

The *limb deformity* mutation disrupts the SHH/FGF-4 feedback loop and regulation of 5' *HoxD* genes during limb pattern formation

Anna G. Haramis, Jennifer M. Brown and Rolf Zeller*

EMBL, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

*Author for correspondence

SUMMARY

Mutations in the murine *limb deformity* (*ld*) gene disrupt differentiation of the Apical Ectodermal Ridge (AER) and patterning of distal limb structures. However, initial outgrowth of the limb bud is not affected, suggesting that early and late functions of the AER are uncoupled. Similarly, activation of the 5' members of the *HoxD* gene cluster (*Hoxd-11* to *Hoxd-13*) is not affected in *ld* mutant posterior limb bud mesenchyme, but the subsequent anteriorization of 5' *HoxD* domains is delayed by about 12 hours and is associated with reduced levels of polarising activity. These results indicate that the *ld* gene products act upstream of 5' *HoxD* genes during patterning of the autopod. Expression of the signalling molecule *Sonic hedgehog* (*Shh*) in the posterior limb bud mesenchyme is initiated normally, but ceases prematurely indicating a

defect in maintenance of *Shh* by the *ld* mutant AER. Furthermore, no *Fgf-4* transcripts are detected in the *ld* mutant AER, whereas *Fgf-8* transcripts remain expressed. However, *Shh* expression can be rescued by heterospecific grafting of *ld* mutant posterior mesenchyme under a wild-type chicken AER. These studies show that the AER defect in *ld* homozygous limb buds causes disruption of the FGF-4/SHH feedback loop and support the proposed essential role for FGF-4 in maintaining *Shh* expression during limb pattern formation.

Key words: epithelial-mesenchymal interactions, FGF-4, FGF-8, *HoxD* genes, limb pattern formation, *limb deformity* (*ld*), SHH, mouse

INTRODUCTION

Growth and patterning of the vertebrate limb is regulated by two main signalling centres (reviewed by Tickle and Eichele, 1994). One, the apical ectodermal ridge (AER), extends along the distal tip of the limb bud and its signals act on the underlying mesenchyme. AER signals promote proximo-distal outgrowth by maintaining the distal limb bud mesenchyme (progress zone) in a proliferative state (Summerbell et al., 1973) and maintain the posterior mesenchymal cells of the polarising region (ZPA) in an active signalling state (Niswander et al., 1993; Todt and Fallon, 1987). The polarising region cells in turn function as a limb bud organiser by emitting signals that control establishment of antero-posterior limb axis polarity (Tabin, 1991; Tickle, 1991). Therefore, limb bud outgrowth and patterning are mutually dependent and coordinated by signals emanating from the AER and polarising region.

Several signalling molecules mediating interactions between AER and the limb bud mesenchyme have been identified. In particular, members of the fibroblast growth factor (FGF) gene family are expressed in the AER during its differentiation and functioning (for details see Crossley and Martin, 1995; Dono and Zeller, 1994; Mahmood et al., 1995; Niswander and Martin, 1992). One member, FGF-4 can promote limb bud outgrowth and maintain activity of polarising region cells

(Niswander and Martin, 1993; Niswander et al., 1993; Vogel and Tickle, 1993). The polarising region cells express *Sonic hedgehog* (*Shh*), a signalling molecule that induces mirror-image digit duplications when expressed ectopically in the anterior limb bud mesenchyme (Riddle et al., 1993; reviewed by Johnson and Tabin, 1995). Experimental manipulation of chicken limb buds has shown that SHH and FGF-4 are part of a positive feedback loop between mesenchyme and AER that regulates limb morphogenesis (Laufer et al., 1994; Niswander et al., 1994).

Furthermore, SHH participates in regulating expression of 5' (posterior) members of the *HoxD* gene cluster (Riddle et al., 1993; Laufer et al., 1994). Genetic analysis has shown that 5' members of both the *HoxD* and *HoxA* gene clusters are involved in determining positional identities of limb bud mesenchymal cells (see e.g. Dollé et al., 1993; Davis and Capecchi, 1994). The establishment of the nested expression domains of the *Hoxd-10* to *Hoxd-13* genes in the limb bud is controlled by their position within the cluster (spatial and temporal colinearity; for details see Duboule, 1994b). Initially, their transcripts are restricted posteriorly (early domains), but subsequently their domains extend anteriorly in the distal part of the limb bud (late domains). Recent studies by Sordino et al. (1995) suggest that the late domains function in morphogenesis of vertebrate autopods (hand- and foot plates). Indeed, inactivation of the most 5' member of the *HoxD* gene cluster

(*Hoxd-13*) causes a neotenic delay in differentiation of all autopodal skeletal elements (Dollé et al., 1993).

Patterning of the autopod is also severely affected by mutations of the murine *limb deformity* (*ld*) locus. Five recessive *ld* alleles have been isolated which cause oligodactyly and syndactyly of autopod skeletal elements (Kleinebrecht et al., 1982; Woychik et al., 1985). Ulna and radius primordia fuse during ossification (secondary fusion), whereas tibia and fibula form as one primordium (Kleinebrecht et al., 1982; Zeller et al., 1989). This phenotype first manifests itself during early limb bud morphogenesis by a failure of the AER cells to differentiate into the characteristic columnar epithelium and leads to severe truncations of the autopod (Zeller et al., 1989). These studies indicated a pivotal role for *ld* gene products in the co-ordination of limb bud outgrowth and patterning. The *ld* gene gives rise to several alternatively spliced transcripts that are expressed during embryogenesis and in specific adult tissues (Maas et al., 1990; Woychik et al., 1990a; Jackson-Grusby et al., 1992). The corresponding Ld proteins (or formins) are nuclear proteins expressed in a variety of cell-types from gastrulation onwards (de la Pompa et al., 1995; Trumpp et al., 1992). Their distribution in the developing limb bud is confined to the differentiating AER and shows a dynamic pattern in the mesenchyme (Trumpp et al., 1992) consistent with roles in both AER differentiation and patterning of the limb bud mesenchyme.

We have used two *ld* alleles (*ld^{ln2}*, Woychik et al., 1990b; *ld^l*, Green, 1962) as genetic tools to investigate the molecular pathways that regulate vertebrate limb pattern formation. In limb buds of *ld* homozygous embryos, the establishment of the anteriorly expanded and distally shifted *Hoxd-11* to *Hoxd-13* expression domains was delayed by approximately 12 hours. Concurrently, polarising activity and *Shh* expression were not maintained in the posterior mesenchyme. Interestingly, FGF-4, the candidate *Shh* maintenance factor, was not expressed by the *ld* mutant AER. However, *Shh* expression could be rescued by heterospecific grafting of *ld* mutant mouse posterior limb bud mesenchyme under a wild-type chicken AER. These results demonstrate that the defect leading to disruption of the FGF-4/SHH feedback loop resides in the *ld* mutant AER. The *ld* gene products act upstream of the *HoxD* genes and interact with both the epithelial and mesenchymal signals that control growth and patterning of the distal limb bud compartment.

MATERIALS AND METHODS

Genetic crosses and staging of mouse embryos

Mice strains were maintained by crossing B6/SJLF1 wild-type mice to either the homozygous or heterozygous *ld^{ls1Gso}* (*ld^{ln2}*, Woychik et al., 1990b; provided by Oak Ridge National Laboratory) or *ld^l* (Green, 1962; provided by Jackson Laboratories) mice. To isolate embryos, timed pregnancies were set and the day of the vaginal plug was considered as gestational day zero. The somites of all isolated embryos were counted and the embryonic stages were determined according to Martin (1990). For all experiments described in this study, age-matched embryos with an equal number of somites (variation: \pm one somite) were used. All results shown are based on analysis of at least three embryos per developmental stage and genotype.

