Evidence that preaxial polydactyly in the Doublefoot mutant is due to ectopic Indian Hedgehog signaling

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Accepted 10 June; published on WWW 21 July 1998

SUMMARY

Patterning of the vertebrate limb along the anterior-posterior axis is controlled by the zone of polarizing activity (ZPA) located at the posterior limb margin. One of the vertebrate Hh family members, Shh, has been shown to be able to mediate the function of the ZPA. Several naturally occurring mouse mutations with the phenotype of preaxial polydactyly exhibit ectopic Shh expression at the anterior limb margin. In this study, we report the molecular characterization of a spontaneous mouse mutation, Doublefoot (Dbf). Dbf is a dominant mutation which maps to chromosome 1. Heterozygous and homozygous embryos display a severe polydactyly with 6 to 8 digits on each limb. We show here that Shh is expressed normally in Dbf mutants. In contrast, a second Hh family member, Indian hedgehog (Ihh) which maps close to Dbf, is ectopically expressed in the distal limb bud. Ectopic Ihh expression in the distal and anterior limb bud results in the ectopic activation of several genes associated with anterior-posterior and proximal-distal patterning (Fgf4, Hoxd13, Bmp2). In addition, specific components in the Hedgehog pathway are either ectopically activated (Ptc, Ptc-2, Gli1) or repressed (Gli2). We propose that misexpression of Ihh, and not a novel Smoothened ligand as recently suggested (Hayes et al., 1998), is responsible for the Dbf phenotype. We consider that Ihh has a similar activity to Shh when expressed in the early Shh-responsive limb bud. To determine whether Dbf maps to the Ihh locus, which is also on chromosome 1, we performed an interspecific backcross. These results demonstrate that Dbf and Ihh are genetically separated by approximately 1.3 centimorgans, suggesting that Dbf mutation may cause an exceptionally long-range disruption of Ihh regulation. Although this leads to ectopic activation of Ihh, normal expression of Ihh in the cartilaginous elements is retained.

Key words: Indian hedgehog, Polydactyly, Doublefoot mutant, Mouse, Limb patterning

INTRODUCTION

The vertebrate limb serves as a model system for studying the control of pattern formation. Classic embryonic studies in the chick limb have revealed three signaling centers, namely the AER (Apical Ectodermal Ridge), ZPA (Zone of Polarizing Activity) and limb bud ectoderm, that control the growth and patterning along the proximal-distal (P-D), anterior-posterior (A-P) and dorsal-ventral (D-V) axes respectively. Insight into the nature of these patterning processes has been revealed through the identification of signaling molecules that appear to mediate the function of these signaling centers. Members of the Fgf (Fibroblast Growth Factor) family such as Fgf2, Fgf4 and Fgf8 (Niswander et al., 1993; Fallon, et al., 1994; Crossley and Martin, 1995) are capable of substituting for the function of the AER. Sonic hedgehog (Shh), a member of the vertebrate Hedgehog (Hh) family (Echelard et al., 1993), can mediate the function of the ZPA (Riddle et al., 1993; Laufer et al., 1994; Niswander et al., 1994; Lopez-Martinez et al., 1995; Yang et al., 1997). Wnt7a, a member of the vertebrate Wnt family, controls the determination of dorsal fate in the distal limb through the regulation of transcription factor Lmx1b, a vertebrate homologue of Drosophila apterous (Parr and McMahon, 1995; Riddle et al., 1995; Cygan et al., 1997). In addition, En-1, a homeobox transcription factor expressed specifically in the ventral ectoderm determines ventral cell fate by suppressing Wnt7a expression (Cygan et al., 1997; Loomis et al., 1998). Moreover, the chick and mouse studies demonstrate that molecules from the three signaling centers interact with each other to coordinately control limb development along the three axes (for review, see Johnson and Tabin, 1997).

