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Lhx2, a vertebrate homologue of *apterous*, regulates vertebrate limb outgrowth

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SUMMARY

***apterous* specifies dorsal cell fate and directs outgrowth of the wing during *Drosophila* wing development. Here we show that, in vertebrates, these functions appear to be performed by two separate proteins. *Lmx-1* is necessary and sufficient to specify dorsal identity and *Lhx2* regulates limb outgrowth. Our results suggest that *Lhx2* is closer to *apterous* than *Lmx-1*, yet, in vertebrates, *Lhx2* does not specify dorsal cell fate. This implies that in vertebrates, unlike *Drosophila*, limb outgrowth can be dissociated from the establishment of the dorsoventral axis.**

Key words: *Lmx-1*, *Lhx2*, *apterous*, Vertebrate, *Drosophila*, Limb outgrowth

INTRODUCTION

The limbs of fruit flies and vertebrates are not only morphologically quite distinct, but also their development proceeds very differently. In *Drosophila*, ectodermal cells destined to give rise to limbs are set aside in the embryo. These cells divide in the larval stages to form invaginated epithelial sacs termed imaginal discs. During metamorphosis this epithelium unfolds and grows outward to form the adult limb (Cohen, 1993). In contrast, vertebrate limbs develop through a continued localised proliferation of the lateral plate mesoderm giving rise first to limb buds and ultimately to the adult limb (Tickle and Eichele, 1994).

The first hint that limb outgrowth in these diverse species might after all be based upon a similar genetic foundation was the finding that a gene known to be involved in limb outgrowth in *Drosophila*, *distal-less* (Cohen et al., 1989; Cohen, 1990) was also expressed in the distal regions of mouse and newt limbs (Beauchemin and Savard, 1992; Dollé et al., 1992). More recently, *distal-less* has been found in the appendages of a wide range of different species across several phyla (see Popadic et al., 1996 and references therein). Subsequently, many other genes that play a role in patterning the *Drosophila* appendages have been identified and shown to play a similar role in the vertebrate limb. These similarities are particularly clear for the genes acting along the anteroposterior and proximodistal axes (for reviews see (Lawrence and Struhl, 1996; Shubin et al., 1997). In contrast, homologies in the genes involved in specifying the dorsoventral (DV) axis are less obvious.

Two of the vertebrates genes that specify DV identity, *Wnt-7a* (Parr and McMahon, 1995; Yang and Niswander, 1995) and *En-1* (Loomis et al., 1996), do not appear to have functional equivalents in *Drosophila*. A third gene, *Lmx-1* (Riddle et al., 1995; Vogel et al., 1995), has been proposed to be a homologue of the *Drosophila apterous* gene, since not only do they share sequence similarity but also both genes are sufficient to specify dorsal cell fate (Blair, 1993; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Blair et al., 1994). Importantly, besides determining dorsality, *apterous* is also necessary for wing outgrowth and contributes to the positioning of the wing margin at the dorsoventral boundary through regulating the expression of the *fringe* and *Serrate* genes (Irvine and Wieschaus, 1994; Kim et al., 1995). It appears that *Lmx-1* does not share this activity since mis-expression of the *Lmx-1* gene has no effect on limb outgrowth, or on apical ectodermal ridge (AER, a key structure required for limb outgrowth) (Summerbell et al., 1973; Todt and Fallon, 1984) formation.

In this paper we show that *Lmx-1* is not only sufficient, but also necessary, to specify dorsal cell fate, since down-regulation of *Lmx-1* activity results in limbs lacking dorsal-specific structures. We also demonstrate that *Lmx-1* is neither able to induce *R-fng* when mis-expressed in the limb or the flank of the embryo, nor *Drosophila fringe* when ectopically expressed in flies. Further, we describe the cloning of another LIM-homeodomain gene, *Lhx2*, that shows higher sequence conservation with *apterous* and, unlike *Lmx-1*, when mis-expressed in the flank of the embryo induces ectopic *R-fringe* expression. *Lhx2* is also able to induce *fringe* and *Wingless* (downstream targets of *apterous*) expression in *Drosophila*. Consistent with these data, down-regulation of *Lhx2* activity causes a down-regulation of genes required for the outgrowth of the limb along its proximodistal axis and consequently

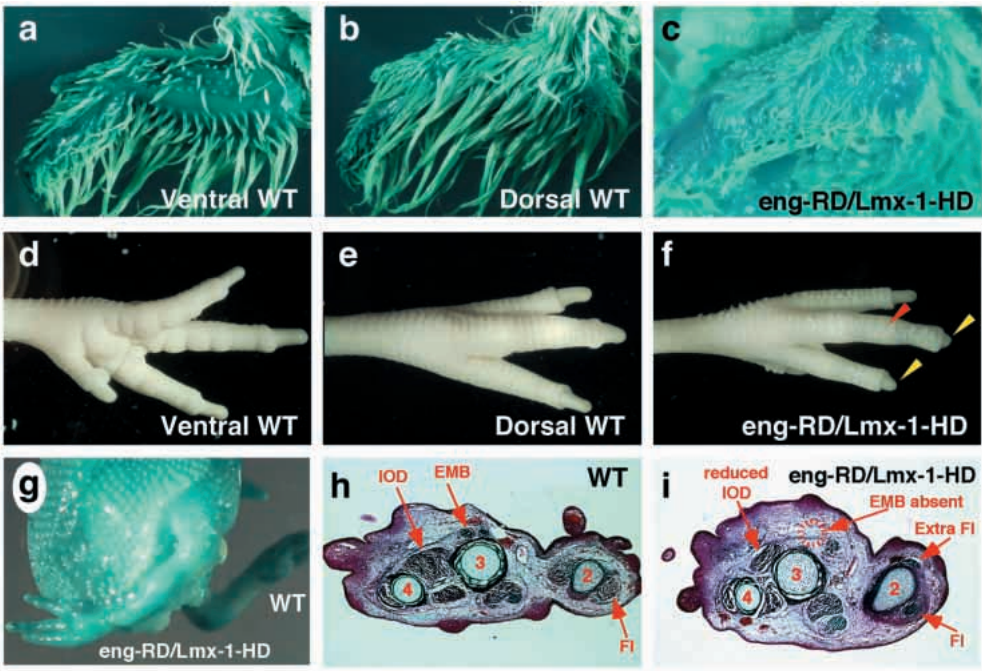

Fig. 1. Competitive inhibition of *Lmx-1* activity inhibits dorsal cell fate. (a) Wild-type wing viewed from the ventral side. (b) Wild-type wing viewed from the dorsal side. (c) Wing infected with the *Eng*-RD/*Lmx-1*-HD construct viewed from the dorsal side. The normal chick wing is more densely feathered on the dorsal surface compared with the ventral surface. Wings infected with the *Eng*-RD/*Lmx-1*-HD retroviral construct display a greatly reduced feather density on their dorsal side. (d) Wild-type leg viewed from the ventral side. (e) Wild-type leg viewed from the dorsal side. (f) Leg infected with the *Eng*-RD/*Lmx-1*-HD construct. The characteristic large scales present on the dorsal side of the wild-type toes 2 and 3 are partially lost, and instead small scales characteristic of the ventral side are present (red arrowhead). In addition, a reduction of the claws is observed (yellow arrowheads). (h) Distal cross section of a wild type wing. (i) Distal cross-section of an *Eng*-HD/*Lmx-1*-HD infected wing. The reduction in the number of feathers is accompanied in some cases by reduction of distal elements and by smaller or absent dorsal muscle *(interosseus dorsalis*, IOD and *extensor medius brevis*, EMB). In some cases, ectopic ventral muscles *(flexor indicis*, FI) were observed on the dorsal side (i). The loss of dorsal muscles would presumably account for abnormal bending towards the ventral side of the infected limb buds (g).