Genotyping of embryos

The genotypes of embryos were determined using genomic DNA

isolated from their extra-embryonic membranes and/or heads (Zeller et al., 1989). The genotypes of embryos from the *ld^l* line were determined using Southern Blot analysis as described by Woychik et al. (1985), whereas embryos from the *ld^{ln2}* strain were genotyped using PCR amplification. To obtain heterozygous and homozygous embryos of the *ld^{ln2}* strain, *ld^{ln2/+}* females were mated with *ld^{ln2/ld^{ln2}}* males, therefore PCR primers were designed to detect the wild-type allele. PCR primers were derived from a subclone of plasmid pLE1-5.5-11, which encompasses the breakpoint of *ld^{ln2}* allele (*ld^{ls1Gso}*; Bultman et al., 1991). A *Pst*I-*Bam*HI fragment from pLE1-5.5-11 was subcloned, partially sequenced and two primers were designed for genotyping (*wt4as* : 5'TGCCATGCTCAGCTAACAGTA^{3'}; *wt4s*: 5'ACTACAAACCTTCCATCCT CC^{3'}). These primers give rise to a 782 bp PCR product marking the wild-type allele. To exclude false negative samples, the integrity of DNA was checked using a second set of primers against a region common to both the wild-type and *ld^{ln2}* alleles (*wt4as*: see above; *JPI*: 5'TCATGGCTATCCTAAACTACC T^{3'}). These primers produced a 300 bp PCR product in all samples.

Whole-mount in situ hybridisation

Whole-mount in situ hybridisation was performed using a protocol adapted and modified from those of Wilkinson (1993; see also Harland 1991). Embryos were fixed in 4% paraformaldehyde and stored until further use as described by Wilkinson (1993). For hybridisations, embryos were rehydrated into PBS plus 0.1% Tween 20 (PBT) and bleached with 6% hydrogen peroxide in PBT for 15 minutes. After three PBT washes, the embryos were digested for 20 minutes with Proteinase K (Merck 10 μ g/ml in PBT) at room temperature. When using *Fgf-4* and *Fgf-8* riboprobes, embryos were digested with only 5 μ g/ml Proteinase K for 4 minutes to preserve the limb ectoderm (Crossley and Martin, 1995). Following postfixation for 20 minutes in 0.2% glutaraldehyde/4% paraformaldehyde in PBT, embryos were prehybridized at 65°C for 4 hours in prehybridisation mix (modified from Wilkinson, 1993 as follows: 2% blocking powder (Boehringer Mannheim), 0.1% Tween 20, 0.5% CHAPS (Sigma) and 5 mM EDTA were added to the prehybridisation mix, whereas SDS was omitted). Following addition of digoxigenin-labelled riboprobes to 1 μ g/ml, the embryos were hybridised overnight at 70°C. Subsequent washes were carried out in the following solutions at 70°C for 5 minutes each: 100% prehybridization mix; 75% prehybridization mix/25% 2 \times SSC pH 4.5; 50% prehybridization mix/50% 2 \times SSC; 25% prehybridization mix/75% 2 \times SSC. Embryos were then washed twice in 2 \times SSC containing 0.1% CHAPS for 30 minutes at 70°C. After RNase A treatment (20 μ g/ml for 1 hour), embryos were washed twice for 10 minutes in 100 mM maleic acid/150 mM NaCl (pH 7.5) at room temperature and twice at 70°C in the same solution. After two washes in PBS (10 minutes at room temperature) and one in PBT, the embryos were preblocked in PBT containing 10% heat-inactivated sheep serum and 1% BSA for 4 hours at room temperature. The anti-digoxigenin Fab alkaline phosphatase conjugate (Boehringer Mannheim) was preabsorbed in a solution containing 0.3% heat-inactivated mouse embryo powder (or 0.2% chicken plus 0.1% mouse embryo powder for chicken embryos with mouse grafts), 10% heat-inactivated sheep serum and 1% BSA in PBT for 4 hours at 4°C. Embryos were incubated overnight with the preabsorbed antibody (4°C), and washed five times for 45 minutes each with PBT containing 0.1% BSA (room temperature) and twice for 30 minutes each with PBT. Subsequently, the detection reaction was performed as described by Wilkinson (1993) and embryos were stored in PBT at 4°C. All embryos directly compared in this study were treated in exactly the same way.

Antisense riboprobes used for whole-mount in situ hybridisation

The murine *Hoxd-11*, *Hoxd-12* and *Hoxd-13* probes were used as described by Dollé et al. (1989). The murine *Shh* riboprobe corresponds to the 0.6 kb partial cDNA clone described by Echelard et al.