A challenge in studying vertebrate limb development is to understand how these signaling molecules are integrated in their physiological context. The existence of several naturally occurring mouse mutants that affect limb development has provided an opportunity to further study the regulatory mechanisms underlying limb patterning. For instance, several naturally occurring mouse mutations with the phenotype of polydactyly have been shown to affect the A-P patterning of
the limb, especially the hindlimb. Their phenotypes have been characterized and the mutations genetically mapped. Among the polydactyly mutants, preaxial polydactyly with malformation of the radius or tibia represents a major group. Studies carried out in several research groups have shown that mouse mutations luxate (lx), Strong’s luxoid (lst), X-linked polydactyly (Xpl), Recombination induced mutant 4 (Rim4), Hemimelic extra toes (Hx) and Extra toes (Xt) (Chan et al., 1995; Buscher et al., 1997; Masuya et al., 1995, 1997) exhibit mirror image duplication of anterior digits. These defects are most likely caused by the anterior formation of an ectopic ZPA, since Shh and Fgf4 are ectopically expressed at the anterior margin of these mutant limbs. Furthermore, tissue grafting experiments show that the anterior limb bud of the lst mutant has polarizing activity (Chan et al., 1995). However, the molecular mechanism of ectopic expression of Shh and Fgf4 is poorly understood except for the case of Xt, which has been shown to be a loss-of-function mutation in the Gli3 gene (Vortkamp et al., 1992; Hui and Joyner, 1993). Ci (Cubitus interruptus, the Drosophila homologue of Gli3) has been shown to inhibit the expression of Hh in the anterior compartment of Drosophila wing disc, consistent with a similar role for Gli3 in vertebrate limb development (Dominguez et al., 1996). Studies of preaxial polydactyly mutants may eventually lead to the identification of the molecular pathway that serves to set up and restrict the endogenous ZPA to the posterior limb margin.

Apart from the mutations that occurred naturally, genetic manipulations in the mouse have revealed other candidates for ZPA regulation. Alx4, a paired-type homeodomain protein expressed in the anterior mesenchyme of the limb, also appears to restrict ZPA formation to the posterior limb margin by inhibiting the expression of Shh at the anterior limb margin (Qu et al., 1997). Hoxb8, a homeobox transcription factor, is a candidate determinant to position the polarizing activity at the posterior margin of the future limb bud. When ectopically expressed in the entire proximal forelimb bud, Hoxb8 induces ectopic Shh expression at the anterior limb margin (Charite et al., 1994). These studies suggest that the restriction of Shh expression to the posterior margin may involve at least two mechanisms: the presence of transcriptional inhibitors and the absence of transcriptional activators of Shh expression at the anterior limb margin.

Two other hedgehog family members have been identified in the mouse, Indian hedgehog (Ihh) and Desert hedgehog (Dhh) (Echelard et al., 1993). Neither gene is expressed in the distal limb bud where patterning occurs, but Ihh is expressed in the condensed cartilage and is required for cartilage development (Vortkamp et al., 1996; B. St-Jacques, M. Hammerschmidt and A. P. McMahon, unpublished data).

Here we describe the molecular characterization of a spontaneous mouse mutation Doublefoot (Dbf) (Lyon et al., 1996), an autosomal dominant mutation mapping to a new gene locus on chromosome 1. Dbf mutation results in extreme polydactyly in all four limbs. Tibia and joint formation are also severely affected in the hindlimbs of both heterozygotes and homozygotes. In addition to these limb defects, the skull is abnormally broad and in homozygotes the maxillary processes fail to fuse. Moreover, the tail is often kinked. We present evidence that these Dbf phenotypes result from ectopic expression of Ihh, but that the Dbf mutation is genetically separable from Ihh, suggesting it has a long-range effect on Ihh regulation. Our results indicate that the two Hh family members, Shh and Ihh, which share overlapping expression in several regions of the mouse embryo, have similar polarizing activities. Our interpretation contrasts with that in a recent report by Hayes et al. (1998) which speculated that Dbf might encode a hitherto undiscovered activator of Smoothened (Smo), a seven-transmembrane protein thought to activate Hedgehog signaling.

MATERIALS AND METHODS

Mice

All animals were maintained under conventional conditions in the animal house of the MRC Mammalian Genetics Unit, Harwell. The Dbf mutant stock was maintained by crosses to 3H1 hybrids, which are (C3H/HeH × 101/H)F1.

For molecular mapping, Dbf+/+ females were crossed to Mus spretus males, and the F1 female offspring backcrossed to C3H/HeH males. All offspring were typed with simple sequence length polymorphism (SSLP) markers.

To produce independent genetic linkage data, 3H1 females were crossed to Mus spretus males, and the F1 female offspring backcrossed to C3H/HeH males.

