have also been mapped to this chromosomal region (MGD, 1998). The similar map position and the overlap in phenotypes of these three mouse mutants, suggest that these phenotypes may be allelic.

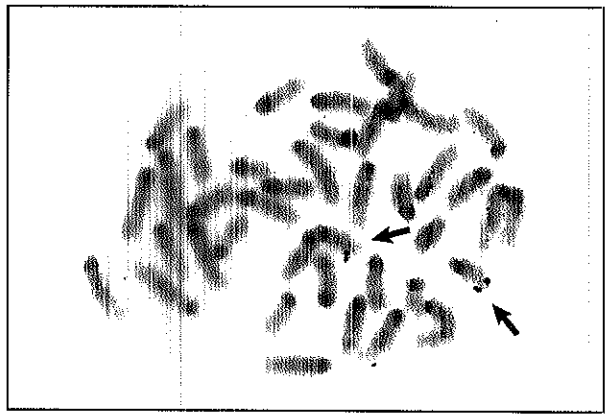

Figure 3 Color-inverted photograph of fluorescent *in situ* hybridization on homozygous *tipsy* mutant metaphase chromosomes with the human rhodopsin transgene. Dots indicated by the black arrows are the fluorescent hybridization signal on mouse chromosome 18qE1.

DISCUSSION

We have described a new mouse model for a recessive form of syndactyly that is characterized by synostosis of the phalanges in the second and third digits. This mutant was observed in an independent line of mice transgenic for the human rhodopsin gene.

The integration of a transgene in genomic DNA can have several effects on the function of genes in close proximity to the integration site. If the transgene has integrated within the an exon of a gene then the structure of this gene could be disrupted. Alternatively, if the transgene has integrated in an intron or regulatory elements of a gene then the transcription level of this gene could be changed. Another possibility is that on transgene integration genomic DNA that contains one or several genes has been deleted from the mouse genome.

We partially characterized the transgene integration site. Three phage clones were identified that, in addition to part of the transgene, also contain mouse genomic DNA that was positioned 5' from the integration site. Sequence information from these clones indicates that the transgene has integrated in non-coding repetitive DNA, and is not likely to disturb an exon of a gene. A complete characterization of the integration site will reveal the exact nature of the disturbance, and lead to identification of the disrupted gene(s).

The syndactyly that has been observed in the *tipsy* mutant is not comparable to any of the human forms of syndactyly that have been categorized by Tentamy and McKusick (1978). However, a family has been reported with a congenital hand malformation that shows remarkable similarity to the mouse mutant phenotype (Percin *et al.*, 1998). The common phenotype in this family is an autosomal dominant form of syndactyly type I, but a more severe

phenotype has been observed in three family members that might be homozygotic for the condition. This severe phenotype is characterized by syndactyly with synostosis of the phalanges in the third and fourth fingers, complete or partial soft tissue syndactyly of the toes, and aplasia/hypoplasia of the halluces and several phalanges.

The comparable form of syndactyly might indicate that this human family and the *tipsy* mutant are mutated in homologous genes. We were able to show that the transgene has integrated on mouse chromosome 18qE1. At this moment investigation whether the family is genetically linked to the region syntenic to mouse chromosome 18qE1 (human chromosome 5q32-33.1) is underway.

Two allelic mutations observed in the *sy* - and the *fp* mouse mutant have been mapped to a region that is close to the rhodopsin transgene integration site in the *tipsy* mutant on chromosome 18qE1 (MGD, 1998) (Lane and Hummel, 1973). Both mutant phenotypes inherit in a recessive manner, and show a form of syndactyly characterized by synostosis of phalanges. The similar chromosomal localization, and the evident overlap in phenotype, indicates that the *tipsy* mutation and the *sy* / *fp* mutations might be allelic.

The *sy* mutant phenotype was found among descendants of an X-ray irradiated male mouse (Hertwig, 1942). Homozygous *sy* mutants may be syndactylous on all four limbs. The mutant phenotype is more severe in the hind limbs, and the forelimbs are often normal. Synostosis of the phalanges can be present in digits two and three, digits three and four, or all three digits simultaneously. These fusions can involve all three – or just the two most distal phalanges, but the metacarpals and metatarsals are never affected.

The remainder of the *sy* mutant skeleton is small in size, and shows deviations in the shape of various bones. The osseous skeleton is less densely constructed than normal, the shafts of the long bones are very thin, and the shape of the scapula and the sacral vertebrae is abnormal (Grüneberg, 1955).

Since the discovery of the *sy* mutant in 1942, several investigators have addressed its abnormal embryonic development. In extensive studies on homozygous *sy* mutant embryos it has been found that the first visible abnormality in limbs can be seen at 12.5 days post coitum (dpc) (Grüneberg, 1955). At that time the shape of the mutant limb paddle deviates from that of the *wildtype* littermates. The distance from pre-axial to post-axial borders of the mutant limb paddles is too small, and the blastemata of digits two, three and four are too close together and tend to overlap distally. This will eventually lead to phalange synostosis.

Most homozygous *sy* mutants die very soon after birth. The ones that do survive are infertile and have a shortened life span. The surviving homozygous *sy* mutants show, along with bone – and limb anomalies, a hearing deficiency, and an abnormal pattern of behavior (Hertwig, 1942; Deol, 1963). These deficiencies might be explained by extensive degeneration of the

membranous labyrinth, the aberrant position and partial degeneration of the equilibrium organ, and abnormalities in the cerebellar lobes of the brain that have been observed (Deol, 1963).

The second syndactylous mutant that has been mapped to mouse chromosome 18qE1, the *fp* mutant, arose spontaneously in strain C3HeB/FeHu (Hummel and Chapman, 1971). The *fp* mutant is officially designated as $sy^{<fp>}$, because the *sy* and *fp* mutations were shown to be allelic (Lane and Hummel, 1973) (MGD, 1998). All homozygous $sy^{<fp>}$ mutants show a fusion of the three central digits of the hind feet, and about half of the mutants show the same abnormality in the fore limbs. Fusions occur between digits two and three, or digits three and four, but were never observed in all three digits simultaneously. Homozygous $sy^{<fp>}$ mutants are viable and fertile, show a normal pattern of behavior, and have a normal sense of hearing.

