Manifestation of the Limb Prepattern:
Limb Development in the Absence of Sonic Hedgehog Function

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The secreted protein encoded by the Sonic hedgehog (Shh) gene is localized to the posterior margin of vertebrate limb buds and is thought to be a key signal in establishing anterior-posterior limb polarity. In the Shh−/− mutant mouse, the development of many embryonic structures, including the limb, is severely compromised. In this study, we report the analysis of Shh−/− mutant limbs in detail. Each mutant embryo has four limbs with recognizable humerus/femur bones that have anterior-posterior polarity. Distal to the elbow/knee joints, skeletal elements representing the zeugopod form but lack identifiable anterior-posterior polarity. Therefore, Shh specifically becomes necessary for normal limb development at or just distal to the stylopod/zeugopod junction (elbow/knee joints) during mouse limb development. The forelimb autopod is represented by a single distal cartilage element, while the hindlimb autopod is invariably composed of a single digit with well-formed interphalangeal joints and a dorsal nail bed at the terminal phalanx. Analysis of GDF5 and Hoxd11-13 expression in the hindlimb autopod suggests that the forming digit has a digit-one identity. This finding is corroborated by the formation of only two phalangeal elements which are unique to digit one on the foot. The apical ectodermal ridge (AER) is induced in the Shh−/− mutant buds with relatively normal morphology. We report that the architecture of the Shh−/− AER is gradually disrupted over developmental time in parallel with a reduction of Fgf8 expression in the ridge. Concomitantly, abnormal cell death in the Shh−/− limb bud occurs in the anterior mesenchyme of both fore- and hindlimb. It is notable that the AER changes and mesodermal cell death occur earlier in the Shh−/− forelimb than the hindlimb bud. This provides an explanation for the hindlimb-specific competence to form autopodial structures in the mutant. Finally, unlike the wild-type mouse limb bud, the Shh−/− mutant posterior limb bud mesoderm does not cause digit duplications when grafted to the anterior border of chick limb buds, and therefore lacks polarizing activity. We propose that a prepattern exists in the limb field for the three axes of the emerging limb bud as well as specific limb skeletal elements. According to this model, the limb bud signaling centers, including the zone of polarizing activity (ZPA) acting through Shh, are required to elaborate upon the axial information provided by the native limb field prepattern.

Key Words: Sonic Hedgehog function; Shh; limb development; limb patterning; zone of polarizing activity; ZPA; stylopod; zeugopod; autopod; limb field.

INTRODUCTION

Limb pattern formation has been defined recently in terms of three signaling centers that control the three limb axes through specific signaling molecules and their downstream targets (reviewed in Ng et al., 1999; Schaller et al., 2001). The dorsal-ventral limb axis is controlled by the dorsal and ventral limb bud ectoderm through expression of Wnt7a and En-1, respectively (reviewed in Zeller and Duboule, 1997); proximal-distal axis elongation is controlled by fibroblast growth factor family members (FGFs) synthesized by the apical ectodermal ridge (AER) (reviewed

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in Martin, 1998); and the anterior–posterior limb axis is controlled by Sonic hedgehog (Shh) synthesized by a small group of mesodermal cells along the postaxial limb bud border called the zone of polarizing activity (ZPA). When grafted beneath the preaxial AER, either ZPA cells or the recombinant amino-terminal product of Shh (SHH-N)
(Lopez-Martinez et al., 1995; Yang et al., 1997) will induce mirror-image duplications in host wings (reviewed in Pearse and Tabin, 1998). This ability to induce extra digits to form is called polarizing activity (summarized in Tanaka et al., 2000) and SHH-N is sufficient to mediate this activity. The three signaling centers appear to be interdependent for both their maintenance and function (reviewed in Johnson and Tabin, 1997).

Presently it is thought that signaling by Shh occurs through the transmembrane proteins patched (Ptc) and smoothened (Smo). In the absence of Shh, Ptc inhibits Smo activity, whereas Shh binding to Ptc relieves this inhibi-
tion. Smo then transduces the signal causing changes in activity of the Gli gene products (vertebrate homologues of the Drosophila cubitus interruptus gene) and mediates downstream gene transcription (reviewed in Pearse and Tabin, 1998). Shh signaling results in the upregulation of Ptch and Gli1 expression in ZPA cells. This is followed by a rapid increase in levels of bone morphogenetic protein 2 (Bmp2) transcripts, and the 5’ Hoxd gene cluster (specifically, Hoxd11, d12, and d13) in the mesoderm adjacent to the ZPA (Nelson et al., 1996). Zuñiga et al. (1999) have shown that Shh signaling regulates the expression of Formin and the BMP antagonist Gremlin in the limb bud mesoderm adjacent to the ZPA and under the AER. These authors propose that the function of Formin is to mediate the expression of Gremlin, which carries out the interaction between the ZPA and the AER. While a feedback loop has been hypothesized between continued Fgf4 expression in the AER and Shh expression by ZPA cells (Pearse and Tabin, 1998), recent evidence from conditional knockouts of Fgf4 in the mouse AER indicates that Shh expression and normal limb development are not dependent on AER Fgf4 function (Moon et al., 2000; Sun et al., 2000).

