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# Retinoid signaling is required for the establishment of a ZPA and for the expression of *Hoxb-8*, a mediator of ZPA formation

**Hui-Chen Lu[1,2], Jean-Pierre Revelli[2], Lisa Goering[2,*], Christina Thaller[2] and Gregor Eichele[1,2,†]**

[1]Developmental Biology Program, and [2]V. and M. McLean Department of Biochemistry, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030, USA

*Present address: Department of Human Genetics, University of Utah, Salt Lake City, UT84112, USA
†Author for correspondence (e-mail: geichele@bcm.tmc.edu)

## SUMMARY

**We show that retinoid receptor antagonists applied to the presumptive wing region block the formation of a zone of polarizing activity (ZPA). This suggests a direct relationship between retinoid signaling and the establishment of the ZPA. We provide evidence that the *Hox* gene, *Hoxb-8,* is a direct target of retinoid signaling since exogenously applied RA rapidly induces this gene in the absence of protein synthesis and, moreover, retinoid receptor antagonists down-regulate *Hoxb-8* expression. In addition, we find that, in the lateral plate mesoderm, the domains of *Hoxb-8* expression and of polarizing activity are coextensive. Taken together, these findings support the hypothesis that retinoids are required for the establishment of a ZPA, and that retinoids act, at least in part, through *Hoxb-8,* a gene associated with ZPA formation (Charité et al., 1994).**

Key words: limb pattern formation,limb development, zone of polarizing activity, retinoid receptor antagonists, homeobox genes, *Hoxb-8*, retinoid, bone morphogenetic protein, sonic hedgehog gene

## INTRODUCTION

Vertebrate limb development begins with the specification of limb position and polarity, and the initiation of a limb bud. Tissue transplantation experiments and fate mapping studies indicate that in the case of the chick limb, position and polarity along the anteroposterior axis are specified as early as Hamburger-Hamilton stages 8 to 11 (Chaube, 1959). An important event during the first phase of limb development is the appearance of polarizing activity in the lateral plate mesoderm (Hornbruch and Wolpert, 1991). Polarizing activity is defined as the ability of tissue to induce additional digits when grafted to the anterior wing bud margin (e.g. Saunders and Gasseling, 1968). By stage 14/15, the lateral plate mesoderm locally condenses and, subsequently, through a ventral involution of the somatopleure identifiable limb buds form around stage 16/17. At this time, the distalmost portion of the ectoderm that encloses the limb bud thickens and forms the apical ectodermal ridge (AER). The AER is required for further limb outgrowth (Saunders, 1948; Summerbell, 1974). By stage 18, polarizing activity becomes concentrated to the posterior mesenchyme of the limb bud, thereby giving rise to the zone of polarizing activity (ZPA). It is believed that the ZPA directs patterning along the anteroposterior limb axis (Tickle et al., 1975) and, in conjunction with signals produced by the AER (Summerbell et al., 1973), promotes a rapid distal elongation at the tip of the limb bud. Around stage 22, differentiated tissues such as cartilage or muscle precursors appear.

Much has been learned about molecules guiding limb bud growth and patterning. The list of important factors includes SHH, FGF-4, BMP-2 and Wnt-7a (Riddle et al., 1993; Francis et al., 1994; Laufer et al., 1994; Niswander et al., 1994; Yang and Niswander, 1995; Duprez et al., 1996; for reviews see Tickle and Eichele, 1994; Tabin, 1995; Tickle, 1995). By contrast, much less is known about the nature of the factors that control the early phase of limb development. It is proposed that *Hox* genes of paralog groups 5 to 8 specify forelimb position, possibly in a combinatorial fashion (Tickle and Eichele, 1994; Burke et al., 1995; Tickle, 1995). It is conceivable that certain *Hox* genes also mediate the development of the polarizing region; the chief evidence for this view is that ectopic expression of *Hoxb-8* at the anterior margin of the forelimb bud induces an ectopic ZPA (Charité et al., 1994). The fibroblast growth factor FGF-8 is implicated in controlling the initial outgrowth of limb buds. The main evidence for this notion is that ectopic FGF-8 applied to the lateral plate evokes the formation of an additional limb and that *fgf-8* is expressed in the intermediate mesoderm at the level of the future limb buds as well as in the presumptive and, later, in the definite AER (Crossley et al., 1996; Vogel et al., 1996). Retinoids act early in limb development because disruption of the retinoid signaling pathway by retinoid receptor antagonists prior to limb bud outgrowth results in truncated limbs (Helms et al., 1996). Furthermore, all*trans*-retinoic acid (RA) locally applied to limb buds, rapidly induces genes implicated in early limb development (*Hoxb-6/8*, Lu et al., 1997) and, at a substantially slower rate, genes of the later phase (*shh, bmp-2, fgf-4*, Riddle et al., 1993; Francis et al., 1994; Helms et al., 1994; Niswander et al., 1994).