results in arrested limb outgrowth. Finally, unlike *Lmx-1*, *Lhx2* does not specify dorsal cell fate.

Our data suggest that *Lhx2* could be a bona fide vertebrate homologue of *apterous* yet, in vertebrates, it does not specify dorsal cell fate. This raises the question of whether in vertebrates, unlike in *Drosophila*, limb outgrowth can be dissociated from the establishment of the DV axis and brings into focus the questions of whether or not vertebrate and *Drosophila* limbs can be considered to be homologous structures and how limbs in flies and vertebrates have evolved to be so different.

MATERIALS AND METHODS

Isolation of *Lhx2*

The open reading frame of the *Drosophila apterous* gene was used to

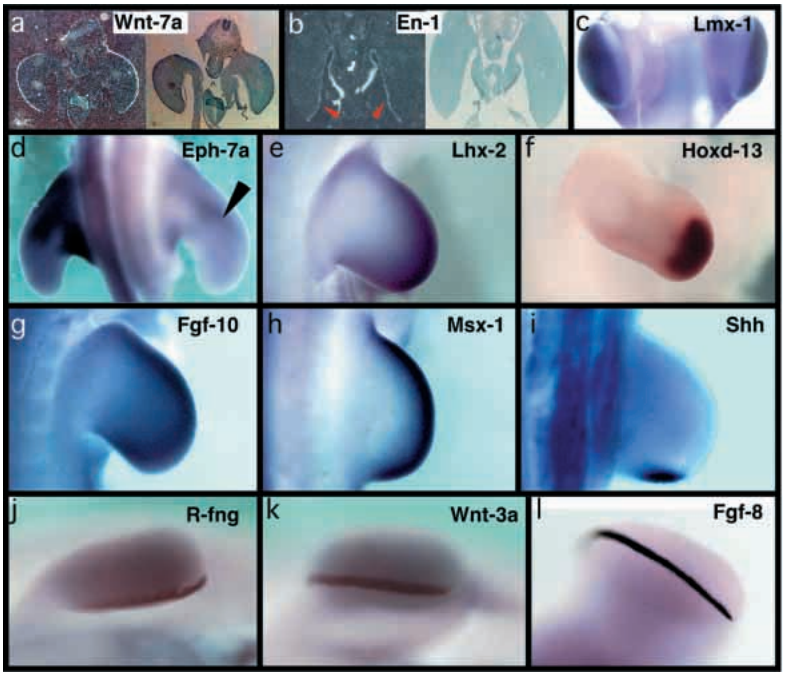

Fig. 2. Gene expression after inhibition of *Lmx-1* activity. 48-66 hours after viral infection with the *Eng*-RD/*Lmx*-HD construct in the limb primordia, embryos were processed either for sectional (a,b) or whole-mount (c-l) in situ hybridisation. With the exception of *Eph-7a* (d) which showed a strong down-regulation (the infected limb is marked with a black arrowhead), none of the genes analyzed that are known to be involved in the patterning and outgrowth of the limb along its different axes was perturbed. In b, the normal ventral staining of *En-1* is marked with a red arrowhead. a and b are split so that the left side of the panel shows a dark field view and the right side a bright field view. The infected limb in a-c appears on the left side of the picture. The rest of the limbs are viewed from the dorsal side.

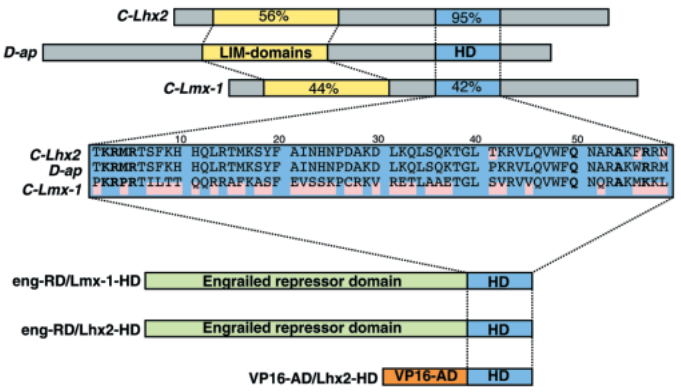

Fig. 3. Comparison of chick *Lhx2*, *Lmx-1* and *Drosophila apterous* genes. The top panel shows a schematic representation indicating the percentage amino acid identity between the LIM and homeodomain motifs of *Lhx2*, *apterous* and *Lmx-1*. The middle panel shows a sequence alignment of the homeobox domains. Amino acids shown in bold have been seen to contact DNA bases in co-crystal structures. Amino acids that differ from *apterous* are highlighted in pink. The lower panel illustrates the chimaeric proteins used in the retroviral mis-expression experiments (see Materials and methods).

screen a stage 20-22 HH cDNA library following standard procedures (Sambrook et al., 1989). The positive clones were sequenced with an automated sequencer.

In situ hybridisation, antibody staining and histology

Whole-mount in situ hybridisation was carried out as described (Wilkinson, 1993) with some minor modifications (Izpisúa Belmonte et al., 1993). Sectional in situ hybridisations were performed as described previously (Izpisúa Belmonte et al., 1991b). The probe used for *Lhx2* (700 bp) encompasses the homeobox and the second LIM domain. The *Wnt-3a* probe (362 bp) was a kind gift of A. MacMahon and C. Tabin. The remaining probes have been described elsewhere: *Wnt-7a* (Dealy et al., 1993), *Eng-1* (Logan et al., 1992), *hoxd-13* (Izpisúa Belmonte et al., 1991a), *Eph-7a* (Kengaku et al., 1998), *Fgf-10* (Ohuchi et al., 1997), *Msx-1* (Robert et al., 1989), *Shh* (Ogura et al., 1996), *Serrate-2* (Myat et al., 1996), *Lmx-1* (Vogel et al., 1995), *Fgf-8* (Vogel et al., 1996) and *R-fng* (Rodriguez-Esteban et al., 1997). *apterous* antibody staining was done according to Capovilla et al. (1994). In some cases the embryos were dehydrated in 30% sucrose, embedded in gelatin, frozen and sectioned with a cryostat. Cartilage and muscle staining were performed as described (Vogel et al., 1996).

