Author’s correction to the print version

The lst allele is \textit{lst} and not \textit{lst} \textit{D}. 
The role of Alx-4 in the establishment of anteroposterior polarity during vertebrate limb development

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SUMMARY

We have determined that Strong’s Luxoid (IstJ) mice have a 16 bp deletion in the homeobox region of the Alx-4 gene. This deletion, which leads to a frame shift and a truncation of the Alx-4 protein, could cause the polydactyly phenotype observed in IstJ mice. We have cloned the chick homologue of Alx-4 and investigated its expression during limb outgrowth. Chick Alx-4 displays an expression pattern complementary to that of shh, a mediator of polarizing activity in the limb bud. Local application of Sonic hedgehog (Shh) and Fibroblast Growth Factor (FGF), in addition to ectodermal apical ridge removal experiments suggest the existence of a negative feedback loop between Alx-4 and Shh during limb outgrowth. Analysis of polydactyly mutants indicate that the interaction between Alx-4 and Shh is independent of Gli3, a negative regulator of Shh in the limb. Our data suggest the existence of a negative feedback loop between Alx-4 and Shh during vertebrate limb outgrowth.

Keys words: Alx-4, Strong’s Luxoid, Polarizing activity, shh, Mouse, Chick, Limb development

INTRODUCTION

The vertebrate limb has served for a long time as a model system for studying the mechanisms by which ‘patterns’ are established and propagated during embryogenesis. One of the most studied events in limb development is the determination of the anteroposterior axis (see Tickle and Eichele, 1994; Johnson and Tabin, 1998; and Schwabe et al., 1998 for reviews). Embryological studies have shown that the anteroposterior axis of the limb bud is determined in the limb primordia before limb induction (Hamburger, 1938; Chaube, 1959; Hornbruch and Wolpert, 1991). However, little is known about the molecular mechanisms that initially establish anteroposterior asymmetries. Two molecules have been shown to play an important role in this process: retinoic acid (RA) and Hoxb-8. Inhibition of RA signaling before limb budding prevents the formation of the zone of polarizing activity (ZPA) and subsequent limb development (Helm et al., 1996; Stratford et al., 1996; Lu et al., 1997; Stratford et al., 1997). The ZPA is a group of cells located at the posterior margin of the limb bud. When transplanted into the anterior region of a host limb bud, the ZPA induces a mirror-image digit duplication (Saunders and Gasseling, 1968). Hoxb-8 seems to be a direct target of RA signaling, since it is rapidly induced by RA and is downregulated by retinoid receptor antagonists and inhibitors of RA synthesis (Lu et al., 1997; Stratford et al., 1997). Furthermore, the spatiotemporal expression pattern of Hoxb-8 correlates well with the distribution of polarizing activity before the limb bud is induced (Hornbruch and Wolpert, 1991; Lu et al., 1997; Stratford et al., 1997), and when ectopically misexpressed in the anterior margin of the mouse limb, Hoxb-8 is capable of inducing mirror-image digit duplications (Charité et al., 1994).

As the limb bud grows, the maximum polarizing activity is associated with cells in the ZPA, which is considered to direct patterning along the anteroposterior axis of the limb (Tickle et al., 1975). Sonic hedgehog (Shh) has been shown to be expressed in the ZPA and is able to mediate polarizing activity (Riddle et al., 1993; López-Martínez et al., 1995; Marti et al., 1995; Yang and Niswander, 1995). The apical ectodermal ridge (AER) is a group of ectodermal cells located at the most distal tip of the limb bud, which both permit limb bud elongation and interact with the ZPA in establishing the anteroposterior axis of the limb. AER removal results in loss of polarizing activity
The cDNA library was made from poly(A)+ RNA purified from Whole-mount in situ hybridization was performed essentially as described (Vogel and Tickle, 1993) and loss of shh expression (Laufer et al., 1994; Niswander et al., 1994). Both polarizing activity and shh expression, as well as limb bud outgrowth, can be rescued by local treatment with several members of the Fibroblast Growth Factor (FGF) family of secreted factors (Niswander et al., 1993; Vogel and Tickle, 1993; Fallon et al., 1994; Laufer et al., 1994; Niswander et al., 1994; Mahmood et al., 1995; Crossley et al., 1996; Vogel et al., 1996). Together, these results have provided a wealth of information about the mechanisms by which the molecular cues located at the posterior margin of the limb bud govern the establishment of the anteroposterior axis. However, less is known about the molecular mechanisms that interpret and/or dictate additional information at the anterior side of the limb (see Bryant and Gardiner, 1992 for a review). Recent experiments have focused on polydactyly. Analysis of several such mouse and chick mutants suggests the presence of an active mechanism, governed by anterior cells, that confines polarizing activity to the posterior cells of the limb bud (Büscher et al., 1997; Chan et al., 1995; MacCabe and Abbott, 1974; MacCabe et al., 1975; Masuya et al., 1995, 1997; Rodriguez et al., 1996). This would argue for reciprocal interactions between anterior and posterior cells in establishing the anteroposterior axis of the vertebrate limb bud. One of these mutations, Extra toes (Xt), has been shown to be the result of a mutation in the Gli3 gene (Schimmang et al., 1992; Hui and Joyner, 1993). However, in most cases, the molecular alterations responsible for the polydactyly phenotypes are unknown.

