Development 121, 1971-1978 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Polydactylous limbs in *Strong's Luxoid* mice result from ectopic polarizing activity

David C. Chan[1,2], Ed Laufer[1], Cliff Tabin[1] and Philip Leder[1,2]

[1]Department of Genetics, Harvard Medical School, and [2]Howard Hughes Medical Institute, 200 Longwood Avenue, Boston, MA 02115, USA

SUMMARY

***Strong's Luxoid* (*lst^D*) is a semidominant mouse mutation in which heterozygotes show preaxial hindlimb polydactyly, and homozygotes show fore- and hindlimb polydactyly. The digit patterns of these polydactylous limbs resemble those caused by polarizing grafts, since additional digits with posterior character are present at the anterior side of the limb. Such observations suggest that *lst^D* limb buds might contain a genetically determined ectopic region of polarizing activity. Accordingly, we show that mutant embryos ectopically express the pattern-determining genes *fibroblast growth factor 4* (*fgf-4*), *sonic hedgehog* (*shh*), and *Hoxd-12* in the anterior region of the limb. Further, we show that anterior mesoderm from mutant limbs exhibits polarizing activity when grafted into host chicken limbs. In contrast to an experimentally derived polydactylous transgenic mouse, forelimbs of homozygotes show a normal pattern of *Hoxb-8* expression, indicating that the duplication of polarizing tissue here occurs downstream or independently of *Hoxb-8*. We suggest that the *lst* gene product is involved in anteroposterior axis formation during normal limb development.**

Key words: *Strong's Luxoid*, limb mutant, limb development, polarizing activity, apical ectodermal ridge, mouse

INTRODUCTION

Classical developmental studies have shown that the anteroposterior axis of the chick limb is determined as early as stage 12, well before the limb primordia are morphologically distinguishable from the surrounding flank (Hamburger, 1938). Furthermore, when prospective limb mesoderm from stage 12 chicks is implanted under the flank ectoderm of a host chick, a supernumerary limb develops, which displays the anteroposterior characteristics of the donor tissue, regardless of its orientation with respect to the host (Saunders and Reuss, 1974). These results indicate that the anteroposterior axis is determined early in limb development and that this axial information resides in the mesoderm. Little, however, is known about the molecules that establish and maintain the polarity of the anteroposterior axis.

One important consequence of the determination of the anteroposterior limb axis is the induction of a zone of polarizing activity (ZPA, or polarizing region) in the posterior mesoderm of the limb (Tabin, 1991; Tickle and Eichele, 1994). When this polarizing region is grafted into the anterior mesoderm of a host chick limb bud, the resulting wing, instead of having the normal IV-III-II (posterior to anterior) digit pattern, has the symmetric pattern of IV-III-II-II′-III′-IV′ (′ indicates duplicated digits; Saunders and Gasseling, 1968). The polarizing region, perhaps by secreting a diffusible morphogen, is thought to provide positional information for mesodermal limb bud cells along the anteroposterior axis (Tickle et al., 1975). Cells close to the polarizing region acquire posterior identities, while cells far from the polarizing region acquire anterior identities.

Sonic hedgehog (*shh*), a vertebrate homolog of the *Drosophila* segment polarity gene *hedgehog*, is likely to be a key player in mediating the polarizing activity of posterior limb mesoderm. *Shh* is expressed in posterior limb mesoderm, in a domain coincident with the polarizing region (Riddle et al., 1993). *Shh* is also expressed in other embryonic tissues that display polarizing activity when implanted into chick limb buds, including the notochord, floor plate and Hensen's node (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994). Tissue culture cells infected or transfected with *shh*-expressing constructs acquire polarizing activity, as demonstrated by their ability to induce mirror-image digit duplications when implanted into the anterior mesoderm of host chick limb buds (Riddle et al., 1993; Chang et al., 1994).

The apical ectodermal ridge (AER), a specialized rim of limb ectoderm that is required for distal outgrowth of the underlying mesoderm (Saunders, 1948), is likely to play an indirect role in patterning the anteroposterior axis through its interactions with the polarizing region. Removal of the AER results in loss of polarizing activity (Vogel and Tickle, 1993) and *shh* expression (Vogel and Tickle, 1993; Laufer et al., 1994) in the posterior mesoderm. In the absence of the AER, maintenance of polarizing activity and *shh* expression can be provided by beads releasing fibroblast growth factor 4 (FGF-4; Vogel and Tickle, 1993; Laufer et al., 1994; Niswander et al., 1994), which is normally expressed in the posterior portion of the AER (Niswander and Martin, 1992).

In spite of these recent advances in our understanding of the patterning of the anteroposterior limb axis, very little is known about the earlier molecular mechanisms that establish and maintain anteroposterior asymmetry. *Hoxb-8* encodes a candidate molecule for playing such a role. This homeodomain-containing gene is expressed in the posterior mesoderm of day 9.5 mouse forelimbs, before the onset of *shh* or *fgf-4* expression, and disappears shortly thereafter (Charité et al., 1994). In transgenic mice, misexpression of *Hoxb-8* in the anterior mesoderm of forelimb buds results in mirror-image duplication of digits (Charité et al., 1994). Such digit patterns are similar to those observed in chick limbs containing a polarizing graft (ZPA graft) in the anterior mesoderm (Saunders and Gasseling, 1968). These ZPA-like duplications are preceded by ectopic expression of *shh* in the anterior limb mesoderm and *fgf-4* in the anterior portion of the AER. These results raise the possibility that *Hoxb-8* may normally be involved in activating the expression of *shh* and *fgf-4*, and thereby establish the polarizing region. However, *Hoxb-8* is clearly not required for maintenance of the polarizing region because its expression in the forelimb disappears shortly after day 9.5, and therefore is absent at the time of maximal polarizing activity. Furthermore, *Hoxb-8* does not appear to have an analogous role in the hindlimbs since its expression there is throughout the mesoderm (Charité et al., 1994). In addition to *Hoxb-8*, *fgf-4*, in cooperation with retinoic acid, may play a role in inducing the expression of *shh* (Laufer et al., 1994; Niswander et al., 1994).