To analyze the embryonic phenotypes of homozygous or heterozygous Dbf mutants, progeny embryos were obtained from the crosses of Dbf+/+ × +/- or Dbf+/+ × Dbf+/+. After 11.5 days, Dbf embryos were picked by the broad limb bud phenotypes. All 10.5 day embryos from the same litter were collected in the same tube.

SSLP markers

Genomic DNA was prepared from spleen and from tail biopsies using proteinase K-phenol/chloroform extraction. The polymerase chain reaction (PCR) was used to detect SSLPs. Primers D1Mit22, D1Mit77, D1Mit24 and D1Mit8 were purchased from Research Genetics (Huntsville, AL., USA). PCR was performed in a total volume of 10 μl containing 50 ng of genomic DNA, 6.6 μM forward and reverse primers, 100 mM Tris-HCl (pH 8.4), 500 mM KCl, 1.5 mM MgCl2, 200 μM dNTPs, 1 unit of Perkin-Elmer Cetus Taq polymerase. Amplifications were performed in a Perkin-Elmer-Cetus thermal cycler for 30 cycles of: 94°C for 60 seconds, 55°C for 90 seconds, 72°C for 90 seconds. Amplification products were separated by electrophoresis in 3% agarose gels.

Southern blot analysis

Ten micrograms of genomic DNA prepared from spleens were digested with the appropriate restriction endonuclease under standard conditions, separated by electrophoresis through 0.8% agarose gel at 2.5V/cm for 12-15 hours, and transferred onto Hybond-N+ membrane (Amersham, Life Sciences). The probes were labeled with [32P]dCTP by nick translation, and hybridizations were performed at 64°C in a solution containing 10% dextran sulphate, 200 μg/ml herring sperm DNA, 1 mM EDTA, 6X SSC, 5X Denhardt’s solution and 1% SDS. After hybridization, blots were washed with increasingly stringent washes ranging from 2X SSC, 20% SDS at 64°C to 0.1X SSC, 20% SDS prior to exposure to X-ray film for 1-3 days.

Gene markers

The Ihh locus was detected by a full length cDNA constructed by adding a NcoI/SacI genomic fragment to the 5’-end of the partial Ihh cDNA clone reported previously (Echelard et al., 1993; Marigo et al., 1995). The 2.2 kb cDNA insert detected a major fragment of 8.5 kb in C3H DNA and a major fragment of 6.0 kb in Mus spretus DNA following digestion with BglII.
The Pax3 probe, an approx. 1.2 kb EcoRI fragment of mouse cDNA, detected RFLV of 3 kb in C3H DNA and of 2.3 kb in Mus spretus DNA following digestion with TaqI (Goulding et al., 1991).

In situ hybridization and vibratome section
Whole-mount in situ hybridization using digoxigenin-labeled antisense RNA was performed as described by Wilkinson and Nieto (1993). The riboprobes used in this study have been described previously: Shh and Ihh (Echelard et al., 1993); Fgf4 (Niswander and Martin, 1992); Bmp2 (Lyons et al., 1989); Ptc (Goodrich et al., 1996) and Hoxd13 (Dolle et al., 1991).

After whole-mount in situ hybridization, one of the limbs was cut off and embedded in 2% agarose (in PBS) at 50°C. The embedded sample was vibratome-sectioned at a thickness of 40 μm.

RESULTS
Limb skeleton abnormalities of Dbf mutants
The Dbf mutation is an autosomal dominant mutation which arose spontaneously. Homozygous Dbf mutants die in utero at around 14.5 days post coitum (d.p.c.). Typical heterozygous Dbf mutants have extreme preaxial polydactyly with 6 to 8 digits on all the four limbs (Fig. 1). In addition, the tibia and fibula are often shortened and bowed with gross twisting of ankles (Lyon et al., 1996). A similar phenotype has been observed in Rim4, Hoxd13 in 11.5 d.p.c. mutants, all of which exhibit preaxial polydactyly (Masuya et al., 1995). Digit duplication was clearly seen around 13.5 d.p.c. (Fig. 1A,B). However, digit 1, which has only two phalanges, is often missing, and there are two or more triphalangeal extra digits. In addition, fusion of digits, webbing of soft tissue and abnormal swelling of the ventral surface have been reported. Since the embryonic limb phenotypes of Dbf+/Dbf and Dbf/+ are indistinguishable (Lyon et al., 1996), most of the Dbf mutants we studied here are heterozygous for the mutant allele.