In this study, we report the discovery of a 16 bp deletion in the homeobox region of the Alx-4 gene in Strong’s Luxoid (lst4) mice. lst4 is a semidominant mutation resulting in preaxial polydactyly. Recently targeted disruption of Alx-4 has been shown to cause a similar phenotype to the lst4 mutant (Qu et al., 1997). We also report the cloning and spatiotemporal pattern of expression of Alx-4 in the chick limb bud and demonstrate its dependence on AER signals. Using mouse and chick limb mutants, as well as ectopic protein delivery assays, we present evidence for the existence of a negative feedback loop between anterior and posterior cells.

The localization of shh in the limb bud is a key event in determining the correct anteroposterior axis of the vertebrate limb.

**RESULTS**

Cloning of chick and mouse Alx-4

Screening of mouse and chick libraries with the paired type homeobox gene resulted in a putative full-length mouse cDNA clone and 40 partial chick cDNA clones. The murine sequence is identical to that recently reported for Alx-4, predicting a protein of 399 amino acids (Qu et al., 1997). Two of the chick clones were found to be the chick homologue of the mouse Alx-4. An alignment of the predicted chick and mouse amino acid sequence shows 100% identity to the mouse Alx-4 in the homeodomain (Fig. 1A, shaded box) and a high degree of amino acid conservation throughout the C-terminal region (dots in Fig. 1A). The consensus sequence motif named the paired-tail is also present at their extreme carboxyl terminus (boxed in Fig. 1A; Mathers et al., 1997). A comparison of different paired type homeodomain proteins (Fig. 1B) indicates a strong conservation among the homeodomains of Alx-4, Alx-3 and Cart-1 (93% and 92%, respectively), and a lesser conservation with Arx, Chx-10, aristless, unc-4, Prx-1 and Prx-2 (87%, 80%, 77%, 70%, 70%, 68%, respectively). Interestingly, the sequence conservation is higher in helix 3/4 among these proteins than in helix 1/2 of the homeodomain (Fig. 1B). Overall, both mouse and chick Alx-4 genes appear to be closer to Arx and Cart-1 than to the other genes shown in Fig. 1B.

**Alx-4 expression during chick limb development**

To further investigate the role of Alx-4, we first analyzed the spatiotemporal pattern of expression of its mRNA during limb bud outgrowth. Alx-4 transcripts are detected in the lateral plate mesoderm prior to limb induction. At stage 17/18, expression is seen throughout the entire lateral plate mesoderm except for the posterior portion of the presumptive limb bud (somites 20-21 in wing bud and somites 29-30 in leg bud, Fig. 2A; see also Fig. 3A). Expression at this stage is already stronger in the most anterior region of the presumptive limb bud. This anterior
pattern of Alx-4 expression sharply contrasts with the pattern of \( shh \) in the posterior region of the limb bud. In Alx-4 null mice, \( shh \) is upregulated in the anterior side of the limb bud, thus suggesting a cross-regulation between \( shh \) and Alx-4 during normal limb outgrowth. Therefore, we compared the spatiotemporal distribution of these two genes during chick limb development. At stage 17/18, no \( shh \) transcripts are yet observed in the limb bud, although Alx-4 is already expressed in the anterior mesoderm of the limb bud (Fig. 2B,C). \( shh \) expression is detectable around stage 18 in the posterior side of the limb bud (Riddle et al., 1993). By stage 19 a gap between \( shh \) and Alx-4 expression is observed in the middle region of the limb bud (Fig. 2D,E). This gap becomes broader as limb outgrowth proceeds (Fig. 2F,G). The anterior expression domain of Alx-4 remains unaltered until stage 24, after which Alx-4 transcripts are confined to the most anteroposterior region (Fig. 2H-J) and begin to disappear by stage 28. Stage 28 is also the stage at which \( shh \) begins to disappear from the posterior limb mesoderm. At stage 26, a weak expression of Alx-4 was observed in the most posterior proximal region. Alx-4 is never detected in the dorsal or ventral ectoderm, or in the AER (Fig. 2H and data not shown).