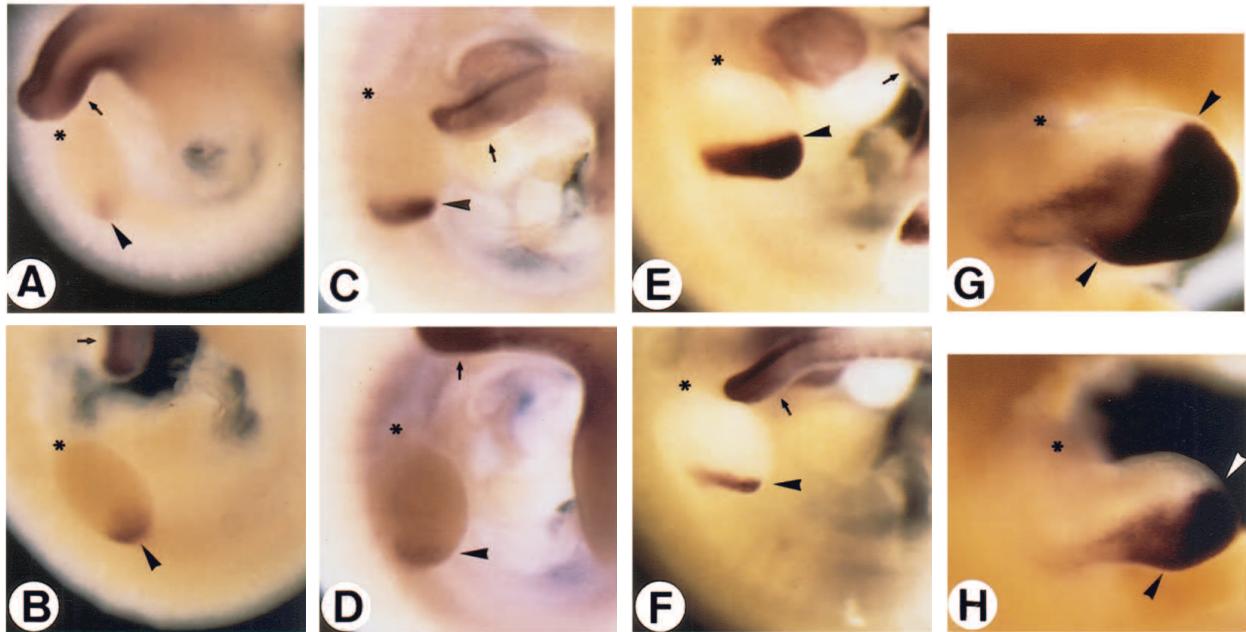


Fig. 1. Up-regulation and establishment of distal *Hoxd-12* expression domains is delayed in *ld* homozygous limb buds. Whole-mount in situ hybridisation study of the *Hoxd-12* transcripts in forelimb buds of embryonic day 10-12. Left panels (A,C,E,G): heterozygous embryos; right panels (B,D,F,H): homozygous embryos. Arrowheads indicate *Hoxd-12* expression domains in the limb bud mesenchyme. Asterisks indicate anterior margins of forelimb buds. Small arrows indicate *Hoxd-12* expression in the tail buds. (A) *ld*^{+/+} embryo (E10.25, 31 somites). (B) *ld*^{ld} embryo (E10.25, 32 somites). Note that transcriptional activation in the limb bud is not affected (arrowhead). (C) *ld*^{+/+} embryo (E10.5, 34 somites). (D) *ld*^{ld} embryo (E10.5, 34 somites). Note that *Hoxd-12* expression in the limb bud (arrowhead) remains at low levels. (E) *ld*^{+/+} embryo (E10.75, 38 somites). Note the onset of anterior expansion in the distal compartment (arrowhead). (F) *ld*^{ld} embryo (E10.75, 38 somites). Note that expression of *Hoxd-12* remains low and posteriorly restricted (arrowhead). (G) *ld*^{+/+} embryo (E12). (H) *ld*^{ld} embryo (E12). Arrowheads in G and H indicate the approximate posterior and anterior boundaries of *Hoxd-12* expression.

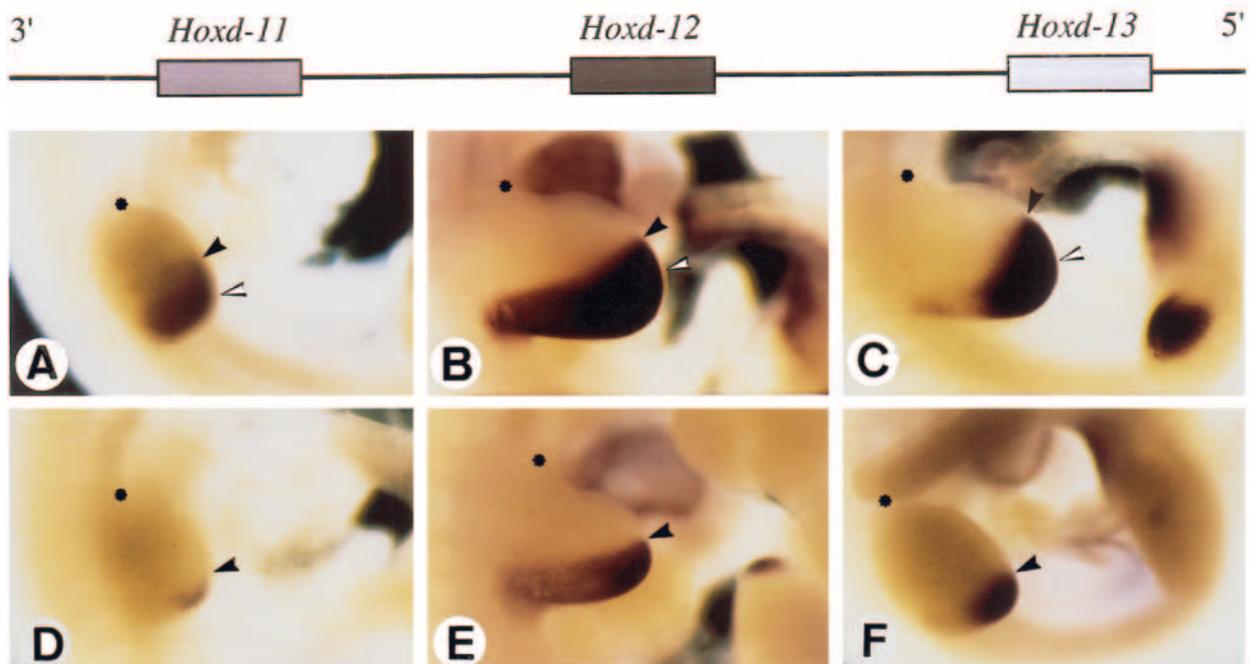


Fig. 2. Up-regulation and onset of anterior expansion of the 5' *HoxD* genes is altered in *ld* homozygous forelimb buds. (A) *Hoxd-11* expression in a *ld*^{+/+} embryo (E10.5, 35 somites). (B) *Hoxd-12* expression in a *ld*^{+/+} embryo (E11, about 40 somites). (C) *Hoxd-13* expression in a *ld*^{+/+} embryo (E11, about 40 somites). (D) *Hoxd-11* expression in a *ld*^{ld} embryo (E10.5, 35 somites). (E) *Hoxd-12* expression in a *ld*^{ld} embryo (E11, about 40 somites). (F) *Hoxd-13* expression in a *ld*^{ld} embryo (E11, about 40 somites). Black arrowheads indicate the anterior boundaries of the expression domains. The white arrowheads in A-C indicate the approximate superimposition of the *ld* mutant anterior boundaries (D-F) onto the wild-type forelimb buds to reveal the delay in anterior expansion. Asterisks indicate the anterior margins of forelimb buds.

(1993). The *Fgf-4* riboprobe was synthesised from a cDNA clone containing the entire coding region (Hebert et al., 1990; Niswander and Martin, 1992). The *Fgf-8* riboprobe was derived from a 400 bp partial cDNA clone containing the 3' UTR and carboxy-terminal coding sequences (Crossley and Martin, 1995). Antisense riboprobes were synthesised as described by Wilkinson (1993).

Grafts of mouse posterior limb bud mesenchyme to chicken limb buds

The posterior halves of mouse limb buds (right side of embryo) were isolated from embryonic day 10.5-11 mouse embryos (age-matched) produced from mating either *ld^{ln2}* or *ld^l* strains. The mouse tissue was placed in 2% trypsin (Sigma) for 1 hour at 4°C. Extraembryonic membranes and heads were frozen in liquid nitrogen to prepare genomic DNA for genotyping. Following trypsinization, the limb bud tissue was placed in minimal essential medium (MEM) plus 10% FCS at 4°C and the ectoderm was removed. To map polarising activity of mouse posterior mesenchyme, pieces of about 200 µm size (corresponding to approximately regions A-D, Fig. 3A) were cut out and individually grafted to the anterior margin of limb buds (right side) of stage 19-20 chicken embryos (Hamburger and Hamilton, 1951). The grafts of mouse mesenchyme were held in a loop made by stretching the AER (for details see Tickle, 1981). The host chicken embryos were incubated in a humidified incubator at 38°C for 6 days and the pattern of cartilage elements was visualised by Alcian green staining. Polarising activity of mouse mesenchyme was scored as described by Vogel and Tickle (1993; see also Tickle, 1981).

To assay *Shh* expression following grafting to chicken limb buds, mouse embryos of embryonic day 10.5-11 were isolated and posterior mesenchyme from right limb buds was grafted as described above. The remaining left side of the mouse embryo was fixed in 4% paraformaldehyde and processed for whole-mount in situ hybridisation. Posterior pieces of limb bud mesenchyme tissue encompassing the *Shh* expression domain (regions A and B, Fig. 3A) were grafted either anteriorly or posteriorly under the AER of host chicken limb buds (see above). Following manipulation, the host chicken embryos were incubated at 38°C for the appropriate time period (6, 12, 15, 18, 21, 24 or 42 hours) and fixed for about 4 hours in 4% paraformaldehyde. Subsequently, the embryos were processed for whole-mount in situ hybridisation with the murine *Shh* riboprobe as described above.