Production of viruses and injection protocols

Chicken embryos, obtained from either MacIntyre Poultry (San Diego, CA) or SPAFAS (Norwich, CT), were infected with RCAS(BP)A viruses containing the following constructs. *Eng*-RD/*Lmx-1*-HD was constructed by fusing the *Drosophila engrailed* repressor domain (aa 1-299) with the homeobox of the *Lmx-1* gene (aa 1-60 in Fig. 3). *Eng*-RD/*Lhx2*-HD was constructed similarly by fusing the *engrailed* repressor domain with the homeobox of *Lhx2* (as shown in Fig. 3). The *Lhx2* viral vector was constructed by inserting the open reading frame of the *Lhx2* into RCAS (BP)A. VP16-AD/*Lhx2*-HD was constructed by fusing the VP16 transactivation domain 78 aa (Kliewer et al., 1992) with the *Lhx2* homeodomain. Virus preparation and injections were performed as described (Morgan et al., 1992). 408 embryos were injected with the *Eng*-RD/*Lmx-1*-HD construct and 16% of them showed an abnormal phenotype; 386 embryos were injected with the *Lhx2* construct, and

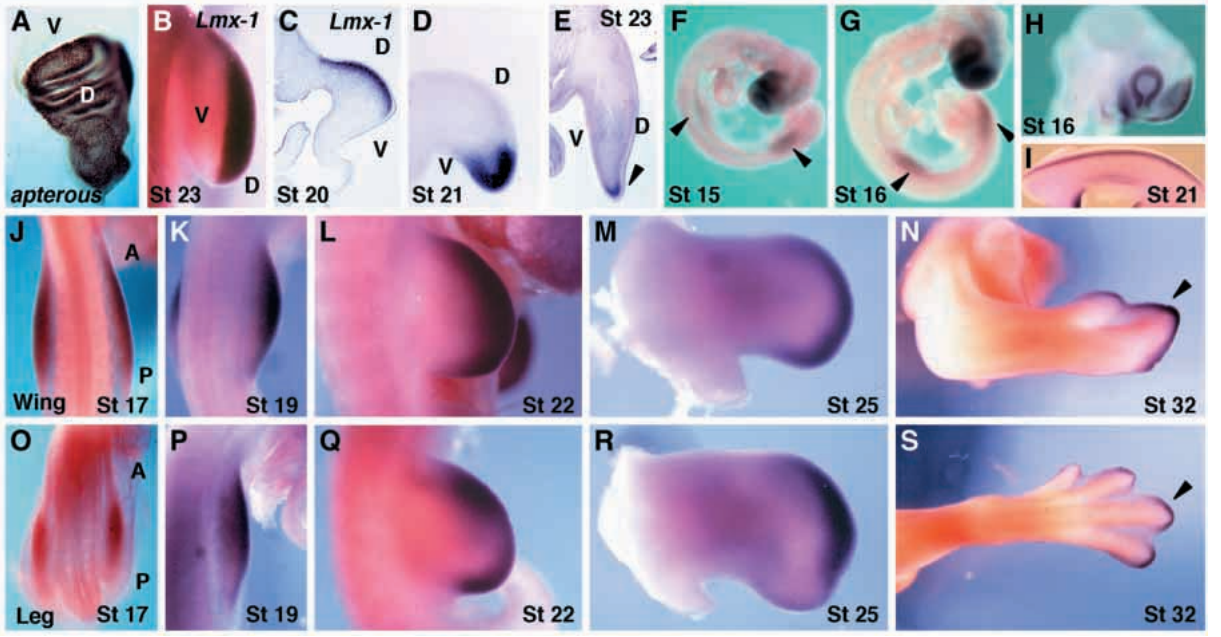

Fig. 4. Expression of *apterous*, *Lmx-1* and *Lhx2* during chick limb development (A) *Drosophila* wing imaginal disc, at the third instar larval stage, showing antibody staining against the dorsally restricted *apterous* protein. (B) Whole-mount in situ hybridisation showing *Lmx-1* transcripts on the dorsal side of the developing chick limb bud (stage 23). (C) Mesodermal dorsal restriction of *Lmx-1* transcripts seen in a transverse section of a limb bud at stage 20. (D-S) Localisation of *Lhx2* transcripts. (D,E) Transverse sections at stages 21 (D) and 23 (E). Note that *Lhx2* expression is restricted to the most distal limb bud mesoderm (arrowhead) with no dorsoventral asymmetry. (F,G) Besides being expressed at the earliest stage of limb budding (arrowheads), *Lhx2* transcripts are also detected in the developing nervous system, nasal epithelia and eye. (H,I) Details showing expression in the eye and nasal epithelia (H) and in the spinal chord (I). (J-S) Expression of *Lhx2* in the wing bud between stages 17 and 32. Whilst at stage 17, *Lhx2* mRNA is found throughout the mesoderm; later on, as the bud elongates, transcripts are confined to the most distal mesoderm just beneath the AER. A similar expression pattern is seen in the developing leg (M,Q). At no stage was *Lhx2* detected in the ectoderm. D, dorsal; V, ventral; A, anterior; P, posterior.

22% of them showed ectopic expression of *R-fng* in the flank; finally, 580 embryos were injected with the *Eng*-RD/*Lhx2*-HD construct and 16% exhibited arrested limb outgrowth, which varied with the extent of the infection.

Fly stocks and expression analysis

The following fly transformants were used: 71B-Gal4 (Brand and Perrimon, 1993), *ap*-Gal4 (Calleja et al., 1996), *ptc*-Gal4 (Bloomington Drosophila Stock Center), C5-Gal4 (Yeh et al., 1995), *fringe-lacZ* (Irvine and Wieschaus, 1994), *UAS:apterous* and *UAS:hLhx2* (D. E. R. and J. B., unpublished). The *UAS:cLmx-1* construct was created by inserting the full-length *cLmx-1* cDNA into the pUAST vector (Brand and Perrimon, 1993). This construct was transformed into *yw* flies following standard procedures (Rubin and Spradling, 1982). For each UAS responder, at least two independent lines were used.

RESULTS

Lmx-1 is necessary for dorsal specification

Whilst the ectopic overexpression of *Lmx-1* indicates that this gene can induce dorsalisation of the ventral side of the limb bud (Riddle et al., 1995; Vogel et al., 1995), this experiment cannot determine whether this is the normal role of *Lmx-1* on the dorsal side of the vertebrate limb, nor does it show if this is the only factor that specifies dorsal cell fate. To start addressing these issues in the chick limb we generated a chimaeric construct between the repressor domain of *engrailed* (kindly provided by J. Jaynes, Thomas Jefferson University, Philadelphia) and the homeodomain of *Lmx-1* (Fig. 3). Since LIM homeodomains have been shown to act as transcriptional activators (German et al., 1992; Bach et al., 1995; Wang and Drucker, 1995; Szeto et al., 1996; Bach et al., 1997), this chimaeric protein, when in excess, would be expected to compete for and repress the genes normally activated by *Lmx-1*.

