Author's correction to the print version

The lst allele is lst^{J} and not lst^{D}.

Development 125, 4417-4425 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV4060

The role of *Alx-4* in the establishment of anteroposterior polarity during vertebrate limb development

Motohisa Takahashi[2,*], Koji Tamura[1,*], Dirk Büscher[1], Hiroshi Masuya[3], Sayuri Yonei-Tamura[1], Ken Matsumoto[2], Mayumi Naitoh-Matsuo[2], Jun Takeuchi[2], Keiko Ogura[2], Toshihiko Shiroishi[3], Toshihiko Ogura[2,‡] and Juan Carlos Izpisúa Belmonte[1]

[1]The Salk Institute for Biological Studies, Gene Expression Laboratory, 10010 N. Torrey Pines Road, La Jolla, CA 92037-1099, USA
[2]Nara Institute of Science and Technology, 8916-5, Takayama, Ikoma, Nara 630-01, Japan
[3]Mammalian Genetics Laboratory, National Institute of Genetics, 1111, Yata, Mishima, Japan
*These two authors contributed equally to this work
[1,2]The laboratories of these two authors contributed equally to this study
‡Author for correspondence (e-mail: ogura@bs.aist-nara)

Accepted 10 September; published on WWW 20 October 1998

SUMMARY

We have determined that *Strong's Luxoid (lst^J)* 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 *lst^J* 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 polydactylous 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 (Helms 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-Martinez 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

(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 polydactylous mutants. 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 polydactylous 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* (lst^J) mice. lst^J is a semidominant mutation resulting in preaxial polydactyly. Recently targeted disruption of *Alx-4* has been shown to cause a similar phenotype to the lst^J 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 Alx-4 and Shh and show that this interaction appears to be independent of Gli3. These results indicate that the anterior localization of *Alx-4* in the limb bud is a key event in determining the correct anteroposterior axis of the vertebrate limb.

MATERIALS AND METHODS

Cloning of chick *Alx-4* and mutant *Alx-4* allele in lst^J mice

A chick cDNA library (random primed) was prepared from poly(A)+ RNA isolated from stages 20-23 chick limb buds using Time Saver cDNA synthesis kit (Pharmacia) and ZAPII vector (Stratagene) as described in the manufacturers' instructions. One million independent plaques were screened with the mouse paired type homeobox gene *Rx* (Mathers et al., 1997). 40 positive clones were isolated, subcloned into plasmids, purified and sequenced (Qiagen). Two clones were found to be the chick homologue of mouse *Alx-4*. The *Strong's Luxoid* mouse cDNA library was made from poly(A)+ RNA purified from lst^J embryo ($lst^J/+$) limb buds as described above. The library was screened with a chick *Alx-4* cDNA probe and positive clones were sequenced.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed essentially as described (Wilkinson, 1993). A plasmid containing chick *Alx-4* was linearized with *Bam*HI and transcribed with T7 RNA polymerase. The chick (a kind gift from Dr Cliff Tabin, Harvard Medical School) and mouse *Gli3* digoxigenin-labeled probes are as described (Marigo et al., 1996; Büscher et al., 1997). The *sonic hedgehog* probe was prepared as described (Yonei et al., 1995). Genotyping of *Xt* embryos was carried out as described previously (Büscher et al., 1997). The *diplopodia-4* chick embryos were a kind gift from Robert Kos and Dr Ursula Abbott (Avian Science Department, UC Davis, CA 95616).

Bead implantations and embryo manipulations

Affigel blue beads (200-250 μm in diameter) were used as carriers for Shh protein (5 μg/μl; Ontogeny, Inc., 45 Moulton Street, Cambridge, MA 02138-1118) and FGF protein (1 μg/μl; R&D Systems, 614 McKinley Pl. N.E., Minneapolis, MN 55413). Beads were anchored to the limb buds by tungsten pins (20 μm in diameter). Staging of chick embryos was done according to Hamburger and Hamilton (1951). Using ophthalmological forceps and electrolytically sharpened tungsten wire needles, Shh beads were implanted at the anterior margin beneath the AER of stage 19/20 wing buds and FGF beads were implanted in the lateral plate mesoderm at somite 23-25 level of stage 15/16 embryos. For the AER removal, the ectoderm of stage 18 or 20 wing buds was stained with Nile blue sulfate (100 μg/ml) and the AER was removed with a tungsten needle.

