**Hoxb-8 has a role in establishing early anterior-posterior polarity in chick forelimb but not hindlimb**

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**SUMMARY**

The distribution of Hoxb-8 transcripts through the chick flank and early forelimb mirrors the distribution of polarizing activity in the flank at these early stages. Polarizing activity displayed by Hoxb-8-expressing tissue is only realised when placed adjacent to the AER and appears to be mediated through Shh induction, suggesting that Hoxb-8 may lie genetically upstream of Shh. Accordingly, Hoxb-8 expression is rapidly induced by retinoic acid (RA) treatment in the anterior of the forelimb in a spatial and temporal manner that is consistent with the induction of Shh and formation of the ZPA. Furthermore, inhibition of RA synthesis in the flank downregulates the expression of endogenous Hoxb-8 and results in the loss of Shh expression. However, once the ZPA has become established the posterior limb mesoderm displays resistance to the induction of Hoxb-8 expression. Grafting of ZPA cells to the anterior of a host limb renders the host anterior tissue resistant to RA-induced Hoxb-8 expression. These results indicate that Hoxb-8 expression may be regulated by the established ZPA through a negative feedback loop. The anterior AER also secretes an inhibitory factor, preventing RA-induced or already established Hoxb-8 expression in the cells immediately underneath the AER.

Consistent with a role for Hoxb-8 in positioning of the forelimb ZPA, Hoxb-8 expression is not seen in RA-induced duplications at the anterior of the hindlimb. However, grafting of Hoxb-8-expressing tissue to the hindlimb can lead to Shh expression and similar duplications, suggesting that factors mediating ZPA formation are very similar in both wing and leg.

Key words: Hoxb-8, limb field, forelimb, hindlimb, ZPA, Shh, AER, FGFs, chick

**INTRODUCTION**

Early studies on the developing vertebrate limb have identified signalling centres that specify the three axes of the limb; the zone of polarizing activity (ZPA), a region of mesoderm at the posterior margin of the limb that specifies the anterior-posterior axis (Saunders and Gasseling, 1968), the apical ectodermal ridge (AER), a specialised ectoderm at the distal tip of the limb that maintains the proximal-distal axis (Saunders, 1948) and the overlying ectoderm that determines the dorsal-ventral (D-V) axis (MacCabe et al., 1974).

In recent years, there has been a dramatic increase in our understanding of the molecular components that mediate the activity from these signalling centres (reviewed by Tickle, 1995). Activity of the ZPA appears to be mediated by the gene sonic hedgehog (Shh), since its expression colocalises with the ZPA (Riddle et al., 1993) and application of SHH protein is sufficient to initiate polarizing activity (Lopez-Martinez et al., 1995; Marti et al., 1995). Other genes thought to be involved in this pathway, Bmp-2 (Francis-West et al., 1994) and Hoxd-11, Hoxd-12 and Hoxd-13 (Izpisua-Belmonte et al., 1991) have also been identified and, when ectopically expressed, Bmp-2 also shows some polarizing activity (Duprez et al., 1996).

Experiments where the AER is substituted with different signals suggest that members of the fibroblast growth factor family (Fgf) mediate its function (Fallon et al., 1994; Niswander et al., 1993; Vogel and Tickle, 1993). Fgf-4 expression is biased to the posterior of the AER (Niswander and Martin, 1992), functioning not only to maintain proliferation in the progress zone, but also to maintain Shh expression and therefore ZPA activity (Laufer et al., 1994; Niswander et al., 1994).

The dorsal ectoderm secretes the signalling molecule Wnt-7A, a homolog of the Drosophila wingless gene, which when homozygous for a null mutation results in the formation of limbs with a double ventral structure (Parr and McMahon, 1995). The downstream target of Wnt-7A, Lmx-1, a LIM homeobox gene, is restricted to the dorsal mesoderm and appears to be sufficient for dorsalizing activity when ectopically expressed on the ventral side of the limb (Riddle et al., 1995; Vogel et al., 1995). It has also been demonstrated that the dorsal signal, Wnt-7A, is required for the maintenance of Shh expression (Parr and McMahon, 1995; Yang and Niswander, 1995). These data provide evidence that all three axes (antero-posterior, proximodistal and dorsoventral) are mutually linked to allow co-ordinated growth and patterning of the limb.