During normal limb development, the dorsal surface of the wing has a higher feather density than that of the ventral side (Fig. 1a,b). In the *Eng*-RD/*Lmx-1*-HD infected wings the feather density on what would normally be the dorsal side of the limb is greatly reduced (Fig. 1c). Furthermore, the limb is generally straighter and in a few cases a reduction in the thickening of the ulna was observed. In this case, the distal tip of digit 2 was missing. The dorsal surface of the tarsometatarsus and toes of the chick leg is covered by large scales or scuta, whilst the ventral surface has small scales or tuberculae (Fig. 1d,e). In the *Eng*-RD/*Lmx-1*-HD infected leg, the large scales of the dorsal side of certain toe areas were not present and, instead, tuberculae-like structures were observed (Fig. 1f). Digits showing this loss of dorsal-type integument also display a reduction of the claws (Fig. 1f), a structure of dorsal origin. Furthermore, instead of the normal dorsal bending of the leg an abnormal ventral curvature of the whole limb was observed (Fig. 1g). In addition, the digits of the infected legs have a more cylindrical morphology than the dorsally flattened digits of control limbs. Sectioning of the infected limbs shows that whilst the ventral muscles and tendons of the infected limbs are normal, the dorsal muscles are smaller or absent; particularly, those associated with digit 4 (*interosseus*, IOD) and digit 3 (*extensor medius brevis*; EMB). In some instances, the *flexor indicis* (FI), a ventral muscle associated with digit 2, was also present on the dorsal side of the limb (Fig. 1h,i). Overall, the changes we observed were a lack of dorsal structures, and only in some cases were additional ventral structures present on the dorsal side of the infected limb buds.

To determine whether these phenotypes are related to changes in ectodermal or mesodermal cell fate, several molecular markers were analyzed at different time after mis-expression of the *engrailed* repressor domain/*Lmx-1* (*Eng*-RD/*Lmx*-HD) homeodomain fusion construct. In situ hybridisation of genes involved in the outgrowth of the limb along its proximodistal and anteroposterior axes, *Lhx2, hoxd-13, Fgf-10, Msx-1* and *Shh* (mesenchyme, Fig. 2e-i) and *R-fng, Wnt-3a, Fgf-8* (ectoderm, Fig. 2j-l) and *Serrate-2* (data not shown), showed that there is no alteration in their spatiotemporal pattern of expression. Similarly, mis-expression of the *Eng*-RD/*Lmx-1*-HD chimera did not affect the three genes known to be involved in the patterning of the limb along its dorsoventral axis. Neither *Wnt-7a*, *Lmx-1* transcripts (normally localized to the dorsal ectoderm and mesoderm, respectively (Dealy et al., 1993; Riddle et al., 1995; Vogel et al., 1995) nor *En-1* (normally present in the ventral ectoderm; Logan et al., 1992) transcripts distribution was altered (Fig. 2a-c). However, in situ hybridisation to detect *Eph-7A* mRNA, a gene expressed in the dorsal mesoderm (Araujo and Nieto, 1997), showed down-regulation in the dorsal side of the infected limb buds (Fig. 2d). This suggests that the reduction of dorsal character observed after mis-expression of the *Eng*-RD/*Lmx-1*-HD construct is mostly attributable to changes in mesenchymal cell fates along the dorsal-ventral limb axis.

Taken together, these results suggest that *Lmx-1* is not only sufficient, as was suggested previously (Riddle et al., 1995; Vogel et al., 1995), but also required to specify dorsal cell fate.

Lhx2, structurally related to *apterous*, is expressed in the distal limb mesoderm

Whilst the sequence similarity (44% identity in the LIM domains and 42% in the homeodomain) prompted us and others (Riddle et al., 1995; Vogel et al., 1995) to suggest that the chick *Lmx-1* gene might be the vertebrate homologue of the *Drosophila apterous* gene, we have now cloned a second LIM/homeodomain gene *Lhx2* that shows significantly greater sequence similarity to *Drosophila apterous* (56% identity in the LIM domains and 96% in the homeodomain; Fig. 3). *Lhx2* appears to be the chick homologue of a rat gene called *LH2* (Xu et al., 1993) and a human gene *(Lhx2)*, with which it shares 93% aa identity in the homeodomain and 57% aa identity in the LIM domains, respectively.

We have characterized the expression of *Lhx2* during chick embryogenesis (Fig. 4). *Lhx2* is expressed in the developing limb buds, nervous system, optic vesicles and nasal placodes, amongst other tissues. The expression pattern in these areas is very similar to that seen for *apterous* in *Drosophila* (Cohen et al., 1992). *Lhx2* transcripts are first detected in the presumptive limb mesoderm at around stages 14-15HH, coincident with the first signs of limb budding. As limb bud outgrowth proceeds, *Lhx2* mRNA is confined to the cells of the progress zone underlying the distal ectoderm, where it remains until the latest stage examined (stage 32, Fig. 4D-S). *Lhx2* transcripts were never detected in the ectoderm of the limb bud. In contrast to *Lmx-1* and *apterous*, which are dorsally restricted (Fig. 4A-C), the expression pattern of *Lhx2* shows no DV asymmetry (Fig. 4D-S).

The sequence and expression data described above suggest

that *Lhx2* could be a vertebrate homologue of the *Drosophila apterous* gene. To determine whether the homology between these two genes extends to functional similarity we performed ectopic experiments in both the chick and fly embryos.

Lhx2, but not *Lmx-1*, is able to induce *R-fng* expression in the flank of the chick embryo

We first mis-expressed chick *Lhx2* in the chick limb primordia using retroviral technology. Whilst no significant morphological perturbations were observed, in situ hybridisations indicate that infection in the flank (but not in the limb bud) leads to ectopic *R-fng* expression (Fig. 5A). The ectopic expression of *R-fng*, which was restricted to the flank ectoderm, was not accompanied in any case by the formation of an ectopic AER. This is supported by the absence of *Fgf-8* expression (data not shown). Similar experiments with *Lmx-1* did not induce ectopic *fringe* expression (Fig. 5B). These results suggest that *Lhx2*, but not *Lmx-1*, may play a role in regulating *R-fng* expression and that some other factor(s), expressed in the limb primordia but not in the flank, cooperate(s) with *Lhx2* in directing AER formation and hence limb outgrowth.