RESULTS

Cloning of chick and mouse *Alx-4*

Screening of mouse and chick libraries with the paired type mouse *Rx* 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, aristaless, 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 anteroproximal 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 manipulations 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 anteroposterior 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 (Crossley

A

```
mouse MNAETCVSYCESPAAAMDAYYSPVSQSREGSSPFRGFPGG          40
chicken
      DKFGTTFLSAGAKGQGFGDAKSRARYGAGQQDLAAPLESS          80
      SGARGSFNKFQPQPPTPQPPPAPPAPPAHLYLQRGACKTP          120
                                              PARP.
      PDGSLKLQEGSGGHNAALQVPCYAKESNLGEPELPPDSEP          160
      .ESN.....-.....G....S..G...S...AD.Q.NTD.
      VGMDNSYLSVKETGAKGPQDRASAEIPSPLEKTDSESNKG          200
      S...S.......A.V.V......TDL...MD.A.......
      KKRRNRTTFTSYQLEELEKVFQKTHYPDVYAREQLAMRTD          240
      .....................................
      LTEARVQVWFQNRRAKWRKRERFGQMQQVRTHFSTAYELP          280
      ..........................................
      LLTRAENYAQIQNPSWIGNNGAASPVPACVVPCDPVPACM          320
      ..................................E...S..
      SPHAHPPGSGASSVSDFLSVSGAGSHVGQTHMGSLFGAAG          360
      ......HAA.--G.....G..ST.G......V.G...T..
      ISPGLNGYEMNGEPDRKTSSIAALRMKAKEHSAAISWAT          399
      .G.S.....L.S.....S.....................
```

B

```
consensus  -RR-RT-F---QL--LE--F---HYPD---RE-LA----L-E-R-QVWFQNRRAK-R--E
                    Helix1          Helix2          Helix3/4
Alx-4      KRRNRTTFTSYQLEELEKVFQKTHYPDVYAREQLAMRTDLTEARVQVWFQNRRAKWRKRE identity(%)
Alx-3      ........STF.......................L.......................... 93
Cart-1     ...H......L...................V.....L..E..................... 92
Arx        Q..Y.............RA.........FT..E....L..................... 87
Chx-10     ...H..I............A.NEA.........M...K.E.P.D.I.............. 80
aristaless Q..Y......F........A.SR......FT..E...KIG.....I.............Q. 77
unc-4      R..T..N.SGW......SA.EAS.....FM..A....L..L.S................. 70
Prx-1      Q.......N.S..QA..R..ER.....AFV..D..R.VN..............F.RN. 70
Prx-2      Q.......N.S..QA..R..ER.....AFV..E..R.VN.S............F.RN. 68
```

Fig. 1. (A). Comparison of amino acid sequence of mouse and chick Alx-4 protein. Identical amino acids are indicated as a dot, otherwise as symbolized using single letter code. The homeodomain is marked by the black box. Areas of high homology outside the homeodomain are underlined (sequence following the homeodomain), or outlined (most of the C-terminal part, the 'pair tail motif' sequence). (B). Comparison of the homeodomain of different Alx-4 related proteins. The percentage of identity indicated on the right refers to the homeodomain only. The sequence areas of the helices are depicted by a box. An overall consensus sequence of the mentioned Alx-4 related proteins is shown above the helices.

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

Based on comparative chromosomal mapping studies, the *lst*^{J} 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; Vogt and Leder, 1996). Targeted disruption of *Alx-4* results in mice with preaxial polydactyly, a phenotype also observed in the *lst*^{J} mice. This suggests a relationship between the *lst*^{J} gene product and *Alx-4* (Qu et al., 1997; Vogt and Leder, 1996).