RESULTS

All five *ld* alleles show complete penetrance of the limb phenotype, whereas the severity of the associated renal agenesis varies in an allele-dependent manner (Maas et al., 1994). Therefore, all our studies were performed using both the *ld^{ln2}* (low penetrance of renal agenesis) and *ld^l* alleles (highest penetrance) to reveal any possible allele-specific differences. The results were the same for both *ld* alleles, except for the two differences mentioned below. All embryos shown in Figs 1-6 carry the *ld^{ln2}* allele, except where mentioned otherwise (Fig. 3).

Establishment of the distal expression domains of the *Hoxd-11* to *Hoxd-13* genes is delayed in *ld* mutant limb buds

To investigate whether the *ld* gene products act up- or downstream of 5' *HoxD* gene products, the distribution of *Hoxd-11* to *Hoxd-13* transcripts in wild-type and *ld* mutant limb buds from embryonic days 9.5-12 (E9.5-E12) was compared using whole-mount in situ hybridisation analysis. The distribution of *Hoxd-12* transcripts in heterozygous (phenotypically wild-

type) and *ld* homozygous forelimb buds from embryonic day 10-12 is shown in Fig. 1 (analysis of hindlimb buds yielded similar results; data not shown). *Hoxd-12* transcription starts in the proximal part of the posterior limb bud and is up-regulated before anterior expansion of its domain (E10-E10.75; arrowheads, Fig. 1A,C,E; see also Dollé et al., 1989; Izpisúa-Belmonte et al., 1991). Anterior expansion of the *Hoxd-12* domain occurs during formation of the autopod simultaneously with its distal restriction of expression (late *Hoxd-12* expression domain; arrowheads, Fig. 1G). Transcriptional activation of the *Hoxd-12* gene was normal in *ld* homozygous limb buds (E10; compare arrowheads, Fig. 1A,B) but subsequent up-regulation and onset of anterior expansion were severely delayed (E10.5-E10.75; compare arrowheads Fig. 1C,D and Fig. 1E,F). In striking contrast to the wild-type pattern, *Hoxd-12* expression remained at low levels and more posteriorly restricted in *ld* mutant limb buds during embryonic days 10.75-11 (arrowheads, Figs 1F, 2E). Only during formation of the malformed *ld* mutant autopod, did expression levels become comparable to the wild-type and the *Hoxd-12* domain expanded anteriorly in the distal compartment (E11.5-E12; compare Fig. 1G with 1H). These results reveal a delay of approximately 12 hours in the up-regulation and establishment of the late *Hoxd-12* expression domains in *ld* mutant limb buds.

Sequential colinear activation of *Hoxd-11* to *Hoxd-13* transcripts occurred normally in *ld* mutant forelimb buds (data not shown). In wild-type limb buds, up-regulation and anterior expansion of the *Hoxd-11* domain (E10.5; Fig. 2A) precedes the *Hoxd-12* and *Hoxd-13* domains (E11, Fig. 2B,C; see also Dollé et al., 1989). In *ld* mutant limb buds up-regulation and anterior expansion of the *Hoxd-11* and *Hoxd-13* expression domains was delayed in a similar manner to *Hoxd-12* (arrowheads; Fig. 2D-F; white arrowheads in Fig. 2A-C superimpose the relative anterior expansion in *ld* mutant limb buds onto their wild-type counterparts). This temporal delay was limb bud specific, since expression of the three 5' *HoxD* genes was normal in all other structures in *ld* homozygous embryos (Fig. 1 and data not shown).

Polarising activity is reduced in *ld* homozygous limb buds

The observed temporal changes in the establishment of the late 5' *HoxD* domains (Figs 1, 2) suggested that the *ld* gene products act upstream of these genes. Previous studies showed that signals from the polarising region regulate expression of 5' *HoxD* genes in the limb bud (Izpisúa-Belmonte et al., 1992; Nohno et al., 1991). Therefore, the signalling activity of posterior limb bud mesenchyme from wild-type (*ld/+*) and *ld* homozygous embryos was determined by grafting donor tissue into recipient chicken limb buds as described in Materials and Methods. Signalling strength of the grafted tissue was scored by its ability to induce duplications of the wing digit pattern. A complete duplication (digit 4) scores 100%, duplication of digit 3 scores 50%, duplication of digit 2 scores 25% and an extra cartilage nodule 12.5% (see Vogel and Tickle, 1993).

Polarising activity was assayed for limb buds of the *ld^{ln2}* and *ld^l* alleles and the results of the analysis between embryonic days 10.5-11 are summarised in Fig. 3. Tissue grafts from heterozygous limb buds revealed that polarising activity was mainly localised in regions A and B of the posterior limb bud mesenchyme (Fig. 3A). Polarising activity was very similar in

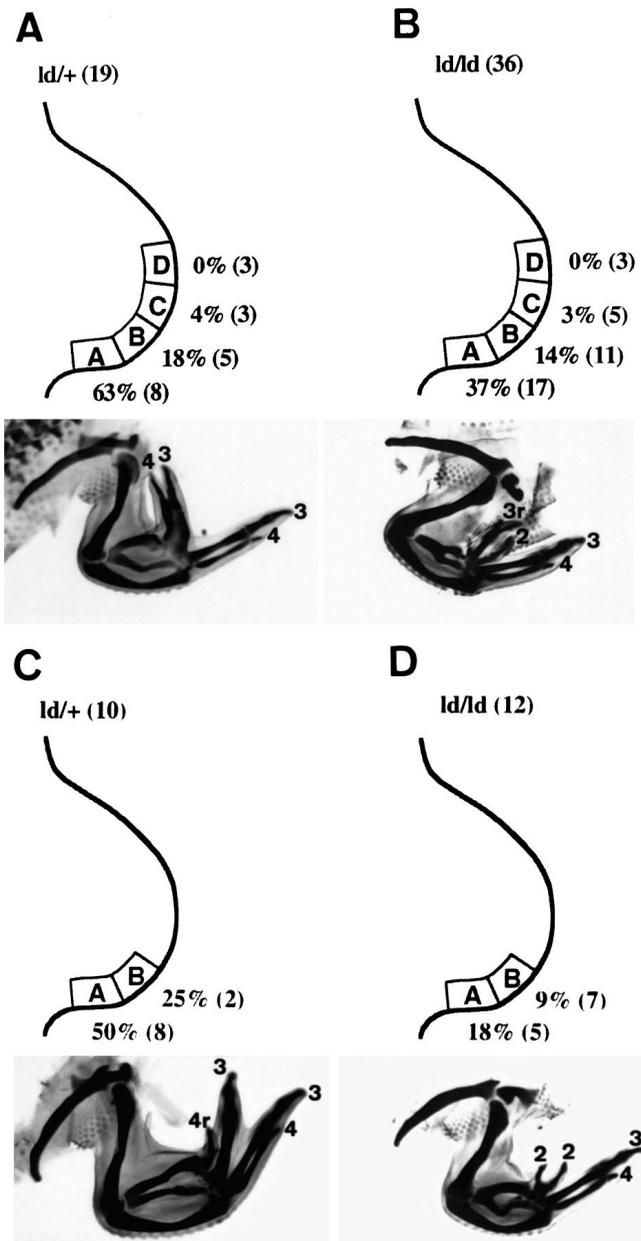


Fig. 3. Polarizing activity is reduced in *ld* homozygous posterior limb bud mesenchyme from embryonic day 10.5-11. Posterior mesenchyme from wild-type (A,C) or *ld* mutant limb buds (B,D) was isolated (see Materials and Methods) and the area from the posterior margin to the apex was divided in four pieces of about 200 μ m prior to grafting (schematically indicated as regions A-D; for details see Materials and Methods). The strength of polarizing activity was determined as described by Vogel and Tickle (1993). (A,B) Summary of the results obtained by analysis of heterozygous and homozygous limb buds of the *ld^{ln2}* allele. Note that most of the polarizing activity is located in regions A and B in both *ld^{ln2}/+* and *ld^{ln2}/ld^{ln2}* tissue. Representative duplications obtained after grafting of either *ld^{ln2}/+* (A) or *ld^{ln2}/ld^{ln2}* posterior mesenchyme (region A) are shown below the schemes. Digit 3r: partial duplication of digit 3. (C,D) Summary of the results obtained by analysis of heterozygous and homozygous limb buds of the *ld^J* allele. Only regions A and B were assayed for polarizing activity. Representative duplications obtained after grafting of either *ld^J/+* (C) or *ld^J/ld^J* posterior mesenchyme (D) are shown below the schemes. Digit 4r, partial duplication of digit 4.