The overlap and differences between the phenotypes of the *sy* -, $sy^{<fp>}$ -, and *tipsy* mutant might be explained by different mutations in a single gene. This gene would have to perform diverse functions in bone patterning and growth, inner ear development, and brain development. Alternatively, it could be that these mutants have defects in an overlapping, but distinct, set of genes in which each gene performs one or more of these functions.

The diverse set of malformations in the *sy* mutant might suggest that more than one gene has been disturbed in this mutant. The *sy* mutant was found in a radiation induced mutagenization experiment, and it is possible that a piece of DNA that contains several genes has been deleted from its genome.

The $sy^{<fp>}$ mutant shows syndactyly that is comparable to that of the *sy* mutant, but in contrast to the *sy* mutant it has normal sized skeletal bones, and normal behavior. It is tempting to think that the $sy^{<fp>}$ mutant has a defect in only one of the genes that might be deleted in the *sy* mutant. With respect to this last remark, it is interesting to notice that the phenotype of the *sy* / $sy^{<fp>}$ compound heterozygote mutant resembles that of the homozygous $sy^{<fp>}$ mutant (Lane and Hummel, 1973). This shows that the $sy^{<fp>}$ mutation can complement the additional abnormalities of the *sy* mutant, and is another indication that the *sy* mutant might be disturbed in several genes.

The *tipsy* mutant shows a phenotype that is somewhat intermediate between the *sy* and $sy^{<fp>}$ mutant. Like in the *sy* mutant, the skeletal bones appear fragile and show abnormalities such as malformations of the sacral vertebrae, and the presence of abnormal bony structures. However, like in the $sy^{<fp>}$ mutant, the sense of hearing and behavioral patterns are not affected.

It is, therefore, possible that the *tipsy* mutant has a defect in more than one gene that resulted from a deletion of genomic DNA at the time the transgene integrated. Alternatively, the differences between the *tipsy* and $sy^{<fp>}$ mutant might be explained by distinct mutations in a single gene.

The syndactyly of the *sy*$^{<fp>}$ mutant can be used as a visible phenotypic marker to position genes, and has been used in genetic linkage mapping studies on mouse chromosome 18 (Lane *et al.*, 1981; MGD, 1998). Two mouse genes map around the *sy*$^{<fp>}$ mutant locus: the *colony stimulating factor 1 receptor* (*Csf1r*) - and the *adrenergenic receptor beta 2* (*Adrb2*) gene (MGD, 1998). These two genes define the candidate region for the syndactyly gene in the mouse, and place it in a region of approximately 4 cM. The human homologues of these genes, *CSF1R* and *ADRB2*, map between polymorphic markers D5S413 and D5S470 (located on chromosome 5q32-33.1) in a region of approximately 2.8 cM (Deloukas *et al.*, 1998) (see figure 4).

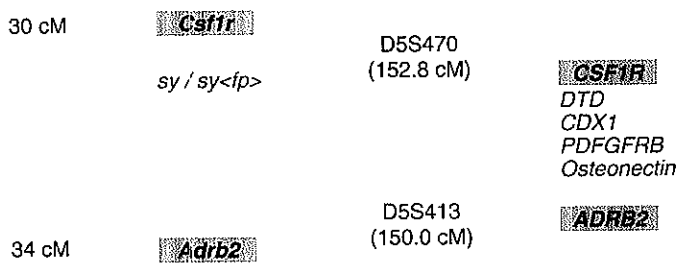

Figure 4 Synteny between chromosomal regions on mouse 18qE1 and human 5q32-33.1 in the regions between *Csf1r* and *Adrb2*.

In this region several interesting candidate genes that are involved in bone patterning and growth have been mapped. These are the caudal type homeobox (*CDX1*) transcription factor, the platelet-derived growth factor receptor beta (*PDGFRB*), the secreted glycoprotein osteonectin (*SPARC*), and the diastrophic dysplasia (*DTD*) sulfate transporter (Deloukas *et al.*, 1998). Specific information on these genes is listed in table 1.

The most interesting candidate gene is the *DTD* gene. The DTD protein is a sulfate transporter that is involved in sulfation of proteoglycans in the cartilage matrix (Hastbacka *et al.*, 1994). Lack of sulfation of these proteoglycans is proposed to lead to premature calcification of cartilage structures (Hastbacka *et al.*, 1996). There are indications that the *DTD* gene could be involved in the mouse and human syndactyly phenotypes.

Mutations in the human *DTD* gene cause recessive disorders, such as diastrophic dysplasia (OMIM 222600), atelogenesis type II (OMIM 256050), and achondrogenesis type 1B (OMIM 600972). Individuals affected with one of these congenital malformations show many bone abnormalities that are comparable to those that have been observed in the *sy* and *tipsy*

mutant. These bone abnormalities include limb anomalies, reduced skeleton size, and malformations of the sacral vertebrae.