In this study, we have explored the role of retinoids during

the early limb development. We provide evidence that *Hoxb-8*, a gene implicated in the establishment of the ZPA (Charité et al., 1994), is a primary response gene to the retinoid signal. We show that the expression domain of *Hoxb-8* prefigures two important features of limb development. (1) Already by stage 8, the rostral boundary of its expression domain falls within the future wing region. (2) The initially broad expression domain of *Hoxb-8* in lateral plate mesoderm develops into a domain co-extensive with the tissue that exhibits polarizing activity. Targeting retinoid receptor antagonists into this domain not only abolishes *Hoxb-8* expression, but also prevents the establishment of a ZPA.

## MATERIALS AND METHODS

### cDNA cloning

RT-PCR was used to generate a chicken *Hoxb-8* fragment containing the homeobox of this gene (Scotting et al., 1990). A chick stage 14-17 embryonic λZAPII cDNA library was screened with this fragment and positive clones were isolated and sequenced. Two overlapping cDNAs defined the complete coding region of the *Hoxb-8*. Sequence comparison was performed using the software Wisconsin Package, version 8, 1994, Genetics Computer Group, Madison, Wisconsin. The GenBank accession number of the *Hoxb-8* sequence is U81801.

### Tissue grafting

White Leghorn chicken embryos were used as donors and hosts. To analyze for polarizing activity, posterior tissue of either normal or antagonist-treated wing buds was transplanted to the anterior margin of a stage 20 embryo wing bud (Fig. 1B). Host embryos were incubated to day 10 and processed as described (Wedden et al., 1990).

### Antagonists and agonist treatment

AG1-X2 ion-exchange beads of 200 μm diameter were soaked for 5 hours in a mixture of 2.4 mg/ml LG754 (RXR selective antagonist; see Lala et al., 1996) and 2.4 mg/ml LG629 (RAR selective antagonist, identical to Ro 41-5253, Apfel et al., 1992). Embryos with 20 to 24 somites (stage 13 to 14, Hamburger and Hamilton, 1951) were slightly stained with Neutral Red and two incisions were made in the lateral plate at somite levels 15 and 20; beads were placed into these slits. The soaking concentration used in the present study was 5 times higher than that used in an earlier study (0.5 mg/ml; Helms et al., 1996). With 2.4 mg/ml, complete hand plate truncations were observed in 67% of wings, 24 % of the wings had just a digit **3** and/or **2** and 9% of the wings were normal ($n$=12). Furthermore 58% of the wings had no ulna. All-*trans*-retinoic acid (RA, agonist, 100 μg/ml), LG754 or LG629 treatment of stage 20 wing buds was performed as previously described (Helms et al., 1994) using AG1-X2 beads of 250-300 μm diameter.

### Antagonist action in vivo of LG629 and LG754

The RXR antagonist LG754 is a potent inhibitor of RXR homodimer-mediated transactivation, but can induce a reporter gene through RXR-RAR heterodimers (Lala et al., 1996). However, the gene expression data reported here show that LG754 and LG629 are not acting agonistically. We applied beads soaked in LG629 and/or LG754 to the anterior margin of stage 20 wing buds and monitored, by in situ hybridization, the expression of *RARβ*, a direct RA target gene (de Thé et al., 1990; Sucov et al., 1990). LG754 and LG629, applied alone or in combination, did not induce *RARβ*. Instead, the combined treatment with LG629 and LG754, or that with LG629 alone, markedly decreased the expression of *RARβ* in the vicinity of the bead (data available upon request).

### Whole-mount in situ hybridization

Synthesis of digoxigenin-tagged riboprobes of *shh* (Riddle et al., 1993; Roelink et al., 1994), *bmp2* (Francis et al., 1994), *fgf-8* (Crossley et al., 1996), *Hoxb-8* (nt -51 to nt 726), *Hoxd-11*, *Hoxd-13* (Izpisúa-Belmonte et al., 1991) and *RARβ* (nt 1 to nt 612, Smith and Eichele, 1991), was carried out with a Stratagene RNA transcription kit following the procedure described in Albrecht et al. (1997). Whole-mount in situ hybridization was performed with digoxigenin-labelled riboprobes as described by Albrecht et al. (1997).

