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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; Strecker 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 ectoderm 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 responsible not only for stimulating the proliferation of progress zone cells, but also for maintaining *Shh* expression and hence the patterning activities of the ZPA (Laufer 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 (Prahlad 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* (Dealy 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).

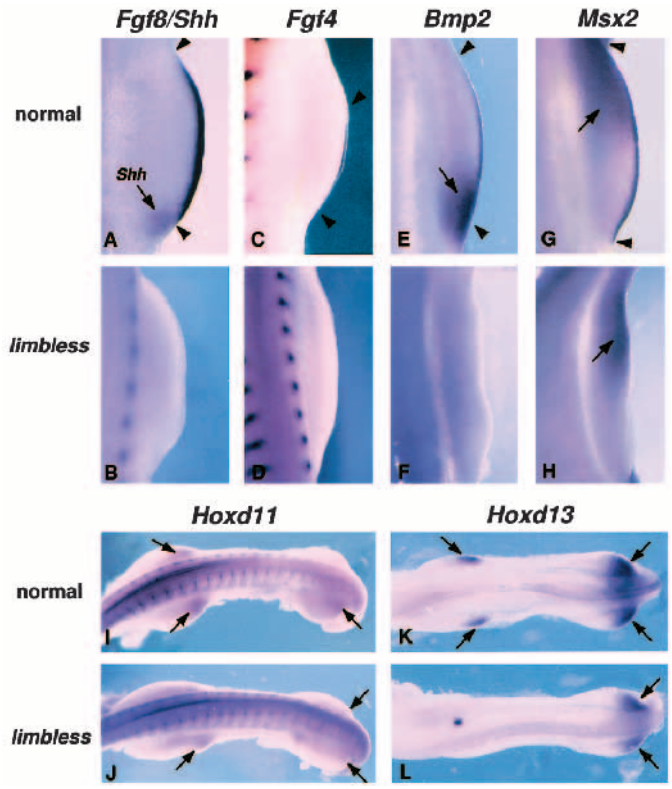

Fig. 1. Comparison of gene expression patterns in normal and *limbless* mutant limb buds at stage 19. Analysis by whole-mount RNA in situ hybridization of the expression of the genes indicated, in normal and *limbless* mutant embryos at stage 19. (A-H) A dorsal view of the right wing bud (anterior is 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 (Dealy 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 *Wnt7a* 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 overlying 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 overlying 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; Laufer 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 FGF4 (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; Laufer 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 the dorsal, but not the ventral wing surface (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; Gardner and Barald, 1992). 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, *Fgf4, Fgf8, Bmp2, Bmp4, Bmp7* and *Msx2,* 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 *Msx2, 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 (Searls 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 (Searls 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.