Gene	Remarks	References
Cdx1	Transcription factor proposed to regulate *Hox* gene expression.	Subramanian *et al.*, 1995
	Cdx1$^{-/-}$ mice show anterior homeotic transformations of the vertebrae but develop normal limbs.	
Pdfgfrb	Receptor for growth factors, may play a role in mesenchymal – epithelial interactions	Betsholtz, 1995
	Expressed during embryonic development in mesenchymal tissues	Shinbrot *et al.*, 1994
	Pdfgfrb$^{-/-}$ mice show cardiovascular, hematological and renal defects, but develop normal limbs	Leveen *et al.*, 1994
Osteonectin	Secreted glycoprotein proposed to regulate cell adhesion and proliferation (specifically binds collagen IV and *Pdfgfrb*).	Lane and Sage, 1994
	Expressed during embryonic development starts at 14.5 dpc and is associated with new matrix of bones.	Nomura *et al.*, 1989
	Osteonectin$^{-/-}$ mice develop severe eye pathology, but show a normal development of the limbs	Gilmour *et al.*, 1998
Dtd	Sulfate transporter proposed to be involved in sulfation of proteoglycans in the cartilage matrix.	Hastbacka *et al.*, 1994
	Mutations in the human *DTD* gene are responsible for diastrophic dysplasia, atelosteo genesis type II, and achondrogenesis type 1B.	Hastbacka *et al.*, 1996

Table 1 Candidate genes for syndactylism between D5S413 and D5S470

Furthermore, there is some similarity between *DTD* associated limb anomalies and the human syndactyly phenotype reported by Percin *et al.* (1998). Both malformations include aplasia of phalanges, and halluces. Syndactyly of phalanges has not been reported in *DTD* associated syndromes, but a duplication of the middle phalanges has been observed in two individuals affected with atelogenesis type II (de la Chapelle *et al.*, 1972).

Whether the *DTD* gene is really involved in the mouse and human syndactyly phenotypes will become clear after the responsible gene has been identified. We are currently, screening a *wildtype* mouse genomic cosmid library with the phage clones that cover the transgene integration site in the *tipsy* mutant. This will allow investigating the exact nature of the transgene integration and the identification of genes that are disrupted by the integration.

Identification of the responsible gene will hopefully provide more insight into the pathogenesis of this form of syndactyly, and the processes that are involved in normal separation of the phalanges during embryonic development.

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CHAPTER 7

General Discussion

Our knowledge of the genetic basis of congenital hand malformations is far from complete, but during the past years tremendous advances have been made in this field. Although the type of work that has been described in this thesis is of a rather fundamental nature, it can have clinical implications in the future. It is not to be expected that prenatal diagnosis will become a routine for couples at risk of getting a child with an isolated hand malformation, or that hand anomalies can be treated without surgery. Hand malformations are, however, often associated with other congenital malformations, and investigating them can provide valuable information about the pathogenesis of these anomalies.

The interaction between fundamental biology and human clinical genetics is beneficial for both disciplines. It is expected that, information on the molecular biology of limb development will increase our insight into the pathogenesis of congenital anomalies. Likewise, functional analysis of genes that are involved in congenital malformations will reveal their roles in normal pattern formation and embryogenesis.

This thesis describes a study of two congenital limb malformations: pre-axial polydactyly (PPD) and syndactyly. These malformations were investigated in:

- families that are affected with PPD type II/III linked to chromosome 7q36
- the *Hammertoe* (*Hm*) - and *Hemimelic extra toes* (*Hx*) mouse mutants that serve as a model for the hand anomalies linked to chromosome 7q36. These mutants are most likely mutated in gene that is homologous to the human gene (Heutink et al., 1994; Tsukurov et al., 1994).
- a new mouse model for a form of syndactyly that is characterized by synostosis of phalanges.

Several hand malformations have been genetically linked to human chromosome 7q36. These malformations include PPD type II/III (Heutink et al., 1994; Hing et al., 1995; Radhakrishna et al., 1996) and PPD type IV, or complex polysyndactyly (Tsukurov et al., 1994).

In this thesis, twelve families from different ethnic backgrounds are reported, that are affected with PPD. These families were ascertained to investigate which forms of polydactyly are linked to chromosome 7q36. A family affected with PPD type I, without the occurrence of PPD type II/III, could be excluded from linkage to 7q36, indicating genetic heterogeneity for this type of polydactyly. All families with PPD type II/III are linked to chromosome 7q36 regardless of their ethnic origin.

The *Hm* and *Hx* mouse mutants have provided valuable information about the pathogenesis of the hand malformations linked to human chromosome 7q36. The work that has been presented in this thesis illustrates that comparative investigation of mouse and human is useful in the study of congenital malformations, which will become increasingly effective as more biological data on mouse and human accumulates.

Within the polydactyly families that are linked to chromosome 7q36 a large phenotypic variation was observed, including PPD type I, post-axial polydactyly, and radial – and tibial

dysplasia/aplasia. Experiments with the *Hx* mutant indicate that these phenotypic variations might be explained by genetic modifiers that influence the phenotype. The *Hx* mutation in its original outbred genetic background also showed a wide phenotypic variation. When this outbred genetic background was replaced by an inbred background the phenotype stabilized. This indicates that other genes in the mouse genome affect the *Hx* mutant phenotype. A comparable effect of genetic modifiers in the human genome might explain the phenotypic variations that are often seen within a family.

One of the phenotypic variations, which has been observed in the original *Hx* mutant outbred strain is radial/tibial dysplasia. By breeding one copy of the *Hx* mutation into a stable genetic background, the radial/tibial dysplasia completely disappeared in this mutant. This indicates that the occurrence of radial/tibial dysplasia in the *Hx* mutant phenotype is strongly influenced by genetic modifiers. When this same stable genetic background was used to breed homozygous *Hx* mutants, the tibial aplasia reappeared, this time with full penetrance. This indicates that influence of genetic background is not the only cause of radial/tibial aplasia, and that mutations in the polydactyly gene can be responsible for this phenotype. Radial/tibial aplasia has been observed in two unrelated individuals affected with PPD linked to chromosome 7q36 (Hing et al., 1995); chapter 3). Because, both individuals are heterozygous for the haplotype that is associated with polydactyly, it is not likely that they are homozygous for a mutation in the polydactyly gene on chromosome 7q36. Instead, their more severe phenotype probably resulted from the influence of genetic modifiers.

Malformations other than those of the limbs were never observed in association with the anomalies in any of the families linked to 7q36 (Zguricas et al., 1994). In agreement with this, no malformations other than that of the limbs were observed in the *Hm* and *Hx* mouse mutants (Knudsen and Kochhar, 1981; Green, 1989); chapter 5). This could indicate that the responsible gene is exclusively involved in limb development. Other options are that the gene function is not affected by the mutations that cause these hand malformations, that is functioning in the rest of the body is of less critical importance, or that the gene's function is redundant.