### Cell culture experiments

Whole limb buds from stage 20 chick embryos were dissociated to single cells by trypsin treatment. Cells were cultured at 37˚C in DMEM containing 10% fetal calf serum (Gibco, BRL) until they had attached to the culture plate. Thereafter, medium containing either 1 μM RA, or 30-50 μM cycloheximide (Sigma), or the combination of RA and cyloheximide was added. Cells were harvested for RNA purification after 6 hours of treatment and total RNA was isolated using RNAzol (Tel-Test). Northern analysis was carried out with 20 μg total RNA per lane (Sambrook et al., 1989). Filters were probed with $^{32}$P-labelled DNA probes corresponding to *Hoxb-8*, *RARβ* and *Pax-3* (Goulding et al., 1994).

## RESULTS

### Retinoid signal transduction is required for the establishment of a ZPA in the limb bud

When retinoid receptor antagonists are applied to the presumptive wing region, the resulting wings lack either all three or the two posteriormost digits and one or both forearm elements (see Material and Methods and Helms et al., 1996). Thus, proximodistal and anteroposterior axes are both affected. Since signals from the ZPA are required for anteroposterior patterning as well as distal growth (reviewed by Tickle and Eichele, 1994; Tabin, 1995; Tickle, 1995), the dual effects of anti-retinoids could best be understood if retinoids are needed for the formation of the ZPA. To test this hypothesis, we assayed for polarizing activity in anti-retinoid-treated wing buds. Antagonists were applied from beads implanted into the lateral plate at the rostral and caudal limits of the wing region opposite somites 15 and 19/20 (Fig. 1A). When treated embryos had reached stage 18, tissue surrounding the posterior bead was excised and grafted to stage 20 host wing buds (Fig. 1B). Of a total of 17 wings that received antagonist-exposed grafts, ten exhibited a normal digit pattern, five had an additional cartilage rod (d) within the hand plate, one had an additional digit **2** and one exhibited a **4334** duplication (Fig. 1C). Thus, with the exception of one specimen, anti-retinoid-treated tissue had its polarizing activity greatly reduced. Control ZPA taken from untreated stage 18 embryos resulted in nine out of ten cases in full digit pattern duplications. This functional assay thus demonstrates that anti-retinoids block the establishment of a ZPA.

The absence of ZPA was further substantiated by an analysis of molecular markers that are normally expressed in posterior limb bud mesenchyme. We first examined how anti-retinoid treatment affected the expression of *shh*, a polarizing signal (Riddle et al., 1993; Chiang et al., 1996). We found that, in six out of seven embryos, *shh* mRNA was not detectable in the treated wing bud while expression was unaffected on the contralateral side (Fig. 1D, arrowhead). The other marker analyzed