In order to investigate this possibility, we sequenced six *Alx-4* clones isolated from an *lst*^{J}/+ 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 homeobox (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*^{J} amino acid sequences shows that neither the functional helix 3/4 of the homeodomain nor C-terminal region would exist in the Alx-4^{lstJ} 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*^{J} 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*^{J} 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*

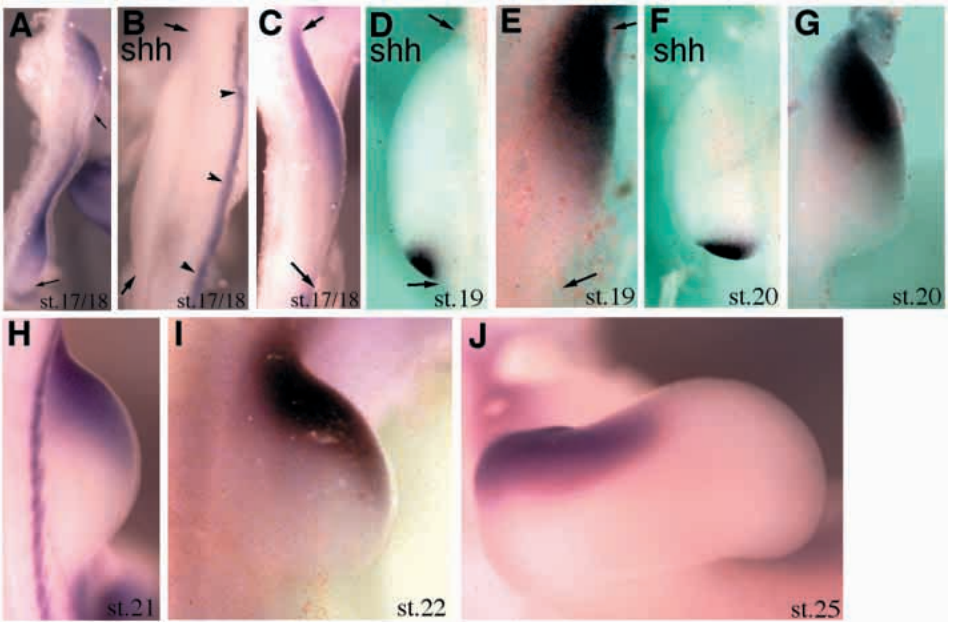

Fig. 2. Expression of *shh* and *Alx-4* during chick limb bud outgrowth. (A) Right side of a stage 17/18 chick stained with *Alx*-4. Arrows indicate lateral plate mesoderm lacking detectable *Alx*-4 expression. (B) Contralateral side of the embryo shown in A stained for *shh* expression. Arrows on the 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).

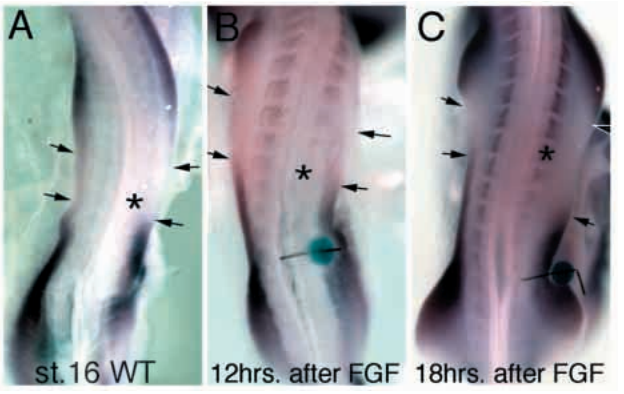

Fig. 3. *Alx-4* expression after FGF-beads implantation. (A) Control embryo at stage 16 showing *Alx*-4 expression. The asterisk marks somite 21. (B,C) FGF-beads were implanted in stage 15/16 embryos and *Alx-4* expression was monitored 12 hours later (B) or 18 hours later (C). The asterisks indicate position of somite 21; the arrows indicate the region in the flank showing no *Alx-4* expression. Note that this region is significantly enlarged in the right side of C.