Many of the molecules discussed above, although clearly...
involved in patterning of the limb axis (or secondary axis), are not expressed early enough to mediate the positioning and polarizing of the limb field with respect to the primary axis. Further, we know nothing about the differences between forelimbs and hindlimbs since the signalling molecules described above are apparently common to both wing and leg. Evidence for the presence of factors that mediate the early limb field polarity in the anteroposterior axis comes from several experiments. Early grafting experiments showed that prospective wing mesoderm taken from a stage 12 embryo and grafted to the flank of a host will develop in the anteroposterior orientation of the donor tissue (Hamburger, 1938). Recent analysis of the limbless mutation in chick showed that Hoxd-11, Hoxd-12 and Hoxd-13 genes were expressed in their normal nested pattern at the posterior of the limb, even though Shh expression was absent from these mutant limbs (Grieshammer et al., 1996; Ros et al., 1996). The members of the Hox gene cluster are good candidates for mediating the polarity of the flank and locating the early limb field, since they are expressed in specific domains that have distinct anterior boundaries and are known to be involved in the anterior-posterior specification of the embryo (McGinnis and Krumlauf, 1992).

Indeed, positioning of the limb bud along the anterior-posterior axis of the vertebrate body would appear to be at least in part determined by Hox gene activity, with mice carrying a null mutation in the Hoxb-5 gene displaying a rostral shift of the shoulder girdle (Rancourt et al., 1995). The distribution of transcripts for another member of the Hoxb gene cluster, Hoxb-8, showed an anterior boundary at the posterior of the mouse forelimb. Moreover, when Hoxb-8 expression is shifted anteriortly to encompass the anterior margin of the forelimb, mirror-image duplications of the forelimb are observed (Charite et al., 1994), suggesting that Hoxb-8 plays a role in determining the position of the ZPA in the mouse forelimb.

Here we report the distribution of the chick homolog of Hoxb-8 and show that it is expressed in the flank and early forelimb of the chick embryo in positions that demonstrate polarizing activity (Hornbruch and Wolpert, 1991) and a capacity to express Shh. Expression of Hoxb-8 is also induced rapidly, and probably directly, when RA-soaked beads are placed at the anterior of the forelimb bud, but not in the equivalent location in the hindlimb. We also show that there are factors inhibitory to Hoxb-8 expression in the AER and the established posterior limb mesoderm, reflecting a possible mechanism by which endogenous expression is depleted from the posterior forelimb. Although Hoxb-8 expression is not induced in the hindlimb, grafting Hoxb-8-expressing tissue to the hindlimb can evoke duplications. This suggests that the regulators and downstream targets of ZPA establishment are conserved between the wing and leg.

**MATERIALS AND METHODS**

**Isolation of chicken Hoxb-8 cDNA clones and in situ probes**

cDNA made from day 2.5 chick embryos was screened for Hoxb-8 clones using 5'-RACE PCR (Loh et al., 1989). Initial PCR amplification was carried out with a 5' primer AGACCTCGATCCCTCCTTGTG designed to previously published Hoxb-8 sequence (Scotting et al., 1990) and the 5'-anchor primer. PCR products were separated on the basis of size and a second round of PCR performed on products from 500 bp to 1500 bp, using the 5'-anchor primer and a nested primer TCCCGGGTCAGGTAGGATTAATAG. PCR products were then cloned into a TA cloning vector (Invitrogen), a 583 bp clone confirmed by sequencing in both directions as chick Hoxb-8 and then subcloned with EcoRI and NotI into pBluescript sk+ (Stratagene).

**Chick embryos**

Fertilised chicken eggs were obtained from Needle Farms Enfield and incubated at 38°C to the required stages (Hamburger and Hamilton, 1951). Embryos were dissected into sterile PBS for RNA isolation or in situ hybridisation as described below.

**Probes used for in situ hybridisation**

RNA probes for Hoxb-8 transcripts were produced from the pBlue-script-Hoxb-8 clone by linearising with NotI and transcribing with T3 (for antisense probes) or linearising with EcoRI and transcribing with T7 (for sense probes).

Antisense RNA probes specific for Shh were synthesised as previously described (Roelink et al., 1994).

**Whole-mount in situ hybridisation**

Embryos were processed and hybridised according to Wilkinson (1993), with blocking and antibody washing steps using maleic acid buffer and blocking reagent (Boehringer Mannheim).