**Alx-4 and the establishment of A-P polarity in FGF-induced limb buds**

The expression of Alx-4 at the first stage of limb induction appears to be absent from the anterior flank mesoderm and confined to the posterior flank mesoderm (Figs 2A,C, 3A) at the start of limb budding. Together with the mutually exclusive expression pattern of \( shh \) and Alx-4 in the limb bud, these data suggest a role for Alx-4 in the establishment of the anteroposterior polarity of the chick limb bud. This issue was investigated by applying FGF beads to the presumptive flank region at stage 15/16 and subsequently analyzing Alx-4 expression at different time points. This surgical manipulation can induce the formation of ectopic limb buds that develop into limbs with a reversed pattern of digits (Cohn et al., 1995; Mahmood et al., 1995; Crossley et al., 1996; Ohuchi et al., 1995, 1997; Vogel et al., 1996). In these ectopic limb buds, \( shh \) expression is found at the anterior margin (Cohn et al., 1995). 12 hours after FGF bead implantation no change is observed in Alx-4 expression (Fig. 3B; \( n=7 \)). However, 18 hours after FGF application (Fig. 3C; \( n=4 \)) the rostral end of the Alx-4 expression domain in the flank region is shifted down caudally from somite 21 to somite 23. This downregulation of Alx-4 and restriction to the future anterior side of the ectopic limb bud precedes the induction of \( shh \), which does not occur until 24 hours post-FGF bead implantation (Cohn et al., 1995 and data not shown). The sequential activation of gene expression in the FGF-induced limbs closely resembles the pattern of gene expression during normal limb outgrowth. These observations suggest that the appearance of polarizing activity in the posterior region of the limb bud could be linked to the absence or downregulation of Alx-4 expression in the presumptive \( shh \)-expressing cells, thus indicating an early role for Alx-4 in the establishment of the anteriorposterior polarity of the vertebrate limb.

The AER is required for the maintenance of Alx-4 expression in the early stages of limb outgrowth

Interactions between the distal-most limb mesoderm and the AER are both required for proper outgrowth of the limb along its proximodistal axis and along its anteroposterior axis (Rowe and Fallon, 1982; Saunders, 1948; Summerbell, 1974; Todt and Fallon, 1984). The arrest in limb outgrowth following AER removal is preceded by a downregulation of known proximodistal and anteroposterior mesodermal gene markers (Ng et al., 1998; Johnson and Tabin, 1998 for reviews). One such marker is \( shh \). Grafting and misexpression experiments have shown that signals from the AER (e.g. FGFs) induce mesodermal expression and maintain \( shh \) expression (Crossey...
et al., 1996; Fallon et al., 1994; Laufer et al., 1994; Mahmood et al., 1995; Niswander et al., 1994; Vogel and Leder, 1996). To determine whether continued Alx-4 expression might also depend upon signals from the AER, we surgically removed the anterior and posterior ridges at stages 18-20. The expression of Alx-4 is not affected by removal of the posterior half of the AER (Fig. 4B; n=4). However, when the anterior half of the AER is removed at stage 18, the expression of Alx-4 is downregulated within the following 24 hours (Fig. 4A; n=3). The AER requirement for Alx-4 expression seems to be time-dependent since removal of the AER at stage 20 does not alter Alx-4 expression (Fig. 4C, n=7; Fig. 4D, n=5).