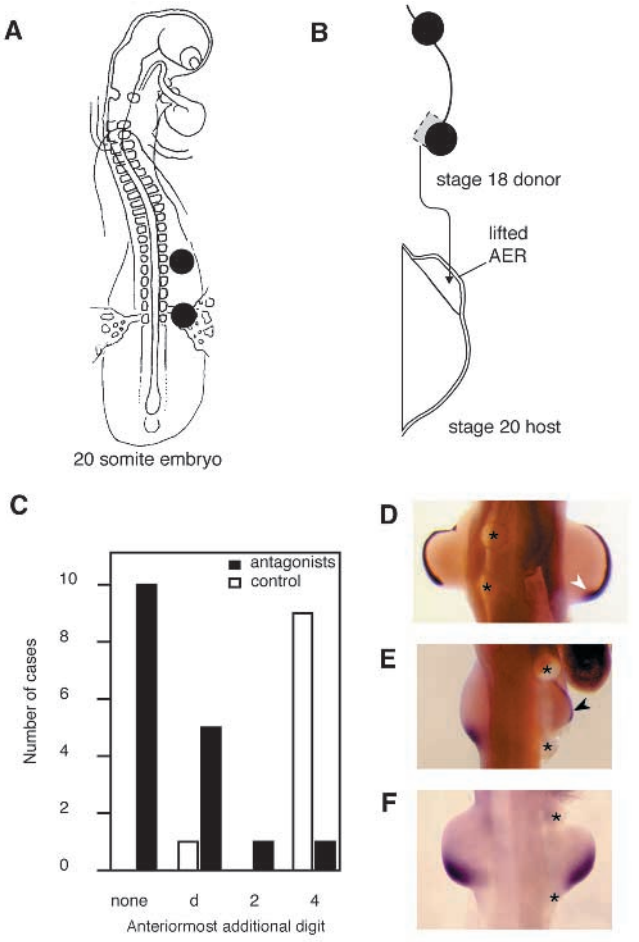


**Fig. 1.** Retinoid receptor antagonists prevent the establishment of a ZPA and the expression of *shh*, and *bmp-2*, but not of *fgf-8* and *Hoxd-13*. (A) Beads presoaked in antagonists LG629 and LG754 were implanted into the lateral plate at the level of somites 15 and 20. (B) Subsequently, posterior mesenchyme together with the overlying ectoderm was grafted to stage 20 host wing buds. (C) Histogram comparing polarizing activity in grafts taken from antagonist-treated wing buds or from control wing buds. Frequently, full digit pattern duplications resulted from such control grafts, while treated tissue most frequently yielded either normal patterns or *d234* patterns, *d* is a cartilage rod. (D) Ventral view of an embryo simultaneously hybridized with *shh* and *fgf-8* riboprobes. To better visualize *fgf-8* and *shh*, the embryo was flipped over so that the treated bud is on the left side of the figure. Note the absence of expression of *shh* but not of *fgf-8*. The untreated bud shows the characteristic small patch of *shh* expression (white arrowhead) adjacent to the *fgf-8*-positive AER. (D-F) Antagonist-releasing beads are indicated by an asterisk. (E) Dorsal view of an embryo hybridized with a *bmp-2* riboprobe. The treated bud is on the right. Anti-retinoids prevent the expression of *bmp-2* in the posterior mesenchyme of the treated wing bud, but *bmp-2* expression in the AER is not affected (black arrowhead). (F). Dorsal view of an embryo hybridized with a *Hoxd-13* riboprobe. The treated bud is on the right. Anti-retinoids do not visibly affect the expression of *Hoxd-13*. Note, wing buds developing from retinoid receptor antagonist-treated wing primordia were narrower than control buds (D-F). Plain beads, which had no effect on the pattern (Helms et al., 1996), also caused such a shape change, presumably by confining the bud between the two beads. Another cause for buds being smaller is the loss of the ZPA as a result of anti-retinoid treatment. Since an ectopic ZPA widens the limb bud (Cooke and Summerbell, 1980), the absence of a ZPA would result in narrower buds.

was *bmp-2*, which is normally expressed in the posterior mesenchyme of limb buds in and around the *shh* expression domain and throughout the AER (Francis et al., 1994). Since ZPA grafts (Francis et al., 1994), ectopic expression of *shh* (Laufer et al., 1994) or RA application (Francis et al., 1994; Helms et al., 1994) activate *bmp-2*, this gene is considered to be a reporter for polarizing signals (Francis et al., 1994; Tabin, 1995). We found that retinoid receptor antagonists applied at stage 13/14 prevented the expression of *bmp-2* in the posterior mesenchyme ($n$=7; Fig. 1E), but had no effect on the expression of *bmp-2* in the AER. The absence of a ZPA and the loss of *shh* and *bmp-2* expression could be accounted for if anti-retinoids abolished posterior mesenchymal cells. This is not the case, since *Hoxd-13*, normally expressed in the posteriormost wing bud mesenchyme (Fig. 1F; see Izpisúa-Belmonte et al., 1991; Nohno et al., 1991) was still expressed in antagonist-treated buds ($n$=13, Fig. 1F). Anti-retinoid-treated embryos were also stained with Nile blue vital dye to inspect for cell death. There was no evidence for an abnormal pattern of cell death in treated embryos at any stage examined (stage 16-22). Taken together, our gene marker studies show that *shh*, a polarizing signal, as well as *bmp-2* are not expressed in retinoid receptor antagonist-treated wing primordia. By contrast, expression of *Hoxd-13* as well as *Hoxd-11* (data not shown) was not significantly affected, arguing against a loss of posteriormost mesenchymal cells.

When wing buds first appear (stage 16/17), the morphology of anti-retinoid-treated and control buds do not differ. The absence of morphologic differences does not preclude that antagonists already by this early stage compromise outgrowth. To investigate this possibility, we examined the expression of *fgf-8*, which is considered to be the prime candidate for initiating limb bud outgrowth (see Introduction). We found that, at any stage examined (stage 16-22), *fgf-8* expression was not visibly affected by retinoid receptor antagonists ($n$=10); however, in the same specimens, expression of *shh* was not detectable (Fig. 1D). Taken together, the absence of change in early limb bud morphology and in the expression of *fgf-8* suggest that anti-retinoids do not directly interfere in any obvious way with limb bud outgrowth. Instead, anti-retinoids effectively block the establishment of the ZPA as revealed by grafting experiments and the loss of expression of *shh* and *bmp-2*. The absence of the ZPA then precludes the establishment of a feed back loop between AER and the mesenchyme (reviewed e.g. by Tabin, 1995) and distal growth is markedly reduced, eventually resulting in an absence of distal skeletal elements.

