The chick limbless mutation causes abnormalities in limb bud dorsal-ventral patterning: implications for the mechanism of apical ridge formation

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SUMMARY

In chick embryos homozygous for the limbless mutation, limb bud outgrowth is initiated, but a morphologically distinct apical ridge does not develop and limbs do not form. Here we report the results of an analysis of gene expression in limbless mutant limb buds. Fgf4, Fgf8, Bmp2 and Msx2, genes that are expressed in the apical ridge of normal limb buds, are not expressed in the mutant limb bud ectoderm, providing molecular support for the hypothesis that limb development fails in the limbless embryo because of the inability of the ectoderm to form a functional ridge. Moreover, Fgf8 expression is not detected in the ectoderm of the prospective limb territory or the early limb bud of limbless embryos. Since the early stages of limb bud outgrowth occur normally in the mutant embryos, this indicates that FGF8 is not required to promote initial limb bud outgrowth. In the absence of FGF8, Shh is also not expressed in the mutant limb buds, although its expression can be induced by application of FGF8-soaked beads. These observations support the hypothesis that Fgf8 is required for the induction of Shh expression during normal limb development. Bmp2 expression was also not detected in mutant limb mesoderm, consistent with the hypothesis that SHH induces its expression. In contrast, SHH is not required for the induction of Hoxd11 or Hoxd13 expression, since expression of both these genes was detected in the mutant limb buds. Thus, some aspects of mesoderm A-P patterning can occur in the absence of SHH and factors normally expressed in the apical ridge. Intriguingly, mutant limbs rescued by local application of FGF displayed a dorsalized feather pattern. Furthermore, the expression of Wnt7a, Lmx1 and En1, genes involved in limb D-V patterning, was found to be abnormal in mutant limb buds. These data suggest that D-V patterning and apical ridge formation are linked, since they show that the limbless mutation affects both processes. We present a model that explains the potential link between D-V positional information and apical ridge formation, and discuss the possible function of the limbless gene in terms of this model.

Key words: AER, apical ectodermal ridge, D-V patterning, EN1, FGF8, limb development, limbless, SHH, Sonic hedgehog, WNT7A

INTRODUCTION

Development of the vertebrate limb depends on the establishment and maintenance of discrete signaling centers within the limb bud: the apical ectodermal ridge (here termed the ‘apical ridge’ or the ‘ridge’), a specialized ectoderm at the distal tip of the limb bud; the zone of polarizing activity (ZPA) in the mesoderm at the limb bud posterior margin; and the non-ridge ectoderm of the limb bud (reviewed by Hinchliffe and Johnson, 1980; Johnson et al., 1994; Tickle and Eichele, 1994; Martin, 1995). The signals that they produce act on mesodermal cells in the ‘progress zone’ at the distal tip of the limb bud (Summerbell et al., 1973) or their descendants, which give rise to most of the mesenchymal elements of the limb. In turn, the progress zone produces signals that maintain the apical ridge (reviewed by Hinchliffe and Johnson, 1980). The functions of the ridge, ZPA, and ectoderm were previously thought to be largely independent of one another, with the ridge providing signals required for outgrowth along the proximal-distal (P-D) axis, the ZPA producing a ‘polarizing’ signal that regulates patterning along the anterior-posterior (A-P) axis and the ectoderm supplying signals involved in patterning along the dorsal-ventral (D-V) axis. However, it is now clear that there are regulatory interactions among the different signaling centers and that their products work cooperatively to regulate limb outgrowth and patterning along all three axes. For example, signals from both the ridge and the dorsal ectoderm are required to maintain the activity of the ZPA and the ZPA in turn influences gene expression in the ridge (reviewed by Johnson et al., 1994; Tickle and Eichele, 1994; Martin, 1995).

Significant progress has been made towards understanding how these signaling centers are established and in identifying the molecules that mediate their activities. Moreover, it has become evident that the basic mechanisms of limb development and the signaling molecules involved have been evolutionarily conserved. In the chick, the signal that initiates limb...
development (the limb inducer) appears to emanate from the intermediate mesoderm at stage 15 (Stephens and McNulty, 1981; Streckler and Stephens, 1983; Geduspan and Solursh, 1992), and may be a member of the FGF gene family (Cohn et al., 1995; Ohuchi et al., 1995), possibly FGF8 (Crossley et al., 1996a; Vogel et al., 1996). One proposed function of this signal is to induce the expression of Fgf8 in the ectoderm overlying the prospective limb territory at stage 16 (Crossley et al., 1996a; Vogel et al., 1996). It has been suggested that ectodermal cells competent to respond to the inducer are localized at or near the border of regions with distinct dorsal and ventral positional values, and thus Fgf8 expression in the ectoderm may depend on appropriate D-V patterning (Crossley et al., 1996a). It has also been proposed (Mahmood et al., 1995; Crossley et al., 1996a; Vogel et al., 1996) that the FGF8 produced by the ectoderm is responsible for the initial outgrowth of the limb bud mesoderm as well as the induction at stage 17/18 of the expression of Sonic hedgehog (Shh), one of several vertebrate homologs of the Drosophila hedgehog gene, which is thought to be the polarizing signal produced by the ZPA (Riddle et al., 1993; Chang et al., 1994; López-Martínez et al., 1995).