**lst** mice have a 16 bp deletion in the homeobox of Alx-4

Based on comparative chromosomal mapping studies, the **lst** mutation has been mapped on chromosome 2, very close to the polymorphic marker D2Mit130. Similarly, Alx-4 has been mapped on chromosome 2 between the polymorphic markers D2Mit15 and D2Mit97, and is inseparable from the polymorphic marker D2Mit130 (Qu et al., 1997; Vogel and Leder, 1996). Targeted disruption of Alx-4 results in mice with preaxial polydactyly, a phenotype also observed in the **lst** mice. This suggests a relationship between the **lst** gene product and Alx-4 (Qu et al., 1997; Vogel and Leder, 1996).

In order to investigate this possibility, we sequenced six Alx-4 clones isolated from an **lst**/+ mouse limb bud cDNA library. Two clones showed 100% identity to the wild-type sequence and four clones showed a 16 bp deletion within the region corresponding to the homebox (Fig. 5A, bold letters; Fig. 5B). This deletion was further confirmed by RT-PCR (12 out of 28 clones were mutant, data not shown) and very recently Qu et al. (1998) have reported that this 16 bp deletion occurs also in genomic DNA. The 16 bp deletion causes a frame shift and potentially produces a truncated protein at amino acid position 332, lacking the last 67 amino acids (Fig. 5A). A comparison of the wild-type and **lst** amino acid sequences shows that neither the functional helix 3/4 of the homeodomain nor C-terminal region would exist in the Alx-4**lst** protein (Fig. 1A). It is unclear whether helix 1 and 2 are sufficient for DNA binding.

**Negative feedback loop between Alx-4 and shh during limb bud outgrowth**

In both the Alx-4 null and in the **lst** mice, shh transcripts are ectopically induced at the anterior margin of the developing limb bud. This has been interpreted to indicate that posterior serves as the default state of the limb, while Alx-4 might repress the expression of posterior genes like shh (Chan et al., 1995; Qu et al., 1997). Alternatively, posterior signals could repress the expression of anterior molecules like Alx-4.

We applied Shh-soaked beads into the anterior margin of stage 20 wing buds and analyzed the expression of Alx-4 at different time points to test this model. This manipulation consistently induced the appearance of extra digits 2 and 3 (100%, n=11; data not shown). 6 hours after Shh bead implantation, Alx-4 transcripts were clearly downregulated in the cells adjacent to the bead (Fig. 6A; n=4). Alx-4 downregulation by Shh was increased after 12 hours (Fig. 6B; n=3) and 24 hours (Fig. 6C; n=4). Together with the ectopic induction of shh in the Alx-4 null and **lst** mice, this immediate response of Alx-4 to Shh indicates the existence of a cross-regulatory interaction between Shh and Alx-4 that could be important for proper establishment of the anteroposterior axis of the limb.

During limb outgrowth, shh and Gli3 transcripts are mutually exclusive (Büscher et al., 1997; Marigo et al., 1996; Masuya et al., 1995, 1997), so that Gli-3 expression is repressed in regions where shh is expressed. Ectopic expression of Shh in the anterior limb mesoderm in the chick causes a downregulation of Gli3 transcripts and Gli3 mutations in the mouse result in ectopic expression of shh. These data suggest a negative feedback loop between Gli3 and Shh during limb outgrowth. To investigate whether the downregulation of Alx-4 expression in the ectopic Shh bead experiments could be mediated by Gli3, we timed the downregulation of both Gli3

**Fig. 2.** Expression of shh and Alx-4 during chick limb bud outgrowth. (A) Right side of a stage 17/18 chick stained with Alx-4. Arrows indicate lateral plate mesoderm lacking detectable Alx-4 expression. (B) Contralateral side of the embryo shown in A stained for shh expression. Arrows on the left mark the anterior and posterior aspect of the limb field. Arrowheads on the right indicate shh expression in the notochord/neural tube. Note that no expression is detected in the presumptive limb region. (C) Higher magnification of the wing region shown in A. Arrows mark the anterior and posterior margin of the wing field. (D) Shh expression in a stage 19 wing bud. The arrows mark the margins of the wing bud. (E) Contralateral wing of the embryo shown in D stained for Alx-4 expression. Arrows again indicate wing margins. (F,G) Contralateral wing buds at stage 20 stained for shh (F) or Alx-4 (G) expression. (H-J) Alx-4 expression in wing buds at stage 21 (H), 22 (I) and 25 (J).
and Alx-4 after ectopic expression of Shh in the anterior chick limb mesoderm. Contrary to Alx-4, no significant changes in Gli3 expression were observed after 6 hours (Fig. 6D; n=3) or 12 hours (Fig. 6E; n=5). Gli3 transcripts started to be downregulated in cells around the Shh bead at 24 hours postsurgery (arrowheads in Fig. 6F; n=3).