**Retinoic acid and disulphiram treatment of chick embryos**

AG1-X2 beads soaked in all-trans retinoic acid (0.005-0.5 mg/ml) in dimethyl sulphoxide were implanted at the anterior margin of stage 20/21 limb buds as previously described (Tickle et al., 1985). At various time intervals, the embryos were dissected in PBS and fixed in 4% (w/v) paraformaldehyde. A group of embryos were also left to develop for a further 7 days, and stained for cartilage.

Embryos were treated with disulphiram (SO-100 mg/ml) impregnated silastin (Stratford et al., 1996). Treatment of embryos was carried out as previously described, with the silastin blocks being placed adjacent to the prospective forelimb region.

**Grafting of lateral plate mesoderm to chick wing buds**

The donor and host embryos were prepared and grafted as described previously in (Hornbruch and Wolpert, 1991). Embryos were collected at various intervals and either processed for whole-mount in situ or stained for cartilage.

**Cartilage staining**

Embryos were fixed in 10% formalin for 24 hours, stained in 0.1% Alcian Blue in 70% ethanol, dehydrated in an ethanol series and then cleared in methyl salicylate.

**RESULTS**

**Distribution of Hoxb-8 transcripts in the developing chick embryo**

The distribution of Hoxb-8 transcripts was determined for chick embryos of stages 10-25. In a stage 10 embryo, Hoxb-8 transcripts can be detected in the posterior of the embryo, spreading anteriorly from the tail bud up to somite 6/7 in the neural ectoderm and somite 12 in the lateral plate mesoderm (LPM) (Fig. 1A). By stage 11, the expression has become stronger, with the neural ectoderm rostral boundary becoming set at somite 6/7, while expression in the LPM extends and tapers out rostrally up to the level of somite 14 (Fig. 1B). As the embryo develops, the rostral boundary of the neural tube remains set at somite 6/7, while Hoxb-8 expression in the LPM is much more dynamic. By stage 14/15, the expression in the LPM around the tail bud has regressed, leaving a domain of expression that no longer extends down to the tail bud of the embryo. There is also a progressive caudal shift of the rostral expression boundary, from opposite somite 14 at stage 11, down to somite 17/18 by stage 15 (Fig. 1C). Hoxb-8 transcripts...
are found in the most extensive and graded domain in the LPM by stage 16/17, extending posteriorly from the posterior of the prospective forelimb at somite 17/18, through the interlimb region and into the anterior prospective hindlimb at somite 26 (Fig. 1D,E). At stage 17/18, some variability in the rostral boundary of expression was observed, which in some specimens extended more anteriorly into the forelimb bud. However, this may reflect a transitory change in regulation of Hoxb-8, as by stage 18, when strong Shh expression is first seen in the forelimb, transcripts are only detected at a low level in the most proximal limb mesenchyme. The expression of Hoxb-8 opposite in the hindlimb is also lost by stage 18, with the expression remaining primarily in the flank. From stage 19 to 25 no expression of Hoxb-8 was detected in the forelimb bud.

In the light of the observations made in the mouse, where expression of Hoxb-8 at the anterior of the forelimb induces polarizing activity (Charite et al., 1994), we compared the distribution of Hoxb-8 expression with that of polarizing activity in the flank of the chick embryo (Hornbruch and Wolpert, 1991). A striking correlation was observed between the two distributions, with regions of the flank expressing Hoxb-8 also displaying polarizing activity (Fig. 1F). The most noticeable similarity was the caudal shift of anterior boundaries for both Hoxb-8 expression and polarizing activity, from opposite somite 12 in a stage 10 embryo to opposite somite 17/18 by stage 16. Furthermore, regions of the flank that showed the highest levels of Hoxb-8 expression, opposite somites 19-22 at stage 16/17, also exhibited high levels of polarizing activity.

Interestingly, Hoxb-8 expression is not detected in the posterior of the hindlimb, even though at stages 16-18 strong polarizing activity can be detected in this region. This adds weight to the suggestion that Hoxb-8 functions in positioning the polarizing signal of the forelimb but not that of the hindlimb. However, the bizarre observation that low levels of polarizing activity are present in the anterior of the prospective hindlimb at stages 16-17 (Hornbruch and Wolpert, 1991), is further supported by the presence of low Hoxb-8 expression in this region (Fig. 1D,E). The significance of this transitory activity in the anterior of the leg bud is not clear.