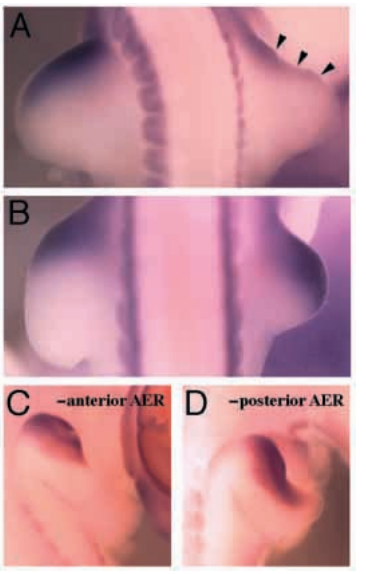

Fig. 4. *Alx-4* expression after AER removal. (A) The anterior AER was removed at stage 18 and the embryo was stained for *Alx-4* 24 hours later. The arrowheads indicate the reduced expression. (B) As in A, only that the posterior AER was removed. Note that expression remains unchanged. (C) The anterior AER was removed at stage 20 and the embryo stained for *Alx*-4 expression 24 hours later. (D) As in C, only that the posterior AER was removed. Note that in both cases (C,D) the anteroproximal expression domain remained unchanged.

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 post-surgery (arrowheads in Fig. 6F; *n*=3).

Gli3 expression in *lst*^{J} 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 *lst*^{J} mouse. Expression of *Gli*3 in 10 dpc *lst*^{J} 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 *Gli*3 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 *lst*^{J} limb buds, *Gli*3 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 *Gli*3 expression pattern, indicating that Gli3 is not downstream of Alx-4 (Fig. 7B, lower limb bud). The lack of *Gli*3 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 *Gli*3 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 polydactylous 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 *Gli*3.

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

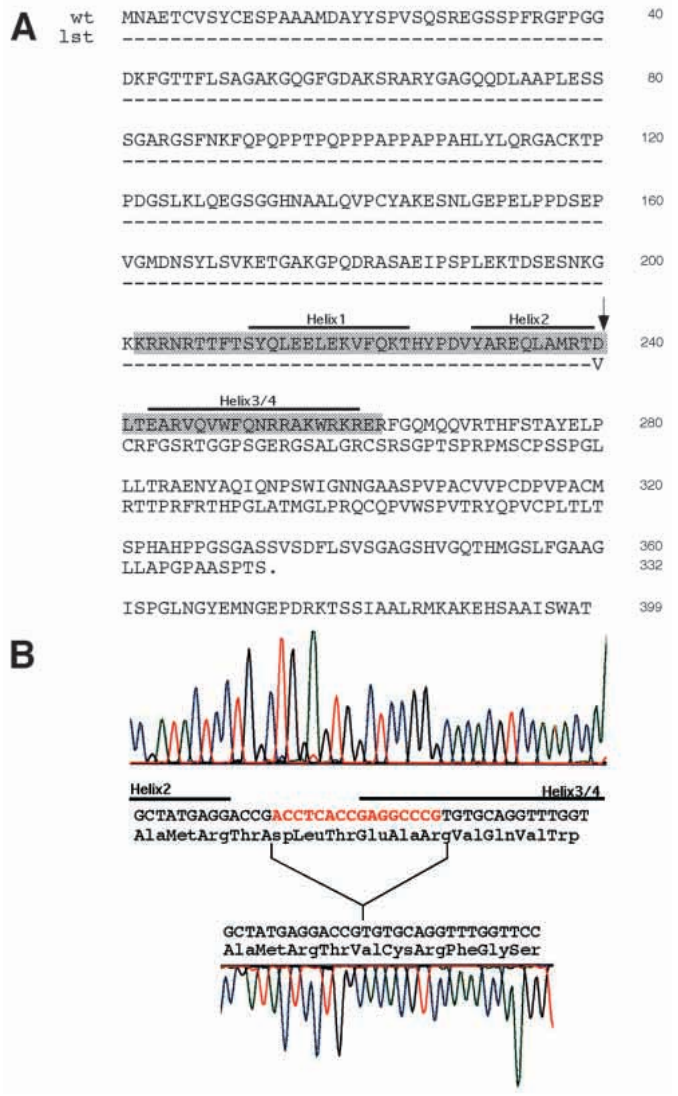

Fig. 5. (A) Comparison of deduced amino acid sequences of wt and Alx-4^{lstJ} proteins. The homeodomain is shaded and the positions of the helixes are indicated above the sequence. The arrow at position 240 marks the beginning of the altered sequence in Alx-4^{lstJ}. Note that no homology exists after helix 2. (B) Nucleotide sequence of wild-type and *lst*^{J} homeoboxes covering the mutated area. The deletion is indicated in red, with the subsequently altered *lst*^{J} sequence shown below.