The apical ridge becomes morphologically distinct in chick limb buds during stage 18 (Todt and Fallon, 1984). Removal of the ridge results in the absence of distal structures: the earlier in limb bud development the removal, the more extensive the truncation of the limb (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982). Thus an intact ridge is essential for continued outgrowth of the limb. Although a number of signaling molecules are expressed in the ridge, ridge-substitution studies have suggested that members of the FGF family of secreted signaling molecules are the key factors in ridge function (Niswander et al., 1993; Vogel and Tickle, 1993; Fallon et al., 1994; Crossley et al., 1996a; Vogel et al., 1996). FGFs produced in the ridge are necessary not only for stimulating the proliferation of progressive zone cells, but also for maintaining Shh expression and hence the patterning activities of the ZPA (Lauper et al., 1994; Niswander et al., 1994; Crossley et al., 1996a; Vogel et al., 1996). Such maintenance of Shh expression also requires a signal from the dorsal ectoderm. Wnt7a, a member of the large family of secreted signaling molecules related to Drosophila wingless, performs this function (Parr and McMahon, 1995; Yang and Niswander, 1995).

Wnt7a is also required for normal dorsal development of the limb, since mice lacking a functional Wnt7a gene have ventralized limbs (Parr and McMahon, 1995). The Lmx1 gene, a LIM homeobox-containing gene that is expressed in the dorsal mesoderm of the limb bud, also appears to play a role in dorsal development (Riddle et al., 1995; Vogel et al., 1995). Retrovirus-mediated ectopic expression of Lmx1 on the ventral side of chick limb buds causes dorsalization of the limb. Lmx1 appears to be a downstream target of Wnt7a signaling, since ectopic expression of Wnt7a in the chick limb bud induces ectopic Lmx1 expression in the ventral mesoderm, but the converse is not true. Moreover, removal of the dorsal ectoderm results in the loss of Lmx1 expression in the underlying mesoderm, suggesting that Wnt7a is required to maintain Lmx1 expression. A third gene that plays a role in D-V patterning of the limb is En1, a homeobox-containing gene related to Drosophila engrailed, which is normally expressed in the limb bud ventral ectoderm. Mice homozygous for a null allele of En1 develop limbs that are dorsalized, indicating that En1 function is required for normal ventral development of the limb (Loomis et al., 1996).

Chick limbless is an autosomal recessive mutation that causes a complete absence of limbs in homozygotes; heterozygotes have normal limbs (Prahllad et al., 1979). The early stages of limb bud formation appear to progress normally in limbless mutant embryos. Outgrowth is first evident at stage 17, and the mutant limb buds are indistinguishable from normal ones until stage 18, when apical ridge development fails. Outgrowth ceases at stage 19/20 and the mutant limb buds soon regress. The mutation appears to affect only the ectoderm, since recombinant limb buds consisting of wild-type ectoderm and mutant mesoderm can develop into a normal limb, whereas recombinants of wild-type mesoderm and mutant ectoderm do not (Fallon et al., 1983; Carrington and Fallon, 1988). Thus, it seems likely that the inability of the ectoderm to form a functional apical ridge is the primary cause of the inability of limbless embryos to form limbs.

Our goal in undertaking an analysis of gene expression in limbless mutant embryos was to test some of the ideas that have recently been proposed on the function of FGF8 in the developing limb bud, including its role in promoting the initial limb bud outgrowth and in inducing the expression of Shh in the limb bud. In the course of this analysis, we made the intriguing observation that mutant limbs rescued by local application of FGF have a dorsal feather pattern on both the dorsal and ventral sides (double-dorsal feather pattern). This prompted us to characterize the expression in mutant limb buds of genes known to play a role in determining D-V polarity, and led to the discovery that the limbless mutation causes abnormal expression of these genes in the early limb bud. This finding raises the possibility that the failure of the apical ridge to form in the mutant limb buds is the direct consequence of inappropriate D-V patterning. We discuss a model to explain how ridge formation may depend on the normal process of D-V patterning and speculate on the function of the gene altered by the limbless mutation.

MATERIALS AND METHODS

Experimental manipulation of chick embryos

Mutant and phenotypically normal embryos were produced by crossing heterozygous carriers of the limbless mutation. In each cross, one parent was inbred (back-crossed to the highly inbred UCD line 003; Abplanalp, 1992) and the other was a non-inbred White Leghorn. This cross provided vigorous embryos in a uniform genetic background.

The eggs were incubated at 38°C and the embryos were staged according to Hamburger and Hamilton (1951). The limbless homozygotes were identified at stage 19/20 by differences in limb bud morphology. Control embryos were the phenotypically normal siblings of the limbless embryos, referred to as ‘normal’ embryos.

Surgeries were performed in ovo, on embryos that had reached the stages indicated. In studies aimed at obtaining mutant limb buds at early stages of development, before they become morphologically distinct from normal limb buds, the membranes were pulled back to reveal a wing bud, and it was amputated using sharpened tungsten needles. The embryos from which the wing bud had been removed were then incubated until they reached stage 19/20, when they could
be identified as mutant or normal. In studies aimed at rescuing mutant limb development by application of FGF, heparin acrylic beads soaked in FGF protein (FGF-beads) were inserted into the mesoderm near the distal end of the wing bud of mutant and normal embryos at stage 18/19 as described in the text. Beads were soaked as previously described (Niswander et al., 1993) in either FGF4 (1 mg/ml in phosphate-buffered saline, kindly provided by Genetics Institute) or FGF8 (0.8 mg/ml, prepared as described by Crossley et al. 1996a and kindly provided by C. MacArthur). Embryos were incubated for 48 hours after bead application and then fixed for in situ hybridization assay of Shh RNA, or they were incubated for 7 or 10 days and then fixed in Bouin’s for histological analysis.