In order to test whether the polarizing activity of Hoxb-8-expressing flank was mediated through Shh, regions of Hoxb-8-expressing flank from stage 16/17 embryos (interlimb region opposite somite 21-24), which would not normally express Shh, were grafted to the anterior of a stage 20/21 host wing bud. When the grafted limbs were incubated for 24 hours and then examined for Shh transcripts, a high level of expression was observed in the graft tissue (Fig. 1G; n=5), while none was detected in the host tissue. When grafted limbs were left to develop for a further 7 days, digit duplications of the type described previously were observed (Fig. 1I).

**Induction of Hoxb-8 expression after retinoic acid treatment**

The application of RA to the anterior margin of the limb bud is capable of inducing an ectopic ZPA (Wanek et al., 1991), leading to a limb with duplicated structure (Tickle et al., 1982). To test whether Hoxb-8 is involved in this induction of an ectopic ZPA, beads soaked in all-trans-retinoic acid (0.5 mg/ml) were placed under the AER at the anterior margin of stage 20/21 limb buds and examined for Hoxb-8 expression at various intervals. The induction of Hoxb-8 expression was very rapid, with detectable levels obtained within 30 minutes of the RA beads being applied (Fig. 2B; n=4). After 4 hours of incubation, staining around the bead was extensively spread throughout the mesoderm and encompassed the anterior third of the limb bud (Fig. 2C; n=6). Expression appeared to be restricted to the mesoderm with no staining detectable in the ectoderm or AER (Fig. 2D). In limbs treated for 18 hours with RA, expression was still detectable in the proximal and posterior mesoderm surrounding the bead (Fig. 2D; n=6), although the domain of expression was not as extensive. However, by 22 hours post-treatment, no Hoxb-8 expression was observed (Fig. 2E; n=5).

When lower concentrations of RA (0.005-0.1 mg/ml) were used, the length of time and, to a certain degree, the domain of Hoxb-8 expression, showed responses in a dose-dependent manner. Beads soaked in RA (0.1 mg/ml) and incubated for 4 hours showed a similar level and domain of expression to that of 0.5 mg/ml (Fig. 2F). However, after 18 hours of incubation with RA (0.1 mg/ml), no Hoxb-8 expression was detectable. Since both concentrations of RA are capable of evoking a similar level of duplication, this suggests that the duration of Hoxb-8 expression, at least after 18 hours, is not critical for the induction of polarizing activity. When the concentration of RA is lowered to the level that only gives the lowest duplication, 0.005 mg/ml (Tickle et al., 1985), only the mesenchyme immediately proximal to the bead showed Hoxb-8 expression after 4 hours of incubation (Fig. 2G).

In contrast to the RA-treated limbs, the untreated contralateral limb (Fig. 2A), and limbs treated with beads soaked in DMSO (data not shown), exhibited no staining in the anterior limb mesoderm.

From these results, the expression of Hoxb-8 appears to respond to RA very rapidly and in a dose-dependent manner, with an increasing number of cells responding as the concentration increases. However, at doses of 0.1 mg/ml and above, the relative number of cells expressing Hoxb-8 dose not increase with RA concentration. Although, the length of time that cells express Hoxb-8 is still dose dependent at the higher concentrations of RA.

**Inhibition of RA synthesis downregulates Hoxb-8 expression in the lateral plate mesoderm**

We have shown previously that blocking RA synthesis in the flank prevents the establishment of Shh expression and a functional ZPA (Stratford et al., 1996). Since RA-induced duplications involve the expression of Hoxb-8 and Hoxb-8 expression is co-extensive with polarizing potential, it seemed likely that blocking RA synthesis would also abolish Hoxb-8 expression in the flank. In order to test this, stage 10/11 embryos were treated with disulphiram and Hoxb-8 expression analysed 24 hours later. In the majority of embryos (75%, n=8), Hoxb-8 expression showed a severe downregulation in the treated flank compared to the control (Figure 3). Control treatments with carrier alone showed no effect on Hoxb-8 expression (data not shown). This result shows that RA is required for endogenous flank Hoxb-8 expression and that the loss of Hoxb-8 expression is associated with a failure to establish a ZPA.