Ihh is ectopically expressed in Dbf mutants
The duplicated digits in Dbf mutants suggest the existence of ectopic polarizing activity in the limb outside the endogenous ZPA. As Shh appears to encode the ZPA activity and ectopic Shh expression underlies preaxial polydactyly in several mutants, we examined Shh expression in Dbf mutants. At 11.5 d.p.c., Dbf mutant embryos exhibit a broadening of limb buds along the anterior-posterior axis. However, expression of Shh in the Dbf mutant is localized to the distal posterior margin in both the fore- and hindlimb in a pattern identical to that of wild-type embryos (Fig. 2A,B).

We next examined the molecular markers induced by polarizing activity such as Fgf4, Bmp2 and Hoxd13 in 11.5 d.p.c. wild-type and Dbf embryos (Fig. 3). In wild-type embryos, Fgf4 expression is detected in the posterior AER and Hoxd13 is expressed at a higher level in the distal posterior mesenchyme. In Dbf mutant embryos, ectopic Fgf4 expression is detected in the anterior AER (Fig. 3B) and Hoxd13 expression extends all the way to the anterior mesenchyme such that the expression domain is symmetric across the A-P axis (Fig. 3D). Bmp2 exhibits a very dynamic expression pattern from 11 to 12.5 d.p.c. At 11 d.p.c. in the wild type, Bmp2 expression is detected in the AER and the posterior and proximal limb margin (Fig. 4A,C) whereas in the Dbf mutant, Bmp2 expression is detected in the distal part of the limb bud throughout the A-P axis in both fore- and hindlimbs (Fig. 4B,D). At 11.5 d.p.c., Bmp2 is expressed in the future metacarpal region, interdigital area, the most distal mesenchyme and the AER in the forelimb of wild-type embryos (Fig. 4E). In the hindlimb, Bmp2 expression is detected in the posterior-distal limb around the ZPA, as well as in the posterior-proximal limb margin and future metacarpal area (Fig. 4G). However, in the anterior forelimb of Dbf mutants, general and broad expression substituted for interdigital expression (Fig. 4F) and in the hindlimb, Bmp2 expression extended all the way to the anterior limb bud (Fig. 4H). These data suggest that unlike all the other preaxial polydactyly mutants characterized to date, the Dbf mutation leads to ectopic polarizing activity outside the endogenous ZPA without altering the expression of Shh. Interestingly, in the metacarpal area at 11.5 d.p.c., the restricted and circular expression pattern of Bmp2 extended posteriorly and anteriorly such that it looks like a thin band. At 12.5 d.p.c., Bmp2 expression is detected exclusively in the interdigital region in the wild-type limb (Fig. 4I,K), but in the mutants, Bmp2

Fig. 1. Preaxial polydactyly in mice heterozygous for the Dbf mutation. At 13.5 d.p.c., each digit primordium can be clearly seen in both fore- and hindlimbs. (A) Wild-type embryos. The five digits in both fore- and hindlimb are numbered. (B) Dbf heterozygous mutant. There are 7 digits in the forelimb and 6 in the hindlimb. (C) X-ray photograph of the resulting forelimb skeleton of an adult Dbf heterozygous mutant. The anterior-most digit, digit 1 with two phalanges, is missing and there are three extra tri-phalangeal digits marked by *.

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expression is missing in some interdigital areas (Fig. 4J,L) where ectopic digits will form.

Hedgehog signaling is associated with the transcriptional activation of Ptc which encodes a Hh receptor (Stone et al., 1996; for review see Tabin and McMahon, 1997). We examined the expression pattern of Ptc in Dbf mutants. At 11.5 d.p.c., Ptc expression extended all the way to the anterior limb margin (Fig. 5B) whereas in wild-type embryos it is localized to the posterior limb mesenchyme (Fig. 5A). In Dbf forelimbs, ectopic Ptc expression is stronger at the distal-anterior limb margin whereas in the hind limb, Ptc is more uniformly expressed across the distal A-P axis. Ptc is also ectopically expressed in the future femur region. These data suggest that ectopic activation of the Hh signaling pathway independent of Shh may be responsible for the observed phenotype. We also examined the expression pattern of transcriptional targets of Hedgehog signaling such as Gli1 and Gli2 and found that Gli1 is similarly ectopically expressed as Ptc (Fig. 5D). However, Gli2 was ectopically repressed in the distal most mesenchyme (Fig. 5H). Recently, a second mouse Ptc gene, Ptc-2, has been identified which shares overlapping expression pattern with Ptc, during several aspects of embryonic development (Motoyama et al., 1998). In the Dbf mutant, Ptc-2 was similarly upregulated and ectopically activated (Fig. 5F) as Ptc, consistent with the view that Ptc-2 may also be a transcriptional target of Hedgehog signaling during A-P patterning of the limb.