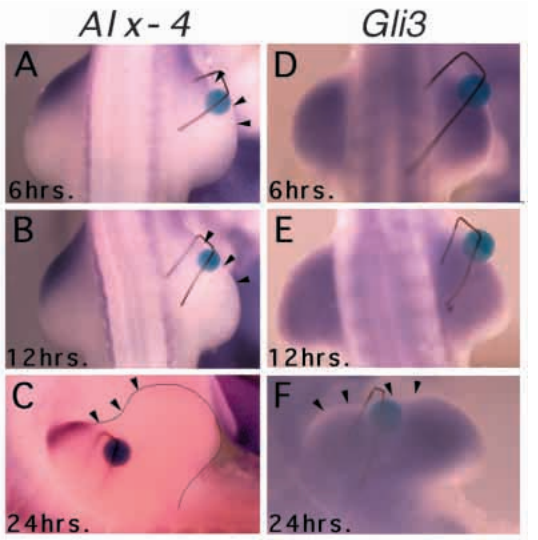

Fig. 6. Beads soaked in Shh protein were implanted in the right wing bud of stage 19/20 chick embryos and stained at different time points for *Alx-4* (A-C) or *Gli3* (D-F) expression. The arrowheads indicate the area in which the expression was reduced. Note that *Alx-4* expression was already reduced after 6 hours (A), whereas a reduction of *Gli3* expression occurred only after 24 hours (F).

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*^{J} 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.

lst^J, 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*^{J} 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*^{J} 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 (Forsthoefel, 1962, 1963). Chromosomal mapping of the *lst*^{J} 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*^{J} 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*^{J} could be a candidate for an allele of *Alx-4*.

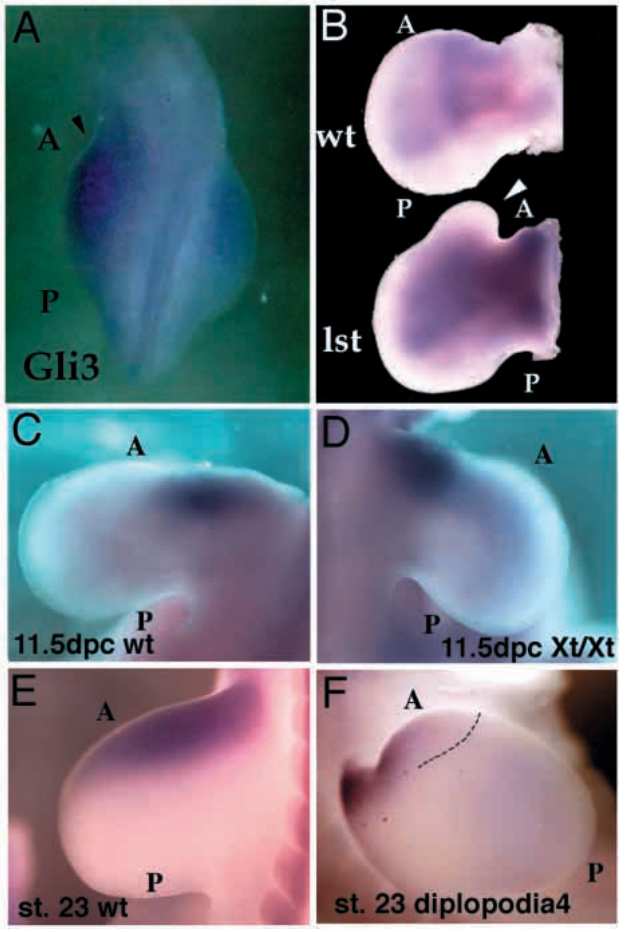

Fig. 7. (A) *Gli3* expression in 10 dpc mouse hindlimbs. The arrowhead indicates high expression in the anterior region. Note that the posterior region is devoid of detectable *Gli3* expression. (B) *Gli3* expression in wild-type (upper) and homozygous *lst*^{J} mouse limb buds (lower) at 12 dpc. The arrowhead indicates lack of detectable *Gli3* expression in the most anterior region of the mutant limb bud. (C-F) *Alx-4* expression in mouse and chick polydactyly mutants. Expression in 11.5 dpc wt (C) or *Xt*/*Xt* (D) mouse forelimbs and stage 23 chick wild-type (E) or *diplopodia4* (F) wing buds. The line in F indicates the area of normal *Alx-4* expression. A, anterior; P, posterior