**Inhibition of Hoxb-8 expression in the posterior limb mesenchyme**

The distribution of Hoxb-8 transcripts in response to RA treatment appears to be biased to particular regions of the mesoderm. Even when the concentration of RA is increased...
fivefold (Fig. 2C compared to F), the domain of expression never spreads into the posterior part of the limb bud. This raises the possibility that not all regions of wing bud mesoderm are competent to express Hoxb-8 at this stage.

In order to further test the competence of posterior wing bud mesoderm to express Hoxb-8, a bead soaked in RA (0.5 mg/ml) was placed at the posterior margin (opposite somite 19/20 border) of a stage 20/21 limb, incubated for 2.5 hours, and analysed for Hoxb-8 expression. In contrast to the result from beads placed at the anterior margin, no expression was detected in the mesoderm.

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**Fig. 1.** Distribution of Hoxb-8 transcripts in whole-mount preparations of chick embryos. (A) A stage 10 embryo showing Hoxb-8 expression in the neural tube at somite 6/7 and in the lateral plate mesoderm (LPM) at somite 12 (arrowhead). (B) Stage 11 embryo showing stronger and more extensive expression in the LPM to somite 14 (arrowhead). (C) Lateral view of stage 15 embryo showing expression in the neural tube (nt) and somitic mesoderm (sm), and expression in the LPM from opposite somite 17 down to somite 24. (D) Dorsal view of a stage 16 embryo (28 somites), showing graded expression in the neural tube from somite 6/7 down to the tail bud. The anterior boundary for expression in the LPM is at somite 17/18, with expression extending through the posterior of the prospective forelimb, interlimb and into the anterior of the prospective leg bud at somite 26. (E) Stage 17 embryo (31 somites) showing expression in the posterior of the forelimb opposite somite 18/19, the interlimb flank and anterior of the leg bud at somite 26. (F) Schematic showing the distribution of polarizing activity in the flank of embryos at the same stages as those in A-E, adapted from Hornbruch and Wolpert (1991). (G) Whole-mount in situ for Shh expression in a stage 24 limb bud, 24 hours after grafting the lateral plate mesoderm opposite somite 23, from a stage 16 embryo, to the anterior margin of the host limb. Ectopic Shh expression can be seen in the grafted tissue (arrowhead). (H) A control limb showing a normal wing digit pattern: 2 3 4. (I) A duplicated digit pattern: 3 2 3 4, resulting from a similar graft to that carried out on limb (G), which has been left for a further 7 days and stained for cartilage.
lying adjacent to the bead (Fig. 4; n=7). Surprisingly however, anterior mesodermal cells at a greater distance from the bead did show detectable levels of Hoxb-8 expression. This result argues against the idea that posterior mesoderm cells lack Hoxb-8 expression because they were not exposed to a high enough concentration of RA, since the non-expressing cells adjacent to the bead are exposed to the highest level of RA when the bead is placed posteriorly.

**Hoxb-8 expression is inhibited by the established ZPA**

From the resistance of posterior cells to express Hoxb-8 in response to RA, and the loss of endogenous posterior Hoxb-8 expression at stage 18, it appeared possible that the ZPA was inhibiting the expression of Hoxb-8. In order to further test this idea, pieces of anterior mesoderm (control grafts), or ZPA from a stage 21 forelimb were grafted under the AER at the anterior margin of a stage 17/18 wing bud, incubated for 24 hours, and then a RA-soaked bead (0.1 mg/ml) was placed at the anterior margin adjacent to the original graft and incubated for a further 3 hours. In control limbs where anterior tissue was grafted to the anterior margin, a strong induction of Hoxb-8 expression in response to RA was observed in the anterior mesoderm (Fig. 5A; n=7). However, in the limbs that received a ZPA graft, Hoxb-8 expression was not detectable in either the grafted tissue or the host anterior mesoderm (Fig. 5B; n=7). This
ability of ZPA grafts to block Hoxb-8 induction in the anterior mesoderm, is strongly suggestive of a role for the ZPA signalling in inhibiting Hoxb-8 expression.