**Gli3 expression in lstJ mutants**

To further investigate the relationship between the Gli3 and Alx-4 genes, we analyzed the expression pattern of Gli3 in the limb buds of the lstJ mouse. Expression of Gli3 in 10 dpc lstJ embryos (Fig. 7A) is similar to the wild-type expression pattern, showing a strong anterior expression (arrowhead in Fig. 7A) and a lack of expression in the most posterior region. In the wild-type limb buds at 12 dpc Gli3 still shows a high level of expression in the anterior region, which gradually fades towards the posterior region (Fig. 7B upper limb bud). In homozygous lstJ limb buds, Gli3 expression is lacking in the most anterior region (Fig. 7B, arrowhead) which has been shown to express shh at that stage (Chan et al., 1995). The remaining limb region displays a normal Gli3 expression pattern, indicating that Gli3 is not downstream of Alx-4 (Fig. 7B, lower limb bud). The lack of Gli3 in the most anterior region results most likely from the ectopic shh expression as seen in Hemimelia Extratoes (Hx; Büscher et al., 1998) and chick (Marigo et al., 1996).

These data, combined with the observation that in the extra toes mutant (a Gli3 mutation) Alx-4 is unaffected (see below), indicate that the downregulation of Alx-4 by Shh may occur independently of Gli3.

**Alx-4 expression is unaltered in mouse extra toes limb buds, but reduced in chick diplopodia4**

To gain more insights into the question of whether the expression of Alx-4 during limb development is dependent on Gli3 expression, we compared Alx-4 expression in homozygous extra toes and wild-type embryos at 10.5, 11.5 and 12.5 dpc (Fig. 7C,D; data not shown). Extra toes is a polydactylos mouse that possesses a mutation in the Gli3 gene (Hui and Joyner, 1993; Schimmang et al., 1992). The expression domain of Alx-4 is normally confined to the anterior-proximal region of the developing limb bud. Expression patterns in wild-type (Fig. 7C) and extra toes limb buds are identical (Fig. 7D). It can, therefore, be concluded that Alx-4 expression occurs independently of Gli3.

The chick diplopodia4 is a sex-linked recessive lethal mutation that has been shown to develop a mirror-image digit duplication without detectable ectopic expression of shh in the anterior region (MacCabe and Abbott, 1974; MacCabe et al., 1975; Rodriguez et al., 1996). However, other posterior limb markers like BMP-2, Fgf-4 and HoxD genes are ectopically expressed (Rodriguez et al., 1996). In situ analysis of Alx-4 expression in normal (Fig. 7E) and diplopodia4 (Fig. 7F) limb buds at stage 23 showed a strong reduction in Alx-4 transcripts in the mutant limb bud. No Alx-4 expression could be detected in the anterior-distal part (Fig. 7F, dotted line) as seen in wild-type limbs (Fig. 7E). The anteriorly enlarged limb bud at stage 23 is clearly visible in the diplopodia4 mutant and comparable to the extended limb bud seen after ectopic shh expression (Riddle et al., 1993). The downregulation of Alx-4 in the diplopodia4 mutant gives further support to the idea of a negative feedback loop mechanism between posterior and anterior limb cells during the establishment of A/P patterning (see also Bryant and Gardiner, 1992).

**DISCUSSION**

Establishment and maintenance of the ZPA: A negative feedback loop between Alx-4 and Shh

The developing chick limb bud provides an amenable model system to study the molecular mechanisms underlying the
establishment of anteroposterior asymmetries of the vertebrate limb. After the initial events leading to limb outgrowth and patterning, the ZPA is established and the anteroposterior coordinates of the limb bud are built on the foundations created by cues initiated in the limb field. The initial gene interactions in the pre-patterned limb mesoderm are subsequently stabilized and augmented, and new interactions appear (Duboule, 1994; Ros et al., 1996). Previously, two genes have been reported to be closely involved in the distribution of polarizing activity, Hoxb-8 and shh (Charité et al., 1994; Lu et al., 1997; Stratford et al., 1997; López-Martinez et al., 1995; Riddle et al., 1993; Marti et al., 1995; Yang and Niswander, 1995). We now present evidence that Alx-4 is implicated in the establishment of the ZPA in the posterior margin of the limb bud.