**RNA in situ hybridization**

For whole-mount RNA in situ hybridizations, embryos were isolated, fixed and processed following the protocol essentially as described by Nieto et al. (1995). For RNA in situ hybridizations on paraffin sections, the protocol of Neubüser and Balling (personal communication) was used. Antisense riboprobes were labeled with UTP-digoxigenin and detected with alkaline phosphatase-coupled anti-digoxigenin antibodies using BM purple (Boehringer Mannheim, Indianapolis, IN) as the substrate.

Antisense riboprobes were prepared using previously published chick clones: Fgf8 (Crossley et al., 1996a), Fgf4 (Niswander et al., 1994), Shh (Riddle et al., 1993; kindly provided by C. Tabin), Msx2 (Coelho et al., 1991a; kindly provided by W. Upholt), Bmp2 and Bmp4 (Francis et al., 1994; kindly provided by P. Brickell), Bmp7 (Houston et al., 1994; kindly provided by B. Houston), Hoxd11 and Hoxd13 (Izpisúa-Belmonte et al., 1991; kindly provided J.-C. Izpisua-Belmonte), Wnt7a (Dealý et al., 1993; kindly provided by A. M. C. Brown); En1 (Logan et al., 1992; kindly provided by A. Joyner) and Lmx1 (Riddle et al., 1995; kindly provided by R. Riddle).

**RESULTS**

**Molecular evidence for the absence of a functional apical ridge in limbless mutant limb buds**

The *limbless* mutant embryos are readily identifiable by the distinctive morphology of their limb buds at stage 19/20. The mutant limb buds not only lack a morphologically identifiable apical ridge but also appear to be flattened or slightly indented at the distal tip (see Fig. 1). As a first step in our analysis, we assayed mutant and normal embryos at stage 19 by whole-mount RNA in situ hybridization for gene expression that marks the normal ridge. Except where noted, the results were similar in both mutant wing and leg buds for all genes assayed. Moreover, in *limbless* embryos expression of each gene was detected in its normal domains (other than limb bud), thus providing a positive control for the assay.

In the normal chick limb bud, Fgf8 expression is detected along the entire A-P length of the apical ridge (Fig. 1A, and Mahmood et al., 1995; Crossley et al., 1996b; Vogel et al., 1996), whereas Fgf4 is expressed in the posterior two-thirds of the ridge (Fig. 1C, and Niswander et al., 1994; Laufer et al., 1994). Neither Fgf8 RNA (Fig. 1B) nor Fgf4 RNA (Fig. 1D) was detected in mutant limb buds. Ridge removal experiments and FGF-bead application studies have provided evidence that FGFs produced in the ridge maintain the expression of Shh (Laufer et al., 1994; Niswander et al., 1994), which is normally detected in the ZPA at the posterior margin of the limb bud (Fig. 1A, and Riddle et al., 1993). Consistent with this conclusion, Shh RNA was not detected in mutant limb buds at stage 19 (Fig. 1B).

**Fig. 1.** Comparison of gene expression patterns in normal and *limbless* mutant limb buds at stage 19. Analysis by whole-mount RNA in situ hybridization of the expression of the genes indicated, in normal and *limbless* mutant embryos at stage 19. (A-H) A dorsal view of the right wing bud (anterior is at the top); (I-L) a dorsal view of the trunk region (anterior is to the left). Arrowheads point to the anterior and posterior limits of gene expression within the apical ridge and arrows point to mesodermal expression domains that are visible in these photographs. Fgf8 and Shh were assayed in the same embryos using the same stain to detect both probes, but their expression domains (Fgf8 in the ridge, Shh in posterior mesoderm) are readily distinguishable. Note the absence of ridge marker expression in mutant limb buds. Expression of Msx2, Hoxd11 and Hoxd13, which is normally detected in mesoderm, is detectable in mutant limb buds.

Three members of the TGFβ superfamily of signaling molecules, Bmp2, Bmp4 and Bmp7, are normally expressed throughout the chick apical ridge (Fig. 1E and data not shown; also Francis et al., 1994). However, no Bmp2, Bmp4 or Bmp7 RNAs were detected in the ectoderm of mutant limb buds (Fig. 1F and data not shown). Likewise, expression of Msx2, a gene that encodes a homeodomain-containing transcription factor, which is normally detected along the length of the ridge (Fig. 1G, and Coelho et al., 1991a; Robert et al., 1991; Yokouchi et al., 1991), was not detected in the ectoderm of mutant limb buds (Fig. 1H). Similar results on Msx2 expression in *limbless* mutants were previously reported by Coelho et al. (1991b) and by Robert et al. (1991). Interestingly, it has been shown that Msx2 expression is induced in *limbless* mutant limb buds cultured in vitro in the presence of IGF-I or insulin; the effects of these growth factors on other molecular markers of the ridge have not been reported (Dealý and Kosher, 1996).