The human limb phenotypes that have been mapped to chromosome 7q36 include distinct combinations of polydactyly and syndactyly. This raises the question whether these hand malformations are caused by mutations in a single gene or by mutations in separate genes. A part of the answer to this question may come from the *Hm* and *Hx* mouse mutants. The *Hm* and *Hx* mutant map so close together on mouse chromosome 5, that 3.664 meioses had to be investigated before one recombination event between the two mutations was observed (Sweet, 1982). Although this indicates that the mutations are not allelic, it could still be that the observed recombination event is intragenic. This thesis presents evidence that the *Hm* and *Hx* mutations are distinct and that they show a genetic interaction (see chapter 5). These observations make it very likely that the *Hm* and *Hx* phenotypes are caused by mutations in a single gene. If the *Hm*

and *Hx* mutants are allelic, then it is likely that the human PPD and complex polysyndactyly phenotypes linked to chromosome 7q36 are also caused by distinct mutations in a single gene.

Several lines of evidence suggest that the genetic classification of polydactyly is in disagreement with the morphological classification. Firstly, the morphologically distinct PPD type II and type III occur within single families, although it is expected that all affected individuals in a family share the same mutation. Secondly, recent studies have shown the PPD type IV is a genetically heterogeneous disorder, that can either be caused by mutations in a gene on chromosome 7q36 (Tsukurov et al., 1994), or by mutations in the *GLI3* gene on chromosome 7p13 (Radhakrishna et al., 1998).

A similar disagreement between morphological and genetical classifications has been found for other congenital malformations. This is nicely illustrated by the genetic classification of craniosynostosis syndromes. The morphologically distinct Apert -, Crouzon -, and Pfeiffer syndromes can be caused by mutations in the *fibroblast growth factor receptor* (*FGFR*) *2* gene (Wilkie et al., 1995). Mutations in the *FGFR1* gene can also cause Pfeiffer syndrome (Muenke et al., 1994; Lajeunie et al., 1995). Furthermore, it was found that Pfeiffer and Crouzon syndrome can be caused by a single mutation in the *FGFR2* gene (Rutland et al., 1995).

Since these examples clearly show that genetic classification systems will not be able to replace morphological classifications of congenital disorders, it is more likely that genetical classifications will arise as a supplement to the morphological ones, and perhaps provide explanations for the great phenotypic variability and overlapping phenotypes.

Studying abnormal limb development in the *Hx* mouse mutant has provided insight into the pathogenesis of PPD linked to chromosome 7q36. The ectopic expression patterns of *Shh*, *Fgf4*, and *HoxD13* in *Hx* mutant limb buds suggest a disturbance of anteroposterior patterning. This disturbance is comparable to that created in the classical ZPA transplantation experiments (Saunders and Gasseling, 1968). Therefore, one can assume that the *Hx* polydactylous phenotype and the human PPD type II/III phenotype are mirror image duplications (see figure 1.4). However, the human - and mouse mutant phenotypes do not appear to be perfect mirror image duplications. This might be explained by the timing of appearance of the ZPA duplication and its range of influence. The expression of *Shh* in the anterior mesenchyme of the *Hx* mutant limb bud has a different timing when compared to the normal posterior *Shh* expression. Also, the size of the ectopic *Shh* expression domain is smaller when compared to the posterior *Shh* expression domain. It has been reported that these factors influence the extent of mirror image duplications in ZPA transplantation experiments in chicken embryos (Tickle et al., 1975; Tickle, 1981).

The function of the PPD gene on chromosome 7q36 is still unclear, but several lines of evidence suggest that it might be similar to that of the *GLI3* transcription factor. Firstly, the

mouse *Gli3* gene is expressed in the anterior mesenchyme of the developing limb bud. Loss of *Gli3* expression in the polydactylous Xt mutant, which has a partial deletion of the *Gli3* gene, results in ectopic ZPA formation (Schimmang et al., 1992) very similar to that seen in *Hx* mutant embryos. This suggests that it is involved in an active ZPA suppression mechanism operating in the anterior limb bud (Masuya et al., 1997). Secondly, mutations in different functional domains of the human *GLI3* gene are associated with distinct combinations of polydactyly and syndactyly (see chapter 1.2.2). Study of the *GLI3* homologue in *Drosophila melagonaster* suggests that the function of the human *GLI3* protein is regulated by proteolytic cleavage. The amino-terminal part of the protein would repress *SHH* and its downstream targets, while the full-length protein would activate them (Biesecker, 1997). In analogy with the *GLI3* gene, mutations and deletions of different functional domains in a single gene, could be responsible for the various forms of polydactyly and syndactyly that are linked to chromosome 7q36. The remarkable overlap in *GLI3* and chromosome 7q36 phenotypes might even suggest that these genes operate in a similar pathway. These questions can not be answered until the responsible gene has been identified.

In 1994, a positional cloning project aimed at identifying the human PPD type II/III gene on chromosome 7q36 was started in our laboratory. At first, identification of the corresponding mouse gene in the *Hm* and *Hx* mutants was considered. However, practical considerations made us decide to explore the human resources instead. We hypothesized that, the large PPD families that were available, would allow refinement of the human candidate region by recombination analysis. Achieving a comparable chance of refining the candidate region in mice would have taken large breeding programs that are labor – and cost intensive. In addition to this, some genomic YAC clones positioned on human chromosome 7q36 were available. These YAC clones could be used as a starting point in the identification of the responsible gene. Finally, we already expected that the amount of information on the human genome would soon exceed that of the mouse several fold.