heterozygous limb buds of both *ld* alleles (Fig. 3A,C) and comparable to the strength previously reported for wild-type murine polarising region grafts (Wanek and Bryant, 1991). In contrast, polarising activity of *ld* homozygous limb buds from both alleles was markedly reduced, but sufficient to induce duplications of mostly digit 2 (*ld^J/ld^J*; Fig. 3D) and sometimes digit 3 (*ld^{ln2}/ld^{ln2}*; Fig. 3B) in chicken wing buds (compare to Fig. 6 and Discussion). Mesenchyme (regions A and B) from *ld^J* homozygous limb buds always exhibited significantly lower polarising activity (Fig. 3D) than the corresponding tissue from *ld^{ln2}* homozygous limb buds (Fig. 3B). Furthermore, these studies showed that the residual polarising activity was correctly localised to the posterior mesenchyme of *ld* homozygous limb buds (compare Fig. 3A,C to B,D).

Shh expression is not maintained in limb buds of *ld* homozygous embryos

The *Shh* expression domain marks polarising region cells (Riddle et al., 1993). In wild-type limb buds, *Shh* was activated in the proximal posterior mesenchyme (E10; arrowhead, Fig. 4A) and extended distally as limb bud outgrowth proceeds (E10.5-E10.75; Fig. 4C,E). Between embryonic day 10.5 and 10.75, the levels of *Shh* transcripts increased in the distal part of the posterior limb bud mesenchyme (arrowheads, Fig. 4C,E). In *ld* mutant limb buds, *Shh* was activated normally (arrowhead, Fig. 4B and data not shown) and began to extend distally by embryonic day 10.5 (arrowhead, Fig. 4D). In contrast to the wild-type, expression decreased rapidly and *Shh* became low or undetectable by embryonic day 10.75-11 (arrowhead, Fig. 4F, see also Fig. 6B). Expression was never detected in limb buds of *ld* homozygous embryos older than day 11 (data not shown). These results show that *Shh* was activated but not maintained in *ld* homozygous limb buds, whereas expression was normal in other embryonic structures (e.g. notochord and floorplate; see arrows Fig. 4).

The defective AER of *ld* homozygous limb buds expresses the FGF-8, but not FGF-4 signalling molecule

FGF-4 has been implicated in maintaining *Shh* expression in the limb bud mesenchyme (Laufer et al., 1994; Niswander et al., 1994), whereas FGF-8 seems to play a role in initiating limb bud outgrowth (Crossley et al., 1995, Mahmood et al., 1995). Because *Shh* expression was not maintained in *ld* mutant limb buds, the distribution of *Fgf-4* and *Fgf-8* transcripts in the AER was investigated between embryonic days 9.5 and 12. Fig. 5 shows the alterations of the *Fgf-4* and *Fgf-8* expression domains in *ld* homozygous limb buds at embryonic day 10.75, by which time the *Shh* transcripts become low or undetectable in *ld* mutant limb buds (Figs 4F and 6B).

No *Fgf-4* transcripts were detected in the *ld* mutant AER during all stages of development (Fig. 5B, and data not shown), indicating that *Fgf-4* is not expressed by the defective AER of *ld* mutant limb buds. In contrast, the temporal distribution of *Fgf-8* transcripts was normal in *ld* mutant limb buds (data not shown), whereas their spatial distribution was affected (compare Fig. 5C to 5D). *Fgf-8* expression was lower and patchy in the anterior part of the *ld* mutant AER (arrowheads, Fig. 5D), whereas higher and more uniform levels were detected posteriorly. In general, the spatial distribution of *Fgf-*

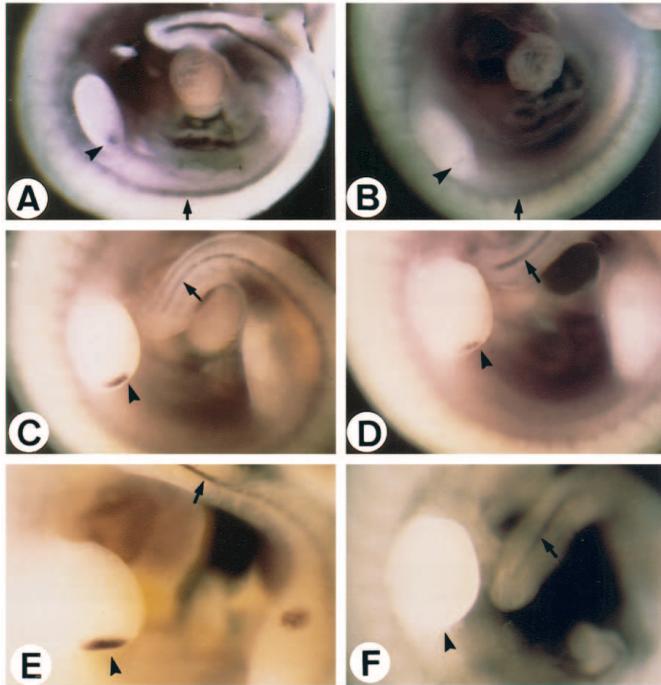


Fig. 4. *Shh* expression is not maintained in *ld* homozygous limb buds. Whole-mount in situ hybridisation to detect *Shh* transcripts. (A,C,E) Wild-type embryos and (B,D,F) homozygous embryos. Arrowheads indicate the *Shh* expression domain in the forelimb buds, whereas arrows indicate *Shh* expression in the notochord and floor plate. (A) *+/+* embryo (E10.25, 32 somites). (B) *ld/ld* embryo (E10.25, 33 somites). Note the activation of *Shh* expression in the posterior mesenchyme (arrowhead). (C) *ld/+* embryo (E10.5, 35 somites). (D) *ld/ld* embryo (E10.5, 35 somites). (E) *ld/+* embryo (E10.75, 38 somites). (F) *ld/ld* embryo (E10.75, 38 somites). *Shh* transcripts were no longer detected (arrowhead indicates the corresponding region).

8 transcripts was slightly more patchy in *ld^l* than *ld^{ln2}* homozygous limb buds (data not shown). The patchy distribution of *Fgf-8* transcripts probably directly reflects the morphology of the *ld* mutant AER. Previous analysis had revealed that the height and dorso-ventral width of the *ld* mutant AER varied, rendering its appearance patchy (Zeller et al., 1989).