### *Hoxb-8* is a molecular link between retinoid signaling and polarizing activity

Our experiments show that retinoid signal transduction is required for the establishment of a ZPA and for the expression of *shh* and *bmp-2*. Although *shh* and *bmp-2* expression are inducible in the limb bud by retinoids (Riddle et al., 1993; Francis et al., 1994; Helms et al., 1994), the activation of both these genes requires ≥20 hours of treatment (Helms et al., 1994). This suggests the existence of intermediate factors that

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cHoxb-8 MSSYFVNSLFSKYKTGDSLRPNYYDCGFAQDLGGRPTVVYGPSTGGTFQH
mHoxb-8 ----------------E-------------------------S--S---

cHoxb-8 PTQIQEFYHGASSLSSSPYQQNPCAVACHGDPSNFYGYDPLQRQSLFSAQ
mHoxb-8 -S--------P----TA--------------G-------------G--

cHoxb-8 ESDLVQYTDCKL.AASGLGEEAESSEQSPSPTQLFPWMRPQ.AAGRRRGR
mHoxb-8 DP-----A----A----------G-----------------A---_____

cHoxb-8 QTYSRYQTLELEKEFLFNPYLTRKRRIEVSHALGLTERQVKIWFQNRRMK
mHoxb-8 ________________________________________________

cHoxb-8 WKKENNKDKFPSSKCEQEELEKQKMERAQEVDEEGEAQKADKK
mHoxb-8 ____----------------- E-L---P-TA-Q-D---G---
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**Fig. 2.** The sequences of the homeodomain (underlined) of chicken and mouse Hoxb-8 are identical and there is 91% identity between the two N-terminal domains. Other paralogues are much more diverged (see Results).

link retinoid signaling with the activation of *shh* and *bmp-2* genes. It has long been proposed that *Hox* genes provide positional information along the anteroposterior body axis (reviewed in McGinnis and Krumlauf, 1992) and *Hox* genes may determine the position of limbs (Tickle and Eichele, 1994; Tabin, 1995; Gérard et al., 1996). Initial studies have shown that *Hoxb-8* is activated in the wing bud by RA within 6 hours (Lu et al., 1997). Moreover, it was shown that the ectopic expression of *Hoxb-8* at the anterior margin of the mouse forelimb bud results in the formation of an ectopic polarizing region (Charité et al., 1994). This finding, and the retinoid responsiveness of *Hoxb-8*, make the gene product one of the candidates for transducing the retinoid signal. In what follows, we provide evidence for this idea.

### RA rapidly induces *Hoxb-8* expression in vivo and in vitro

A *Hoxb-8* cDNA encompassing the entire protein coding region was isolated as described in Material and Methods. Sequence comparison with the mouse *Hoxb-8* gene confirmed that the isolated chick cDNA indeed encoded *Hoxb-8* (Fig. 2). The homeodomains of mouse Hoxb-8 and our chicken protein are identical, and the N-terminal domains exhibit 91% identity. By contrast, the identity between our sequence and the N-terminal domains of mouse paralogues Hoxc-8 and Hoxd-8 is only 55% and 42%. Therefore, we conclude that the isolated chicken cDNA encodes *Hoxb-8*.

The anterior wing bud margin of a stage 20 embryo is a convenient model system for testing the inducibility of limb patterning genes by polarizing agents. We used this feature to assess the time course of inducibility of *Hoxb-8* by RA. We found that RA released from an implanted bead, rapidly and transiently induced the expression of *Hoxb-8* in the mesenchyme surrounding the implant (Fig. 3A-C). The loss of *Hoxb-8* expression is not due to degradation of RA; earlier studies have shown that, in the presence of a bead continuously releasing RA, this compound persists in the wing bud for at least 20 hours (Eichele et al., 1985). We note that *Hoxb-8* transcripts are found throughout the mesenchyme, bordering the RA source and at a distance from the AER (Fig. 3A,B, the AER straddles the bead). This is in contrast with the RA-induced ectopic expression domain of *shh*, *bmp-2* and *Hoxd* genes, which is limited to tissue distal to the bead and adjacent to the AER (Helms et al., 1994, 1996). This suggests that unlike *shh*, *bmp-2* and *Hoxd*, *Hoxb-8* expression is independent of signals from the AER. ZPA grafts present for 6 hours (Fig. 3E, arrowhead), or even 12, 18 or 24 hours (data not shown) do not induce *Hoxb-8*, suggesting that this gene is not responding to, but is upstream of, the polarizing signal. Intriguingly, the induction of *Hoxb-8* by RA is site-specific since anteriorly but not posteriorly implanted beads induce this gene (Fig. 3B). This lack of *Hoxb-8* induction is not due to a inability of posterior tissue to respond to RA, since *RARβ* is rapidly induced in posterior cells by exogenously applied RA (data not shown). More likely, posterior cells, as a result of having become a ZPA, are prevented from activating a gene potentially involved in the induction of a ZPA.