The Dbf mutation has been previously mapped to mouse chromosome 1, close to the Ihh locus. This prompted us to examine whether Ihh expression is affected by the Dbf mutation. Strong ectopic expression of Ihh is detected in the distal limb bud across the A-P axis of the Dbf mutant at 11.5 d.p.c. (Fig. 6B). As no ectopic Ihh expression is detected in Dbf mutants at 10.5 d.p.c. (data not shown), we infer that the onset of ectopic Ihh expression lies between 10.5 and 11.5 d.p.c. By 12.5 d.p.c., ectopic Ihh expression has decreased and becomes localized to the anterior limb bud (Fig. 6C). Strong ectopic Ihh expression can also be observed in the future femur region where Ptc and Ptc-2 are also ectopically expressed (Fig. 7F,I).

We also noticed that in both fore- and hindlimbs of Dbf mutants, Ihh is not ectopically expressed in the distal posterior limb margin where Shh is expressed (Fig. 6B). Examination of limb bud sections indicates that ectopic Ihh expression is restricted to the distal limb mesenchyme directly beneath the surface ectoderm (Fig. 6D,E). Taken together, these results suggest that ectopic expression of Ihh establishes an ectopic polarizing activity which perturbs the normal A-P limb patterning by the endogenous ZPA. As the distal mesenchyme is responsible for zeugopod and autopod development in the hindlimb after 10.5 d.p.c. (Milaire and Goffard, 1995), it seems likely that the abnormality of the tibia and fibula in Dbf mutants is also caused by ectopic Ihh expression. Ectopic Ihh expression is also detected in the metacarpal regions where expression of Ptc and Bmp2 are also altered (Fig. 7D,E,G,H). This may cause the abnormally expanded and ventrally smooth handplate.

As joint abnormalities are seen in Dbf mutants, this raised the possibility that dorsal-ventral polarity may be reversed in certain regions of the limbs. To test this, whole-mount in situ hybridization was performed on 12.5 d.p.c. Dbf mutant embryos with Lmx1b, a dorsal determinant (Riddle et al., 1995; Cygan et al., 1997). Lmx1b expression is unaltered in Dbf mutant limbs when compared with wild-type embryos (data not shown), suggesting that the observed alteration along the D-V axis in Dbf mutants are independent of Lmx1b.
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Dbf mutants also have facial abnormalities and kinky tails suggesting that these phenotypes may also result from ectopic Ihh expression. We detected weak ectopic Ihh expression in the branchial arches and tail bud of Dbf mutant (Fig. 6B) at 11.5 d.p.c. At 12.5 d.p.c., Dbf mutants have broader facial processes and ectopic activation of Ihh was more obvious (Fig. 7E,F) accompanied by elevated expression of Ptc, Ptc-2, Gli1 (Fig. 5B,D,F) and decreased expression of Gli2 (Fig. 5H) in the branchial arches. However, no ectopic expression of Ptc was observed in the tail bud (Fig. 5B).

**Molecular mapping of Dbf mutation**

As a first attempt to determine the relationship between Ihh and the Dbf mutation, the Dbf mutation was further mapped using molecular markers. A total of 204 mice were typed with D1Mit22, D1Mit24, D1Mit77 and D1Mit8 microsatellite markers. The results indicate a complete co-segregation of Dbf with both D1Mit77 and D1Mit24. Miller et al. (1996) found a 4.6 centimorgan (cM) interval between D1Mit77 and D1Mit24. To check if the cosegregation we observed was due to an effect of the Dbf mutation, 79 mice from a (3H1 × Mus spretus)F1 ×...