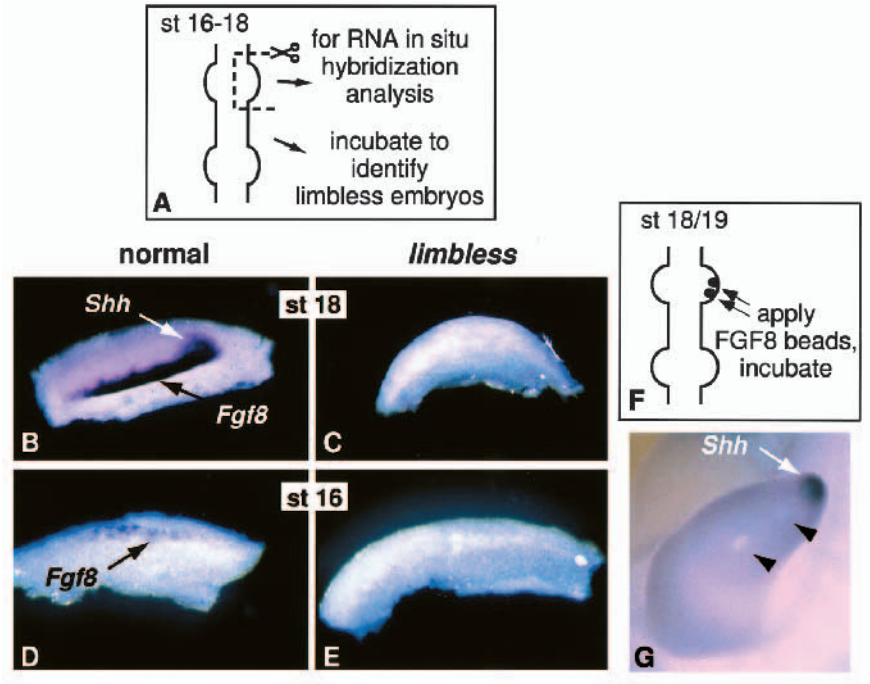

Fig. 2. *Fgf8* and *Shh* RNAs are not detected in *limbless* mutant limb buds at the early stages of outgrowth and FGF8 protein can rescue *Shh* expression in mutant limb bud mesoderm. (A) Diagram illustrating the method used to identify the mutant wing buds among those that were collected at stages 16-18, prior to the morphological manifestation of the *limbless* phenotype. (B-E) At the stages indicated, the right wing bud was surgically removed from embryos *in ovo* and processed for whole-mount in situ hybridization. The manipulated embryos were incubated until stage 19 or later, at which time mutant embryos could be identified by the morphology of the remaining wing and leg buds. Wing buds collected at stage 18 were hybridized with probes for both *Fgf8* and *Shh* (B,C), whereas those collected at stage 16 were hybridized with a probe for *Fgf8* alone (D,E). The expression domains of *Fgf8* and *Shh* in the normal embryos are indicated by arrows. No *Fgf8* or *Shh* expression was detected in any of the mutant wing buds analyzed. (F) Diagram illustrating the placement of FGF8-beads under the ectoderm of mutant limb buds. A slit was made through the ectoderm and into the mesoderm near the distal tip of the right wing bud of stage 18/19 embryos. Two beads soaked in FGF8 protein were placed into the slit at the locations indicated by filled circles. (G) The embryos were incubated for 48 hours, at which time the homozygous mutant embryos were easily identified and the rescued wing bud was analyzed for *Shh* expression by whole-mount in situ hybridization. The arrowheads points to the FGF8-beads in a rescued mutant wing bud.

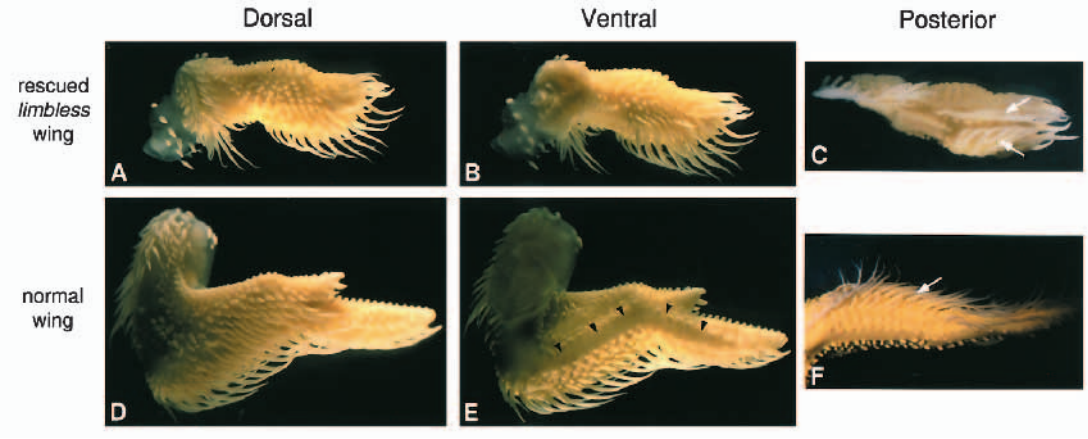

Fig. 3. The feather pattern of rescued *limbless* wings is double-dorsal. (A-C) The pattern of feather growth at stage 39 on a *limbless* mutant wing rescued by treatment with FGF4 beads is shown from the dorsal, ventral and posterior sides. The arrows in C point to two rows of primary flight feathers in the mutant wing, one originating on the dorsal and the other on the ventral surface. (D-F) Comparable views of a normal wing. The arrowheads in E, point to regions of the ventral wing surface that lack feather buds. In the view from the posterior side, one row of primary flight feathers, originating on the dorsal surface, is clearly visible (arrow in F). Note that the dorsal (A) and the ventral (B) surfaces of the mutant wing resemble each other, and that there is no area devoid of feather buds on the ventral surface of the mutant limb, as there is in the normal limb (E).