Thus six genes normally expressed in the apical ridge, representing three different gene families, are apparently not
expressed in mutant limb bud ectoderm. It is very unlikely that these negative results are due to a non-specific cause, such as degeneration of the ectoderm in the regressing limb bud, since Wnt17a expression was detected in mutant limb bud ectoderm (see Fig. 4). These data provide molecular evidence that limbless mutant limb buds fail to form a functional apical ridge.

**Evidence that early stages of mutant limb bud outgrowth occur in the absence of Fgf8 expression**

Based on expression and functional studies, it has been proposed that the initial outgrowth of the limb buds at stage 17 is stimulated by FGF8 produced in the ectoderm overlaying the prospective limb territories (Mahmood et al., 1995; Crossley et al., 1996a; Vogel et al., 1996). In normal embryos, Fgf8 expression is first detected in this ectoderm at stage 16. The fact that outgrowth of limb buds appears to be initiated normally in mutant embryos, but that Fgf8 is not detected in mutant limb buds at stage 19 raises the possibility that their initial outgrowth occurs in the absence of Fgf8 expression. Alternatively, Fgf8 could be responsible for the initial outgrowth of limb buds if it is transiently expressed in limbless mutant limb buds at or prior to the stage when limb bud outgrowth is initiated. To distinguish between these alternatives, we assayed for Fgf8 expression in prospective limb territories and early limb buds collected from mutant embryos.

As noted above, it is not possible to distinguish mutant from normal embryos until there has been substantial development of the limb buds. To circumvent this problem and to obtain mutant tissue at the appropriate stages for this analysis, we surgically removed the prospective forelimb territory (at stage 16) or the nascent wing bud (at stages 17-18) from one side of the embryo in ovo and fixed the amputated tissue for subsequent analysis of gene expression. The embryos from which tissue was removed were incubated to stage 19 or later in order to determine which were mutant homozygotes (see Fig. 2A).

In samples collected at stage 18, Fgf8 RNA was not detected in mutant wing buds (n=4), but it was detected in the nascent apical ridge of all normal wing buds examined (n=20; Fig. 2B,C). Similarly, in wing buds collected at stage 17, shortly after the initiation of outgrowth, no Fgf8 RNA was detected in any of the mutant limb buds assayed (n=6), whereas it was detected in most (10/13) normal wing buds (data not shown). In samples collected at the earliest stage of limb bud outgrowth (stage 16/17), no Fgf8 RNA was detected in any of the mutant wing buds (n=4) assayed (Fig. 2E). Since Fgf8 RNA was detected in only half (8/14) of the normal wing buds collected at this stage (Fig. 2D, and data not shown) this is presumably the stage at which Fgf8 expression is being initiated in the normal wing bud. These data suggest that there is no transient expression of Fgf8 in limbless mutant limb buds, and thus indicate that FGF8 is not required for the initial phase of limb bud outgrowth.

**FGF8 protein induces Shh expression in limbless mutant limb buds**

It has been proposed that Fgf8 expression in the ectoderm overlaying the nascent limb buds is required (in conjunction with other as yet unidentified factors) for the induction of Shh expression in the posterior limb mesoderm (Crossley et al., 1996a; Vogel et al., 1996). Since mutant limb buds apparently never express Fgf8, they provide a means of testing this hypothesis. As described above, we have found that Shh RNA is not detected in mutant limb buds at stage 19 (see Fig. 1B). To determine whether Shh might be transiently expressed in mutant limb buds at earlier stages of development, the amputated mutant wing buds described above were also assayed for Shh RNA (Fig. 2). To maximize the sensitivity of the assay for both genes, we used the same stain to detect Fgf8- and Shh-expressing cells, and relied on the fact that Fgf8 expression in the developing limb bud is restricted to the ectoderm and Shh to the mesoderm to distinguish the expression patterns of the two genes.

In previous studies, Shh expression in posterior mesoderm was first detected at stage 17/18 (Riddle et al., 1993; Lauder et al., 1994). In our experiments, Shh RNA was detected in only 9/24 normal wing buds collected at those stages (Fig. 2B, and data not shown). In contrast, no Shh RNA was detected in any of the 10 mutant wing buds collected at those stages (Fig. 2C). These data are consistent with the idea that Fgf8 gene expression is required to induce Shh expression during normal limb development.

To demonstrate that FGF8 is sufficient to induce Shh expression in mutant limb buds, we placed two beads soaked in recombinant FGF8 protein directly beneath the ectoderm of stage 18/19 wing buds, one at the distal tip and one on the posterior side (Fig. 2F). After 48 hours incubation, the treated mutant wing buds displayed substantial outgrowth and Shh RNA was readily detected in mesoderm near the wing bud distal tip (n=2; Fig. 2G). Bead application had no effect on Shh expression in normal wing buds (data not shown). Similar results were obtained with beads soaked in recombinant FGFR (n=4 mutant wing buds; all grew but only three expressed Shh; data not shown), a result consistent with the fact that both proteins have similar activities in different induction assays (Crossley et al., 1996a,b). These data provide support for the hypothesis that FGF8 is the endogenous inducer of Shh expression during normal limb bud development.