Lhx2, but not *Lmx-1*, is able to induce *fringe* and *Wingless* expression in *Drosophila* wing imaginal discs

To support our proposal that *Lhx2* might be the vertebrate homologue of *apterous* we misexpressed *apterous*, *Lhx2* and *Lmx-1* on the ventral side of the *Drosophila* wing imaginal disc (using the 71B-GAL4 driver; Fig. 5C) (Brand and Perrimon, 1993) and probed for changes in the expression of *fringe* and *Wingless*, downstream targets of *apterous*. Following mis-expression of the three UAS responders, the expression of the *fringe* gene was monitored using a *lacZ* enhancer detector inserted in the *fringe* locus (Irvine and Wieschaus, 1994). Fig. 5D shows the wild-type dorsal restriction of *fringe* during late third instar larva. Following mis-expression of *apterous* (Fig. 5E) and *Lhx2* (Fig. 5F), but not *Lmx-1* (Fig. 5G), the *fringe-lacZ* marker is ectopically activated on the ventral side of the imaginal disc. Furthermore, the expression of *Wingless*, which is a well known marker of the wing margin, was also monitored using a *wglacZ* enhancer detector present in the *CyOwglacZ* balancer chromosome. Fig. 5H shows the wild-type expression of *Wingless* along the DV compartment boundary during third instar larva. After mis-expression of the UAS constructs, the expression of the *Wingless-lacZ* marker is ectopically extended toward the ventral compartment only with *apterous* (Fig. 5I) and *Lhx2* (Fig. 5J), but not with *Lmx-1* (Fig. 5K). This agrees with the fact that in these crosses only *apterous* and *Lhx2* cause ablation of wing tissues in adult flies (Fig. 5M,N). In contrast, *Lmx-1* does not disturb the wing morphology at all and only causes the appearance of 1-3 ectopic bristles in the vein 3 (Fig. 5O). We know that this *UAS:Lmx-1* transgene is functional because it causes lethality with other GAL4 drivers such as *apterous*-GAL4 and *patched*-GAL4 (data not shown). In addition, when the C5-GAL4 wing driver (Yeh et al., 1995) was used, *apterous* and *Lhx2* caused defects in the wing margin, whereas *Lmx-1* only produced venation anomalies (data not shown). Thus, like *apterous*, *Lhx2*, but not *Lmx-1*, plays a role in regulating *fringe* and *Wingless* expression, and hence wing outgrowth.

Competitive inhibition of *Lhx2* activity leads to arrested limb outgrowth

Since overexpression of *Lhx2* has no phenotype in developing chick limbs, we sought to repress *Lhx2* function by using a similar strategy to that which we describe for *Lmx-1* above. We mis-expressed a dominant negative *Eng*-RD/*Lhx2*-HD hybrid construct in the chick limb primordia at stages 8-12 (see Fig. 3). Infected limb buds failed to develop a normal AER and consequently limb outgrowth was arrested (Fig. 6A-C). In most cases, the truncations occurred in the most distal element (showing an absence of digits and reduction in the size of the radius and the ulna), but in some cases, truncated wings at the level of the humerus were observed (Fig. 6D-G).

Since the *Lhx2* dominant negative chimaera is able to perturb AER formation, we would expect that this is preceded by changes in gene expression, both in the ectoderm and in the underlying limb bud mesoderm. In situ hybridisation of embryos infected with the *Eng*-RD/*Lhx2*-HD hybrid construct using riboprobes for the *En-1, Wnt-7a* and *Lmx-1* genes showed, just as was seen with the *Eng*-RD/*Lmx1*-HD construct, no alteration in their expression pattern (Fig. 7a-c). Furthermore, and contrary to the effects observed with the *Eng*-RD/*Lmx-1*-HD construct, mis-expression of the *Eng*-RD/*Lhx2*-HD construct did not alter the transcript distribution of *Eph-7a* (Fig. 7d). In situ hybridisation of embryos using probes for mesodermal genes involved in the outgrowth of the limb *hoxd-13* (Fig. 7e), *Msx-1* (Fig. 7f), *Shh* (Fig. 7g), *Fgf-10* and *NF-kB* (data not shown) showed a down-regulation in their transcript levels. None of these changes in gene expression were observed after mis-expression of the *Eng*-RD/*Lmx-1*-HD construct (see Fig. 2).

However, and contrary to the effects observed with the *Eng*-RD/*Lmx-1*-HD construct, in situ hybridisation after mis-expression of the *Eng*-RD/*Lhx2*-HD chimaera using probes for *R-fng* indicate that *R-fng* expression is indeed abolished in the infected limbs (Fig. 7h). Furthermore, transcripts for other ectodermal genes involved in limb outgrowth, *Fgf-8* (Fig. 7i), *Wnt-3a* (Fig. 7j), and *Serrate-2* (Fig. 7k) are also absent or downregulated.

The limb truncations observed following mis-expression of the *Eng*-RD/*Lhx2*-HD chimaera raise the concern that the fusion construct might repress genes other than those normally regulated by *Lhx2*. Very recently a second *Lhx2* gene (*Lhx2B*) has been cloned (Nohno et al., 1997). The expression of *Lhx2B* is confined to the most anterior region of the limb bud, whereas *Lhx2* is expressed in both the anterior and posterior mesoderm of the developing limb. Since the most frequent phenotype observed, following mis-expression of dominant negative forms of *Lhx2*, is the loss of posterior limb elements, it seems reasonable to conclude that these phenotypes are likely to be a result of the down-regulation of *Lhx2* rather than interference with the function of *Lhx2B*. However, it is of course possible that the activity of both genes is down-regulated.

To further understand the properties of *Lhx2* we made a second fusion construct, this time with the activation domain from VP16. Similarly to the *Eng*-RD/*Lhx2*-HD construct, mis-expression in the flank at stage 16 induced ectopic *R-fng* expression (data not shown). Mis-expression of this VP16-AD/*Lhx2*-HD chimaera in the limb primordia (stages 8-12) gave no abnormal phenotype.