**Inhibition of Hoxb-8 expression by the anterior AER**

In addition to the inhibition of Hoxb-8 in posterior mesenchyme, a further inhibitory influence was noted. At all concentrations of RA that were tested, Hoxb-8 expression was never observed in a strip of mesodermal cells immediately underlying the AER (Fig. 2D,F). The location of these non-induced Hoxb-8 cells, immediately underlying the AER, implies that a signal from the AER may mediate a local inhibition of Hoxb-8 expression. In order to test this, the anterior AER was removed, left to stand for 2 hours and then a bead soaked in RA (0.5 mg/ml) placed at the anterior margin. After 4 hours of incubation, strong Hoxb-8 expression in the anterior mesoderm was observed. However, the expression now extended to the anterior margin and encompassed the cells that had been lying directly adjacent to the AER (Fig. 6A; n=8), thus suggesting the suggestion that a signal from the AER prevents adjacent peripheral mesoderm cells from expressing Hoxb-8. Furthermore, experiments where the anterior AER was removed and an RA (0.5 mg/ml) bead placed at the posterior margin showed an even greater induction of Hoxb-8 expression in the anterior mesoderm after 2.5 hours (Fig. 6B; compare Fig. 4A). Control limbs that had only the anterior AER removed, or the anterior AER removed plus DMSO-soaked beads placed at the anterior margin, showed no ectopic expression of Hoxb-8 (data not shown).

Since signals from the AER were capable of inhibiting RA-induced Hoxb-8 expression, it was of interest to determine if cells with established Hoxb-8 expression would downregulate expression when placed adjacent to the AER. To test this, 100 μm pieces of the lateral plate mesoderm from opposite somite 21-24 were grafted from a stage 16/17 embryo to under the anterior AER of a host limb bud. When embryos were harvested after 4 hours and analysed, they showed no Hoxb-8 expression in the grafted tissue (Fig. 6C; n=6), while embryos left for 24 hours showed strong expression of Shh in the grafted AER (Fig. 1G; data not shown). Furthermore, when grafted pieces of LPM were increased in size to 200 μm and analysed after 4 hours, Hoxb-8 expression was found only in the grafted cells furthest from the AER (Fig. 6D; n=7).

It appears from these results that the lack of Hoxb-8 expression in the small group of peripheral cells at the anterior margin is due to factor(s) present in the AER. However, these factors would seem to act only over a short distance, since only the cells that lie a few cell diameters from the AER are affected.

**Hoxb-8 is only involved in forelimb ZPA establishment; but can function in the hindlimb to induce a duplication**

In contrast to the distribution of Hoxb-8 transcripts in the posterior of the prospective forelimb, the prospective hindlimb in both mouse (Charite et al., 1994) and chick lacks Hoxb-8 expression at the posterior margin. To test if Hoxb-8 has a role in establishing the ZPA of the hindlimb, beads soaked in RA (0.1 mg/ml) were placed at the anterior margin of a stage 20 wing and leg bud in the same embryo. Analysis of Hoxb-8 transcripts after 4 hours incubation revealed a high level of expression in the anterior forelimb mesoderm, while hindlimb anterior mesoderm had no detectable expression (Fig. 7A; n=6). However, when a group of these embryos were left for a further 7 days, mirror-image duplications were observed for both forelimbs and hindlimbs (data not shown; Wilde et al., 1987). This suggests that an RA-induced ZPA in the hindlimb does not require Hoxb-8 expression, in contrast to the events that occur at the anterior forelimb, which appear to always involve the expression of Hoxb-8.

Since the expression of Hoxb-8 at the anterior margin of the forelimb, either by graft of expressing tissue or expression driven by a transgene, can lead to duplicated structures, it was interesting to test if cells expressing Hoxb-8 would induce duplications when placed at the anterior margin of a stage 20 hindlimb. Regions of interlimb flank (opposite somite 21-24) were grafted as 200 μm pieces from stage 16/17 embryos to the anterior margin of a stage 20 hindlimb and analysed for Hoxb-8 transcripts after 4 hours. The grafts showed an identical response to those placed at the forelimb, with cells closest to the AER showing a downregulation of Hoxb-8 expression (Fig. 7B). Furthermore, grafts left to develop for a further 24 hours showed a high level of Shh expression (Fig. 7C; n=5) and duplicated digits when left for a further 7 days (Fig. 7D; n=6).