**Evidence that Sonic hedgehog is not required for the induction of Hoxd11 and Hoxd13 expression in the early limb bud**

Ectopic expression studies have demonstrated that Sonic hedgehog can induce the expression of Bmp2 (Laufer et al., 1994), as well as two HOX gene family members, Hoxd11 and Hoxd13 (Riddle et al., 1993; Lauder et al., 1994; Chang et al. 1994; López-Martínez et al., 1995) that are required for normal patterning of the limb skeleton (Dollé et al., 1993; Davis and Capecchi, 1994, 1996; Favier et al., 1995) and which are normally expressed in limb bud posterior mesoderm (Izpisúa-Belmonte and Duboule, 1992; Nelson et al., 1996). These results have led to the suggestion that expression of these genes is induced by SHH in the normal limb bud. Since mutant limb buds apparently do not express Shh, they provide a means of investigating whether Shh is required for the expression of Bmp2, Hoxd11 and Hoxd13.

In limb buds assayed at stages 19-22, Bmp2 RNA was detected in normal limb bud posterior mesoderm (Fig. 1E, and Francis et al., 1994) but was not detected in mutant limb buds (Fig. 1F), consistent with the hypothesis that expression of this gene is induced by SHH. The results of assays for Hoxd11 and Hoxd13 led to a different conclusion. Both Hoxd11 and Hoxd13 RNAs were detected in posterior mesoderm in normal
limb buds, with the level of Hoxd11 RNA being significantly higher in wing than in leg bud (Fig. 1I). Conversely, Hoxd13 RNA was more abundant in leg than in wing bud (Fig. 1K). In mutant limb buds, Hoxd11 RNA was readily detected in wing bud posterior mesoderm, and was detected, albeit at very low levels, in leg bud posterior mesoderm (n=7 embryos; Fig. 1J). Hoxd13 RNA was readily detected in mutant leg bud posterior mesoderm, but was not detected in wing buds (n=6 embryos; Fig. 1L). These data strongly suggest that Shh is not required for the induction of Hoxd11 and Hoxd13 expression in the early limb bud.

It is also noteworthy that although Msx2 RNA was not detected in the mutant limb bud ectoderm, it was detected in its normal domain in mutant limb bud anterior mesoderm (Fig. 1H and Coelho et al., 1991b; Robert et al., 1991). Together with our data showing Hoxd11 and Hoxd13 expression in posterior mesoderm, these results indicate that, despite the lack of a functional apical ridge and Shh expression, some aspects of A-P patterning of the early limb bud can occur in the limbless embryos.

The limbless mutation causes defects in limb bud D-V patterning

Since FGF4-beads are capable of substituting for the apical ridge in wild-type embryos (Niswander et al., 1993), we anticipated that they would rescue mutant limb buds. As expected, there was substantial development of mutant wing buds in embryos incubated for 7 or 10 days (to stages 36 or 39, n=2) after bead implantation. Analysis of the gross morphology (Fig. 3A-C) and transverse sections of rescued wings (data not shown) indicated that, in one case, a humerus and severely truncated radius and ulna formed, whereas in the other, the radius and ulna were more complete. The failure to form complete wings with digits can probably be accounted for by the observation that the FGF-beads generally did not remain at the distal tip of the wing buds as they grew out, but were displaced deep into the mesoderm (see Fig. 2G). In previous studies in which FGF-beads were applied to wild-type wing buds following ridge removal, complete distal development occurred only when the beads remained at the distal tip of the treated wing buds (Niswander et al., 1993, and unpublished observations).

The most striking feature of the rescued wings was that they appeared to be double-dorsal, at least with respect to feather formation. In the normal wing, feather buds are evenly distributed on the dorsal surface, whereas regions of the ventral surface lack feather buds (compare Fig. 3D and E). Furthermore, long primary flight feathers form at the posterior margin of both the dorsal and ventral mesoderm (Fig. 3F). In rescued limbless wings, the feather pattern on the ventral side appeared to be very similar to that on the dorsal side (compare Fig. 3A and B). For example, feathers resembling primary flight feathers were found at the posterior margins of both the dorsal and ventral surfaces (arrows in Fig. 3C). Perturbations of this type were never observed when FGF4-beads were used in ridge-replacement experiments in wild-type embryos (Niswander et al., 1993, and unpublished data).

These observations prompted us to assay unmanipulated mutant limb buds for the expression of genes involved in the specification of limb D-V patterning. In the chick limb bud, En1 expression is normally detected throughout the ventral ectoderm and in the ventral half of the ridge (Fig. 4A, and Davis et al., 1991; Davis et al., 1993). Expression of Wnt7a (Fig. 4B, and Dealy et al., 1993; Riddle et al., 1995; Vogel et al., 1995) and Lmx1 (Fig. 4C, and Riddle et al., 1995; Vogel et al., 1995) in normal embryos is restricted to the dorsal ectoderm and dorsal mesoderm, respectively. In stage 19 limbless mutant limb buds, we found that the expression of each of these genes was abnormal. En1 RNA was not detected in mutant limb buds, although expression at other sites in mutant embryos (e.g. the somites) appeared normal (Fig. 4D and data not shown). Wnt7a was detected in both dorsal and ventral ectoderm (Fig. 4E), and Lmx1 was detected in the dorsal and ventral mesoderm (Fig. 4F). These data indicate that the limbless mutation affects a gene that is required, directly or indirectly, for normal D-V patterning in the limb.