The distribution of Alx-4 transcripts at the initial stages of limb induction is opposite to the distribution of polarizing activity reported by Hornbruch and Wolpert (1991) (i.e. Alx-4 transcripts are less abundant in cells with high polarizing activity and, conversely, regions with less polarizing activity display high levels of Alx-4 mRNA; see Fig. 2). Alx-4 is first expressed in the anterior presumptive wing field at the time when the expression of Hoxb-8 in the same cells has receded and is confined to the presumptive posterior wing margin. Alx-4 is never detected in the anterior flank, a region that shows both high levels of polarizing activity and Hoxb-8 mRNA expression. Finally, anterior expression of Alx-4 precedes the appearance of shh mRNA at the posterior margin of both the forelimb and hindlimb. Overall, the spatiotemporal pattern of Alx-4 expression suggests it has a role in establishing anteroposterior cues before limb budding.

Together with the expression pattern of Alx-4, two lines of evidence suggest that Alx-4 plays a role in the initiation and establishment of the ZPA at the posterior side of the vertebrate limb bud. First, local application of FGFs to the presumptive chick flank induces an ectopic limb with a reversed anteroposterior polarity and hence reversed shh expression (Cohn et al., 1995, 1997; Mahmood et al., 1995; Ohuchi et al., 1995, 1997; Crossley et al., 1996; Vogel et al., 1996). Subsequent to FGF bead implantation, and several hours before shh expression is induced, Alx-4 expression in the flank is downregulated and confined to the presumptive anterior cells of the ectopic limb bud. Alx-4 downregulation precedes the induction of shh in the future posterior cells of the new limb bud, thus recapitulating the spatiotemporal pattern of gene expression observed during normal limb outgrowth. Second, in both lst/ and Alx-4 null mice, an ectopic ZPA is observed at the anterior region, which is accompanied by ectopic shh expression (Chan et al., 1995; Qu et al., 1997). In addition, misexpression of Shh...
at the anterior region of the chick limb bud leads to a downregulation of Alx-4. Taken together, these results suggest that Alx-4 acts downstream of the early events that establish anteroposterior gene asymmetries in the lateral plate mesoderm, but is required to prevent ZPA formation in the wrong position.

It is clear that Hoxb-8 gene ablation and misexpression experiments, study of Alx-4 expression in shh knockout mice, and Alx-4 misexpression experiments are needed to further ascertain putative genetic interactions between these three genes. Nonetheless, our data, combined with those previously reported (Charité et al., 1994; Lu et al., 1997; Stratford et al., 1997; Chan et al., 1995; Qu et al., 1997), support the idea of the existence of an inhibitory feedback loop mechanism between Alx-4 and Shh during the establishment of the ZPA.

**Ist**, a 16 base pair deletion in the Alx-4 homeobox

Mutants provide one of the most useful tools to elucidate the relationship between different factors in developmental processes. A number of mutations that alter the anteroposterior patterning of the vertebrate limb have been reported and mapped to different chromosomes. The lst mutant has been classified within the luxoid/hemimelic class of mouse mutants, which is characterized by alterations in the long bones of the limb. Mice heterozygous for lst develop ectopic anterior digits in the hindlimb. Homozygous mice display a more severe phenotype: both hind and forelimbs have preaxial ectopic digits, which are accompanied by alterations in the radius and tibia (Forsthoefer, 1962, 1963). Chromosomal mapping of the lst mutation shows that it resides in chromosome 2, very close to the polymorphic marker D2Mit130. The mouse Alx-4 gene has also been mapped on chromosome 2, inseparable from the polymorphic marker D2Mit130 and between D2Mit15 and D2Mit97 (Vogt and Leder, 1996; Qu et al., 1997). Both Alx-4 null and lst mice display ectopic expression of known posterior limb markers (shh, Fgf-4 and hoxd-13) in the anterior region of the limb bud. It has been shown that anterior mesoderm from the mutated limbs exhibits polarizing activity when grafted into host chick limb buds (Chan et al., 1995; Qu et al., 1997). The genetic linkage, the polydactyly, the ectopic ZPA and the ectopic expression of posterior limb markers all suggested that lst could be a candidate for an allele of Alx-4.