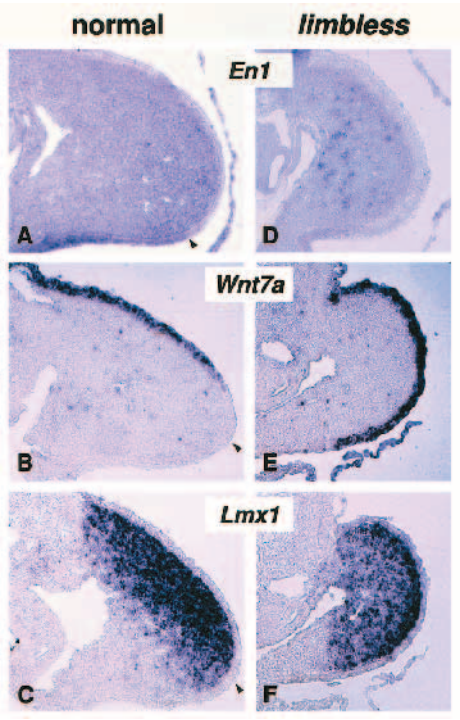

Fig. 4. Molecular markers of D-V identity indicate that *limbless* mutant limb buds are dorsalized at stage 19. RNA in situ hybridization was performed on transverse sections at the level of the wing bud of a normal (A-C) and a mutant (D-F) embryo at stage 19. Dorsal is up in all panels. The sections were hybridized with probes for *En*-1 (A,D), *Wnt7a* (B,E), and *Lmx-1* (C,F). The arrowheads in A-C point to the apical ridge. Note that expression of *Wnt7a* and *Lmx1* is restricted to the dorsal ectoderm and mesoderm of the normal limb bud, respectively. *En1* RNA is detected throughout the ventral ectoderm and in the ventral half of the ridge. In contrast, in the mutant limb bud, *En1* expression is not detectable, and *Wnt7a* expression in ectoderm and *Lmx-1* expression in mesoderm is extended ventrally, albeit not all the way to the proximal limit of the limb bud on the ventral side.

Ortega and C. Basilico, personal communication). Furthermore, beads soaked in FGF8 protein can fulfill the requirement for FGF activity in the ectopic activation of *Shh* expression by retinoic acid (Crossley et al., 1996a). In this study, we have found that *Shh* is not expressed in early limb buds of *limbless* mutant embryos, which do not express *Fgf8*. In addition, we have shown that *Shh* expression is induced in the mutant limb buds following application of beads soaked in FGF8 protein to the distal tip mesoderm. Together, these data support the idea that the *Fgf8* gene plays a key role in the initiation of *Shh* expression during normal limb development.

However, FGF8-mediated induction of *Shh* expression must involve as yet unknown posteriorizing factor(s), since in the normal limb bud *Shh* expression is restricted to posterior mesoderm whereas *Fgf8* is expressed along the length of the ridge. Since FGF protein is sufficient to induce *Shh* expression in mutant limb buds, it appears that they express the necessary posteriorizing factors. This conclusion is supported by the observation that mutant limb bud posterior mesoderm has the potential to express polarizing activity when grafted to host limb bud anterior mesoderm (Fallon et al., 1983; Ros et al., 1996).

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* (Laufer et al., 1994; López-Martínez et al., 1995). In contrast, studies of chick embryos homozygous for the *talpid*^{3} 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 (Laufer 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 *En1*, *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 *En1*) are expressed in their normal domains in mice homozygous for a null allele of *Wnt7a* (Parr and McMahon, 1995). The function of *En1* 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 *En1* mutant mice. Although the appropriate markers are not yet available, our data on the expression in *limbless* embryos of *En1*, *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 *En1* 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*