DISCUSSION

In this study, we have performed an analysis of gene expression in the limb buds of chick embryos homozygous for the limbless mutation. We found that a number of markers of the apical ridge, Fgf8, Fgfl, Bmp2, Bmp4, Bmp7 and Mx2, are not expressed in mutant limb bud ectoderm, providing support for the hypothesis that limb formation fails in the limbless embryo because of the inability of the ectoderm to form a functional ridge. We also obtained evidence that Fgf8 is never expressed in the ectoderm and that Shh is never expressed in the mesoderm of the nascent mutant limb buds. As discussed below, these observations have provided insight into the functions of FGF8 in early limb development, as well as the role played by SHH signaling in establishing patterns of gene expression in the limb bud. Moreover, the finding that Mx2, Hoxd11 and Hoxd13 are expressed in mutant limb bud mesoderm indicates that some aspects of A-P patterning are not affected by the mutation. More importantly, in the course of these experiments, we discovered an unexpected defect in the D-V patterning of mutant limb buds. We discuss a model that explains how limb bud D-V positional information and apical ridge formation may be linked. While this manuscript was being reviewed, similar results were published by Ros et al. (1996).

The role of FGF8 in early limb development

The initiation of limb bud outgrowth appears to involve the maintenance of a high rate of mesoderm cell proliferation in limb-forming regions and a concomitant decrease elsewhere along the length of the lateral plate mesoderm (Sears and Janners, 1971). The question of whether the signal(s) that regulate this differential growth are produced in the mesoderm itself or the overlying ectoderm cannot be answered by studying the effects of ectoderm removal, since the ectoderm rapidly regenerates at these early stages of limb bud formation (Sears and Zwilling, 1964). Recently, it has been observed that Fgf8 is expressed in the surface ectoderm just prior to the first sign of limb bud outgrowth, and there is a strong correlation between the domains of Fgf8 expression and the regions in which limb outgrowth occurs. Moreover, beads soaked in FGF8 protein can stimulate outgrowth of the lateral plate mesoderm in the interlimb region. These data identified FGF8 as a good candidate for a regulator of the initial outgrowth of
the limb bud, before the apical ridge assumes that function (Mahmood et al., 1995; Crossley et al., 1996a; Vogel et al., 1996).

However, we were unable to detect Fgf8 expression in either wing-forming territory or early limb buds of limbless mutant embryos. Since early outgrowth of the mutant limb buds is indistinguishable from that of normal limb buds, these data provide evidence that FGF8 is not required for the initial phase of limb bud outgrowth in limbless embryos. Although it is possible that Fgf8 is expressed at an extremely low level, or that the limbless mutation obviates the normal requirement for Fgf8 in the ectoderm, a more likely explanation is that some other molecule, possibly another FGF, produced in ectoderm and/or mesoderm regulates the initial outgrowth of the limb bud.

It has also been hypothesized that FGF8 expressed in the surface ectoderm overlying the nascent limb bud functions to induce Shh expression in posterior limb bud mesoderm. The evidence that induction of Shh expression is dependent on FGF comes from studies showing that Shh can be induced in anterior mesoderm of the established limb bud in response to retinoic acid only when an intact apical ridge or a source of FGF is present (Niswander et al., 1994). Among the FGFs known to be expressed in the early limb bud, FGF8 is the best candidate to date for the normal inducer of Shh expression. Its expression in surface ectoderm precedes Shh expression in mesoderm during development of normal and FGF-induced ectopic limb buds, whereas Fgf4 is expressed only after Shh expression is induced. FGF2 is apparently not required for any aspect of limb development, since the limbs are normal in mice homozygous for a null allele of Fgf2 (S. U. Grieshammer and others).
The role of Shh in regulating gene expression during early limb development

Based primarily on the results of studies in which Shh is ectopically expressed on the anterior side of the limb bud, it has been hypothesized that SHH induces the early phases of expression of at least two members of the HOX gene family, Hoxd11 and Hoxd13 (Lauf et al., 1994; López-Martínez et al., 1995). In contrast, studies of chick embryos homozygous for the talpid3 mutation have indicated that Hoxd13 is expressed in mutant limb anterior mesoderm in the absence of detectable Shh expression (Francis-West et al., 1995). Our studies of limbless embryos show that expression of not only Hoxd13, but also of Hoxd11, can occur in the absence of Shh expression. However, since the levels of Hoxd11 and Hoxd13 expression were substantially lower in mutant than in normal limb buds, the possibility remains that SHH may play a role in upregulating or maintaining expression of these genes.

It has also been suggested that SHH induces the expression of Bmp2 in posterior limb bud mesoderm (Lauf et al., 1994). Consistent with this hypothesis, we have found that Bmp2 is not expressed in limbless mutant limb buds, in which Shh is not expressed. Although these results are very suggestive, they do not provide conclusive evidence that SHH alone induces the expression of Bmp2 because other key signaling molecules, particularly FGF4 and FGF8, are also absent in mutant limb buds.