One approach towards uncovering early patterning mechanisms in the limb is to study the molecular embryology of mouse mutants that show abnormalities in limb patterning. We have been analyzing the semidominant mouse mutant *Strong's Luxoid* (*lst*^{D}) (Forsthoefel, 1962, 1963). Mice heterozygous for *lst*^{D} have preaxial polydactyly (extra digits are anterior) in the hindlimbs. Mice homozygous for *lst*^{D} have preaxial polydactyly in all four limbs, as well as abnormalities in the preaxial long bones (radius and tibia) of the limb. We present evidence that polydactyly in *lst*^{D} mice results from the induction of additional AER containing posterior characteristics and a new polarizing region in the anterior region of *lst*^{D} limb buds. These results suggest that the *lst* gene product plays an important role in early anteroposterior patterning.

MATERIALS AND METHODS

Cartilage staining

Heterozygous mating pairs of *lst*^{D} mice were checked daily for vaginal plugs, with noon of the day of plug appearance designated as day 0.5. Embryos were dissected from extraembryonic membranes, fixed overnight in 4% paraformaldehyde, and stained with Alcian Green as described previously (Riddle et al., 1993), except that staining was done overnight. After clearing in methylsalicylate, limbs were dissected from the body to facilitate photography.

In situ hybridization

Embryos were obtained from matings between heterozygous *lst*^{D} parents and staged as described above. Whole-mount in situ hybridizations with digoxigenin-labeled riboprobes were performed as previously described (Riddle et al., 1993), except that 0.1% Tween 20 was used in the post-antibody washes. The in situ riboprobes have also been described previously: *fgf-4* (Hébert et al., 1990), *shh* (Echelard et al., 1993), *Hoxd-12* (Dollé et al., 1989), and *Hoxb-8* (Charité et al., 1994). Embryos were genotyped using a CA dinucleotide-repeat assay (Chan and Leder, unpublished results) on DNA extracted from extraembryonic membranes. The CA-repeat resides in the *limb deformity* locus and shows <10% recombination with *lst*^{D}. Control experiments (Chan and Leder, unpublished results) showed that genotypes could be reliably determined by morphological examination (Forsthoefel, 1963) of the limbs of day 11.5 and older embryos. Younger embryos show no morphological distinctions and must be genotyped molecularly. For the *Hoxb-8* studies, at least 5 embryos identified as homozygotes, using the CA-repeat assay, were examined for each stage (days 9.5, 9.75, 10.0, and 10.5). Since the CA marker shows <10% recombination with the *lst*^{D} locus, there is >99.9% probability that a homozygote was examined for each stage.

Polarizing grafts into chick limbs

Anterior mesoderm from day 11.5 mouse limb buds was transplanted into a slit made in the anterior region of stage 18-20 host chicken wing buds. Homozygous *lst*^{D} embryos were identified morphologically in litters from heterozygous parents. Homozygous forelimbs at this stage have a distinct anterior bulge (Forsthoefel, 1963), and mesoderm from the anterior margin of this bulge was used for the transplantations, since in situ hybridization (Fig. 2) showed *shh* expression there. As a positive control, grafts were performed using posterior mesoderm from these limbs. As a negative control, grafts were also performed with anterior limb mesoderm from embryos resulting from wild-type matings. For in situ hybridization, the chick embryos were harvested 36 hours after grafting and processed as described above. For skeletal analysis, embryos were harvested 7 days after grafting and stained with Alcian Green.

RESULTS

ZPA-like duplications in *lst*^{D} limbs

Mice heterozygous for the semidominant mutation *Strong's Luxoid* (*lst*^{D}) have preaxial polydactyly usually limited to the hindlimbs, while homozygotes have a more severe preaxial polydactyly in both the fore- and hindlimbs (Forsthoefel, 1962) (Fig. 1). To examine their digit patterns in more detail, we used Alcian Green to stain the cartilage of day 15.5 embryos obtained from matings between heterozygous *lst*^{D} parents. Our results are consistent with previous skeletal analyses of *lst*^{D} mice (Forsthoefel, 1962). The normal mouse foot has five digits, numbered V, IV, III, II, and I (posterior to anterior direction). Digits V to II each have three phalanges, whereas digit I has two phalanges. In heterozygous *lst*^{D} embryos, the most common hindlimb digit pattern is V, IV, III, II, I, II′ (Fig. 1B,E; ′ denotes extra digit). The forelimbs are generally normal (not shown). The exact identity of the extra digits in homozygotes often could not be determined, but forelimbs of homozygotes usually contain one or more triphalangeal digits positioned anterior to the biphalangeal pollex, or digit I (Fig. 1C,F). Homozygous hindlimbs usually lack digit I and contain six or more triphalangeal digits (Fig. 1D). These digit patterns, in which posterior digits are duplicated in the anterior portion of the limb, resemble those obtained after grafts of polarizing tissue are transplanted into the anterior mesoderm of chick limb buds (Saunders and Gasseling, 1968).