The induction of *Hoxb-8* is as rapid as that of *RARβ*, a direct retinoid target gene (compare Fig. 3A and D; de Thé et al., 1990; Sucov et al., 1990). It is thus plausible that like *RARβ*, *Hoxb-8* is a direct response gene. To investigate this possibility, we dissociated stage 20 wing buds into single cells and exposed these primary cultures to 1 μM RA for 6 hours in the presence of cycloheximide. Optimal concentrations of cycloheximide not affecting cell viability were empirically determined as 30 μM to 50 μM. Fig. 4 shows that, in the absence of RA, *RARβ* mRNA levels are virtually undetectable (lane 1).

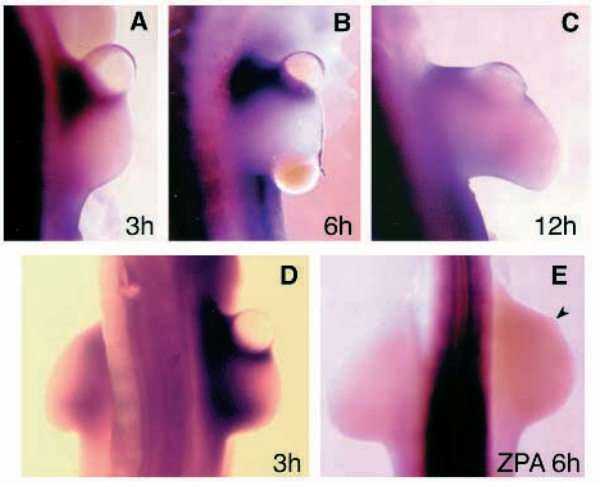


**Fig. 3.** *Hoxb-8* and *RARβ* are both rapidly induced by RA at the anterior margin of a chick wing bud. (A-C) The ectopic expression of *Hoxb-8* can be detected after 3 hours (A) and 6 hours (B) of RA treatment. Ectopic expression was dramatically decreased at 12 hours (C) of RA treatment. Ectopic RA released from the posterior wing bud margin (B) fails to induce *Hoxb-8* expression. (D) The induction of *RARβ* expression by RA after 3 hours of treatment. (E) ZPA graft (arrowhead) can not induce ectopic expression of *Hoxb-8* in the anterior wing bud margin, the specimen shown was analyzed 6 hours after the graft was implanted.

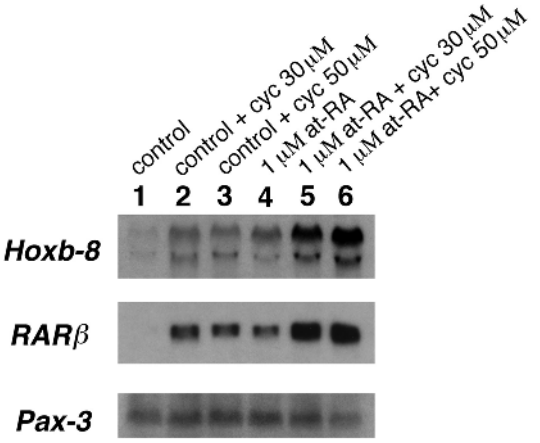


**Fig. 4.** Northern analysis shows induction of *Hoxb-8* and *RARβ* by RA in primary limb bud culture. Such induction occurs in the presence of cycloheximide, a protein synthesis inhibitor. (Lane 1) Medium only; lane 2, medium with 30 μM cycloheximide; lane 3, medium with 50 μM cycloheximide; lane 4, medium with 1 μM RA; lane 5, medium with 1 μM RA and 30 μM cycloheximide; lane 6, medium with 1 μM RA and 50 μM cycloheximide. *Pax-3* expression is used as a standard for comparing RNA loading.