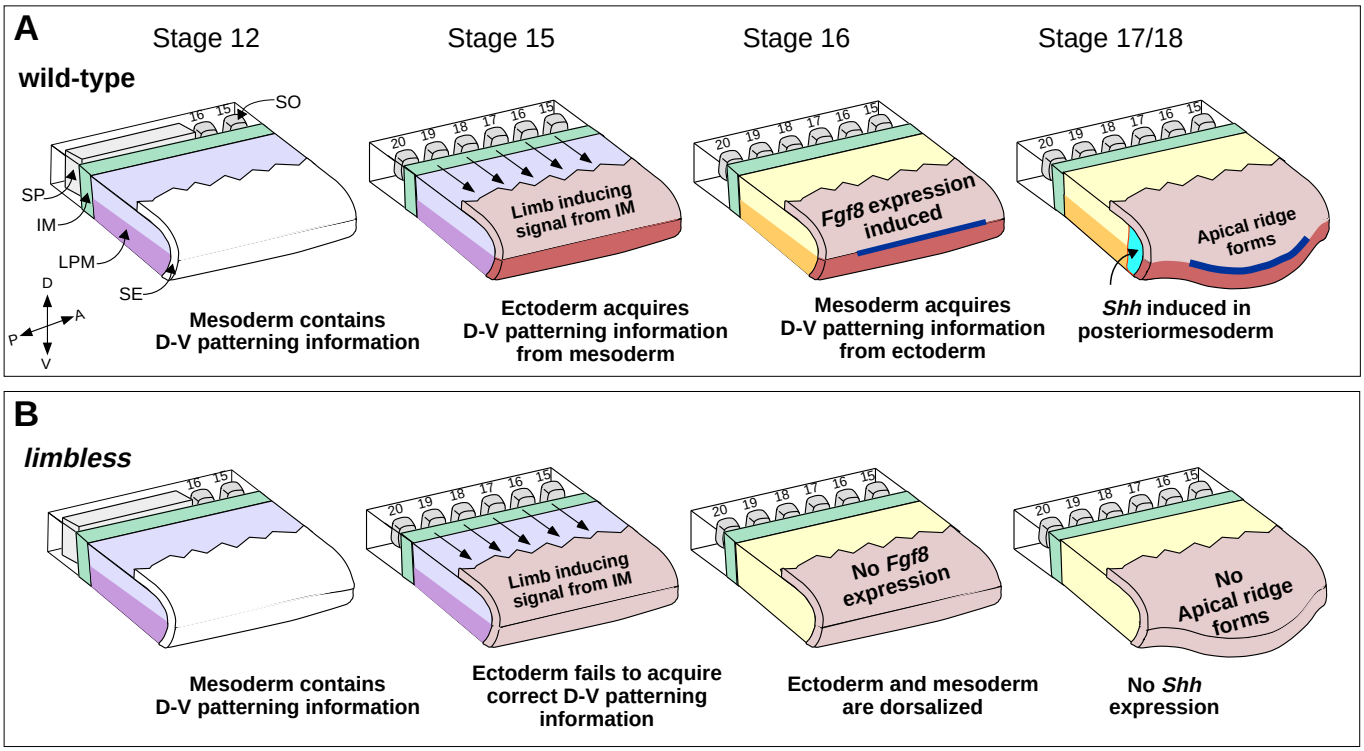

Fig. 5. A model describing limb bud dorsal-ventral patterning and its relationship to apical ridge formation in normal and *limbless* embryos. The diagrams illustrate the prospective forelimb territory (at the level of somites [SO] 15-20) at stages 12 through 17/18. At stage 12, somites 17-20 have not 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.

gene is required for the acquisition of the appropriate D-V patterning information by the ectoderm beginning at stage 14. Based on the finding that at later stages the ectoderm of mutant limb buds is dorsalized (no *En1*, and ectopic ventral *Wnt7a* expression), we further suggest that it is the acquisition of ventral information that is defective. In the absence of a border between cells with appropriate dorsal and ventral identities in the prospective limb bud ectoderm, there is no 'zone of competence,' and the ectoderm cells cannot respond to the signal from the underlying mesoderm that normally induces *Fgf8* expression at stage 16. The observed failure to express *Fgf8* in mutant limb buds is presumably an early manifestation of the ultimate failure of apical ridge formation, and apparently leads to a failure to express *Shh* as well as the genes that are downstream of it. The abnormal D-V patterning observed in mutant limb bud mesoderm at stage 19 (i.e. ectopic *Lmx1* expression) presumably reflects the 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.

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