The first step in any positional cloning approach is to define the smallest genomic region that is shared by all affected individuals. This candidate region must contain the gene that is responsible for the disease phenotype. By investigating recombination events in PPD type II/III families with available polymorphic markers, the responsible gene was initially mapped to a 1.9 cM region (see chapter 3). There were few polymorphic markers positioned in this candidate region and good physical maps of chromosome 7q36 were not available. Therefore, a detailed physical map covering 1.200 kb of the candidate region was constructed. This allowed the identification of new polymorphic markers and a further refinement of the candidate region to approximately 450 kb (see chapter 4). The recombination event that defines the candidate region on the centromeric side was identified in a non-affected individual, and would be incorrect if this person carries a mutation without showing a hand phenotype. However, the penetrance of the

polydactylous phenotype linked to chromosome 7q36 is nearly complete (see chapter 3), and detailed examination of this individual showed no abnormalities of hands and feet.

The physical map of the candidate region was used to identify candidate genes with a combination of direct cDNA selection, exon trapping analysis and computer-based methods such as homology searches and gene mapping. Not all of these gene identification methods performed equally well.

The cDNA selection experiment was done on a P1 clone and two YAC clones that contain genomic DNA from the candidate region. Although the YAC clones span a much larger genomic region when compared to the P1 clone, half of the cDNA clones that were obtained belong to a single gene that maps to the P1 clone (see chapter 4). Presumably for technical reasons, such as the inability to precisely separate the YAC from the other yeast chromosomes, the experiment worked less efficient on the YAC clones.

About half of the transcripts that were identified by other methods were found with exon trapping. However, 45 putative exons could not be assigned to any previously identified transcript, and we were unable to find corresponding cDNA clones in several cDNA libraries. This could mean that these putative exons are the result of cryptic splicing events (as discussed in chapter 2.1.1), or that the corresponding transcripts are not represented in the cDNA libraries.

Four candidate genes map in the refined PPD candidate region of 450 kb. The most intriguing candidate gene is the homeobox containing transcription factor *HLXB9*. As has been discussed in the first chapter of this thesis, most hand malformations for which the responsible gene has been identified are caused by mutations in transcription factors. A detailed search for mutations in individuals affected with PPD type II/III did not show any pathogenic sequence alterations in the coding region and intron-exon boundaries of the HLXB9 gene. Although this indicates that *HLXB9* is not involved in hand malformations, there is still the possibility of mutations in regulatory elements, which have not been investigated.

Evidence that makes mutations in regulatory elements less likely comes from another congenital malformation that is linked to chromosome 7q36. Recently, it was shown that mutations in *HLXB9* are responsible for an autosomal dominant form of sacral agenesis (OMIM 176450) (Ross et al., 1998). One of the mutations that has been reported is a nonsense mutation at position 4213 in the homeobox, replacing glutamate codon 261 (CAG) with the TAG termination codon. Such a termination is likely to prevent *HLXB9* from binding DNA and therefore interferes with its transcription regulating capabilities. Because there are no hand malformations associated with sacral agenesis, it is unlikely that the hand malformations linked to chromosome 7q36 are caused by mutations in *HLXB9* regulatory elements that result in loss of half of its activity.

At this moment, the effort to identify the PPD gene on chromosome 7q36 continues. The candidate region is being sequenced by a large-scale genomic sequencing centre that is using

genomic clones from our physical map. It is expected that the refined candidate region and the candidate genes that have been investigated already, will contribute to the quick identification of the responsible gene. Furthermore, the putative exons that could not be matched to any corresponding cDNA might speed up the identification of coding sequences.

The availability of 165 kb of genomic sequence has resulted in the identification of a new candidate gene. It might be that this gene has not been identified previously because its expression levels are low or restricted to certain tissues. This shows that identifying genes from genomic sequence is less dependent on gene expression when compared to cDNA selection and exon trapping. Both cDNA selection and exon trapping depend on gene expression. The cDNA selection method is directly dependent on expression, while exon trapping is independent of expression until the putative exons have been identified. After this first step cDNA libraries will have to be screened to find a corresponding cDNA clone. It will be interesting to see if any of the putative exons that could not be matched to a transcript are part of the newly identified gene. This can not be tested until the entire coding region of the new gene has been identified.

Analysis of genomic sequence of the entire candidate region will perhaps lead to the identification of more new candidate genes. Furthermore, there is the possibility that additional coding regions that have not been identified on previous occasions will be found in known candidate genes. The genomic sequence will also make it possible to identify and investigate regulatory regions, like promoters and enhancers, in candidate genes.

Recently, a limb malformation was reported with a form of syndactyly that does not fit the present classification of syndactyly (Percin et al., 1998). The family was affected with an autosomal dominant form of syndactyly type I. Three individuals show a more severe limb phenotype, that is characterized by synostosis of the phalanges in the third and fourth fingers, complete or partial soft tissue syndactyly of the toes, and aplasia/hypoplasia of the halluces and several phalanges. These individuals might be homozygous for the condition.

We have described a new mouse model for a recessive form of syndactyly that is comparable to this human phenotype. The mutant phenotype is characterized by synostosis of the phalanges in the second and third digits. This mutant was observed in an independent line of mice transgenic for the human rhodopsin gene, and is therefore named the transgene induced phalange synostosis (tipsy) mutant. In order to identify the gene responsible for the syndactyly phenotype in this mutant, the transgene integration site has been characterized. Non-coding repetitive DNA has been found on the 5' side of the transgene integration. Therefore, it is not likely that the transgene disturbs an exon of a gene. Instead, it is more likely that the mutant phenotype is caused by integration of the transgene in an intron or a regulatory sequence, or that upon integration one or several genes have been deleted from the mouse genome.

It was shown that the transgene has integrated on mouse chromosome 18qE1. On this same position two allelic mouse mutants with a comparable form of recessive syndactyly, have

been mapped. The comparable map position and phenotype of these three mutants suggests that they might be allelic. However, the mutant phenotypes also show large differences (see chapter 6), which suggests that they are caused by separate defects in a single gene, or alternatively by defects in an overlapping but distinct set of genes. A detailed characterization of the transgene integration site will hopefully clarify the exact nature of the disruption, and lead to identification of the gene that is involved in the syndactyly phenotype.