A variety of experiments have been carried out in chick and mouse embryos to attempt to determine what effect the loss of the ZPA or Shh expression would have on limb development. Studies using chemical treatment or microsurgical manipulation (e.g., Bell et al., 1999; Pagan et al., 1996; Stratford et al., 1996) were not as informative as hoped because of potential nonspecific effects of the chemical treatment used and because microsurgical techniques may damage the AER and/or reduce the amount of mesoderm below a critical mass required for normal development. However, targeted disruption of the Shh gene also has been reported (Chiang et al., 1996); this provides the definitive opportunity to study the role of Shh in limb development without the caveats associated with microsurgery or chemical treatment. It was reported that Shh homozygous embryos show dramatic and specific developmental defects that correlate with the spatial and temporal expression of Shh and its proposed role in signaling networks. In the limbs, distal defects were noted. Here, we report in detail on the development of Shh-mutant mouse limbs.

**MATERIALS AND METHODS**

**Animals**

The generation and identification of Shh homozygous mutant mice and embryos are as described in Chiang et al. (1996). White Leghorn chick embryos of the Babcock strain were maintained at the University of Wisconsin. Chick embryos were staged according to the Hamburger and Hamilton series (Hamburger and Hamilton, 1951). Skeletal Staining and Histological Sections

The skin and viscera of 18.5-dpc embryos were removed and the embryos fixed in 95% ethanol. Cartilage and bone were stained with Alcian blue and alizarin red as described (Kochhar, 1973). For autopod histology, 18.5-dpc wild-type and Shh-/- fore- and hindlimb digits were fixed in Bouin’s fixative, embedded in JB-4 medium, and sectioned at 4-μm thickness with a JB4 Sorvall micromachine using glass knives. Sections mounted on glass slides were stained with methylene blue, azure II in 1.0% aqueous borax, cover slipped, and viewed. Early limb buds from 10.5-, 11.5-, and 12.5-dpc wild-type and Shh-/- mutant mice were fixed and processed in a similar manner. Sections of 1-μm thickness were made in a transverse cross section to the AER in anterior, mid, and posterior locations to permit visualization of anterior–posterior changes in AER structure.

**BrdU and TUNEL Analysis**

Forelimbs and hindlimbs of 10.5- and 11.5-dpc embryos were dissected with attached lateral tissue, which serves as a reference for anterior–posterior orientation of the limb. Limbs were dehydrated and embedded in paraffin. Serial sections, parallel to the proximal–distal axis of the limb, were collected onto glass slides. TUNEL and BrdU labelings were performed as previously described (Litingtung et al., 1998). Apoptotic cells were visualized by TUNEL according to the manufacturer’s specification (Intergen, New York).

**Whole-Mount In Situ Hybridization**

Embryos at 10.5 dpc were dissected from their extraembryonic membranes and fixed in 4% paraformaldehyde in phosphate-buffered saline. Whole-mount in situ hybridization was performed essentially as previously described (Henrique et al., 1995). The following probes were used: BMP2 (B. Hogan); Bmp4 and Gdf5 (S. Lee); Fgf4 and Fgf8 (G. Martin); Mxs2 (R. Maxson); Ptc1 (M. Scott); Ptc2, Gli1, and Gli3 (C-c. Hui); Hoxd11, Hoxd12, and Hoxd13 (D. Duboule); En-1 (A. Joyner); Wnt7A (A. McMahon); Formin (P. Leder); and Mxs1 (B. Robert).

**Zone of Polarizing Activity Grafts**

Mesoderm tissue was dissected from the posterior border of 10.5-dpc Shh-/- limb buds. A small piece of mesoderm tissue was then grafted under the anterior apical ridge of stage-18–20 chick host limb buds. Host embryos were allowed to develop to 10 days, fixed in 10% formalin, stained with Victoria blue, and cleared in methyl salicylate to visualize the cartilage patterns (Ros et al., 2000).

**RESULTS**

All vertebrate limbs have a similar structure composed of three proximal-to-distal segments: the stylopod (humerus or femur), the zeugopod (radius/ulna or tibia/fibula), and theautopod (wrist/hand or ankle/foot) (Stocum, 1995). While the proximal two limb segments exhibit relatively little variation across tetrapods, the autopod is highly variable.
\textbf{Shh^{-/-} Embryo Limb Morphology}

External morphology, terminal phalanx, and dorsal-ventral polarity. Fifteen Shh^{-/-} mutant embryos were compared with age-matched wild-type siblings (representative specimens shown in Figs. 1A and 1B). The mutant embryos were always smaller than wild-type littermates but each had four short appendages at the correct anatomical locations on the body. In every limb, at the gross level of observation, there appeared to be a single terminal phalanx that was similar to that of the wild-type digits (compare Figs. 1C and 1G with Figs. 1E and 1I).