Ectopic expression of *shh*, *fgf-4*, and *Hoxd-12*

To explore the possibility that *lst*^{D} limb buds might contain an ectopic polarizing region, we used whole-mount in situ hybrid-

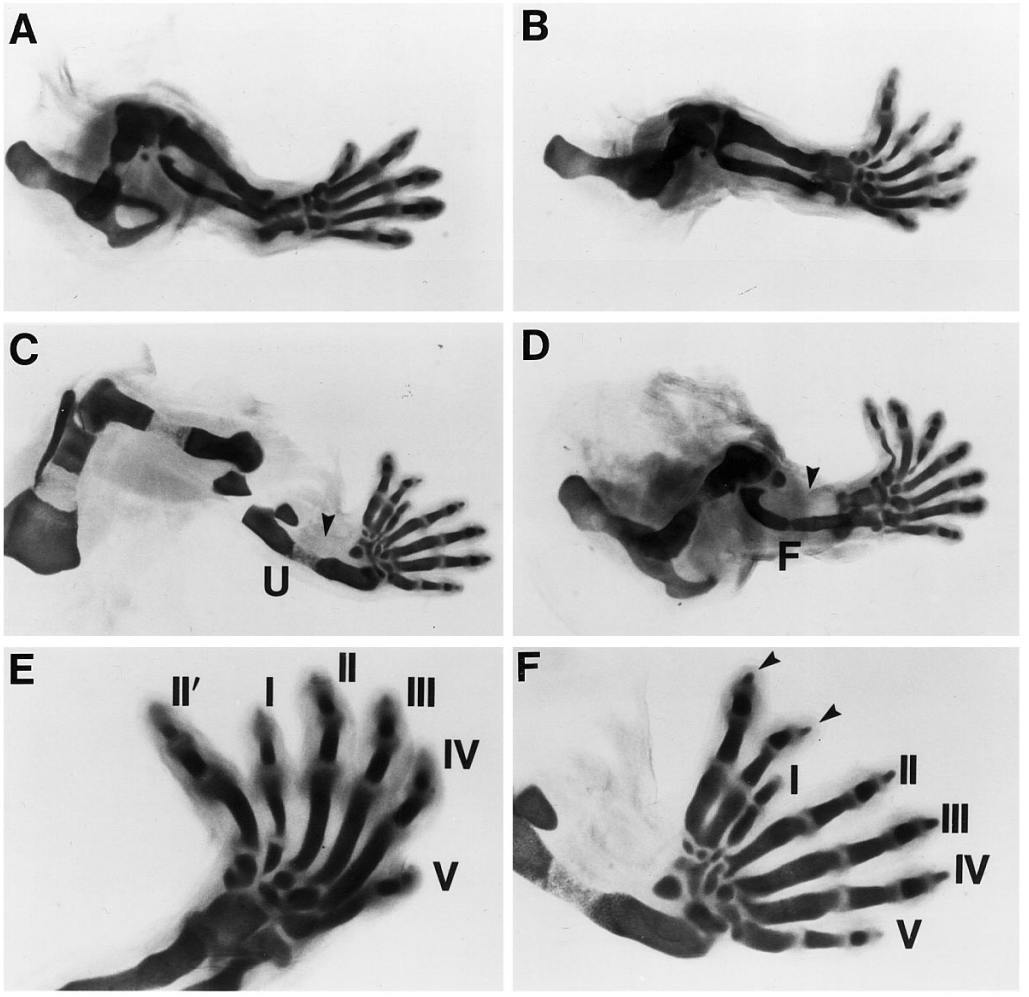

Fig. 1. Polydactylous limbs in *lst*^{D} embryos exhibit extra anterior digits with posterior identity. Alcian Green was used to stain the cartilage of day-15.5 embryonic limbs. (A), Wild-type hindlimb. (B) Heterozygous hindlimb. (C) Homozygous forelimb. Arrowhead indicates reduction of radius, which is common in homozygotes. (D) Homozygous hindlimb. Arrowhead indicates reduction of tibia. (E) Higher magnification of B, with digit identities, as deduced by examination of carpal elements (Hinchcliffe and Griffiths, 1983), labeled with Roman numerals. (F) Higher magnification of C, showing two triphalangeal digits (arrowheads) positioned anterior to digit I. U, ulna; F, fibula. The embryos shown are littermates.

ization on mutant and wild-type embryos to examine the expression of *fgf-4* (Niswander and Martin, 1992), *shh* (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al., 1994), and *Hoxd-12* (Dolle et al., 1989), which are molecular markers for, respectively, the posterior apical ectodermal ridge (AER), the zone of polarizing activity (ZPA), and posterior limb mesoderm. With these probes, we found three in situ staining patterns resulting from the three genotypes generated from matings between heterozygous *lst*^{D} parents. Wild-type embryos showed staining patterns consistent with published reports. *Fgf-4* is expressed in the central and posterior portion of the AER, most intensely at embryonic day 10.5 (not shown) and fading by day 11.5 (Niswander and Martin, 1992) (Fig. 2A). *Shh* is expressed in posterior limb mesoderm coincidently with the ZPA (Echelard et al., 1993; Riddle et al., 1993; Fig. 2A); *Hoxd-12* is restricted to posterior and distal mesoderm (Dollé et al., 1989; Fig. 2H).

Heterozygous *lst*^{D} embryos show normal staining patterns for these genes in the forelimbs and in the posterior half of the hindlimbs. However, they show ectopic expression of *fgf-4*, *shh*, and *Hoxd-12* in the anterior region of the hindlimbs, starting at day 11.5 (Fig. 2B,I). *Fgf-4* is ectopically expressed in a strip of anterior AER in day 11.5 hindlimbs, by which time the normal expression of *fgf-4* in the posterior AER has started to disappear (Fig. 2B and data not shown). At the same time, *shh* is ectopically expressed in a small patch of anterior mesoderm that underlies the strip of *fgf-4* ectopic expression (Fig. 2B and data not shown). *Hoxd-12* is ectopically expressed in a broader region of the anterior mesoderm (Fig. 2I).