The transgene integration site on mouse chromosome 18qE1 corresponds to human chromosome 5q32-33.1, where several interesting candidate genes that are involved in bone patterning and growth have been mapped (see chapter 6). The most promising candidate is the diastrophic dysplasia (DTD) gene, a sulfate transporter that is involved in sulfation of proteoglycans in the cartilage matrix. Mutations in the human DTD gene cause several recessive congenital disorders of the skeleton, in which the bones of the limbs are always affected but syndactyly has never been observed. However, the DTD associated skeletal disorders include anomalies of the sacral vertebrae, which have been observed in the sy and tipsy mutant, and a reduced skeleton size that has been observed in the sy mutant. This indicates that mutations in the mouse Dtd gene could be involved in the pathogenesis of the sy and tipsy mutant phenotypes, and might even be responsible for syndactyly in all three mouse mutants.

The human syndactyly phenotype reported by (Percin et al., 1998) shows a remarkable similarity with the *DTD* associated limb malformations and the syndactyly mouse mutants. At this moment investigation whether this family is genetically linked to chromosome 5q32-33.1 is underway.

Identification of the genes responsible for the *tipsy* mouse mutant phenotype and the PPD gene on human chromosome 7q36 will make functional analysis of these genes possible. This will help us reveal their role in limb development and lead to more insight into the pathogenesis of these malformations.

Once the responsible genes have been identified it is possible to perform further studies. In fact, definite answers to many of the questions that were posed above can not be presented until the genes have been identified. The ultimate goal is to understand their exact role in the pathogenesis of the limb malformations, and its role in normal embryonic development. There are several ways to investigate the function of the responsible genes.

The presence of a genotype – phenotype correlation is the first thing that can be investigated. It will be interesting to see whether PPD type II/III and type IV are caused by mutations in the same gene, and if the different phenotypes can be explained by the mutations that underlie them. Mutation analysis on the corresponding mouse gene will hopefully clarify the genetic interaction that has been observed between the *Hm* and *Hx* mouse mutants. The role of the gene in the developing limb bud can also be investigated by examining its expression pattern. The *Hm* – and *Hx* mutant can be used to study the effect of mutations in the PPD gene on

expression and interaction with other genes during embryonic limb development. Later on, it may become interesting to create a targeted null mutation or to study the effect of specific mutations on limb development. Interaction with (pathways of) other genes can be investigated by methods like two-hybrid screening, or study of homologues genes in model organisms such as *Drosophila melagonaster*. This will provide more information on the function, and might even explain the phenotypic variations that have been observed.

In the coming years, large-scale gene mapping projects and genomic sequencing, will add to the power of the positional candidate approach. The availability of biological information will no longer be the rate limiting step in gene identification. Instead, it will become more important to filter the significant information out of all the available data. It is therefore expected that positional cloning projects will increasingly depend on knowledge of biological processes and tools to search, process and visualize huge amounts of biological data. Many of these tools are still under development today. It is to be expected that in the next decade a shift will take place from identifying genes towards the study of the function, regulation and interaction of proteins.

Inevitably, the majority of the genetic factors involved in limb development will be discovered in the near future. It is expected that interaction between fundamental and clinical research will continue to play an essential role in investigating the etiology, pathogenesis, prevention, diagnosis and treatment of congenital (limb) malformations.

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SUMMARY

Malformations of the limbs are the most frequently found congenital malformations in newborns. They can occur in an isolated form or as part of a syndrome and are caused by environmental or genetic factors. Many of the syndromic forms also show other anatomical malformations. This indicates that the processes that regulate embryonic limb development are also involved in the development of other parts of the body. Therefore, studying the genes involved in limb malformations generates knowledge about normal and abnormal embryonic development. In this study we investigated two hand malformations. Firstly, complete or partial duplication of the fingers, also called polydactyly. Secondly, webbing of fingers, or syndactyly. These malformations have been investigated in human and in mouse that can be used as a model system for human development.

A start was made with the identification of the genetic defect that is responsible for a form of polydactyly on the thumb side of the hand in a Dutch family. The hand malformation in this family has been genetically linked to the short arm of chromosome 7 (7q36). A comparable form of polydactyly in Cuban, English and Turkish families was shown to be linked to chromosome 7q36 as well. This makes it very likely that these families have a defect in the same gene as the Dutch family. Furthermore, it shows that defects in this gene could be responsible for a substantial part of congenital polydactyly cases.

The chromosomal region is the only starting point to identify the gene defect. Therefore, a positional cloning approach was started. The polydactyly families have been used to define the minimal region in which the gene could be localised. Subsequently, a number of techniques have been used to identify candidate genes in the critical region. These candidate genes have been investigated for defects in the polydactyly families, but the responsible gene has not been identified yet.

In addition to polydactyly, another congenital hand malformation called complex polysyndactyly, has been linked to chromosome 7q36. Complex polysyndactyly is characterised by syndactyly with webbing of the soft tissue in between the digits, that can occur in combination with polydactyly. The phenotypic resemblance and the similar chromosomal location might indicate that polydactyly and complex polysyndactyly are caused by defects in the same gene.

To learn more about these hand malformations we investigated the *Hemimelic extra toes* (*Hx*) and the *Hammertoe* (*Hm*) mouse mutants. These mouse mutants are a model system for the hand malformations linked to 7q36 for two reasons. Firstly, the mutant phenotype is comparable to that of the human hand malformations; the *Hx* mutant is characterised by polydactyly and the *Hm* mutant is characterised by syndactyly. Secondly, the genetic defects responsible for the *Hx* and *Hm* mutant phenotypes have been localised on mouse chromosome 5, in a region that is syntenic to human chromosome 7q36. Therefore, it is likely that the *Hx* and *Hm* mutant have a

defect in the homolog of the gene that is responsible for congenital hand malformations in our families. The *Hx* and *Hm* mutant are mutants that arose spontaneously. Therefore it is unknown which genetic defects are responsible for the malformations. It is known that the *Hx* and *Hm* mutations are positioned at a small distance from eachother. Cross breeding the *Hx* and *Hm* mutant demonstrates that the mutations most likely reside within a single gene. This indicates that the hand malformations in the families might also be caused by mutations in a single gene.