The mutant forelimb terminal phalanx appeared conical when compared with the more claw-like wild-type forelimb structure (compare Fig. 1C with Fig. 1E). Histological sections (compare Fig. 1D with Fig. 1F) showed that, in fact, the mutant forelimb terminated in a single cartilaginous carpal-like element that did not form a joint with the next most proximal element and no nail bed was present. The distal element did not form a connection with the proximal element, as it could be separated when the skin of the forelimb was removed during preparation for skeletal staining. There were no external distinguishing dorsal-ventral characteristics visible on the mutant forelimb, however, in histological sections, as reported previously (Chiang et al., 1999; St-Jacques et al., 1998), there were incipient hair follicles marking the dorsal skin that were absent on the ventral surface.

The Shh^{-/-} mutant hindlimb terminal phalanx was similar to the corresponding wild-type structure (compare Fig. 1G with Fig. 1I). There appeared to be a ventral curvature to the digit and at least one ventral pad (VP; Fig. 1I) was always present; some specimens had two or three ventral pads. Histological sections (compare Fig. 1H with Fig. 1I) confirmed that there was a joined terminal phalanx, with a proximal nail fold, beginning nail growth, and other obvious dorsal-ventral characters including incipient dorsal hair follicles, dermal cell concentrations embodying the ventral pads, associated gland structures, and ventral tendons. A recent publication by Kraus et al. (2001) describes similar anatomy of the terminal element of the fore- and hindlimb of the Shh^{-/-} mutant mouse. Additionally, the authors show evidence that the dorsal epidermis of the hindlimb terminal phalanx expresses keratins specific to nail and hair keratinocytes. This complements the histological descriptions presented here identifying the terminal dorsal structure as a nail.

Forelimb skeletal anatomy. The limb skeletal patterns of seven mutant and wild-type 17.5-18.5-dpc embryos were analyzed (compare Figs. 2A and 2C with Figs. 2B and 2E). The pectoral girdle of the Shh^{-/-} mutant appeared to be normally formed. An identifiable scapula with coracoid process (not shown) and clavicle was present in all mutant specimens. The proximal part of the stylopod was identifiable as a humerus with a deltoid tuberosity and a humeral head that articulated with the scapula. No mutant specimens had a joint at the elbow; however, each forelimb showed a bend where the elbow is expected. The region of the bend was composed of cartilage while the single elements proximal and distal to the bend were ossified.

Hindlimb skeletal anatomy. The Shh^{-/-} mutant girdle appeared to be normally formed (compare Fig. 2C with Fig. 2E) with an ossifying os, pubis, and ischium. The mutant femur appeared normal with well-defined head, neck, shaft, and two condyles (cd). Two distinct but incomplete elements represented the Shh^{-/-} mutant zeugopod, one larger (Z-2) than the other (Z-1). The proximal ends of these elements were cartilaginous and formed a joint with femoral condyles. This was best seen in a ventral view (Fig. 2G). The middle of the two zeugopod elements invariably showed a cartilage fusion (asterisk, Fig. 2G) linking the two; the distal part of the larger element (Z-2) had begun ossification. In the context of the knee joint, these two truncated elements appear to represent the tibia (Z-2) and fibula (Z-1) bones. A single digit that consisted of a tarsal bone (t; Fig. 2E), the metatarsal (mt), and two phalanges represented the autopod of the leg (Figs. 2E and 2H). The proximal autopod elements had not begun ossification. However, the mutant terminal phalanx invariably showed ossification (compare Figs. 2C and 2D with Figs. 2E and 2F) that was morphologically similar to the wild-type terminal phalanx.

\textbf{Molecular Analysis of Digit Formation}

To analyze the character of the autopod structures further, we took two approaches. The first of these was to compare the expression of Gdf5, a bone morphogenetic protein family member whose expression occurs in the interphalangeal cells of the forming digital joints (Storm and Kingsley, 1996). In outgrowth of the mutant embryo forelimb, there was no detectable expression of Gdf5 at 14.5 dpc, at a stage when there is robust expression in the wild-type presumptive joints (compare Figs. 3A and 3B). This is consistent with our observations of the 18.5-dpc histology in which the terminal element of forelimb cartilage did not appear to have a typical jointed relationship with the proximal limb skeleton. The mutant hindlimb autopod interphalangeal cells, however, did express Gdf5 at 14.5 dpc (compare Figs. 3C and 3D). Interestingly, Gdf5 is only expressed in two domains in the forming digit, similar to wild-type digit one. The Gdf5 expression data provide a molecular foundation for the interpretation that the mutant hindlimb forms a digit and suggest that the forming digit represents digit one. We further demonstrated that Msx1 expression, which marks the nail bed cells on the dorsum of the terminal phalanx (Reginelli et al., 1995), was expressed similarly in normal and mutant hindlimb digits (Figs. 3G and 3H): Msx1 was undetectable in the mutant forelimb digit (Figs. 3E and 3F). We conclude that stylopod, zeugopod, and autopod elements form in Shh^{-/-} limbs.