In this study, we showed that the phenotype of *lst*^{J} is indeed likely to be caused by a deletion in the homeobox of *Alx-4*. Sequence analysis of a *lst*^{J}/+ cDNA limb library revealed a 16 bp deletion in the *Alx-4* gene. This deletion results in a frame-shift 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*^{J} versus the recessive phenotype in the *Alx-4* null mice argues for a dominant negative Alx-4^{lstJ} 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^{lstJ} binding to DNA is abolished. The phenotypic differences between *Alx-4* null and *lst*^{J} mutant mice suggest the presence of strain-specific modifier genes. Whilst detailed biochemical analysis of Alx-4^{lstJ} 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 (Rodríguez 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 *lst*^{J} 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 bud.

We thank Bruce Blumberg, Javier Capdevila and Jennifer Ng for discussion and invaluable comments. We thank Lorraine Hooks for her skills and dedication in assembling this manuscript. We thank Ulrich Rüther and Lars Grotewold for providing *Xt* embryos and Robert Kos and Ursula Abbott for providing the *diplopodia4* chick mutant. K. T. and S. Y. T. were supported by the JSPS. D. B. was supported by a fellowship from the Deutsche Forschungsgemeinschaft. The initial stage of this research was supported by a grant from Sumitomo Zaidan to T. O. Work in the laboratories of T. O. and J. C. I. B. was supported by a grant from the HFSPO, and a grant from the G. Harold and Leyla Y. Mathers Charitable Foundation to J. C. I. B who is a Pew Scholar.

REFERENCES

Bryant, S. V. and Gardiner, D. M. (1992). Retinoic acid, local cell-cell interactions, and pattern formation in vertebrate limbs. *Dev. Biol.* **152**, 1-25

Büscher, D., Bosse, B., Heymer, J. and Rüther, U. (1997). Evidence for genetic control of Sonic hedgehog by Gli3 in mouse limb development. *Mech. Dev.* **62**, 175-182.

Büscher, D., Grotewold, L. and Rüther, U. (1998). The Xt^{J} allele generates a *Gli3* fusion transcript. *Mamm. Genome*, In press.

Chan, D. C., Laufer, E., Tabin, C. and Leder, P. (1995). Polydactylous limbs in *Strong's Luxoid* mice result from ectopic polarizing activity. *Development* **121,** 1971-1978.

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

Chaube, S. (1959). On axiation and symmetry in the transplanted wing of the chick. *J. Exp. Zool.* **140**, 29-77.

Cohn, M., Izpisúa Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995). FGF-2 application can induce additional limb formation from the flank of chick embryos. *Cell* **80,** 739-746.

Cohn, M. J., Patel, K., Krumlauf, R., Wilkinson, D. G., Clarke, J. D. and Tickle, C. (1997). Hox9 genes and vertebrate limb specification. *Nature* **387**, 97-101. (1997).

Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R. (1996). Roles for FGF-8 in the induction, initiation and maintenance of chick limb development. *Cell* **84,** 127-136.

Duboule, D. (1994). How to make a limb? *Science* **266,** 575-576.

Fallon, J. F., López, A., Ros, M. A., Savage, M. P., Olwin, B. B. and Simandl, B. K. (1994). FGF-2: Apical ectodermal ridge growth signal for chick limb development. *Science* **264,** 104-107.

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). Responses to selection for plus and minus modifiers of some effects of *Strong's luxoid* gene on the mouse skeleton. *Teratology* **1,** 339-352.

Gehring, W. J., Qian, Y. Q., Billeter, M., Furukubo-Tokunaga, K., Schier, A. F., Resendez-Perez, D., Affolter, M., Otting, G. and Wuthrich, K. (1994). Homeodomain-DNA recognition. *Cell* **78,** 211-223.

Hamburger, V. (1938). Morphogenic and axial self-differentiation of transplanted limb primordia of 2-day chick embryos. *J. Exp. Zool.* **77,** 379-400.