Homozygotes show ectopic expression of limb markers in the anterior portion of all limbs (Fig. 2C,J), whereas expression patterns in the posterior portion of the limbs are normal. Ectopic expression of *fgf-4* is first observed at day 10.5 in an anterior strip of AER in the forelimb, resulting in two discontinuous strips of *fgf-4* production (Fig. 2D). As in heterozygotes, these homozygotes do not show ectopic expression of *fgf-4* in the hindlimb until day 11.5 (Fig. 2C,F), probably reflecting the normally delayed development of murine hindlimbs relative to forelimbs. By late day 11.5, the homozygous *lst*^{D} limb buds show a distinct anterior outgrowth from which the extra digit(s) will form (Forsthoefel, 1963). At this time, *fgf-4* is expressed in the AER at the anterior border of this bulge (Fig. 2E,F), whereas the wild-type expression in the posterior AER has largely faded. Mutant fore- and hindlimbs also express *shh* ectopically in a small patch of anterior mesoderm that directly underlies and extends anterior to the ectoderm expressing *fgf-4* (Fig. 2E,G), such that this juxtaposition of *fgf-4* and *shh* is in mirror-image relationship to their normal day 10.5 expression domains in the posterior region (Laufer et al., 1994). The onset of *shh* expression is delayed

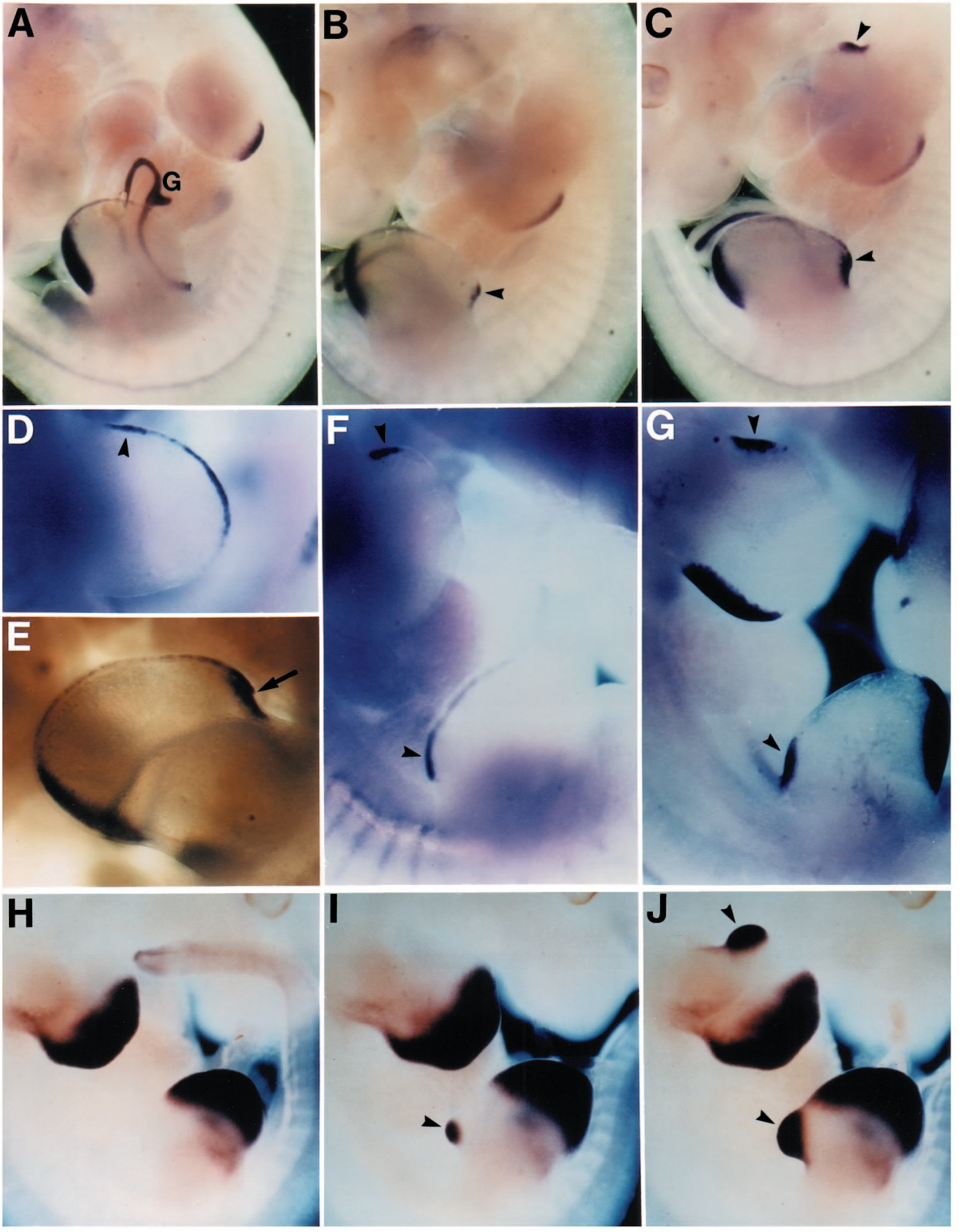
A
G
B
C
D
E
F
G
H
I
J

Fig. 2. The anterior limb region of *lst*^{D} embryos ectopically expresses *fgf-4*, *shh*, and *Hoxd-12*. The expression of these genes was examined by whole-mount in situ hybridization. In A, B, and C day 11.5 embryos were hybridized simultaneously to riboprobes for *fgf-4* and *shh*. (A) Wild-type embryo, with expression limited to the posterior region of the fore- and hindlimbs. G, gut. (B) Heterozygote showing ectopic expression (arrowhead) of both *fgf-4* and *shh* in the hindlimb. (C) Homozygote showing ectopic expression (arrowheads) of *fgf-4* and *shh* in both the fore- and hindlimbs. (D) Forelimb of day-10.5 homozygote stained with a riboprobe for *fgf-4*. A strip of anterior ectoderm (arrowhead) shows expression. (E) Higher magnification of hindlimb from the *lst*^{D} homozygote in C, showing expression of *shh* in the mesoderm directly underlying and extending anteriorly to the ectoderm expressing *fgf-4*. The anterior limit of *fgf-4* expression in the ectoderm is marked by the arrow. Although this limb bud has been hybridized simultaneously with both *fgf-4* and *shh* riboprobes, the individual staining patterns of these two genes can be unambiguously identified because *fgf-4* is restricted exclusively to the ectoderm, whereas *shh* is restricted to the mesoderm (for example, see F and G). (F) Day 11.5 homozygote stained with *fgf-4* riboprobe alone. Arrowheads indicate ectopic expression in the anterior portion of the AER. (G) Day 11.5 homozygote stained with *shh* riboprobe alone. Arrowheads indicate ectopic expression in the anterior mesoderm of the limb bud. In H, I and J, day 12.0 embryos were stained with *Hoxd-12*. (H) Wild-type embryo; (I) heterozygote showing ectopic expression (arrowhead) in the hindlimb; (J) homozygote showing ectopic expression (arrowheads) in both the fore- and hindlimbs.

relative to *fgf-4* (data not shown), raising the possibility that *fgf-4* may play a role in inducing *shh* expression (Laufer et al., 1994; Niswander et al., 1994). Interestingly, the ectopic staining patterns in homozygous hindlimbs were significantly more intense than in heterozygous hindlimbs (compare Fig. 2B with 2C, and Fig. 2I with 2J), consistent with the more severe polydactyly in homozygotes. *Hoxd-11* (not shown) and *Hoxd-12* are ectopically expressed in a broad region of anterior mesoderm in day 11.5 limbs (not shown), and in later embryos are expressed throughout the anterior bulge (Fig. 2J).

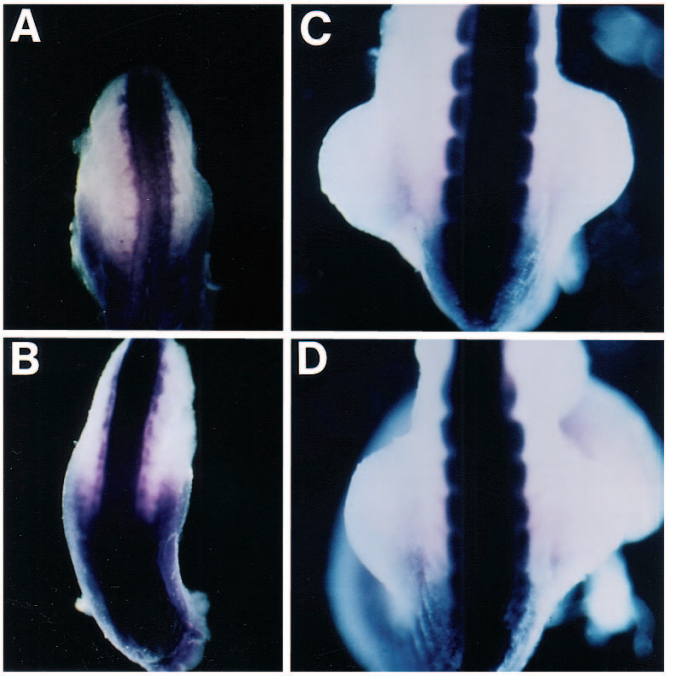

Fig. 3. *lst*^{D} limbs do not ectopically express *Hoxb-8*. Wild-type (top panel) and homozygous *lst*^{D} (bottom panel) embryos were examined for *Hoxb-8* expression by whole-mount in situ hybridization. (A,B) Day 9.5; (C,D) day 10.0 embryos. Photographs show a dorsal view of the forelimbs. Note that expression of *Hoxb-8* in the posterior mesoderm of the forelimb is only apparent in early (day 9.5) embryos (Charité et al., 1994), and disappears thereafter, regardless of genotype.

Normal expression of *Hoxb-8*

Recent transgenic mouse experiments have shown that ectopic expression of *Hoxb-8* in early stage anterior limb mesoderm causes anterior ectopic expression of *fgf-4* and *shh* and results in ZPA-like transformations in the forelimb (Charité et al.,