**Fig. 4.** Dynamic expression patterns of Bmp2 in the developing limb buds are altered in Dbf heterozygous mutant mice. (A, E, I) Bmp2 expression in the forelimbs of wild-type mice at 11, 11.5 and 12.5 d.p.c. respectively. (B, F, J) Bmp2 expression in the forelimbs of stage-matched Dbf heterozygous mutants. (C, G, K) Bmp2 expression in the hindlimbs of wild-type mice at 11, 11.5 and 12.5 d.p.c. respectively. (D, H, L) Bmp2 expression in the hindlimbs of stage matched Dbf mutants. In the Dbf heterozygous mutants, ectopic Bmp2 expression is detected in a broad band across the AP axis at the distal part of both the forelimb (B) and hindlimb (D, H). At 11.5 d.p.c., digit primordia can be seen in the forelimbs and Bmp2 expression starts to be detected in the interdigital area shown in E. At this stage, Bmp2 can also be detected in the metacarpal region of both the fore- and hindlimbs. In the forelimb of same stage mutant (F), Bmp2 expression can still be detected in a band-like domain in the anterior distal limb bud, where extra limb outgrowth is obvious and expression in the metacarpal region is narrowed and extends to both the anterior and posterior ends as a thin band. At 12.5 d.p.c., Bmp2 expression is detected in the interdigital area of both the wild-type fore- and hind-limbs (I, K) whereas in the mutants, Bmp2 expression is missing in some interdigital area shown by * (J, L). Arrows point to ectopic Bmp2 expression.

**Fig. 5.** Hedgehog targets are ectopically activated or repressed in the limb bud and branchial arches of embryos heterozygous for the Dbf mutation. Whole-mount in situ hybridization to 11.5 d.p.c. mouse embryos. (A, E, G) Wild-type embryos. (B, F, H) Dbf mutant embryos. Ptc, Ptc-2, Gli-1 are ectopically activated and Gli2 is ectopically repressed in the facial processes, the distal limb across the A-P axis and in the future femur region. Black arrows point to the ectopic activation (B, D, F) or repression (H). The yellow arrow in G points to the branchial arches where Gli-2 is strongly expressed.

Wild-type

Dbf/+
**DISCUSSION**

**Polydactyly and Ihh expression**

All of the previously characterized preaxial polydactyly mutants contain an ectopic anterior ZPA characterized by ectopic Shh expression. Here we show the first example of a polydactyly mutant, Dbf, which does not result from Shh expression. Rather, ectopic expression of another Hedgehog family member, Ihh, is detected in the distal limb mesenchyme. Thus, in agreement with studies in the chick (Vortkamp et al., 1996), Ihh, which is normally expressed later in limb development in association with cartilage development, is capable of altering limb polarity resulting in the formation of extra digits along the A-P axis, similar to those resulting from ectopic Shh activity.

All three Hh family members share a high degree of sequence similarity in the N-terminal region of the proteins. However, their comparative activities have not been rigorously addressed. For Shh, it has been shown in several assay systems that the signaling activity resides in the N-terminal part of the protein (Marti et al., 1995; Ericson et al., 1995; Roelink et al., 1996), which appears to encode the Shh and Ptc-2 sequence similarity in the N-terminal region of the proteins. However, their comparative activities have not been rigorously addressed. For Shh, it has been shown in several assay systems that the signaling activity resides in the N-terminal part of the protein (Marti et al., 1995; Ericson et al., 1995; Roelink et al., 1996), which appears to encode the Shh and Ptc-2 orthologue Hh, induce the expression of Ptc (Marigo et al., 1996; Marigo and Tabin, 1996; Goodrich et al., 1996; Bitgood and McMahon, 1995) which induces the expression of Ptc (Marigo et al., 1996; Marigo and Tabin, 1996; Goodrich et al., 1996; Bitgood and McMahon, 1995) which appears to encode the Shh and probably general Hh receptor (Stone et al., 1996; for review see Tabin and McMahon, 1997). Here we show that Ptc-2 is ectopically expressed in the Dbf mutant in a similar fashion as Ptc. As ectopic presentation of Shh protein to later limb cultures is able to influence cartilage development in a manner similar to Ihh (Vortkamp et al., 1996), it seems very likely that at least for Shh and Ihh, their activities may be similar, if not equivalent. This result may have some significance as Shh and Ihh expression overlap at several sites in the developing
Ectopic Ihh expression causes Dbf phenotypes