These results would appear to suggest that, although Hoxb-8 is not normally expressed or induced by RA in the hindlimb, cells expressing Hoxb-8 can function to form an ectopic ZPA when grafted to the hindlimb.

**DISCUSSION**

Hox genes are known to play important roles in specifying identity in the anterior-posterior (primary) axis of the developing embryo (Krumlauf, 1994). Many Hox genes are also expressed within the developing limb bud (secondary axis; Izpisua-Belmonte et al., 1991; Nelson et al., 1996) acting in both the anterior-posterior (Morgan et al., 1992) and proximal-distal axis (Davis et al., 1995). In the mouse, one of these genes, Hoxb-8, has been shown to be involved not only in the primary axis, but also to affect the anterior-posterior polarity of the limb (secondary axis; Charite et al., 1994). Here we have isolated the chick homolog of Hoxb-8 and have shown that its distribution and activity in the flank and early limb bud are consistent with a role in determining early forelimb polarity.

**Hoxb-8 is co-expressed with regions of the flank displaying polarizing activity**

The distribution of polarizing activity within the flank and prospective forelimb field strongly correlates with the distribution of Hoxb-8 transcripts at the same stage. The level of polarizing activity is also consistent with the intensity of Hoxb-8 staining, with regions of the highest staining also recording the highest level of polarizing activity. Furthermore, the detection of low levels of Hoxb-8 transcripts within the anterior of the leg bud at stages 16/17 coincides with the surprising observation of polarizing activity in this region (Hornbruch and Wolpert, 1991), although the absence of Hoxb-8 expression from the posterior of the leg bud appears to support previous suggestions that Hoxb-8 is not involved in determining leg bud polarity (Charite et al., 1994). Further support for this comes from our experiments, where an RA-soaked bead fails to induce Hoxb-8 when placed in the anterior of the hindlimb bud even though limb duplications are induced.

The polarizing activity that is displayed by grafts expressing
**Hoxb-8 expression is downregulated by the established ZPA**

Our results show that the anterior and posterior limb mesenchyme differ in their competence to express Hoxb-8 in response to RA treatment: anterior cells can be induced to express Hoxb-8 whereas posterior cells cannot. This raises the possibility that a signal from the polarizing region is mediating this effect. Consistent with this idea, when a ZPA is grafted to the anterior margin, the anterior mesoderm also becomes unable to express Hoxb-8 in response to RA treatment. The fact that the inhibition of expression is not confined to the graft only, but is widely spread through the host anterior tissue suggests several possibilities. Either, the inhibitory signal is expressed in the graft and diffuses widely into the surrounding host tissue, or the inhibitor is induced in response to the graft throughout the host tissue. However, clearly the molecule(s) responsible for the inhibition is not a local signal from the graft.

This inhibitory mechanism may also operate earlier, depleting the endogenous Hoxb-8 expression from the posterior of the limb. Consistent with this idea, Shh expression is first detected at stage 17/18 (Crossley et al., 1996; Riddle et al., 1993), a time that coincides with the loss of Hoxb-8 expression from the posterior limb mesenchyme.

These data support the idea that Hoxb-8 expression is regulated by the ZPA through a negative feedback loop. A model for this type of pathway is shown (Fig. 8), where Hoxb-8 would lie upstream of Shh and the ZPA. We see from this model that a co-ordinated signal from the AER, probably Fgf-8 together with Hoxb-8 from the mesoderm, would be required to establish Shh expression and a polarizing region. Once the ZPA is established its activity is maintained by previously characterised FGF-4 and WNT-7A pathways (Laufer et al., 1994; Niswander et al., 1994; Parr and McMahon, 1995; Yang and Niswander, 1995), while a consequence of establishing a stable polarising region is to release factor(s) that inhibits Hoxb-8 expression and prevent activation of a further aberrant polarizing centre.