To further explore the possible digit-one identity of the hindlimb digit suggested by Gdf5 results, we extended our analysis of digit formation by looking at the expression of...
Hoxd11–13 in the forming autopodial structures of wild-type and mutant limbs. Hoxd11–13 are expressed in a Shh-dependent fashion in the forming autopod of chicken and mice (Nelson et al., 1996; Shubin et al., 1997) and are thought to impart a dose-dependent mechanism for proliferation and growth of forming phalangeal structures (Zákány and Duboule, 1999; Zákány et al., 1997). Analysis of Hoxd11 and -d12 expression in 11.5- and 12.5-dpc wild-type fore- and hindlimbs show the characteristic phase II Hoxd gene expression along the posterior presumptive zeugopod (Figs. 4A, 4E, 4I and 4M) and initial autopod expression characteristic of phase III Hoxd gene (Nelson et al., 1996). A comparison with mutant limbs at this stage demonstrates an absence of Hoxd12 and -d13 phase II expression in both mutant fore- and hindlimbs (Figs. 4J, 4N, 4R, and 4V). Hoxd11 expression, however, is maintained at reduced levels in both fore- and hindlimbs. The absence of Shh-dependent Hoxd12 and d13 phase II expression correlates

**FIG. 3.** Molecular analysis of autopod pattern in: Gdf5 and Msx1. Expression of Gdf5 in the forming joints of forelimbs (A, B) and hindlimbs (C, D) in wild-type (A, C) and Shh<sup>−/−</sup> mutant (B, D) limbs at 15.5 dpc. Expression of Msx1 in the nail beds of forelimbs (E, F) and hindlimbs (G, H) in wild-type (E, G) and Shh<sup>−/−</sup> mutant (F, H) at 17.5 dpc. Note the patterned expression of Gdf5 and Msx1 in Shh<sup>−/−</sup> hindlimbs.
FIG. 4. Molecular analysis of autopod patterning: Hoxd11–13. Autopod expression of Hoxd11–13 in Shh−/− limbs. Whole-mount in situ hybridization of wild-type and Shh−/− mutant limbs with probes against Hoxd11 (A–H), Hoxd12 (I–P), and Hoxd13 (Q–Y) at 11.5 and 12.5 dpc. Hoxd11–13 genes are differentially expressed in the autopod of both wild-type and mutant limbs. The expression of Hoxd11 and -d12 genes does not extend to the most anterior digit one of wild-type limbs (A, E, C, G and I, M, K, O) while Hoxd13 expression is extended across the whole autopod (Q, U, S, X). Mutant forelimbs show no Hoxd11–13 expression in the autopod (B, D, J, L, R, and T). In contrast, mutant hindlimbs show extensive Hoxd13 expression throughout the autopod (V, Y) and lack detectable Hoxd11 and have reduced Hoxd12 (F, H, N, P).
with the anatomical loss of anterior–posterior polarization of the zeugopodial structures of both mutant limbs (noted above). The expression of Hoxd13 in the forming autopod of 12.5-dpc wild-type limbs encompasses the digit-one primordia (Figs. 4S and 4X), whereas Hoxd11 and -d12 expression is restricted posteriorly to encompass digits two through five (Figs. 4C, 4G, 4K, and 4O). In the mutant, Hoxd13 is expressed throughout the entire hindlimb autopod, but not in the mutant forelimb. In contrast, only distal low levels of Hoxd12 are seen in the mutant hindlimb and Hoxd11 is not detected. These data complement the Gdf5 analysis of joint formation and support the conclusion that the hindlimb digit is specified and represents digit one. The mutant forelimb shows no autopodial expression of Hox d11–13 consistent with its failure to realize distal phalangeal fates.