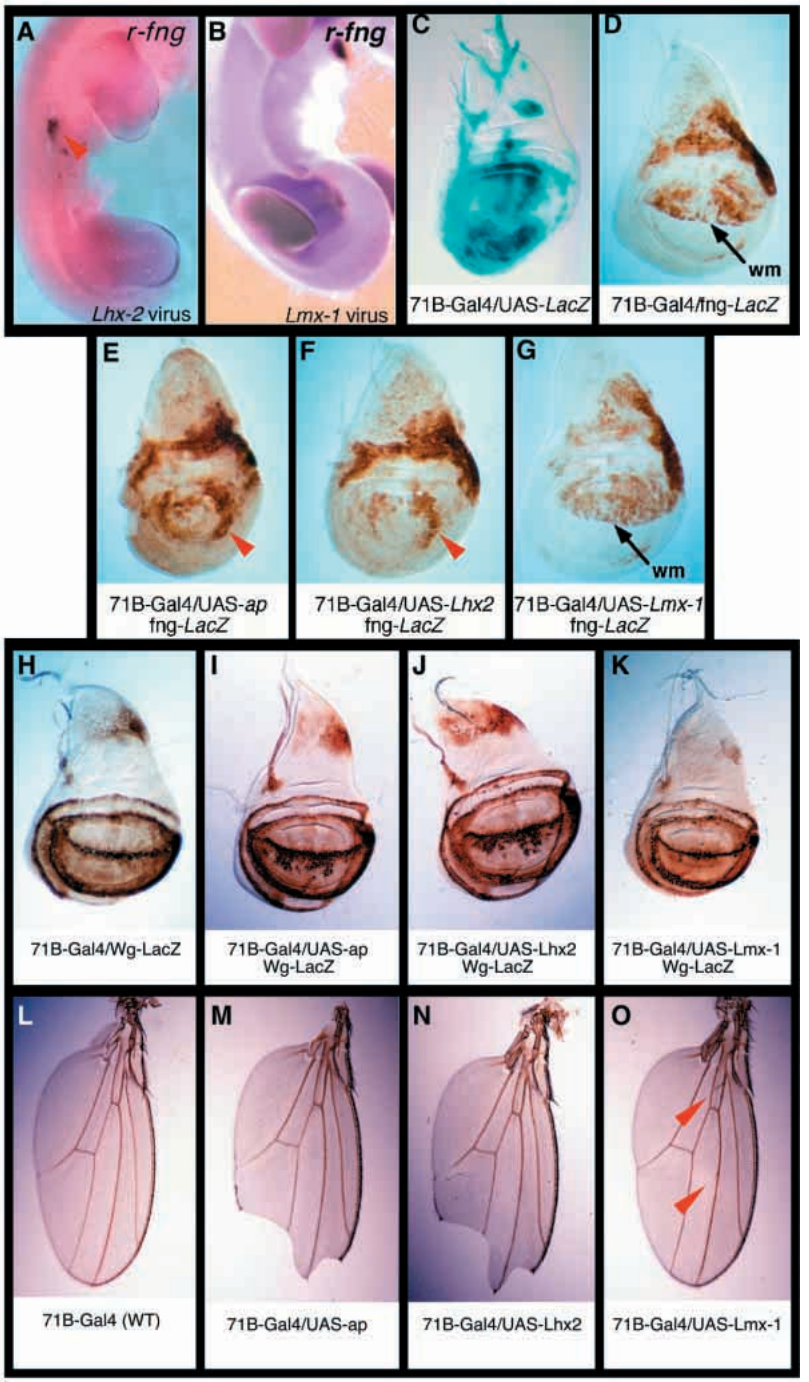

Fig. 5. *Lhx2* and *apterous*, but not *Lmx-1*, induce *R-fringe* in chick and *Wingless* expression in *Drosophila*. At stage 13 the presumptive flank region of the chick was injected with retroviral constructs containing the *Lhx2* or the *Lmx-1* gene. Embryos at stage 23 were processed for whole-mount in situ hybridisation using the *R-fng* probe. Whilst *Lhx2* was able to induce *R-fng* expression in the flank of the embryo (red arrowhead in A), *Lmx-1* mis-expression did not result in ectopic *R-fng* staining (B). In no case did the small outgrowths caused as a result of ectopic *Lhx2* expression exhibit *Fgf-8* expression or AER formation. (C) X-Gal staining of a wing imaginal disc expressing a *UAS:lacZ* transgene under the control of the 71B-Gal4 driver (Brand and Perrimon, 1993). This driver was used to direct expression of the indicated UAS-responder transgenes. (D) Immunochemical staining of a 71B-Gal4, *fringe-lacZ* wild-type wing disc showing *lacZ* expression under the control of the *fringe* regulatory elements. Note that in the wing pouch the *lacZ* marker is restricted to the dorsal compartment, resembling endogenous *fringe* expression (Irvine and Wieschaus, 1994), with a sharp boundary of expression at the wing margin (wm). (E-G) Immunochemical staining showing expression of the *fringe-lacZ* marker in wing discs carrying the 71B-Gal4 driver and the UAS responders expressing either *apterous* (E), human *Lhx2* (F) or *Lmx-1* (G). Note that the *fringe-lacZ* marker is ectopically activated in the ventral compartment only if *apterous* and *Lhx2* are used as responders (red arrowheads). Arrow points to the expression along the DV compartment boundary, which will give rise to the wing margin (wm). (H) Immunochemical staining of a 71B-Gal4, *wglacZ* wild-type wing disc showing *lacZ* expression under the control of the *Wingless* regulatory elements. (I-K) Immunochemical staining showing expression of the *wglacZ* marker in wing discs carrying the 71B-Gal4 driver and the UAS responders expressing either *apterous* (I), human *Lhx2* (J) or *Lmx-1* (K). Note that as well as *fringe-lacZ*, the *wglacZ* marker is ectopically expressed in the ventral compartment only when *apterous* and *Lhx2* are used as responders. (L) Normal morphology of a wild-type wing carrying the 71B-Gal4 driver. (M-O) Wings from flies carrying the 71B-Gal4 driver and the *UAS:apterous* (M), *UAS:Lhx2* (N) and *UAS:Lmx-1* (O) responders. Note that *apterous* and *Lhx2* led to similar ablation of the distal region of the wing, whereas *Lmx-1* only causes the generation of ectopic bristles in the vein 3 (arrowheads).

DISCUSSION

The different roles of *apterous, Lmx-1* and *Lhx2*

As discussed above, *apterous* plays several roles during *Drosophila* wing development. Its role in wing margin formation and wing outgrowth is thought to be realized through the activation of *fringe* and *Serrate* expression (Irvine and Wieschaus, 1994; Kim et al., 1995; De Celis et al., 1996; Panin et al., 1997). Indeed, it has been suggested that *apterous* may serve directly as a transcriptional regulator for the *fringe* gene (Irvine and Wieschaus, 1994). Additionally, *apterous* acts as a selector gene specifying the dorsal wing disk compartment, although the exact molecular pathway is not yet established (Blair, 1993; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Blair et al., 1994).

Our data indicate that in vertebrates these functions are executed by at least two proteins. We have shown that expression of *Lmx-1* in the dorsal mesoderm is both necessary (this work) and sufficient (Riddle et al., 1995; Vogel et al., 1995) to define dorsal cell fate, but it plays no role in regulating gene expression required for limb outgrowth. *Lhx2* on the other hand, which based on sequence comparison is more similar to *Drosophila apterous*, does not appear to specify dorsal cell fate and, moreover, is expressed with no dorsoventral asymmetry.