Hamburger, V. and Hamilton, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88,** 49-92.

Helms, J. A., Kim, C. H., Eichele, G. and Thaller, C. (1996). Retinoic acid signaling is required during early chick limb development. *Development* **122**, 1385-1394.

Hornbruch, A. and Wolpert, L. (1991). The spatial and temporal distribution of polarizing activity in the flank of the pre-limb-bud stages in the chick embryo. *Development* **111,** 725-731.

Hui, C. C. and Joyner, A. L. (1993). A mouse model of Greig cephalopolysyndactyly syndrome: the extra toes mutation contains an intragenic deletion of the *Gli3* gene. *Nature Genet.* **3,** 241-246.

Johnson, R. L. and Tabin, C. J. (1998). Molecular models for vertebrate limb development. *Cell* **90,** 979-990.

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.

López-Martinez, A., Chang, D. T., Chiang, C., Porter, J. A., Ros, M. A., Simandl, B. K., Beachy, P. A. and Fallon, J. F. (1995). Limb-patterning activity and restricted posterior localization of the amino-terminal product of Sonic hedgehog cleavage. *Current Biology* **5,** 791-796.

Lu, H.-C., Revelli, J.-P., Goering, L., Thaller, C. and Eichele, G. (1997). Retinoid signaling is required for the establishment of a ZPA and for the expression of *hoxb-8*, a mediator of ZPA formation. *Development* **124,** 1643-1651.

MacCabe, J. A. and Abbott, U. K. (1974). Polarizing and maintenance activities in two polydactylous mutants of the fowl: *diplopodia1* and *talpid2*. *J. Embryol. Exp. Morph.* **31,** 734-746.

MacCabe, J. A., MacCabe, A. B., Abbott, U. K. and McCarrey, J. R. (1975). Limb development in *Diplopodia:* A polydactylous mutation in the chicken. *J. Exp. Zool.* **191,** 383-394.

Mahmood, R., Bresnick, J., Hornbruch, A., Mahony, C., Morton, N., Colquhoun, K., Martin, P., Lumsden, A., Dickson, C. and Mason, I. (1995). A role for FGF8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Curr. Biol.* **5,** 797-806.

Marigo, V., Johnson, R. L., Vortkamp, A. and Tabin, C. J. (1996). Sonic hedgehog differentially regulates expression of *GLI* and *GLI3* during limb development. *Development* **180**, 273-283.

Martí, E., Takada, R., Bumcrot, D. A., Sasaki, H. and McMahon, A. P. (1995). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. *Development* **121**, 2537-2547.

Masuya, H., Sagai, T., Morikawi, K. and Shiroishi, T. (1997). Multigenic control of the localization of the zone of polarizing activity in limb morphogenesis in the mouse. *Dev. Biol.* **182,** 42-51.

Masuya, H., Sagai, T., Wakana, S., Moriwaki, K. and Shiroishi, T. (1995). A duplicated zone of polarizing activity in polydactylous mouse mutants. *Genes Dev.* **9,** 1645-1653.

Mathers, P. H., Grinberg, A., Mahon, K. A. and Jamrich, M. (1997). The Rx homeobox gene is essential for vertebrate eye development. *Nature* **387,** 603-607.

Ng, J. K., Tamura, K., Büscher, D. and Izpisúa Belmonte, J. C. (1998). Molecular and cellular basis of pattern formation during vertebrate limb

development. In *Current Topics in Dev. Biol.* San Diego: Academic Press. (In Press).

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

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N. and Noji, S. (1997). The mesenchymal factor, FGF-10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* **124,** 2235-2244.

Ohuchi, H., Nakagawa, T., Yamauchi, M., Ohata, T., Yoshioka, H., Kuwana, T., Mima, T., Miwaka, T., Nohno, T. and Noji, S. (1995). An additional limb can be induced from the flank of the chick embryo by FGF4. *Biochem. Biophys. Res. Comm*. **209**, 809-816**.**

Peltenburg, L. T. C. and Murre, C. (1997). Specific residues in the Pbx homeodomain differentially modulate the DNA-binding activity of Hox and Engrailed proteins. *Development* **124,** 1089-1098.