A number of genes that are important for embryonic limb development have been identified. Two of these genes, *Sonic hedgehog* (*Shh*) and *Fibroblast growth factor 4* (*Fgf4*), are involved in pattern formation along the anteroposterior axis that runs from the thumb to the little finger. The expression of both genes is disrupted during limb development in *Hx* embryos. *Shh* and *Fgf4*, which are normally only expressed at the side of the little finger of the developing limb, are also expressed on the thumb side in *Hx* embryos. This causes disturbance of the anteroposterior axis that will eventually lead to polydactyly. It is therefore very likely that the responsible gene normally plays a role in formation of the anteroposterior axis. How defects in such a gene could cause syndactyly is unclear at the moment.

Very recently, a mouse mutant with a rare form of syndactyly has been found in a line of transgenic mice. This mutant is characterised by the fusion of the bones in digits two and three of the hind limbs. The transgene has most likely integrated into a gene that is involved in bone development. The transgene integration makes it relatively simple to identify the responsible gene. We have performed a description of the morphological abnormalities of the mutant and initial studies to localise and identify the responsible gene by characterisation of the transgene integration point. Identification of the responsible gene will hopefully lead to a better understanding of syndactyly and bone development in mouse and human.

SAMENVATTING

Aangeboren afwijkingen aan de ledematen zijn de meest voorkomende congenitale afwijkingen bij pasgeborenen en kunnen door zowel omgevings als erfelijke factoren veroorzaakt worden. Deze afwijkingen kunnen geïsoleerd of als onderdeel van een syndroom voorkomen. Bij de syndromatische vormen kunnen ook andere lichaamsstructuren anatomische afwijkingen vertonen. Dit geeft aan dat de processen die de embryonale ledemaatontwikkeling sturen ook een rol spelen in de ontwikkeling van andere delen van het lichaam. Het bestuderen van de genen die betrokken zijn bij erfelijke ledemaatafwijkingen kan dan ook belangrijke kennis opleveren over de normale en afwijkende ontwikkeling van het embryo. In dit proefschrift zijn twee handafwijkingen bestudeerd. Ten eerste, een geheel of gedeeltelijke duplicatie van de vingers, ook wel polydactylie genoemd. Ten tweede, het aaneengroeien van de vingers, ook wel syndactylie genoemd. Dit is gedaan door zowel naar de mens als naar een modelsysteem voor de ontwikkeling van de mens, de muis, te kijken.

Er is een start gemaakt met de identificatie van het gendefect dat in een Nederlandse familie een erfelijke vorm van polydactylie aan de duimkant veroorzaakt. Bij deze handafwijking was al eerder aangetoond dat het defecte gen op de korte arm van chromosoom 7 (7q36) ligt. Een vergelijkbare vorm van polydactylie in Cubaanse, Engelse en Turkse families kon ook aan chromosoom 7q36 gekoppeld worden. Dit maakt het zeer waarschijnlijk dat deze families een defect hebben in hetzelfde gen als de Nederlandse familie. Verder geeft dit aan dat defecten in dit gen voor een groot gedeelte van aangeboren polydactylie verantwoordelijk zouden kunnen zijn.

Behalve de chromosomale positie is er niets bekend dat zou kunnen helpen bij de identificatie van het defecte gen. Daarom is positioneel kloneren de enige manier om het defecte gen te vinden. Met behulp van de families is het kritieke gebied, het gebied waarin het gen moet liggen, precies bepaald. Hierna is dit gebied kunstmatig nagebouwd en zijn er met een aantal technieken kandidaatgenen geïdentificeerd. Deze kandidaatgenen zijn in de polydactylie families op defecten onderzocht. Ondanks deze inspanningen is het defecte gen nog niet gevonden.

Naast polydactylie is de aangeboren handafwijking complexe polysyndactylie aan hetzelfde gebied op chromosoom 7q36 gekoppeld. Complexe polysyndactylie wordt gekarakteriseerd door syndactylie, waarbij het zachte weefsel tussen de vingers aaneen gegroeid is, die kan voorkomen in combinatie met polydactylie. De overeenkomst in fenotype en chromosomale lokalisatie doet vermoeden dat polydactylie en complexe polysyndactylie door defecten in hetzelfde gen veroorzaakt worden.

Om meer van de bovengenoemde handafwijkingen te weten te komen zijn de muis mutanten *Hemimelic extra toes* (*Hx*) en *Hammertoe* (*Hm*) onderzocht. Deze muizen zijn een modelsysteem voor de handafwijkingen op chromosoom 7q36 om de volgende redenen. Ten eerste vertonen ze een fenotype dat vergelijkbaar is met dat van de mens; de *Hx* mutant wordt

gekarakteriseerd door polydactylie en de Hm wordt gekarakteriseerd door syndactylie. Ten tweede zijn de genetische defecten in de *Hx* en *Hm* mutanten op muis chromosoom 5, in een regio die overeenkomt met mens chromosoom 7q36, gelokaliseerd. Dit maakt het waarschijnlijk dat de *Hx* en *Hm* mutant een defect vertonen in een homoloog van het gen dat in de families de handafwijkingen veroorzaakt. De *Hx* en *Hm* mutant zijn op spontane wijze ontstaan. Daarom is, net als in de mens, niet bekend welk genetisch defect aan de afwijkingen ten grondslag ligt. Wel is bekend dat deze defecten heel erg dicht bij elkaar liggen. Kruisingen tussen de *Hx* en *Hm* mutant tonen aan dat ze zeer waarschijnlijk in hetzelfde gen liggen. Dit maakt het aannemelijk dat de handafwijkingen in de families ook door defecten in een enkel gen veroorzaakt worden.