Shh^{−/−} Limb Buds Do Not Have Polarizing Activity

Polarizing activity can occur in the absence of Shh expression. For example, tissues expressing Indian hedgehog (Ihh) (Yang et al., 1998), as in the Doublefoot mutant mouse limb bud, will cause mirror-image duplications when grafted to a chick embryo host wing bud anterior borders (Hayes et al., 1998). We therefore examined whether the Shh^{−/−} mutant mesoderm had polarizing activity when grafted to a host chick limb bud. In controls, wild-type mouse ZPA grafted to the chick induced mirror-image duplications in 9 of 20 cases (45%); grafts of mouse ZPA tissue are less efficient in producing mirror-image duplications than chick ZPA grafts to chick wing buds (Fallon and Crosby, 1975; Tanaka et al., 2000). The digit sequence in response to wild-type mouse ZPA grafts in increasing order of complexity included four cases of 2234, two cases of 32234, one case of 2324, and two cases of 432234 (see Fig. 5A). In the Shh^{−/−} posterior border grafts, 14 grafts were made and all (100%) showed a normal digit pattern of 234 at day 10 (see Fig. 5B). These data indicate that the Shh^{−/−} limb buds do not have polarizing activity and that the zeugopod and autopod elements that form in the Shh^{−/−} mutant do so without the input of polarizing activity.

Polarized Gene Expression in the Limb Bud Does Not Require Shh Function

Shh activity in the posterior margin of the limb bud has been implicated in the establishment of polarized expression of 5’Hoxd genes in the mesoderm and Fgf4 in the AER. Ectopic expression of Shh protein in the anterior margins of chick limb buds induces ectopic mesodermal expression of 5’Hoxd and AER expression of Fgf4 (Pearse and Tabin, 1998). Interestingly, the limb buds of the chick limbless mutant do not express Shh, but express Hoxd11–13 in an asymmetric pattern (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996a). We therefore examined expression of genes implicated in Shh signaling to determine the effect of the Shh mutation on the molecular differentiation in the early limb bud. Several AER markers, including Fgf8 (Figs. 6C and 6D), Bmp4 (Figs. 6E and 6F), and Msx2 (data not shown), are expressed in 10.5-dpc Shh^{−/−} mutant limbs, although Fgf8 expression becomes reduced as development proceeds (see below). Fgf4 expression, which is restricted to the posterior two-thirds of the AER, is also present in the mutant (Figs. 6A and 6B; see also Zürišga et al., 1999), although its expression is detectable only in the hindlimb at 10.5 dpc in our mutant line.

Some of the genes normally expressed in the limb mesoderm were also present in the mutant, but generally did not have a normal distribution or normal levels of expression, whereas the expression of other genes was absent. Bmp4 is expressed in preaxial and postaxial Shh^{−/−} mutant limbs and shows a distribution comparable to that of wild type, but at reduced levels (Figs. 6E and 6F). Bmp2 expression is detectable in the mutant forelimb postaxial mesoderm, but at substantially reduced levels (Figs. 6G and 6H). In addition, the AER expression of Bmp2 is not detected in Shh^{−/−} mutant limbs (Figs. 6G and 6H). Similar to limbless limb buds, Hoxd11 and Hoxd12 are present in the postaxial mesoderm (Figs. 6I and 6J; and data not shown), but the extent of expression is limited to the postaxial border mesoderm of the mutant buds. We were not able to detect Hoxd13 in the Shh^{−/−} mutant forelimb buds, but it was detected at reduced levels and with greatly reduced distribution in the hindlimb postaxial mesoderm (Figs. 6K and 6L). Along with the 5’Hoxd genes, the proposed downstream targets of Shh signaling showed modified expression patterns. Ptc1 (Figs. 6M and 6N), Ptc2 (not shown), and Gli1 (Figs. 6O and 6P) were not detectable in the Shh^{−/−} buds. Formin, normally expressed in the posterior limb bud mesoderm (Fig. 6Q) and implicated in AER maintenance, was not expressed in Shh^{−/−} mutant limbs (Fig. 6R). This confirms a recent report by Zürišga et al. (1999). Gli3 also shows a modified expression pattern in mutant buds in that expression was detected throughout the bud extending to the border of the postaxial mesoderm (Figs. 6S and 6T), suggesting Shh normally represses Gli3 expression in the posterior mesoderm. A similar negative role of Shh on Gli3 transcription has been reported in chick micromass culture (Wang et al., 2000).

Dorsal–ventral polarity is controlled by the dorsal and ventral limb bud epithelia through the expression of Wnt7a and En-1, respectively. Both of these genes are expressed normally by the mutant dorsal and ventral epithelia (data not shown; see also Kraus et al., 2001). This was further indicated by the incipient dorsal hair follicles on both autopod structures as well as the ventral surface protrusions and tendons of the hindlimb digit that are described above (see Figs. 1F and 1J).