In Dbf mutants, the domain of ectopic Ihh expression is much broader and stronger than that of ectopic Shh expression at the anterior margin of other preaxial polydactyly mutants such as lst and Xt (Chan et al., 1995; Masuya et al., 1995). This difference most likely explains the difference between the Dbf phenotype and the phenotypes of all the other preaxial polydactyly mutations in the following aspects. (1) In Dbf mutants, there are generally more digits. It has been shown that a piece of ZPA tissue grafted to the anterior margin can lead to excessive growth of anterior mesenchyme in the chick limb (Cooke and Summerbell, 1981). It is thus likely that a broader polarizing activity due to the broader Ihh expression causes more extensive growth in the distal mesenchyme, eventually leading to the formation of larger number of supernumerary digits. (2) Compared to ectopic Shh expression in other preaxial polydactyly mutants such as Xt and Hx (Buscher and Ruther, 1998), ectopic Ihh expression in the Dbf mutant is broader and stronger. This may explain why Gli2 is ectopically repressed in the distal limb region whereas no change in Gli2 expression was observed in either Xt or Hx mutants (Buscher and Ruther, 1998). This is also consistent with the expression of Gli2 in normal limb which is absent from the Shh-expressing ZPA. Moreover, Gli2+/−:Gli3+/+ compound mutants have a more profound preaxial polydactyly when compared to Gli3+/− (Mo et al., 1997) embryos, indicating that Gli2 and Gli3 may share similar functions in Hedgehog signaling in the limb. (3) All of the digits in Dbf heterozygotes are arranged in a single group which is symmetric along the A-P axis. In contrast, in the heterozygotes of other preaxial polydactyly mutants the duplicated digits are often located anterior to the endogenous digit 1. Ihh ectopic expression appears to be uniform along the A-P axis at 11.5 d.p.c. except in the region of the endogenous ZPA where Shh itself is expressed. As recent evidence indicates that the concentration of Shh is the primary determinant of digit identity (Yang et al., 1997), approximately equivalent expression of similar polarizing activity across the A-P axis would predict the generation of digits with similar pattern. However, at 12-12.5 d.p.c., Ihh ectopic expression is stronger at the anterior margin. Therefore, the endogenous and ectopic polarizing activity appears to affect the posterior and anterior margin more than the apical limb tip. This conclusion is consistent with the observed pattern of digit duplication in

embryo, most notably in the gut endoderm (Bitgood and McMahon, 1995).

Fig. 7. Endogenous Ihh expression is not changed in both Dbf heterozygous and homozygous mutants. Whole-mount in situ hybridization was performed on embryos at 12.5 d.p.c. (A-C) Wild-type embryo; (A) lateral view, (B) ventral view, (C) hindlimb. At this stage, expression of Ihh in the hindlimb is very weak. (D-F) Dbf heterozygous embryo; (D) lateral view, (E) ventral view, (F) hindlimb. (G-I) Dbf homozygous embryo; (G) lateral view, (H) ventral view, (I) hindlimb. In both heterozygous and homozygous embryos, Ihh is still expressed in the cartilage as in wild-type embryos. Normal Ihh expression in the cartilage is indicated by black arrows. Ihh is also ectopically expressed in the branchial arches, dorsal and ventral metacarpal region and future elbow, indicated by purple arrows. The heterozygotes and homozygotes have broader and shorter facial processes as compared to wild-type embryos.
which the most anterior digit appears to adopt a posterior fate (Digit 5 fate). The other additional anterior digits all appear similar in morphology. (4) The development of hindlimb zeugopod and ankle joint is more severely affected. Both the tibia (anterior bone) and the fibula (posterior bone) are shortened and bent. Ectopic Ihh expression in the hindlimb bud of the Dbf mutant embryo extends more posteriorly and proximally than that of ectopic Shh in other preaxial polydactyly mutants, consequently, the fibula is also affected in the Dbf mutant.

Alterations in D-V polarity are associated with abnormal joint formation, but we observed no change in the expression of Lmx1b, a dorsal determinant (Riddle et al., 1995; Cygan et al., 1997) in Dbf mutants. Based on this observation, it is most likely that the joint abnormality in Dbf mutants is due to unbalanced A-P growth, such that the feet are twisted along the D-V axis. However, we can not rule out the possibility that expression of an unknown gene downstream of Lmx1b, which also serves to pattern the D-V axis, is altered in Dbf mutants.