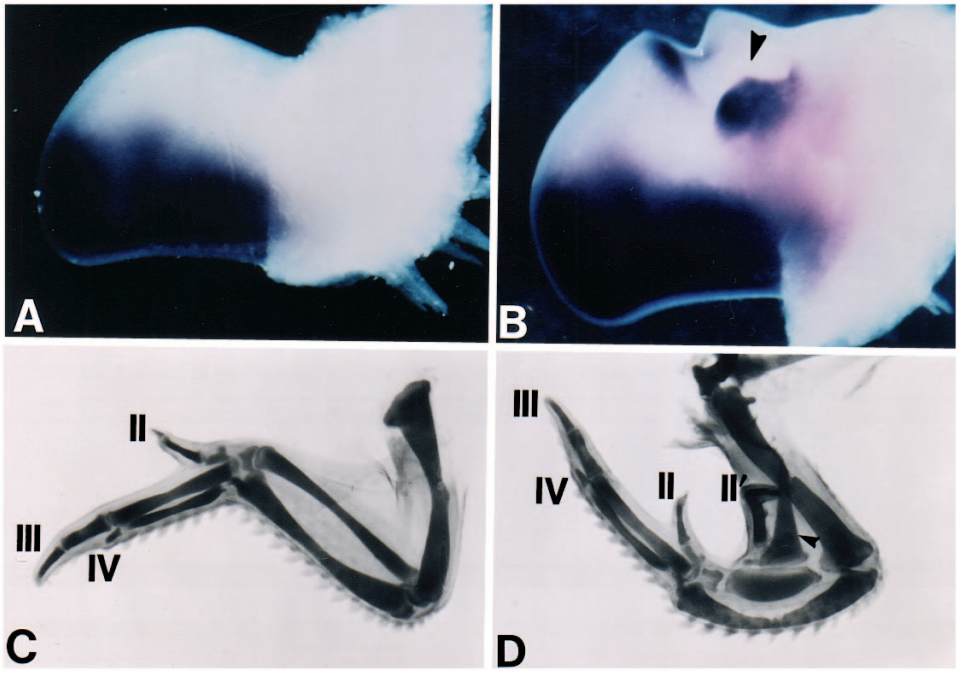

Fig. 4. Anterior mesoderm from *lst*^{D} limbs shows polarizing activity. Host chicken limbs containing grafts of mouse anterior mesoderm from either (A) wild-type limbs or (B) homozygous *lst*^{D} limbs were fixed 36 hours after grafting and stained with a species-specific chicken *Hoxd-11* riboprobe. The arrowhead in B indicates *Hoxd-11* expression induced at the site of the graft. In C and D, host chicken limbs containing anterior mesoderm from either (C) wild-type limbs or (D) homozygous limbs were harvested one week after grafting and stained for cartilage using Alcian Green. Roman numerals indicate digit identity; II′ in D indicates duplicated digit II, and arrowhead in D indicates a duplicated humerus.

1994). This observation raises the possibility that the ectopic expression of *fgf-4* and *shh* observed in *lst*^{D} limbs may result from prior ectopic expression of *Hoxb-8*. Since ectopic *fgf-4* is detectable in homozygous *lst*^{D} forelimbs by day 10.5, we examined mutant embryos at days 9.5, 9.75 (not shown), 10.0, and 10.5 (not shown) for ectopic *Hoxb-8* expression (Fig. 3). Homozygous mutant embryos show no misexpression of *Hoxb-8* in the forelimb at any stage examined, suggesting that the *lst*^{D} mutation causes digit duplications either by acting downstream of *Hoxb-8* or by functioning through a *Hoxb-8*-independent pathway.

Duplication of polarizing activity in *lst*^{D} limbs

To test directly the idea that *lst*^{D} limb buds contain an ectopic ZPA, we transplanted anterior mesoderm from day 11.5 *lst*^{D} limb buds into host chick limb buds. At day 11.5, homozygous *lst*^{D} embryos can be identified in litters from heterozygous parents because their forelimbs contain a distinct bulge in the anterior region. We decided to transplant anterior mesoderm from homozygous limbs since homozygotes contain considerably higher levels of ectopic *shh* expression than heterozygotes (compare Fig. 2B with 2C). As a negative control, we used anterior limb mesoderm from day 11.5 embryos generated from wild-type parents. Transplants of *lst*^{D} anterior mesoderm harvested 36 hours later showed clear induction of ectopic chick *Hoxd-11* expression at the site of the graft, as is typical for grafts containing polarizing activity (Izpisúa-Belmonte et al., 1991; Nohno et al., 1991) (3 of 6 grafts; Fig. 4B). Grafts of anterior mesoderm from wild-type embryos produced no induction of *Hoxd-11* (0 of 6 grafts; Fig. 4A). Furthermore, ten percent of *lst*^{D} transplants that were allowed to develop for a week showed an extra digit II or other skeletal abnormality (4 of 39 grafts; Fig. 4D), whereas transplants of wild-type anterior mesoderm showed no abnormality (0 of 20 grafts; Fig. 2C). Since we were able to obtain more extensive duplications (up to digit III) by grafting mouse posterior limb mesoderm (not shown), *lst*^{D} limb buds appear to contain lower levels of polarizing activity in the anterior limb mesoderm relative to the normal polarizing activity in the posterior mesoderm. The extent of digit duplication obtained with *lst*^{D} anterior limb mesoderm indicates weak ZPA activity (Tickle, 1981), consistent with the relatively lower levels of *shh* expression in the anterior mesoderm of *lst*^{D} limb buds (Fig. 2C,G) and with the limited number of digits duplicated in *lst*^{D} mice (Fig. 1).

DISCUSSION

An ectopic polarizing region in *lst*^{D} limbs

FGF-4 and SHH have been shown to cooperate in patterning the anteroposterior and proximodistal axes of the limb. Each factor may play a role in inducing and maintaining the expression of the other (Laufer et al., 1994; Niswander et al., 1994). Maintenance of polarizing activity (Niswander et al., 1993; Vogel and Tickle, 1993) and *shh* expression (Laufer et al., 1994; Niswander et al., 1994) requires the presence of an intact AER, and this requirement can be largely replaced by FGF-4. The anterior margins of *lst*^{D} mutant limb buds ectopically express both *fgf-4* and *shh* in adjacent cell layers and would therefore appear to have the necessary conditions to support polarizing activity. Consistent with this idea, *lst*^{D} embryos show expression of *Hoxd* cluster genes in the outgrowth of mesoderm that gives rise to the extra digits (Fig. 2I,J); *Hoxd* genes are induced in response to grafts of polarizing tissue into chick anterior mesoderm (Izpisua-Belmonte et al., 1991; Nohno et al., 1991). Finally, anterior mesoderm from *lst*^{D} homozygous limb buds show polarizing activity when transplanted into host chick wing buds.

Taken together, these results show that *lst*^{D} limb buds contain a duplicated polarizing region and posterior AER. The duplication of the AER has been observed previously by histological analysis of *lst*^{D} embryos (Forsthoefel, 1963), and was suggested to result from duplication of an AER maintenance center. Alternatively, it is equally possible that the *lst*^{D} mutation causes the extension of an existing AER and reverses the polarity of the anterior portion. It is likely that the ZPA-like digit duplications seen in *lst*^{D} limbs result from the influence of the ectopic polarizing region that we have demonstrated.