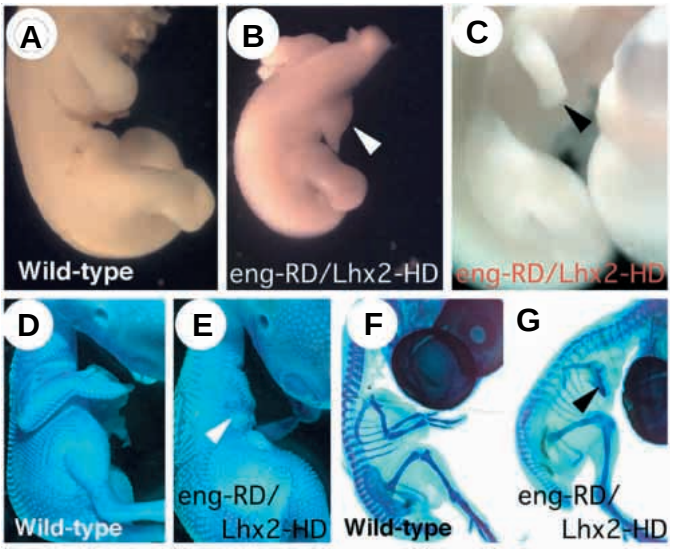

Fig. 6. Competitive inhibition of *Lhx2* activity inhibits limb outgrowth. A retroviral vector containing the *Eng*-RD/*Lhx2*-HD chimaera was injected into the wing or leg primordia of chick embryos at stages 8-12. Embryos were examined at different stages following infection. (A) Stage-27 wild-type embryo. Infected limb buds at the same stage fail to develop a normal AER, resulting in reduction of limb bud outgrowth (B,C, arrowheads). (D-G) 8 days after infection embryos exhibit reduced or no wings (arrowheads in E and G). F and G are the same as D and E after cartilage staining.

However, *Lhx2* does appear to perform the second role of *apterous*, i.e. it regulates gene expression involved in AER formation and hence limb outgrowth. Furthermore, mis-expression of vertebrate *Lmx-1* in *Drosophila* wing imaginal discs is neither able to rescue *apterous* mutant flies (lacking wings; data not shown), nor to induce *fringe* expression in the imaginal disc. Taken together with the fact that vertebrate *Lhx2* is able to rescue *apterous* mutant flies (i.e. generating normal *fringe* and *Wingless* (downstream target of *apterous*) expression, wing morphology and other phenotypes due to lack of *apterous* (this work and our own unpublished results), it would appear that *Lhx2* is functionally closer to *Drosophila apterous* than *Lmx-1*.

Not only does the appearance of *Lhx2* transcripts precede AER formation, but they are also maintained throughout limb bud development. Several lines of evidence suggest that *Lhx2* is involved in both induction and maintenance of the AER, and hence in limb outgrowth. First, ectopic *Lhx2* expression induces *R-fng* expression, *Fgf-8* and *Wnt-3a*, genes involved in ridge formation. Second, surgical removal of the AER results in loss of *Lhx2* expression (our own unpublished results). Third, *Fgf-8* expression maintains *Lhx2* expression. Fourth, down-regulation of *Lhx2* activity perturbs AER formation and results in arrested limb outgrowth at different stages of development. Thus, *Lhx2* may not only be required for continuous limb outgrowth, but may also have a role in positioning the AER at the dorsoventral limb boundary.

Like *apterous* in *Drosophila*, the role of *Lhx2* in AER formation could be mediated through *R-fng*. It is important to note, however, that whilst in *Drosophila apterous* is expressed in the same tissue as *fringe*, in vertebrates *Lhx2* is expressed in the mesoderm. Since it is unlikely that *Lhx2* directly regulates *R-fringe* transcription in the ectoderm, there must be an indirect mechanism that allows *Lhx2* to activate *R-fringe* expression on the ectoderm. This could be achieved via diffusible factors present in the progress zone (i.e. *Fgf-10*, a gene able to induce a complete limb and initiate the cascade of ectodermal gene expression required for limb outgrowth (Ohuchi et al., 1997). By analogy, it would seem possible that in *Drosophila apterous* does not directly regulate *fringe* expression either, and thus may use a similar indirect mechanism.

The fact that *Lhx2* expression in the flank induces *R-fng* expression, but does not result in AER formation or limb outgrowth, suggests that *R-fng* expression in the flank is not sufficient to induce an AER. Previous data suggest that the AER develops at the DV boundary in the limb bud where cells

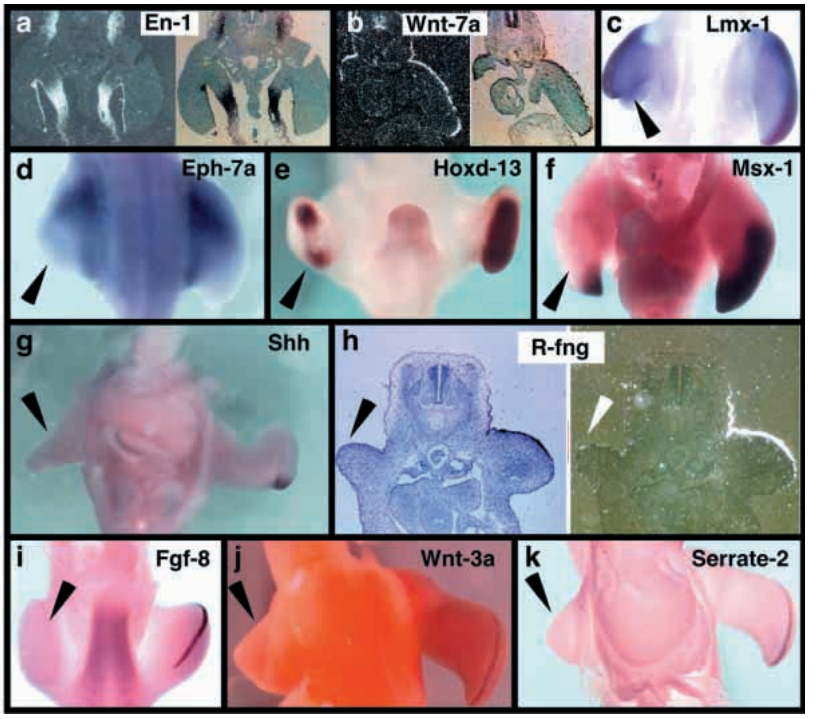

Fig. 7. Gene expression after inhibition of *Lhx2* activity. Mis-expression of the *Eng*-RD/*Lhx2*-HD construct leads to arrest in limb outgrowth that is preceded by changes in the expression of genes involved in the outgrowth of the limb along its proximodistal and anteroposterior axes. Genes involved in patterning the limb along its dorsoventral axis, *En-1* (a), *Wnt-7a* (b), *Lmx* (c) and *Eph-7a* (d) are unaffected. The left side of (a) and (b) are dark field views of sectioned in situ hybridisations using probes for *En-1* and *Wnt-7a*. Bright field views are on the right. Note the reduced size of the infected limbs in all cases but specially in (b). Arrowheads in (c) and (d) point to the reduced infected limb bud. Note that the dorsal expression of neither *Lmx-1* or *Eph-7a* is perturbed. On the contrary, expression of *hoxd-13* (e), *Msx-1* (f), *Shh* (g), *R-fng* (h), *Fgf-8* (i), *Wnt-3a* (j) and *Serrate-2* (k) is absent or down-regulated. The infected limb in all panels is on the left (arrowhead).

expressing *R-fng* are adjacent to cells lacking *R-fng* (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). However, our data and those reported in the mouse (Forbes et al., 1997) suggest that some other factor(s) expressed in the limb primordia but not in the flank, such as *Fgf-10* (Ohuchi et al., 1997), *NF-kB* (Bushdid et al., 1998; Kanegae et al., 1998) or *Msx-1* (see Tickle and Eichele, 1994 for a review), cooperate(s) with *Lhx2* in directing AER formation and driving limb outgrowth.