Adding cycloheximide augmented mRNA levels presumably as result of increasing the stability of mRNA (Lund et al., 1991). RA by itself induced *RARβ*, but combination treatment with RA and cycloheximide strongly induced *RARβ* expression. *Hoxb-8* induction exhibited a very similar behavior (Fig. 4). Untreated cells showed low levels of transcript, cycloheximide and RA-augmented expression. Combination treatments strongly induced *Hoxb-8* expression. Taken together, these in vitro and in vivo studies demonstrate that the induction of *Hoxb-8* by RA is rapid and does not require protein synthesis, a behavior also typical for direct target genes such as *RARβ*.

## The expression domain of *Hoxb-8* correlates with the domain of polarizing activity in lateral plate

So far, we have shown that ectopic RA rapidly induced *Hoxb-8* in the anterior mesenchyme of stage 20 wing buds, some of which will subsequently express *shh* andbecome a ZPA (Noji et al., 1991; Wanek et al., 1991; Helms et al., 1994). This raises the possibility that *Hoxb-8* transduces the retinoid signal that is required for the formation of a ZPA during normal development. The pattern of expression of *Hoxb-8* in the lateral plate supports this idea.

The expression profile of *Hoxb-8* can be divided into five phases (Figs 5, 6).

### Phase I (stage 5-9⁻)

*Hoxb-8* mRNA appears by stages 5 and 6 in the posterior third of the primitive streak (Fig. 5A) in a region, part of which gives rise to the lateral plate (Psychoyos and Stern, 1996). After the formation of the first few somites (Fig. 5B), *Hoxb-8* transcripts appear in the neuroectoderm in which a distinct expression boundary at the border between somites 6 and 7 is established (arrow in Fig. 5B). This boundary is maintained throughout development (Fig. 5 and data not shown). There is a high level of *Hoxb-8* expression in the region surrounding Hensen's node,

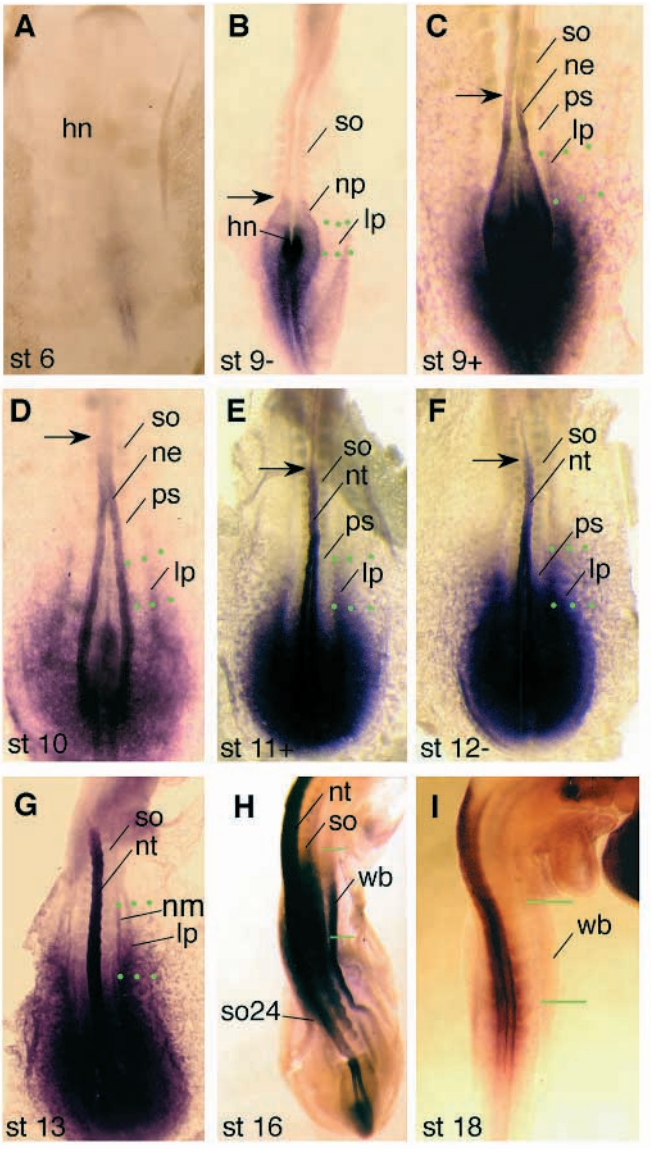


**Fig. 5.** *Hoxb-8* expression pattern during embryogenesis, stages are indicated. Green dots delineate the anterior and posterior boundaries of the wing region as elucidated by Chaube (1959). Green lines demarcate the anterior and posterior boundaries of the wing bud. Arrows point to the anterior expression boundary of *Hoxb-8* in the neural tube. For details see Text. Abbreviations: hn, Hensen's node; lp, lateral plate; ne, neural ectoderm; nm, nephrogenic mesoderm; np, neural plate; nt, neural tube; ps, presomitic mesoderm; so, somite; wb, wing bud.

a previously identified source of retinoic acid (Chen et al., 1992; Hogan et al., 1992). By early stage 9, very low levels of expression are also seen in the lateral plate (Fig. 5B).