Shh^{−/−} Apical Ectodermal Ridge Structure and Fgf8 Expression

We next examined the interrelationship between Shh and AER maintenance by looking at a time course of Fgf8
expression in concert with histological analysis of AER structure during limb outgrowth. As described above in Figs. 6C and 6D (see also Kraus et al., 2001; Sun et al., 2000), Fgf8 was present in the AER of both the fore- and hindlimb of Shh mutants at 10.5 dpc. We extended this analysis up to 12.5 dpc to assess the relationship of Fgf8 expression and AER ridge structure in Shh mutant limb buds. Although Fgf8 expression was detected in mutant 11.5-dpc limbs, there was a distinct change in the height and organization of the AER when compared to control sections. This disorganization began in the AER as early as 10.5 dpc of both fore and hind mutant limb buds (Fig. 6D, and data not shown). In both the fore- and hindlimb, the reduction of Fgf8 expression correlated temporally with the change in AER morphology (compare Figs. 7I, 7M, 7K, and 7O with Figs. 7J, 7N, 7L, and 7P). This is exemplified by the 12.5-dpc mutant forelimb where regions of reduced Fgf8 expression in the middle bud exhibit no apparent AER structure (Figs. 7J and 7N), Sections of the posterior AER of the 12.5-dpc limb bud, showing persistent expression of Fgf8, maintained a stratified squamous AER morphology (Fallon and Kelley, 1977; data not shown). A comparison between fore- and hindlimb 12.5-dpc AER clearly showed that the AER was maintained longer in the mutant hindlimb and correlated with the formation of a complete digit (compare Figs. 7J and 7N, and Figs. 7L and 7P). At the same time, loss of ridge structure and signaling in the forelimb correlated with limb truncation.

Cell Death and Proliferation in Shh Mutant Limbs

In addition to the early reduction of AER structure in the mutant forelimb, we noticed a decrease in anterior expression of Fgf8 in both fore- and hindlimb buds of the Shh−/− mutant (arrow in Figs. 7B and 7D). It was thought that both of these observations could represent changes in either cell proliferation and/or cell death in the mutant limb that may affect positioning and/or maintenance of the AER and subsequently distal patterning of the limb. Therefore, we looked at the incorporation of BrdU as well as TUNEL labeling in Shh−/− and wild-type 10.5- and 11.5-dpc limb buds. Although there was a general decrease in proliferation in the mutant at 11.5 dpc (data not shown), there was not an obvious asymmetry in proliferation during these stages. However, mutant forelimb buds did show a significant increase in cell death in 10.5-dpc limb buds over either the hindlimb or wild-type limbs at comparable stages (Figs. 8A, 8B, 8E, and 8F). The TUNEL data are supported by the presence of apoptotic bodies in the 10.5-dpc mutant forelimb seen in histological sections (data not shown). By 11.5 dpc, the mutant hindlimb showed a similar increase in cell death as seen in the forelimb (Fig. 8H). Cell death in both mutant limb buds was concentrated in the anterior mesoderm, and coincided with asymmetric loss of Fgf8 expression in the mutant AER. No appreciable cell death was detected in wild-type limb buds (Figs. 8A, 8C, 8E, and 8G).

DISCUSSION

It is notable that Shh−/− mutant limbs show little variation in skeletal pattern from one embryo to another, indicating that a precise limb developmental program is replicated in all the Shh−/− mutants. Because of this, we propose that the skeletal elements present in the Shh−/− mutant limbs are specified in the limb-field mesoderm as a prepattern and subsequently are determined by the permissive action of the AER. It follows that specification of the three limb axes is initiated in the limb field in the absence of Shh. In this model, realization of the normal wild-type limb phenotype depends on the action of the three limb signaling centers in the context of the limb-field prepattern.

The early mutant limb buds appear morphologically normal, but the posterior border mesoderm does not express Ptc1, Ptc2, and Gli1, nor does it induce extra digits in a chick limb bud bioassay for hedgehog family members. Nevertheless, Hoxd11, -d12, and -d13 are asymmetrically expressed in the 10.5-dpc mutant postaxial limb bud mesoderm. The Shh−/− mutant limb bud mesoderm has the ability to induce and maintain an AER that permits elongation of the limb bud. The mutant AER transiently expresses Fgf4 (Zúñiga et al., 1999) and weakly expresses Fgf9 and -17 (Sun et al., 2000). However, Fgf8 expression appears normal in the early limb bud (Kraus et al., 2001; Sun et al., 2000; this report) and then declines coincident with the thinning and disruption of AER morphology. Given the apparent similarity of various Fgf activities in the limb, an integrated view of Fgf family expression will be required to understand the relationship between Fgf signaling and AER function (cf., Lewandoski et al., 2000; Moon et al., 2000; Moon and Capecchi, 2000; Sun et al., 2000).

Morphologically, the limb defect in Shh−/− is noticeable by 11.5 dpc, where both fore- and hindlimbs have a relatively narrow and pointed appearance (cf. Fig. 7). Previous studies have suggested that Shh may function as a survival factor in the neural tube, lung, and head mesenchyme (Ahlgren and Bronner-Fraser, 1999; Borycki et al., 1999; Litingtung et al., 1998). Similarly, in the limb, the absence of Shh leads to an increase in cell death primarily in the anterior region of the forming limb bud. This anterior cell death is reminiscent of the extensive anterior cell death in chick limb buds following removal of posterior mesoderm including the ZPA (Todt and Fallon, 1987). Interestingly, Sanz-Ezquerro and Tickle (2000) have reported that releasing SHH-N from a bead into the chick anterior limb bud mesoderm prevents normally occurring anterior necrotic zone cell death. These observations together with the cell death in the anterior mesoderm of the Shh−/− limb buds point to a role for the ZPA and Shh in anterior limb bud mesoderm cell survival.