Finally, it would seem likely that in *Drosophila* dorsal restriction of *fringe* expression is achieved simply by the dorsal expression pattern of *apterous*. In the vertebrate limb *R-fringe* expression is also dorsally restricted. However, since *Lhx2* is expressed symmetrically on both sides of the dorsoventral limb boundary, some other contribution is needed to define the *R-fringe* expression domain. We and others have shown previously that *En-1* represses *R-fringe* expression (Laufer et al., 1997; Rodriguez-Esteban et al., 1997). Since *En-1* is expressed in the ventral ectoderm at the time that *R-fringe* expression is induced by *Lhx2*, it seems likely that the expression domain of *R-fringe* is defined by the combination of *Lhx2* and *En-1*.

Is limb outgrowth independent of dorsoventral signalling?

Current models of *Drosophila* wing development propose that formation of the wing margin and subsequent wing outgrowth along the proximodistal axis depend upon the previous establishment of a boundary at the interface of the dorsal and ventral compartments. This view is based on a number of different studies (Bryant, 1970; Garcia-Bellido et al., 1973, 1976; see also Lawrence and Struhl, 1996, for a review), but primarily on the fact that *apterous* not only specifies dorsal cell fate (Blair, 1993; Diaz-Benjumea and Cohen, 1993; Williams et al., 1993; Blair et al., 1994) but also induces the dorsal expression of the *fringe* and *Serrate* genes which, in turn, induce initiation of wing outgrowth at the dorsoventral boundary (Irvine and Wieschaus, 1994; Kim et al., 1995). Thus, these two processes cannot be readily dissociated since they are controlled by a single molecule.

In vertebrates, however, whilst *Lhx2* induces *R-fringe* expression, it does not specify dorsal cell fate. Since the two functions are dissociated, it raises the question of whether or not limb outgrowth is dependent upon previous dorsoventral patterning. Many different experiments indicate that disruption of limb outgrowth correlates with perturbation of the dorsoventral axis. Mis-expression of *R-fringe* on the ventral side of the limb induces ectopic ventral outgrowths (Laufer et al., 1997; Rodriguez-Esteban et al., 1997); mis-expression of *En-1* on the dorsal side of the limb induces ectopic dorsal outgrowths (Laufer et al., 1997; Rodriguez-Esteban et al., 1997) and mis-expression of either *Wnt-7a* or *Lmx-1* on the ventral side of the limb can cause the appearance of ectopic digits (Riddle et al., 1995; Vogel et al., 1995). In *En-1* minus mice the AER is expanded ventrally, associated with the later formation of ectopic posterior digits (Loomis et al., 1996). In mice lacking *Wnt-7a*, although an AER is present, it appears to be abnormal since *Fgf-4* (normally expressed in the posterior region of the ridge) is absent, accounting for the subsequent loss of posterior skeletal elements (Parr and McMahon, 1995; see also Cygan et al., 1997).

Finally, the correlation of DV patterning and limb outgrowth can also be observed in two naturally occurring chick mutants. The developing limb buds of *eudiplopodia* mutant chicks have an ectopic AER on the dorsal side of the limb bud and the resulting ectopic outgrowth has a bi-dorsal character (Goetinck, 1964; Fraser and Abbott, 1971). In chick *limbless* mutants bi-dorsal limb buds fail to develop an AER and outgrowth is arrested (Prahlad et al., 1979; Fallon et al., 1983).

Together, these data strongly suggest that normal limb outgrowth requires the asymmetric expression of some of the molecules that play a role in dorsoventral patterning and that limb outgrowth is intimately linked to DV patterning (Grieshammer et al., 1996; Noramly et al., 1996; Ros et al., 1996). However, this does not mean that the molecular events that *initiate* limb development, at earlier stages, do not precede the establishment of dorsoventral polarity. Indeed, elegant tissue recombination and fate-map experiments (Kieny, 1971; Altabef et al., 1997; Michaud, 1997), indicate that signals from the limb field mesoderm (before dorsoventral polarity is determined), commit the overlying ectoderm to form an AER. This suggests that limb outgrowth is initiated at very early stages, but that at later stages dorsoventral asymmetry is needed to fine-tune the positioning of the AER, accounting for the perturbations in limb outgrowth that result from disturbed dorsoventral patterning. It is interesting that some recent experiments suggest that *Drosophila* wing development may similarly be initiated prior to the interactions between the dorsal and ventral compartments that establish the wing margin (Klein et al., 1998). In conclusion, whilst it is likely that the initiation of limb outgrowth in vertebrates is independent of DV signalling (see (Zeller and Duboule, 1997) for a review), it seems clear that continued limb development requires dorsoventral asymmetry.

Whilst it is evident that at the morphological level, both during embryogenesis and in the adult stage, the limbs of *Drosophila* and vertebrates are quite distinct, the similarities at the molecular level, during early embryogenesis, suggest that they are evolutionarily related. Evidence that genes controlling the development of *Drosophila* and vertebrate limbs pre-existed as a functional genetic program (directing pattern formation) is now apparent from the similar and recurring expression patterns of several genes. Nonetheless, despite the genetic similarities between vertebrate and invertebrate limbs, the genetic modus operandi seems in some instances to be different. It seems likely that these differences hold the key to understanding how evolution has used conserved genetic programs to build novel structures.

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NOTE ADDED IN PROOF

Whilst this manuscript was under revision, the knockout of *Lmx1b* was reported by Chen et al. (1998). The phenotype of these mutant mice entirely supports our conclusions that *Lmx1* is necessary for dorsal limb specification. This report thus also provides support for the validity of dominant-negative approaches to study chick limb development.

Chen, H., Lun, Y., Ovchinnikov, D., Kokubo, H., Oberg, K. C., Pepicelli, C. V., Gan, L., Lee, B., and Johnson, R. L. (1998). Limb and kidney defects in *Lmx1b* mutant mice suggest an involvement of LMX1B in human nail patella syndrome. *Nat. Genet.* **19**, 51-55.