In this study, we showed that the phenotype of lst is indeed likely to be caused by a deletion in the homeobox of Alx-4. Sequence analysis of a lst+ cDNA limb library revealed a 16 bp deletion in the Alx-4 gene. This deletion results in a frameshift mutation that potentially produces a truncated Alx-4 protein missing its 67 C-terminal amino acids. The mutated protein lacks helix 3 and 4 of the homeodomain as well as the C-terminal consensus sequence motif termed the paired tail. These two regions are highly conserved among Alx-4-related proteins. The semidominant phenotype of lst versus the recessive phenotype in the Alx-4 null mice argues for a dominant negative Alx-4 protein, although this effect does not necessarily have to be coupled to DNA binding. How this function is achieved remains undetermined. One possibility could be that, whilst specific DNA-binding occurs (due to the presence of an intact helix 1 and 2 in the homeodomain), transactivation or transrepression cannot occur due to the absent C-terminal region. The lack of the recognition helices 3 and 4 of the homeodomain, which are located in the major groove where most of the intermolecular contact occurs (for review see Gehring et al., 1994), suggests that Alx-4 binding to DNA is abolished. The phenotypic differences between Alx-4 null and lst mutant mice suggest the presence of strain-specific modifier genes. Whilst detailed biochemical analysis of Alx-4 binding to DNA is needed to further understand transcriptional regulation by this homeodomain protein, it is possible that the Alx-4 truncated protein (which localizes in the nucleus; data not shown) interacts with putative Alx-4-binding proteins, thereby sequestering and preventing them from interacting with the wild-type Alx-4 protein. A similar observation has been reported for the interaction between Pbx and engrailed (Peltenburg and Murre, 1997). Mutations in helix 1 and 2 of the Pbx homeodomain abolish heterodimerization ability, which is not affected after mutations in helix 3 or 4.

**Alx-4 and Shh reciprocal interaction is independent of Gli3**

It is clear that we are far from a complete understanding of the
molecular details of the genetic interactions that occur during the establishment of the anteroposterior limb axis. Nonetheless, expression pattern studies in various limb mutants might be useful. The chick *diplopodia4* mutant is characterized by the presence of ectopic digits (MacCabe and Abbott, 1974; MacCabe et al., 1975) without ectopic expression of *shh* (Rodriguez et al., 1996). Although the molecular basis of this mutation is not known, we have shown that several genes linked to the polarizing activity phenomenon (i.e. *Bmp-2, Fgf-4* and *Hoxd* genes) are ectopically expressed in *diplopodia4* limb buds (Rodriguez et al., 1996). This suggests that the limb phenotype observed in *diplopodia4* embryos is caused by activation of downstream components of the Shh signaling pathway. Activation of the Shh signaling pathway at the anterior margin of the limb could be the cause of *Alx-4* mRNA downregulation (Fig. 3). On the other hand, Gli3 expression is downregulated in the anterior *lsl* limb buds (Fig. 7) and *Alx-4* expression in *Xt/Xt* mice (a *Gli3* mutant) is unchanged (Fig. 7). Together with the observation that downregulation of *Alx-4* by Shh occurs earlier than downregulation of Gli3 (Fig. 6), these data suggest that *Alx-4* and Gli3 could act in parallel pathways, and that the reciprocal negative interactions between *Alx-4* and Shh during the anteroposterior patterning of the limb bud are mediated by some other factors.

Overall the results presented here indicate that *Alx-4* is a critical component of the molecular mechanisms that establishes the anteroposterior patterning of the vertebrate limb. Although further investigations are needed, our data support the idea of a reciprocal interaction between the anterior and posterior cells of the developing vertebrate limb.

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**NOTES ADDED IN PROOF**

Whilst this manuscript was under revision, similar results describing the 16 bp deletion in the homeodomain of *Alx-4* were reported by:


After the acceptance of our manuscript we have completed the full length sequence of chick Alx-4 which has been deposited under GenBank database, accession no. AF092538.