With regard to patterning of limb elements, it appears that the zeugopod (humerus/femur) is completely specified, including anterior–posterior polarity, in the absence of Shh function. The autopod also develop in the absence of Shh, but are incomplete and lack normal
FIG. 5. Polarizing activity assay. (A) A stage-20 host chick wing bud received a sub ridge graft of wild-type mouse ZPA and formed a mirror-image duplication shown at 10 days after Victoria blue staining. The distal radius (arrow) is duplicated and the digital pattern is 4-3-2-2-3-4. (B) A normal wing that developed after Shh \(^{-2}\) postaxial border mesoderm was grafted under the host AER. The digital pattern is 2-3-4.

Our detailed analysis revealed that Shh \(^{-2}\) limbs, relative to normal limbs, are specified along the anterior–posterior, dorsal–ventral, and proximal–distal axes through the entire stylopod and become deficient in anterior–posterior patterning distally with only proximal–distal/dorsal–ventral polarity in the zeugopod and autopod. This indicates the necessary nature of Shh input at or just distal to the stylopod/zeugopod transition (elbow/knee levels) during limb development. The role of Shh in normal limb development would be to stabilize and expand the limb-field prepattern specifying those elements not found in the Shh \(^{-2}\) mutant buds. To do this, Shh must stabilize and amplify asymmetric gene expression arising from the limb field [e.g., Hoxd11, -d12 and -d13, -DHand (Charité et al., 2000; Fernandez-Teran et al., 2000); Formin, Gremlin (Zúñiga et al., 1999)] and add others [e.g., Hox11-13 paralogous genes (Nelson et al., 1996)]. Together with Shh-dependent proliferation, the regulation of patterning genes by Shh would result in the expansion of the limb prepattern to the full three-dimensional limb skeleton of the zeugopod and autopod. A similar phase-in of axial polarity control has been proposed for dorsal–ventral limb axis determination. Analysis of tissue-manipulation experiments, cell-lineage tracing in the chick, and of Imx1b-null mice has led to the proposal that stylopod dorsal–ventral limb polarity is determined before the limb bud forms. Subsequently, during the limb bud stages, the ectoderm controls zeugopod and autopod dorsal–ventral polarity through Wnt7a expression (reviewed in Chen and Johnson, 1999). Nelson et al. (1996) also proposed that the stylopod is a Shh-independent segment of the limb. Interestingly, these authors show a correlation in the expression of Shh and the differential and unique Hox paralogue gene expressions in chick zeugopod and autopod. This supports the notion of a context-dependent response of each limb segment to the Shh signal (Nelson et al., 1996).

The data from the Shh \(^{-2}\) mutant make it apparent that Shh is not necessary for the limb bud to emerge from the lateral plate. Similarly, Shh expression is not detectable in the limb buds of the limbless chick mutant. However,
limbless does not form an AER (no Fgf4 or Fgf8 expression) and is a bidorsal bud without a dorsal-ventral interface (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996b). The lack of an AER results in complete elimination of the limbless bud mesoderm by cell death after initial budding (Carrington and Fallon, 1988). This is a clear indication that the asymmetrically patterned limbless bud is unstable. If a wild-type AER (Carrington and Fallon, 1988) or exogenous FGFs (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996a) are supplied to the limbless bud, the limbless mesoderm is stabilized, Shh is expressed, and a normal limb skeleton develops. In both Shh-mutant and limbless mutants, there are anterior-posterior asymmetries of gene expression in the emergent limb. In addition, the postaxial limb bud border in the limbless mutant has the competence to express Shh when supplied with FGF; this is also a molecular and functional asymmetry of the emergent limb bud mesoderm. The observations on limbless chick and Shh-mutant mouse point to the conclusion that initial axial organization and emergence of the limb bud are indepen-

Fig. 6. AER and mesoderm gene expression in Shh-mutant limbs. Whole-mount in situ hybridization of 10.5-dpc wild-type (A, C, E, G, I, K, M, O, Q, S) and Shh-mutant (B, D, F, H, J, L, N, P, R, T) limbs with probe against Fgf4 (A, B), Fgf8 (C, D), Bmp4 (E, F), Bmp2 (G, H), Hoxd12 (I, J), Hoxd13 (K, L), Ptc1 (M, N), Gli1 (O, P), Formin (Q, R), and Gli3 (S, T). Note the arrowhead in (B) marks Fgf4 expression in the Shh-mutant AER. The arrow in (H) and (J) indicates the polarized expression of Bmp2 and Hoxd12, respectively, in the Shh-mutant limbs. The arrow in (Q) is to indicate the normal Formin expression in the wild-type forelimb. In (S), the asterisk marks the posterior mesoderm devoid of Gli3 expression in the hindlimb. The insets in (B) and (L) represent a higher magnification of the hindlimbs, while the insets in (E), (F), and (H) are of the forelimbs.
dent of the three signaling centers that are the hallmark of later limb development.