**Inhibition of Hoxb-8 expression by the AER**

The results also demonstrate the presence of an inhibitory signal from the AER which is able to not only suppress the...
RA-induced expression of Hoxb-8, but also inhibits established expression in tissue that is grafted underneath the AER. This result initially appears contradictory to a role for Hoxb-8 in establishing the ZPA, since it is known that the establishment of the ZPA requires a signal from the AER (Laufer et al., 1994; Niswander et al., 1994). However, the observation that the inhibition of Hoxb-8 is only effective over a few cell diameters, and that Hoxb-8 is expressed in mesoderm adjacent to the anterior edge of the AER (Fig. 2F), suggest the inhibition may perform a role in positioning of the ZPA. Analysis of the position of the ZPA by Shh expression, in relation to the position of the AER signals (Fgf-4 and Fgf-8) (Bueno et al., 1996), reveals that the ZPA marks the boundary of the AER and that only a small leading edge of the ZPA lies directly beneath the AER. It is therefore possible that the inhibitory properties of the AER plays a role in positioning the expression of Hoxb-8 at the posterior edge of the AER and results in the formation of the ZPA at the posterior boundary of the AER.

Experiments that were carried out to identify the factor(s) mediating the inhibition were unsuccessful (FGF-4, BMP-2 and BMP-4 beads showed no effect). However, the possibility still remains that one of these factors acts in conjunction with others to inhibit Hoxb-8 expression and that the same inhibitory factors may be acting in both the AER and the posterior limb mesoderm.

**Hoxb-8 is not required for establishing polarity of the hindlimb**

It has been suggested from previous experiments that due to the lack of expression in the hindlimb, Hoxb-8 is not involved in the establishment of the hindlimb ZPA. Further support for this comes from the observation that RA beads placed at the anterior of the leg bud, which lead to induction of Shh expression and a duplicated structure (Wilde et al., 1987), do not induce any detectable Hoxb-8 expression. These results are consistent with the proposal that Hoxb-8 plays a role in positioning of forelimb ZPA with respect to the primary axis. Therefore, the positioning of the hindlimb ZPA, at a more posterior axial position, should involve the activity of another more 5′ Hox gene with a more posterior boundary (Duboule and Dolle, 1989; Graham et al., 1989). A possible candidate gene for such an activity is the recently described Hoxb-13 (Zelster et al., 1996), with an anterior boundary of expression at the level of the tail bud of the mouse embryo, although initial analysis has failed to show any expression in the limbs.

Further evidence for this type of role for Hox genes comes from the analysis of Hox group 9 paralogues in ectopically induced limbs from the chick flank (Cohn et al., 1995, 1997). By using an FGF signal to induce ectopic wings or legs from the same group of flank cells, early changes in Hox gene expression in the flank were seen that reflected the type of limb that was formed. These results, taken together with those from the Hoxb-5 knockout mouse, which displays an anterior shift of the shoulder girdle, support the proposal that expression of
Hox genes in the flank are involved in positioning and polarizing the limbs with respect to the body axis.

The observation that grafts of Hoxb-8-expressing flank cells are able to induce duplications in the hindlimb is not inconsistent with this model of Hoxb-8 function. Since many key signals involved in limb patterning are common to both fore- and hindlimb e.g. Fgfs, Shh and Bmps, it is not particularly surprising that Hoxb-8-expressing flank can be activated to express Shh by common signals when placed into the hindlimb environment. Moreover, ectopically induced limbs from the flank display anterior Shh expression and a reversal in digit polarity (Cohn et al., 1995; Crossley et al., 1996; Vogel et al., 1996). The flank cells that are recruited to form the ZPA of these limbs are from the anterior interlimb flank, expressing a high level of Hoxb-8, regardless of whether it is a wing or a leg that is formed. The lack of hindlimb duplications in the Hoxb-8 transgenic mouse might be explained by the level of transgenic Hoxb-8 expression in the anterior of the hindlimb being lower than that of the forelimb.

The results from early grafting experiments have demonstrated a degree of asymmetry and complexity in the limb field which is not apparent from its uniform morphology. Our results, and those from other recent papers, have identified molecules involved in establishing some of these early positioning and patterning events within the limb field. The distribution and activity of many of these genes suggest that initial steps of patterning the limb are set in the limb field (Duboule, 1994), and subsequently the traditional signaling centres of the limb bud are positioned from these activities. The specificity of the limb field is determined to the level of forelimb or hindlimb identity before limb-bud formation occurs (Hamburger, 1938; Lewis and Wolpert, 1976). Even though Hoxb-8 and T-box genes (Gibson-Brown et al., 1996) show differences in forelimb and hindlimb distribution, their activities alone would appear insufficient to confer forelimb or hindlimb morphology. It is therefore likely that the identity of other key regulators for determining limb field identity, polarity and number still remains to be discovered.

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