Since we cannot rule out the possibility that there is a small amount of Shh expression in mutant limb buds (enough to induce expression of Hoxd11 and Hoxd13, but not of Bmp2), confirmation of these conclusions must await the analysis of animals homozygous for a null allele of Shh. With this caveat in mind, what is particularly striking about our data is that they indicate that at least some aspects of A-P patterning can occur in the absence of both SHH and the signaling molecules that are normally expressed in the apical ridge.

A proposed link between D-V patterning and formation of the apical ridge

The failure of limb development in limbless embryos appears to be due to the inability of the ectoderm to form a functional apical ridge at stage 18. However, our molecular analysis shows that mutant limb buds are already abnormal at stage 16, when there is no induction of Fgf8 expression, a presumed marker of the prospective apical ridge, in the ectoderm overlying the limb territory. Since limb formation can be rescued in limbless embryos by substituting wild-type for mutant ectoderm at stage 15, the defect presumably resides in the mutant ectoderm (Fallon et al., 1983; Carrington and Fallon, 1988). One possible reason for this defect is a lack of competence to respond to the signal that normally induces Fgf8 expression in the ectoderm.

Previously, we suggested that, in the normal embryo, competence to express Fgf8 is restricted within the surface ectoderm to cells at or near the border of domains that have different D-V positional values (Crossley et al., 1996a). This hypothesis was based on the ‘boundary model’ for vertebrate limb development proposed by Meinhardt (1983a,b). Reasoning from what was known about limb formation in insects, as well as theoretical considerations, he postulated that signaling centers that play a key role in the control of limb...
development (e.g. the apical ridge) form only at boundaries between cells in differently determined territories, and that signal production is dependent on cooperative interactions between cells in the two different territories. Consistent with this idea, we observed that from the time it is first induced, Fgf8 expression in the ectoderm is restricted to a stripe that runs along the A-P axis of the embryo in a plane perpendicular to its D-V axis (i.e. at a potential border between a dorsal and a ventral domain). Moreover, when an FGF-bead is implanted in the interlimb region mesoderm, the Fgf8 expression that is consequently induced in the interlimb ectoderm is restricted to the same plane as the normal limb Fgf8 expression domain. Since the inducing signal, i.e. FGF from the bead, is not restricted to that plane, this suggests that the cells that are competent to respond to the signal are restricted within the surface ectoderm.

What is known about D-V patterning in the limb and our model for its role in normal limb bud development is summarized in Fig. 5A. We propose that normal D-V positional information is required for induction of Fgf8 expression, which may be an essential step in apical ridge formation. It is known that the D-V information that patterns the limb initially is derived from the D-V pattern of the mesoderm along the primary body axis. Thus, if stage 12 lateral plate mesoderm from the prospective wing territory is rotated 180° around its D-V axis and placed in the prospective interlimb region, a limb forms with D-V polarity that conforms to the orientation of the rotated mesodermal graft (Saunders and Reuss, 1974). In contrast, rotation of the ectoderm at stage 14 results in the development of limbs with normal D-V polarity (Geduspan and MacCabe, 1987). Between stages 14 and 16, the ectoderm in the prospective limb territory acquires D-V positional information from the underlying mesoderm. During these stages the mesoderm loses its capacity to program the D-V polarity of the ectoderm and also becomes responsive to cues from the overlying ectoderm. As the limb grows out, its D-V patterning is under the control of the limb bud ectoderm, which programs the underlying mesoderm as it differentiates into the mesenchymal elements of the limb. Thus ectodermal reversal at stage 16 results in formation of a limb with reversed D-V polarity. However, it is important to note that these effects of ectoderm on mesoderm D-V patterning are limited to the distal limb (Geduspan and MacCabe, 1987, 1989).

A key tenet of the model proposed here is that only those cells at or near the border between the dorsal and ventral domains are competent to respond to a limb-inducing signal, which originates in the intermediate mesoderm, and acts, directly or indirectly, to induce Fgf8 expression in the ectoderm (Crossley et al., 1996a). In accord with Meinhardt’s boundary model, this competence is presumably the consequence of local interactions between cells with dorsal and ventral identities. Once FGF8 is expressed in the ectoderm, it participates in the initiation of Shh expression in the mesoderm, which then induces the expression of genes such as Bmp2 in the mesoderm. By stage 18, the apical ridge, which is presumably composed of cells that began to express Fgf8 at stage 16, becomes morphologically distinct.

One prediction of this model is that, in limbless mutant embryos, there would be abnormal expression of the molecules responsible for the initial specification of D-V polarity in the ectoderm. This prediction cannot be tested at present since such molecules have not yet been identified. At present, the only genes that are known to function in the establishment of limb D-V polarity are Enl, Wnt7a and its downstream target Lmx1. The latter two genes clearly act at a late stage in this process, presumably in the transfer of D-V positional information from ectoderm to mesoderm. Wnt7a plays no role in specifying the D-V polarity of limb bud ectoderm, since molecular markers of dorsal and ventral limb ectoderm (such as Wnt7a and Enl) are expressed in their normal domains in mice homozygous for a null allele of Wnt7a (Parr and McMahon, 1995). The function of Enl in specifying D-V polarity in limb ectoderm is less clear, but it evidently acts downstream of the initial expression of apical ridge markers such as Fgf8 and Bmp2, and plays some role in regulating their expression in the ventral ectoderm (Loomis et al., 1996). In terms of our model, the concept that these genes play roles only in the late phase of D-V patterning is consistent with the observation that apical ridge formation and function appears to be relatively normal in Wnt7a and Enl mutant mice. Although the appropriate markers are not yet available, our data on the expression in limbless embryos of Enl, Wnt7a and Lmx1 do demonstrate that D-V patterning is perturbed in mutant limb buds. Specifically, we found that at stage 19, Wnt7a is expressed throughout most of the mutant ectoderm and Lmx1 is expressed throughout the mesoderm underlying the Wnt7a-expressing ectoderm cells, although it is perhaps noteworthy that the Wnt7a and Lmx1 expression domains do not extend to the proximal limit of the limb bud on the ventral side (see Fig. 4E,F), suggesting that there may be some residual ventral patterning in the mutant embryos. However, no Enl expression was detected in mutant limb bud ectoderm. These data thus are consistent with our model, in so far as they show that the limbless mutation affects both D-V patterning and apical ridge formation.