The conditions and the molecular mechanisms that determine the specifications of the limb-field prepattern are a major question for future studies; clearly, cues from the central body axis must play some role in the determination of the limb field (Coates and Cohn, 1998; Cohn et al., 1997). The hypothesis has been proposed that the expression of the Hox9 paralogous group of transcription factors is a critical component in this process. Cohn et al. (1997) show that prospective limb-field territories are marked within the lateral plate mesoderm by overlapping expression of Hox9 paralogues. This proposal is supported by experiments in which Hox9 expression patterns are altered when supern umery limbs are induced in the flank by grafting beads loaded with FGFs (Cohn et al., 1995; Ohuchi et al., 1995). Recently, Kawakami et al. (2001) reported that Wnt-2b and Wnt-8c are expressed in mesoderm medial to the chick wing and leg limb fields, respectively, and control Fgf10 expression in the emerging limb fields. Relating this information to how the particular limb specification events proposed here are achieved, i.e., the prepattern, is not presently apparent.

A recent study on the role of Shh in zebrafish pectoral fin development provides interesting confirmation and contrast to the data reported here. Shh is expressed along the posterior border of the zebrafish pectoral fin bud with associated expression of genes such as Ptch1 and -2, BMP2 (Akimenko and Ekker, 1995; Neumann et al., 1999), and 5′ Hoxd and Hoxa paralogues (Sordino et al., 1995). These are similar to patterns for tetrapod limb bud mesoderm as is the expression of BMP2 and Fgf8 in the AER analog of the fin bud called the apical ectodermal fold (Neumann et al., 1999). The latter authors have shown the sonic you mutant zebrafish, which lacks fin bud Shh expression, has transient anterior-posterior fin bud polarity in that Hoxd11 and -12 and Hoxa11 and -12 are asymmetrically expressed, but the Hox13 genes are not expressed. These data show some similarity to our observations in the Shh−/− mouse limb. However, in the sonic you mutant fin bud, the apical

FIG. 7. Fgf8 expression and structure of the AER. Fgf8 whole-mount in situ hybridization of 11.5- and 12.5-dpc wild-type (A, C, I, K) and Shh−/− (B, D, J, L) limb buds. Whole-mount hybridization specimens are compared to histological cross sections of AERs from comparably staged wild-type (E, G, M, O) and Shh−/− (F, H, N, P) mouse limbs. The approximate location of section is the middle of each bud. Forelimb (11.5 dpc: A, B, E, F; 12.5 dpc: I, J, M, N) and hindlimb (11.5 dpc: C, D, G, H; 12.5 dpc: K, L, O, P) are compared. Arrows in (B) and (D) indicate anterior regions where Fgf8 is not detected in the mutant compared to the wild-type sibling limbs. The asterisk in (J) marks posterior Fgf8 expression that maintains a tall ridge morphology.
ectodermal fold fails to form, resulting in the absence of endoskeleton formation of the pectoral fin (Neumann et al., 1999). In contrast, we have shown that, although its structure eventually is compromised, mouse AER formation is independent of Shh expression and its maintenance in the Shh$^{-/-}$ mutant permits the stabilization of limb skeletal elements. However, in the absence of Shh, expansion of the distal anterior–posterior axis of the limb bud fails to occur.

In summary, we propose that the ZPA, through the actions of Shh, in conjunction with the AER expands the limb-field prepattern along the limb bud anterior–posterior axis; Shh function becomes necessary at or just distal to the prospective elbow and knee joints. There is now a significant body of data that demonstrates the emerging limb bud is a triaxially polarized structure that requires an FGF or FGFs to become a stable entity. While the three-limb bud-organizing centers normally begin expression very early, even before bud emergence, they are not necessary for the initial triaxial limb bud organization. Moreover, several lines of evidence indicate that the stylopod is completely specified in the limb field. Because there is also competence to form recognizable but imperfect zeugopod and autopod elements without Shh input, the role of Shh in stimulating cell proliferation and survival of limb bud mesoderm assumes critical importance. It will be of great interest to dissect the integration of Shh, Wnts, Fgfs, and the Hox genes in permitting the realization of zeugopod and autopod development. This will lead to an understanding of how the competence to form initial zeugopod and autopod skeletal elements, determined in the limb field, is expanded to give these segments complete anterior–posterior polarity and identity.

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