Broad ectopic expression of Ihh in the distal mesenchyme and tail bud may also be the cause of other aspects of the phenotype such as swollen and smooth ventral limb surface and kinky tails. Indeed, we observed ectopic Ihh expression in the metacarpal region of all four limbs in Dbf mutants. One possible explanation is that the normal balance between growth and patterning is disrupted in the presence of ectopic Ihh. It has been shown that Bmp signaling in the interdigital area may regulate interdigital cell death (Zou and Niswander, 1996; Ganan et al., 1996; Yokouchi et al., 1996). Since in Dbf mutants Bmp2 expression in the distal anterior mesenchyme is changed and the interdigital expression disappeared in some areas, it is possible that this leads to the soft tissue webbing of the anterior digits or the formation of extra digits. In all of the Dbf mutant embryos we have analyzed, none displayed ectopic Ihh expression in the brain. Thus, the reported brain phenotype (Lyon et al., 1996) may be unrelated to the mis-regulation of Ihh or may result from ectopic expression at a different stage of development. Analysis of Dbf and Ihh double mutants will be required to further demonstrate that the phenotypes of Dbf mutants are solely due to Ihh ectopic expression.

Our genetic mapping data localized Dbf to a region approximately 1.3 cM away from the Ihh locus. In the mouse, this genetic distance corresponds to a physical distance of approximately 2600 kb (Lyon et al., 1996). The observation that ectopic Ihh expression is responsible for extra digit formation in Dbf mutants further suggests that the mutation is unlikely to be contained within the coding region of Ihh and we were unable to detect any restriction fragment length polymorphism after analyzing about 8 kb 5' and 8 kb 3' of the Ihh gene (data not shown). Thus it is not clear how the Dbf mutation causes ectopic expression of Ihh. Several possible models could explain the results. For example, Dbf may contain a DNA rearrangement which acts in cis to place Ihh transcription under the control of an enhancer element in a neighboring gene normally expressed in the distal limb, facial processes and tail. A precedent exists in which mutations affect gene expression over a long range. For example, it has been shown that two mutant alleles of steel (Sl), Steel-panda (Slpan) and Steel-contrasted (Sconf), contain DNA rearrangements located 100-200 kb upstream of the coding sequences (Bedell et al., 1995). Such a rearrangement would be consistent with the dominant phenotype. Alternatively, a cis-acting rearrangement may lead to the disruption of a negative regulatory region controlling Ihh expression. Dbf may also act in trans by inactivating a transcription factor which normally inhibits the expression of Ihh in the distal limb. Given the dominant phenotype, repression at the Ihh locus would have to be dose-sensitive in this model. Alternatively, ectopic expression of a transcription factor which directs the activation of Ihh could explain the results. Further mapping and characterization of Dbf will be required to distinguish between these possibilities. However, in view of the large genetic distances involved, and the strong likelihood that Dbf may result in a regulatory mutation, this may be a difficult task.
Interestingly, as the endogenous Ihh expression pattern does not seem to be affected by the Dbf mutation, it is likely that Dbf results in ectopic Ihh expression while leaving normal Ihh regulation intact.

In conclusion, our data suggest that the biological mediator of the Dbf limb phenotype (and probably the face as well) is ectopic Ihh signaling which at this stage mimics the polarizing activity of Shh. Whilst our study was under review, Hayes et al. (1998) published a study on Dbf mutants. Although both studies agree substantially on most of the data, they differ in the key data, which is, Ihh ectopic expression and interpretation. Hayes et al. invoked a novel (as yet unidentified) ligand for Smoothened to account for the ectopic polarizing activity, failing to address the possibility of another Hedgehog member’s involvement. In our view, ectopic Ihh activity simply explains both sets of data, including the non-cell autonomy of ectopic polarizing activity reported by Hayes et al. (1998).

We thank Benoit St-Jacques for molecular mapping of the Ihh locus, Karen Steel for providing the Pax3 probe and Sue Morse for animal care. We also thank Pao-Tien Chuang and Jenny Cygan for critical reading of the manuscript. Yingzi Yang is supported by a postdoctoral fellowship from the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation. This research is supported by EU contract no. CHRX-CT93-0181 and work in A. P. McMahon’s laboratory is supported by an NIH grant NS33642.

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