***Shh* expression in *ld* mutant posterior mesenchyme is rescued by wild-type AER signals**

The lack of *Fgf-4* expression in the *ld* mutant AER suggested that the defect leading to loss of mesenchymal *Shh* expression resides in the AER. This hypothesis was tested by grafting pieces of posterior mesodermal tissue from heterozygous and *ld* mutant limb buds (E10.5-E11) to either the posterior or anterior margin of host chicken wing buds (stage 19-20; for details see Materials and Methods). Initial experiments showed that *Shh* was not detectable in murine tissue 6 hours after grafting, but was re-expressed after 12 hours (data not shown). *Shh* expression was assessed 12-21 hours after grafting and representative results are shown in Fig. 6. Heterozygous (E10.75; Fig. 6A) and *ld* homozygous limb bud mesenchyme (E10.75; Fig. 6B) was isolated and pieces of posterior tissue (corresponding to approximately regions A or B; Fig. 3) were grafted individually either anteriorly (Fig. 6C,D) or posteriorly

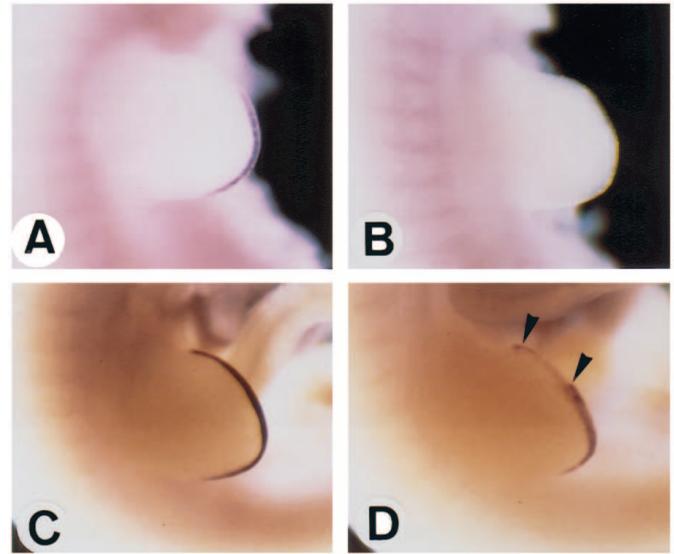


Fig. 5. The defective AER of *ld* mutant limb buds fails to express *Fgf-4*, whereas *Fgf-8* transcripts remain expressed. *Fgf-4* (A,B) and *Fgf-8* (C,D) expression in the AER of heterozygous and homozygous forelimb buds during embryonic day 10.75 (about 38 somites). The embryos shown in A and B were dissected prior to photography. (A) *ld/+* embryo. (B) *ld/ld* embryo. (C) *ld/+* embryo. (D) *ld/ld* embryo. Arrowheads indicate the patchy *Fgf-8* distribution in the anterior part of the *ld* mutant AER.

(Fig. 6E,F) to host chicken wing buds and analysed about 12 hours following grafting. *Shh* was expressed at similar levels in anterior grafts of both heterozygous (arrowhead, Fig. 6C) and *ld* mutant mouse polarising region tissue (arrowhead, Fig. 6D). Heterozygous or *ld* mutant tissue grafted under the posterior AER expressed similar levels of murine *Shh* transcripts as anterior grafts (compare arrowheads, Figs 6C,D to E,F). However, a few heterozygous grafts placed posteriorly expressed higher levels of *Shh* transcripts than compared *ld* mutant grafts (data not shown). In the grafted mouse tissue, the highest *Shh* levels were detected in the distal cells located closest to the host AER, whereas grafts placed away from the AER never expressed *Shh* transcripts (data not shown). These results indicated that *ld* mutant posterior mesenchyme is competent to respond to signals from a wild-type chicken AER that led to rescue of *Shh* expression.

DISCUSSION

A role for *ld* gene products in the establishment of 5' *HoxD* domains and formation of the vertebrate autopod

The *ld* alleles affect patterning of the distal-most limb skeletal structures of the autopod (Kleinebrecht et al., 1992; Zeller et al., 1989). The most 5' members of the *HoxD* gene cluster (*Hoxd-11* to *Hoxd-13*) have been implicated in morphogenesis of distal limb structures by genetic alterations of mouse (Dollé et al., 1993; Davis and Capecchi, 1994; Favier et al., 1995) and chicken embryos (Morgan et al., 1992). Expression of 5' *HoxD* genes during limb morphogenesis can be divided into two distinct phases. Their initially nested and posteriorly

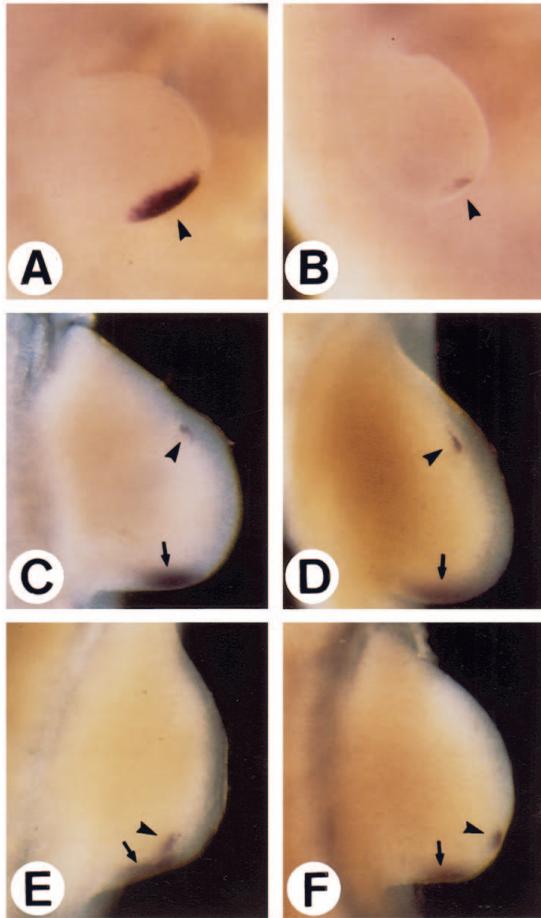


Fig. 6. Signals from a wild-type chicken AER rescue *Shh* expression in *ld* mutant posterior mesenchyme. (A,B) Representative levels of *Shh* expression in donor mouse forelimb buds (E10.75, 38 somites). (A) *ld*^{+/+} forelimb bud. (B) *ld*^{ld} forelimb bud. Arrowheads in A and B indicate the *Shh* domains in the posterior limb bud mesenchyme. (C-F) Expression of the murine *Shh* transcripts in grafts of mouse posterior limb bud mesenchyme to chicken wing buds after 12 to 15 hours (see Materials and Methods). Note that the *Shh* probe detects both the murine and chicken transcripts. Arrowheads indicate *Shh* expression in the grafted mouse posterior limb bud mesenchyme. Small arrows indicate the endogenous chicken *Shh* transcripts located in the posterior wing bud mesenchyme. (C) Anterior graft of *ld*^{+/+} polarising region tissue (derived from the embryo shown in A). (D) Anterior graft of *ld*^{ld} polarising region tissue (derived from the embryo shown in B). (E) Posterior graft of *ld*^{+/+} polarising region tissue (38 somites). (F) Posterior graft of *ld*^{ld} polarising region tissue (also derived from the embryo shown in B)

restricted domains (or early domains) correlate with patterning of proximal axial structures, whereas the subsequent anterior expansion of the distal domains (or late domains) seem to function in patterning of the postaxial structures forming the autopod (reviewed by Duboule, 1994a). Little is known about the regulation of this biphasic expression, but Sordino et al. (1995) have provided evidence that sustained AER signalling is important for establishing the late 5' *HoxD* domains in vertebrate autopods. The late AER signalling functions are impaired in limb buds of *ld* mutant embryos (see below). Our study shows that the temporal transition from early to late 5' *HoxD* domains is substantially delayed. These results indicate

that, in the limb bud, the *ld* gene products act upstream of the 5' *HoxD* genes. They seem necessary to correctly initiate the temporal transition from early to late 5' *HoxD* expression domains during patterning of the autopod. Duboule (1994b) postulated that the timing of colinear 5' *HoxD* gene expression is regulated by the mitotic rates of progenitor cells. Interestingly, previous morphological analysis of *ld* homozygous limb buds provided evidence that the observed patterning defects could be a consequence of affecting proliferation of mesenchymal cells (Zeller et al., 1989). It is therefore possible that the observed temporal alterations of the late 5' *HoxD* domains in *ld* mutant limb buds reflect altered mitotic rates of the undetermined mesenchymal progenitors.