Qu, S., Niswender, K. D., Ji, Q., van der Meer, R., Keeney, D., Magnuson, M. A. and Wisdom, R. (1997). Polydactyly and ectopic ZPA formation in *Alx-4* mutant mice. *Development* **124,** 3999-4008.

Qu, S., Tucker, S. C., Ehrlich, J. S., Levorse, J. M., Flaherty, L. A., Wisdom, R. and Vogt, T. (1998). Mutations in mouse *Aristaless-like4* cause *Strong's luxoid* polydactyly. *Development* **125**, 2711-2721.

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.

Rodríguez, C., Kos, R., Macías, D., Abbott, K. and Izpisúa Belmonte, J. C. (1996). Shh, HoxD, Bmp-2, and Fgf-4 gene expression during development of the polydactylous talpid2, diplopodia1, and diplopodia4 mutant chick limb buds. *Dev. Genetics* **19,** 26-32.

Ros, M. A., López-Martinez, A., Simandl, B. K., Rodríguez, C., Izpisúa Belmonte, J. C., Dahn, R. and Fallon, J. (1996). The limb field mesoderm determines initial limb bud anteroposterior asymmetry and budding independent of *sonic* hedgehog or apical ectodermal gene expressions. *Development* **122,** 2319-2330.

Rowe, D. A. and Fallon, J. F. (1982). The proximodistal determination of skeletal parts in the developing chick leg. *J. Embryol. Exp. Morph.* **68,** 1-7.

Saunders, J. W. and Gasseling, M. T. (1968). Ectodermal-mesenchymal interactions in the origin of wing symmetry. In *Epithelial-Mesenchymal Interactions*. (ed. R. Fleischmajer and R. E. Billingham). pp. 78-97. Baltimore: Williams and Wilkins.

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

Schwabe, J. W. R., Rodríguez-Esteban, C. and Izpisúa Belmonte, J. C. (1998). Limbs are moving...where are they going? *Trends in Genetics* **14**, 229-235.

Schimmang, T., Lemaistre, M., Vortkamp, A. and Rüther, U. (1992). Expression of the zinc finger gene *Gli3* is affected in the morphogenetic mouse mutant extra-toes *(Xt)*. *Development* **116,** 799-804.

Stratford, T., Horton, C. and Maden, M. (1996). Retinoic acid is required for the initiation of outgrowth in the chick limb bud. *Curr. Biol*. **6**, 1124-1133.

Stratford, T. H., Kostakopoulou, K. and Maden, M. (1997). *hoxb-8* has a role in establishing early anterior-posterior polarity in chick forelimb but not hindlimb. *Development* **124,** 4225-4234.

Summerbell, D. (1974). A quantitative analysis of the effect of excision of the AER from the chick limb bud. *J. Embryol. Exp. Morph*. **32,** 651-660.

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

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

Todt, W. and Fallon, J. F. (1984). Development of the apical ectodermal ridge in the chick wing bud. *J. Embryol. Exp. Morph.* **80,** 21-41.

Vogel, A., Rodríguez, C. and Izpisúa Belmonte, J. C. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122,** 1737-1750.

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

Vogt, T. and Leder, P. (1996). Polydactyly in the *Strong's luxoid* mouse is suppressed by limb deformity alleles. *Dev. Genet.* **19,** 33-42.

Wilkinson, D. G. (1993). Whole mount *in situ* hybridisation of vertebrate embryos. In *In Situ Hybridisation*. (ed. D. G. Wilkinson). Oxford: Oxford University Press.

Yang, Y. Z. and Niswander, L. (1995). Interaction between the signaling molecules *Wnt7a* and *shh* during vertebrate limb development; dorsal signals regulate anteroposterior patterning. *Cell* **80**, 939-947.

Yonei, S., Tamura, K., Ohsugi, K. and Ide, H. (1995). MRC-5 cells induce the AER prior to the duplicated pattern formation in chick limb bud. *Dev. Biol.* **170,** 542-552.

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:

Qu, S., Tucker, S. C., Ehrlich, J. S., Levorse, J. M., Flaherty, L. A., Wisdom, R. and Vogt, T. (1998). Mutations in mouse *Aristaless-like4* cause *Strong's luxoid* polydactyly. *Development* **125**, 2711-2721.

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.