Relationship of *lst*^{D} to other limb mutants

The *lst* locus is closely linked to the mouse *limb deformity* (*ld*) gene. Mice homozygous for the recessive *ld* mutation display reduction and fusion of digits and fusion of the distal long bones (radius/ulna, tibia/fibula; Kleinebrecht et al., 1982; Woychik et al., 1985). Thus, in a general sense, *ld* mice have a phenotype opposite to that of *lst*^{D} mice. This observation has led to the suggestion that *ld* and *lst*^{D} may be mutations in the same gene (Woychik et al., 1985). It has been proposed that *lst*^{D}, possibly a gain-of-function mutation, leads to increased gene activity and polydactyly, while *ld*, being a loss-of-function mutation, leads to reduced gene activity and syndactyly. The *ld* gene has been cloned (Woychik et al., 1985) and encodes novel protein isoforms termed formins (Woychik et al., 1990). In situ hybridization studies show that *lst*^{D} embryos have normal expression of *ld* transcripts (Chan and Leder, unpublished observations). Furthermore, western blots with anti-formin antibodies show no gross differences between formins in *lst*^{D} and wild-type mice (Chan and Leder, unpublished observations). Therefore, we currently favor the view that *lst* and *ld* represent two separate genes that function in similar pathways in limb development.

The *lst*^{D} mouse belongs to a class of luxoid/hemimelic mouse mutants (characterized by deficiencies in part or all of the long bones of the limbs) that includes *Green's luxoid* and *Carter's luxate* (Forsthoefel, 1962). These three mutants display polydactyly when heterozygous and hemimelia when homozygous. The genes affected in these mutations are thought to act in independent biochemical pathways, since mice carrying two or more of these mutations show additive effects in limb abnormalities (Forsthoefel, 1962). In future studies, it will be interesting to examine the effect of these other luxoid mutations on the AER and polarizing region.

Models of *lst* function

We propose that the *lst* gene product acts early in anteroposterior patterning. Two general models for the function of *lst* can be proposed, depending on whether the *lst*^{D} mutation is a gain-of-function or loss-of-function mutation (Fig. 5). In the former case, *lst* may normally function in specifying the proper regional expression of *fgf-4* and *shh* in the posterior portion of the limb bud (Fig. 5A). Similar to the role proposed for *Hoxb-*

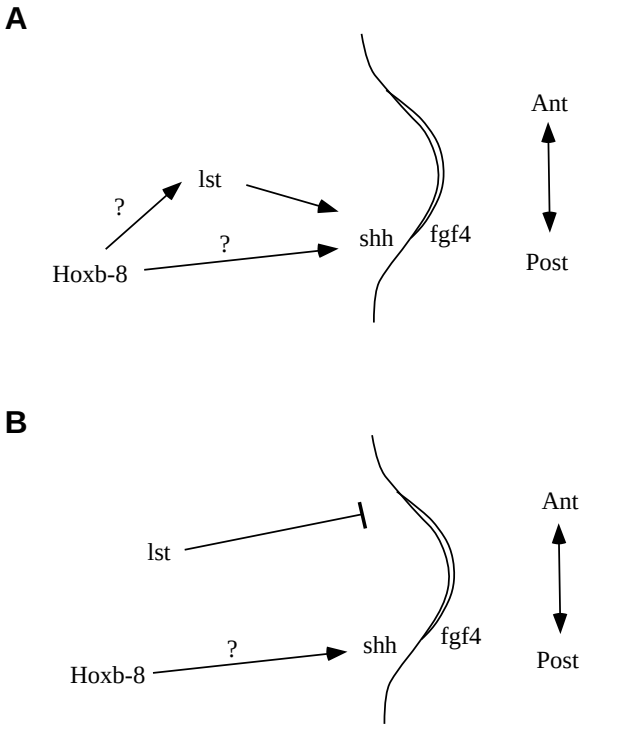

Fig. 5. Two models of *lst* function in normal limb development. (A) Model in which *lst*^{D} is a gain-of-function mutation. As indicated by the arrows, the *lst* gene product might normally function upstream of the polarizing region, perhaps by specifying the regional expression of *shh* and *fgf-4* in the posterior portion of the limb. *Hoxb-8* may function either further upstream or in an independent pathway. (B) Model in which *lst*^{D} is a loss-of-function mutation. Here, the *lst* product is required for establishment or maintenance of anteroposterior asymmetry. In the absence of *lst* product, genes such as *fgf-4* and *shh* are symmetrically expressed, in a dosage-dependent manner. The *lst* product may normally prevent the formation of a polarizing region in the anterior of the limb by repressing the expression of *shh* and *fgf-4*, as indicated by the bar. In both A and B, the proposed function of *Hoxb-8* in activation of the polarizing region applies only to the forelimbs and not in the hindlimbs (Charité et al., 1994). Ant, anterior; Post, posterior.

8 (Charité et al., 1994), *lst* may be involved in the activation of the polarizing region, acting either downstream or independently of *Hoxb-8*. Alternatively, if *lst*^{D} is a loss-of-function mutation, the *lst* gene product would be required for the formation or maintenance of anteroposterior asymmetry in the limb; in its absence, the limb takes on a default state in which there is mirror-image symmetry along the anteroposterior axis (Fig. 5B). This requirement for the *lst* product is dosage-dependent, since heterozygotes show a milder manifestation of the digit duplications found in homozygotes. In this model, *lst* may normally repress the expression of molecules such as *shh* or *fgf-4* in the anterior portion of the limb. In both models, *lst* is likely to play an important role in early anteroposterior patterning. Although other mouse mutants also exhibit preaxial polydactyly (Grüneberg, 1963), *lst*^{D} mutants appear unique in their consistent display of ZPA-like duplications, and hence may yield critical information about early anteroposterior patterning mechanisms in the mouse limb bud.

We are grateful to Anne Burke, Andrew McMahon, and Gail Martin for providing probes. We thank members of the Leder and Tabin labs for helpful advice and comments.