***Shh* expression and polarising activity in *ld* mutant limb bud mesenchyme**

The results of this study show that *Shh* expression is not maintained in posterior mesenchyme of *ld* homozygous limb buds. These results seem to match the reduced levels of polarising activity observed following heterospecific grafting of *ld* mutant posterior limb bud mesenchyme to chicken limb buds. However, analysis of *Shh* expression revealed similar transcript levels in both *ld* mutant and wild-type mesenchyme about 12 to 21 hours following grafting (see below). This apparent discrepancy between *Shh* levels and polarising activity can be explained if the *ld* mutations also affect mesenchymal response to SHH signalling (Laufer et al., 1994). Indeed, the *ld* gene products are expressed by both limb bud compartments (Jackson-Grusby et al., 1992; Trumpp et al., 1992; Zeller et al., 1989) and the molecularly characterised *ld* alleles were shown to truncate all known Ld protein isoforms (*ld*^{ln2}, *ld*^{TgHd}, Maas et al., 1990; *ld*^{TgBri}, Vogt et al., 1992; James and Zeller, unpublished). Therefore, the nuclear Ld proteins most likely function in both the AER and mesenchyme and the *ld* mutations affect both limb bud compartments.

The SHH/FGF-4 feedback loop is disrupted by the defect in differentiation of the *ld* mutant AER

Shh expression is activated in the posterior limb bud mesenchyme during early limb bud outgrowth in response to unknown factors. SHH signalling induces *Fgf-4* expression in the posterior part of the AER, which in turn maintains *Shh* expression in the underlying mesenchyme and establishes a positive feedback loop (Riddle et al., 1993; Laufer et al., 1994; Niswander et al., 1994). The results of the present study show that defective AER in *ld* mutant limb buds does not express *Fgf-4* and that *Shh* is activated but not maintained in the posterior mesenchyme. Heterospecific grafting experiments revealed that wild-type AER signals can be received by *ld* mutant posterior mesenchyme to rescue *Shh* expression. These results indicate that the defect causing disruption of the SHH/FGF-4 feedback loop resides in the ectodermal compartment of *ld* mutant limb buds.

However, some functional signalling between the defective AER and the distal limb bud mesenchyme must still occur in *ld* mutant limb buds for the following reasons. The *ld* mutations predominantly disrupt formation of the autopod, whereas initial limb bud outgrowth and patterning of proximal structures are unaffected (Kleinebrecht et al., 1982; Zeller et al., 1989). Furthermore, the defective AER continues to express the FGF-8 signalling molecule, which

seems to be involved in triggering limb bud outgrowth (Cohn et al., 1995; Crossley and Martin, 1995; Mahmood et al., 1995). These results provide molecular evidence that signalling by the *ld* mutant AER is unaffected during early morphogenesis (i.e. FGF-8), whereas the late signalling functions are impaired (i.e. FGF-4). Incomplete AER differentiation could impair its cell to cell communication (Tickle et al., 1989) and polarisation by signals from the underlying mesenchyme (i.e. SHH; Laufer et al., 1994; Niswander et al., 1994). This failure would prevent localised *Fgf-4* expression in the AER and establishment of the SHH/FGF-4 feedback loop. This study supports a proposed essential role for FGF-4 in maintaining *Shh* expression and polarising activity in the posterior limb bud mesenchyme (Niswander et al., 1993; Vogel and Tickle, 1993; Laufer et al., 1994; Niswander et al., 1994). Furthermore, FGF-8 seems unable to compensate for the lack of FGF-4 signalling in *ld* homozygous limb buds, which provides evidence for distinct, non-complementary functions of these two FGFs during limb bud morphogenesis. Interestingly, exogenously applied FGF-8 is unable to maintain *Shh* expression in chicken limb bud mesenchyme after AER removal (Mahmood et al. 1995). Taken together, the results of our study provide insight into the molecular defects causing the *ld* limb phenotype and show that the *ld* gene products interact with the molecular signals that control patterning of the autopod. Therefore, the existing *ld* alleles provide a valuable tool for the continued investigation of the roles of the Ld proteins in AER function and the signalling cascades that co-ordinate growth and patterning of postaxial limb structures.

We thank Andreas Trumpp for initiating the establishment of several *ld* alleles at EMBL and Rick Woychik for making the *Id^{ln2}* (*Id^{lslGso}*) mice and corresponding DNA clones available to us. We are indebted to Denis Duboule and Rein Aasland for providing the *Hoxd-11*, *Hoxd-12* and *Hoxd-13* probes, to Andy McMahon for the *Shh* probe and to Gail Martin and Lee Niswander for the *Fgf-4* and *Fgf-8* probes and conditions for their use. We are grateful to Andrés E. Carrasco for helpful advice concerning the whole-mount *in situ* hybridisation procedure. We thank Steve Cohen, Rosanna Dono, Dominic James, Kent Lloyd, Kelly McNagny, Peter Uetz and Aimée Zúñiga Mejía Borja for stimulating discussions and critical reading of the manuscript. The EMBL photolab is acknowledged for expert assistance in preparing the photographic reproductions. Anna G. Haramis is the recipient of an EMBL predoctoral fellowship and Jennifer M. Brown of an EMBO long-term postdoctoral fellowship. This study was supported by EMBL and EU grant no. CT-93-0017.