Er zijn een aantal genen bekend die een belangrijke rol spelen in de embryonale handontwikkeling. Twee van deze genen, *Sonic hedgehog* (*Shh*) en *Fibroblast growth factor 4* (*Fgf4*), zijn betrokken bij de patroonvorming over de duim-pink as. In de *Hx* mutant is de expressie van beide genen tijdens de embryonale pootontwikkeling verstoord. *Shh* en *Fgf4*, die tijdens de normale ontwikkeling alleen aan de pink kant tot expressie komen, komen in de *Hx* mutant ook aan de duimkant tot expressie. Hierdoor wordt de patroonvorming over de duim-pink as verstoord en ontstaat de polydactylie. Het is dus zeer waarschijnlijk dat het verantwoordelijke gen een rol speelt in de totstandkoming of het onderhoud van de duim-pink as. Hoe defecten in een dergelijk gen ook tot syndactylie kunnen leiden is op dit moment nog niet duidelijk.

Zeer recent is er een muis mutant met een zeldzame vorm van syndactylie gevonden in een lijn van transgene muizen. Deze mutant wordt gekarakteriseerd door het aaneengroeien van de botten in de tweede en derde teen. Waarschijnlijk is het transgen in een ander gen dat betrokken is bij botontwikkeling terecht gekomen. De integratie van het transgen maakt het relatief eenvoudig om het verantwoordelijke gen te identificeren. We hebben een beschrijving van de morfologische afwijkingen van de mutant gemaakt en zijn begonnen met de identificatie van het verantwoordelijke gen door het transgeen-integratiepunt te kloneren. Identificatie van dit gen leidt hopelijk tot meer begrip van het ontstaan van syndactylie en botvorming in de muis en de mens.

Curriculum Vitae

Henk Heus werd op 27 april 1970 geboren in Rotterdam. In 1986 haalde hij het MAVO diploma aan de Zuidermavo school in Rotterdam. Hij volgde hij twee jaar middelbaar laboratorium onderwijs aan het Van 't Hoff instituut in Rotterdam om vervolgens door te stromen naar het hoger laboratorium onderwijs aan het Van Leeuwenhoek instituut in Delft. Na een onderzoeksstage bij het centrum voor fytotechnologie (TNO) in Leiden behaalde hij in 1992 een diploma in de studierichting biotechnologie. Aansluitend studeerde hij chemie aan de Rijksuniversiteit van Leiden. Hij liep een onderzoeksstage bij de vakgroep biotechnologie aan de Rijksuniversiteit Leiden onder begeleiding van prof.dr. Herman de Boer. In juni 1994 behaalde hij het doctoraal diploma scheikunde. In augustus 1994 begon hij met een promotie-onderzoek op afdeling Klinische Genetica van de Erasmus Universiteit Rotterdam. Hier voerde hij onder begeleiding van prof.dr. Ben Oostra en dr. Peter Heutink het in dit proefschrift beschreven onderzoek uit. Sinds januari 1999 is hij als wetenschappelijk onderzoeker bio-informatica werkzaam bij N.V. Organon in Oss.

LIST OF PUBLICATIONS

Julia Zguricas, Wendela F. Bakker, Henk C. Heus, Dick Lindhout, Peter Heutink, Steven E.R. Hovius. Genetics of limb development and congenital hand malformations. *Plast. Reconstr. Surg.* (1998) 101: 1126-35.

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Stellingen behorende bij het proefschrift

Congenital Limb Malformations
A study of mice and man

I

De handafwijkingen op chromosoom 7q36 worden veroorzaakt door verschillende mutaties die de functie van één enkel gen beïnvloeden.

Dit proefschrift

II

Op basis van *in silico* experimenten is de muizenhomoloog van het *DTD* gen het beste kandidaatgen voor de *sy*, *fp* en *tipsy* muismutanten.

Dit proefschrift

III

De zichtbaarheid van congenitale handafwijkingen draagt bij aan de relatief hoge incidentie ten opzichte van andere ontwikkelingsstoornissen.

IV

Depletie van Pin1 in de hersenen van Alzheimers patiënten suggereert dat herstarten van de mitotische cyclus de oorzaak is van neurodegeneratie.

Lu et al., Nature (1999) 399, 784-788

V

Uit de beoordeling van fokgegevens van muizen zou geconcludeerd kunnen worden dat tibiale aplasie en infertiliteit genetisch gekoppeld zijn.

VI

Veel embryonale ontwikkelingsgenen hebben ook een belangrijke functie in het volgroeide organisme en zijn daarom dankbare onderzoeksonderwerpen voor de medische wereld en de farmaceutische industrie.

VII

Wanneer de genoom sequentie van de muis bekend is zal blijken dat, op een paar onschuldige polymorfismen na, de muis eigenlijk ook een mens is.

VIII

Het met zekerheid kunnen voorspellen van coderende sequenties in genomisch DNA zal pas werken nadat alle genen op een andere manier geïdentificeerd zijn.

IX

Nu er nog relatief weinig genen zijn waarvan de functie bekend is, is het automatisch annoteren van nieuwe genen op basis van homologie vaker misleidend dan informatief.

X

'High-throughput' analyse van DNA, mRNA, eiwitten en metabolieten zal leiden tot een nieuwe revolutie in de medische biologie.

XI

Veronderstellingen als absolute juistheid, absolute precisie en volledige waarheid zijn uitvindingen van de verbeelding en zijn per definitie niet wetenschappelijk.

Vrij naar Max Born

XII

Nu aankopen in de supermarkt per mobiele telefoon worden besproken, moet men de telecommunicatie-industrie verplichten onderzoek te doen naar de gevolgen voor de volksgezondheid.

XIII

De Engelse zin "Biology is the science of life, although biologists don't seem to have any." is niet in het Nederlands te vertalen zonder aan betekenis en kracht in te boeten.

XIV

Wanneer Microsoft werkelijk meent dat ze een innoverend bedrijf zijn, moeten ze Linux gaan propageren.