Speculations on the function of the gene altered by the limbless mutation

Although there is as yet no direct evidence for the hypothesis that the limbless gene plays a role in D-V patterning prior to the stage at which Fgf8 expression is normally induced, it is tempting to speculate on the function of limbless in normal limb development and where it might fit into the model described above (compare Fig. 5A and B). One possibility is that mutant mesoderm is defective during the earliest phase of limb D-V patterning. There are two reasons to think that this is not the case, although neither is compelling. First, recombinants between prospective limb bud mesoderm from mutant embryos at stage 15 and wild-type ectoderm develop into normal limbs (Fallon et al., 1983; Carrington and Fallon, 1988). Thus, at a stage when the mesoderm still has the capacity to influence the ectoderm, mutant mesoderm does not prevent the rescue of the limb by wild-type ectoderm. Second, it has been suggested that the D-V positional information in the mesoderm at early stages is defined by factors that determine the dorsal and ventral domains of the primary embryonic axis (Meinhardt, 1983a). However, other than the lack of limbs, limbless embryos do not display any defects that might be expected to occur if D-V patterning of the primary axis were abnormal (Prahlad et al., 1979).

In the model shown in Fig. 5B, we suggest that the limbless
For the sake of clarity, it is illustrated as being similar at all stages. Also note that the topographical relationship of dorsal and ventral domains changes between stages 12 and 16, but, induced. As a result, the appropriate D-V pattern makes the ectoderm incapable of responding to the limb-inducing signal from the IM and normal responsiveness of the mesoderm to patterning by the overlying ectoderm (which ectopically expresses Wnt7a) at this stage. As a consequence of this abnormal patterning, the limbs that do form when the mutant limb buds are rescued by application of FGF-beads are dorsalized. Although these ideas must remain speculative until the limbless gene is isolated, they provide a framework for designing experiments aimed at testing the role of D-V patterning information in the control of apical ridge formation.

We are extremely grateful to Han-Sung Jung and our laboratory colleagues for many helpful discussions and critical readings of the manuscript. We also thank Valerie Head and Linda Prentice for excellent technical assistance, and Nick Martin for assistance with digital imaging. This work was supported by the NIH Program of Excellence in Molecular Biology (G. R. M.), and funds from the Genetics Resources Conservation Program of the University of California. U. G. was supported by a fellowship from the California Division of the American Cancer Society. G. M. was supported by a Clinical Investigator Development Award from the National Cancer Institute.

Fig. 5. A model describing limb bud dorsal-ventral patterning and its relationship to apical ridge formation in normal and limbless embryos. The diagrams illustrate the prospective forelimb territory (at the level of somites [SO] 15-20) at stages 12 through 17/18. At stage 12, somites 17-20 have not yet differentiated from the segmental plate (SP). (A) In the normal limb bud at stage 12, the lateral plate mesoderm (LPM) has distinct dorsal and ventral identities (indicated by differences in shading). At this stage, the surface ectoderm (SE) has no D-V patterning information. At stage 15, the ectoderm is in the process of acquiring D-V information from the mesoderm. At this stage, a signal for limb induction emanates from the intermediate mesoderm (IM). At stage 16, this signal induces expression of Fgf8 in the ectoderm at or near the D-V border. At this stage, the mesoderm begins to acquire D-V patterning information from the ectoderm (depicted by a change in the colors of the mesoderm). At stage 17/18, limb outgrowth has been initiated and FGF8 induces Shh expression in posterior mesoderm. At stage 18, the apical ridge becomes morphologically distinct and expresses Fgf8. (B) The prospective limb territory of the limbless mutant embryo is indistinguishable from that of the normal embryo at stage 12. At stage 15, the ectoderm fails to acquire correct D-V patterning information from the mesoderm and is dorsalized. Consequently, at stage 16, the mesoderm is likewise dorsalized. The failure to establish appropriate D-V pattern makes the ectoderm incapable of responding to the limb-inducing signal from the IM and Fgf8 expression is not induced. As a result, Shh expression is not induced. The apical ridge fails to form. It is important to note that D-V patterning is a dynamic process and that this schematic diagram should not be interpreted as implying that the various steps in the process are necessarily complete at the stages indicated. Also note that the topographical relationship of dorsal and ventral domains changes between stages 12 and 16, but, for the sake of clarity, it is illustrated as being similar at all stages.
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(Accepted 2 September 1996)