REFERENCES

Chang, D. T., Lopez, A., Kessler, D. P. v., Chiang, C., Simandl, B. K., Zhao, R., Seldin, M. F., Fallon, J. F. and Beachy, P. A. (1994). Products, genetic linkage and limb patterning activity of a murine *hedgehog* gene. *Development* **120**, 3339-3353.

Charite', J., Graaff, W. d., Shen, S. and Deschamps, J. (1994). Ectopic expression of *Hoxb-8* causes duplication of the ZPA in the forelimb and homeotic transformation of axial structures. *Cell* **78**, 589-601.

Dollé, P., Izpisua-Belmonte, J. C., Falkenstein, H., Renucci, A. and Duboule, D. (1989). Coordinate expression of the murine Hox-5 complex homeobox-containing genes during limb pattern formation. *Nature* **342**, 767-772.

Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. A. and McMahon, A. P. (1993). *Sonic hedgehog*, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.

Forsthoefel, P. F. (1962). Genetics and manifold effects of Strong's luxoid gene in the mouse, including its interactions with Green's luxoid and Carter's luxate genes. *J. Morphol.* **110**, 391-420.

Forsthoefel, P. F. (1963). The embryological development of the effects of Strong's luxoid gene in the mouse. *J. Morphol.* **113**, 427-452.

Grüneberg, H. (1963). *The Pathology of Development.* Oxford: Blackwell Scientific Publications.

Hamburger, V. (1938). Morphogenetic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. *J. Exp. Zool.* **77**, 379-399.

Hébert, J. M., Basilico, C., Goldfarb, M., Haub, O. and Martin, G. (1990). Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression patterns during embryogenesis. *Dev. Biol.* **138**, 454-463.

Hinchcliffe, J. R. and Griffiths, P. J. (1983). The prechondrogenic patterns in tetrapod limb development and their phylogenetic significance. In *Development and Evolution* (ed. B. Goodwin, N. Holder and C. Wylie), Cambridge: Cambridge University Press.

Izpisúa-Belmonte, J. C., Tickle, C., Dollé, P., Wolpert, L. and Duboule, D. (1991). Expression of the homeobox Hox-4 genes and the specification of position in chick wing development. *Nature* **350**, 585-589.

Kleinebrecht, J., Selow, J. and Winkler, W. (1982). The mouse mutant limb-deformity (ld). *Anatomischer Anzeiger* **152**, 313-324.

Krauss, S., Concordet, J.-P. and Ingham, P. W. (1993). A functionally conserved homolog of the Drosophila segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75**, 1431-1444.

Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994). *Sonic hedgehog* and *fgf-4* act through a signaling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.

Niswander, L., Jeffrey, S., Martin, G. R. and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb. *Nature* **371**, 609-612.

Niswander, L. and Martin, G. R. (1992). FGF-4 expression during gastrulation, myogenesis, limb, and tooth development in the mouse. *Development* **114**, 755-768.

Niswander, L., Tickle, C., Vogel, A., Booth, I. and Martin, G. R. (1993). FGF-4 replaces the apical ectodermal ridge and directs limb outgrowth and patterning of the limb. *Cell* **75**, 579-587.

Nohno, T., Noji, S., Koyama, E., Ohyama, K., Myokai, F., Kuroiwa, A., Saito, T. and Tanaguchi, S. (1991). Involvement of the Chox-4 chicken homeobox genes in determination of anteroposterior axial polarity during limb development. *Cell* **64**, 1197-1205.

Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.

Roelink, H., Augsburger, A., Heemskerk, J., Korzh, V., Norlin, S., Ruiz i Altaba, A., Tanabe, Y., Placzek, M., Edlund, T., Jessell, T. M. and Dodd, T. (1994). Floor plate and motor neuron induction by *vhh-1*, a vertebrate homolog of *hedgehog* expressed by the notochord. *Cell* **76**, 761-775.

Saunders, J. W. (1948). The proximo-distal sequence of origin of the parts of the chick wing and the role of the ectoderm. *J. Exp. Zool.* **108**, 363-404.

Saunders, J. W. and Gasseling, M. (1968). Ectodermal-mesenchymal

interaction in the origin of limb symmetry. In *Epithelial-Mesenchymal Interaction* (ed. R. Fleischmayer and R. E. Billingham), pp. 78-97. Baltimore: Williams and Wilkins.

Saunders, J. W. and Reuss, C. (1974). Inductive and axial properties of perspective wing-bud mesoderm in the chick embryo. *Dev. Biol.* **38**, 41-50.

Tabin, C. J. (1991). Retinoids, homeoboxes, and growth factors: toward molecular models for limb development. *Cell* **38**, 627-637.

Tickle, C. (1981). The number of polarizing region cells required to specify additional digits in the developing chick wing. *Nature* **289**, 295-298.

Tickle, C. and Eichele, G. (1994). Vertebrate limb development. *Ann. Rev. Cell Biol.* **10**, 121-152.

Tickle, C., Summerbell, D. and Wolpert, L. (1975). Positional signalling and specification of digits in chick limb morphogenesis. *Nature* **254**, 199-202.

Vogel, A. and Tickle, C. (1993). FGF-4 maintains polarizing activity of posterior limb bud cells in vivo and in vitro. *Development* **119**, 199-206.

Woychik, R. P., Maas, R. L., Zeller, R., Vogt, T. F. and Leder, P. (1990). 'Formins': proteins deduced from the alternative transcripts of the *limb deformity* gene. *Nature* **346**, 850-853.

Woychik, R. P., Stewart, T. A., Davis, L. G., D'Eustachio, P. and Leder, P. (1985). An inherited limb deformity created by insertional mutagenesis in a transgenic mouse. *Nature* **318**, 36-40.

(Accepted 21 March 1995)