REFERENCES

- Bultman, S. J., Russell, L. B., Gutierrez-Espeleta, G. A. and Woychik, R. P. (1991). Molecular characterisation of a region of DNA associated with mutations in the agouti locus in the mouse. *Proc. Natl. Acad. Sci. USA* **88**, 8062-8066.
- Cohn, M. J., Izpisua-Belmonte, J.-C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739-746.
- Crossley, P. H. and Martin, G. R. (1995). The mouse *Fgf8* gene encodes a family of polypeptides and is expressed in regions that direct outgrowth and patterning in the developing embryo. *Development* **121**, 439-451.
- Davis, A. P. and Capecchi, M. R. (1994). Axial development and appendicular skeleton defects in mice with a targeted disruption of *hoxd-11*. *Development* **120**, 2187-2196.
- De la Pompa, J.-L., James, D. and Zeller, R. (1995). The limb deformity proteins during avian neurulation and sense organ development. *Developmental Dynamics (in press)*
- 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.
- Dollé, P., Dierich, A., LeMeur, M., Schimmang, T., Schuhbauer, B., Chambon, P. and Duboule, D. (1993). Disruption of the Hoxd-13 gene induces localized heterochrony leading to mice with neotenic limbs. *Cell* **75**, 431-441.
- Dono, R. and Zeller, R. (1994). Cell-type-specific nuclear translocation of fibroblast growth factor-2 isoforms during chicken kidney and limb morphogenesis. *Dev. Biol.* **163**, 316-330.
- Duboule, D. (1994a). How to make a limb. *Science* **226**, 575-578.
- Duboule, D. (1994b). Temporal colinearity and the phylotypic progression: a basis for the stability of a vertebrate Bauplan and the evolution of morphologies through heterochrony. *Development* **1994 Supplement**, 135-142.
- Echelard, Y., Epstein, D. J., St-Jacques, B., Shen, L., Mohler, J., McMahon, J. and Mc Mahon, A. P. (1993). Sonic hedgehog, a member of a family of putative signalling molecules, is implicated in the regulation of CNS polarity. *Cell* **75**, 1417-1430.
- Favier, B., LeMeur, M., Chambon, P. and Dollé, P. (1995). Axial skeleton homeosis and forelimb malformations in *Hoxd-11* mutant mice. *Proc. Natl. Acad. Sci. USA* **92**, 310-314.
- Green, M. C. (1962). Mouse news-letter. *Harwell* **26**, 34.
- Hamburger, V. and Hamilton, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49-92.
- Harland, R. M. (1991). In situ hybridisation: an improved whole-mount method for *Xenopus* embryos. *Methods Cell Biology* **36**, 685-695.
- Hebert, J. M., Basilico, C., Goldfarb, M., Haub, O. and Martin, G. R. (1990). Isolation of cDNAs encoding four mouse FGF family members and characterization of their expression pattern during embryogenesis. *Dev. Biol.* **138**, 454-463.
- Izpisua-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.
- Izpisua-Belmonte, J.-C., Brown, J. M., Crawley, A., Duboule, D. and Tickle, C. (1992). Hox-4 gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms. *Development* **115**, 553-560.
- Jackson-Grusby, L., Kuo, A. and Leder, P. (1992). A variant limb deformity transcript expressed in the embryonic mouse limb defines a novel formin. *Genes Dev.* **6**, 29-37.
- Johnson, R. L. and Tabin, C. (1995). The long and short of hedgehog signalling. *Cell* **81**, 313-316.
- Kleinebrecht, J., Selow, J. and Winkler, W. (1982). The mouse mutant limb-deformity (*ld*). *Anat. Anz.* **152**, 313-324.
- Laufer, E., Nelson, C. E., Johnson, R. L., Morgan, B. A. and Tabin, C. (1994). Sonic hedgehog and *Fgf-4* act through a signalling cascade and feedback loop to integrate growth and patterning of the developing limb bud. *Cell* **79**, 993-1003.
- Maas, R. L., Zeller, R., Woychik, R. P., Vogt, T. F. and Leder, P. (1990). Disruption of formin-encoding transcripts in two mutant limb deformity alleles. *Nature* **346**, 853-855.
- Maas, R., Elfering, S., Glaser, T. and Jepeal, L. (1994). Deficient outgrowth of the ureteric bud underlies the renal agenesis phenotype in mice manifesting the limb deformity (*ld*) mutation. *Development. Dynamics* **199**, 214-228.
- 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 FGF-8 in the initiation and maintenance of vertebrate limb bud outgrowth. *Current Biology* **5**, 797-806.
- Martin, P. (1990). Tissue patterning in the developing mouse limb. *Int. J. Dev. Biol.* **34**, 323-336.
- Morgan, B., Izpisua-Belmonte, J.-C., Duboule, D. and Tabin, C. (1992). Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. *Nature* **358**, 236-239.
- 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. and Martin, G. R. (1993). FGF-4 and BMP-2 have opposite effects on limb growth. *Nature* **361**, 68-71.
- Niswander, L., Tickle, C., Vogel, A., Booth, I. and Martin, G. R. (1993).

- FGF-4 replaces the apical ectodermal ridge and directs the outgrowth and patterning of the limb. *Cell* **75**, 579-587.
- 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.
- Nohno, T., Noji, S., Koyama, E., Myokai, F., Kuroiwa, A., Saito, T. and Taniguchi, S.** (1991). Involvement of the Chox4-chicken homeobox genes in determination of antero-posterior axial polarity during limb development. *Cell* **64**, 1197-1205.
- Riddle, R., Johnson, R. L., Laufer, E. and Tabin, C.** (1993). *Sonic hedgehog* mediates the polarizing activity of the ZPA. *Cell* **75**, 1401-1416.
- Sordino, P., van der Hoeven, F. and Duboule, D.** (1995). *Hox* gene expression in teleost fins and the origin of vertebrate digits. *Nature* **375**, 678-681.
- Summerbell, D., Lewis, J. H. and Wolpert, L.** (1973). Positional information in chick limb morphogenesis. *Nature* **224**, 492-496.
- Tabin, C. J.** (1991). Retinoids, homeoboxes, and growth factors: toward the molecular models for limb development. *Cell* **66**, 199-217.
- 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., Crawley, A. and Farrar, J.** (1989). Retinoic acid application to chick wing buds leads to a dose dependant reorganisation of the apical ectodermal ridge that is mediated by the mesenchyme. *Development* **106**, 691-705.
- Tickle, C.** (1991). Retinoic acid and chick limb bud development. *Development* **1991 Supplement 1**, 113-121.
- Tickle, C. and Eichele, G.** (1994). Vertebrate limb development. *Annu. Rev. Cell Biol.* **10**, 121-52.
- Todt, W. L. and Fallon, J. F.** (1987). Posterior apical ectodermal ridge removal in the chick wing bud triggers a series of events resulting in defective anterior pattern formation. *Development* **101**, 501-515.
- Trumpp, A., Blundell, P. A., de la Pompa, J. L. and Zeller, R.** (1992). The chicken *limb deformity* gene encodes nuclear proteins expressed in specific cell types during morphogenesis. *Genes Dev.* **6**, 14-28.
- 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.
- Vogt, T. F., Jackson-Grusby, L., Wynshaw-Boris, A. J., Chan, D. C. and Leder, P.** (1992). The same genomic region is disrupted in two transgene-induced limb deformity alleles. *Mammalian Genome* **3**, 431-437.
- Wanek, N. and Bryant, S. V.** (1991). Temporal pattern of posterior positional identity in mouse limb buds. *Dev. Biol.* **147**, 480-484.
- Wilkinson, D. G.** (1993). *In Situ Hybridisation*, pp. 257-276. Oxford: IRL Press.
- 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.
- Woychik, R. P., Maas, R. L., Zeller, R., Vogt, T. F. and Leder, P.** (1990a). 'Formins': Proteins deduced from the alternative transcripts of the *limb deformity* gene. *Nature* **346**, 850-853.
- Woychik, R. P., Generoso, W. M., Russell, L. B., Cain, K. T., Cacheiro, N. L. A., Bultman, S. J., Selby, P. B., Dickinson, M. E., Hogan, B. L. M. and Rutledge, J. C.** (1990b). Molecular and genetic characterisation of a radiation-induced structural rearrangement in mouse chromosome 2 causing mutations at the limb deformity and agouti loci. *Proc. Natl. Acad. Sci. USA* **87**, 2588-2592.
- Zeller, R., Jackson-Grusby, L. and Leder, P.** (1989). The *limb deformity* gene is required for apical ectodermal ridge differentiation and antero-posterior limb pattern formation. *Genes Dev.* **3**, 1481-1492.

(Accepted 29 August 1995)