### Phase II (stage 9-12)

This phase is characterized by a significant up-regulation of *Hoxb-8* expression in the lateral plate (Figs 5C-F, 6A). Initially (Fig. 5C,D) expression in this tissue extends slightly anterior of the rostral limit of the wing field (the boundaries of this field are indicated by green dots; see Chaube, 1959). Subsequently, expression in the lateral plate slightly retracts and the anterior

boundary of expression now coincides with the anterior border of the wing region (Fig. 5E,F). The expression of *Hoxb-8* in the lateral plate is graded with the gradient diminishing in a posterior-to-anterior direction (e.g. Fig. 5E). During phase II, the anterior expression boundary of *Hoxb-8* in the presomitic mesoderm shifts rostrally (Fig. 5E,F). Transverse sections (not shown) illustrate the presence of *Hoxb-8* mRNA throughout the mesoderm of the somatopleure and splanchnopleure.

### Phase III (stages 13-15)

The anterior boundary of *Hoxb-8* expression in the lateral plate mesoderm retracts towards the level of somite 17 (center of wing field, Figs 5G, 6B) and the expression in posterior mesoderm around the tail bud begins to decrease (data not shown).

### Phase IV (stage 16, 17)

A wing bud appears (Fig. 5H, between green lines) and, in the lateral plate, marked anterior and posterior *Hoxb-8* expression boundaries are established at the level of somites 17 and 24, respectively (Fig. 6C). *Hoxb-8* is also expressed in somitic mesoderm from somite 16 on, back to somite 25/26.

### Phase V (stage 18 and later)

This phase is characterized by rapid disappearance of *Hoxb-8* expression in the lateral plate and in wing bud mesoderm (Fig. 5I), a reduction of expression in the somites, and the definition of a posterior boundary of *Hoxb-8* expression in the neural tube at the level of somite 24. Temporally, the down-regulation of *Hoxb-8* expression in the nascent wing bud and the lateral plate posterior to it coincides with the initial activation of the *shh* gene (Riddle et al., 1993). *Hoxb-8* mRNA is just disappearing at the time when *shh* transcripts are detectable by in situ hybridization. The dynamic expression of *Hoxb-8* relative to anatomical markers of the wing field and to the maps of polarizing activity is summarized in Fig. 6.

The expression pattern of *Hoxb-8* in the lateral plate is remarkable for two reasons. First, the anterior boundary of *Hoxb-8* expression is located within the wing region (red box in Fig. 6) at a time when this region is being defined. Transplantation studies (Chaube, 1959) show that the anteroposterior axis of the wing region is irreversibly specified as early as stages 8 to 11, a period when *Hoxb-8* expression undergoes retraction and an anterior boundary within the wing region is established (Figs 5C-F, 6A,B). These observations suggest a correlation between the establishment of an anterior expression boundary of *Hoxb-8* and the determination of the anteroposterior axis within the wing field. Second, Hornbruch and Wolpert (1991) have mapped the appearance of polarizing activity in the lateral plate mesoderm of the chick. These studies were based on grafting blocks of lateral plate tissue to the anterior wing bud margin of a host embryo, followed by analysis of digit pattern duplications. While very weak polarizing activity was demonstrated in the lateral plate mesoderm as early as stage 81. Such activity begins to augment between stages 11 and 13 and eventually encompasses the entire wing field. Subsequently polarizing activity begins to recede to the posterior third of the wing bud (between phases III, IV, Fig. 6B, C). During phases III and IV, this dynamic distribution of polarizing activity closely reflects the spatio-temporal expression pattern of *Hoxb-8* (Fig. 6B,C, compare yellow and purple areas in the lateral plate). We conclude that the anterior boundary of *Hoxb-8* expression in the lateral plate prefigures the establishment of a wing field in much the same way as e.g. the anterior boundary of *Hoxa-1* and *b-1* expression anticipates the cranial boundary of rhombomere 4 in the hindbrain (Sundin and Eichele, 1990; Murphy and Hill, 1991). What is more, the expression domain of *Hoxb-8* coincides, at least during phases III and IV, and within the accuracy of the available maps, with the domain of polarizing activity in the wing region.

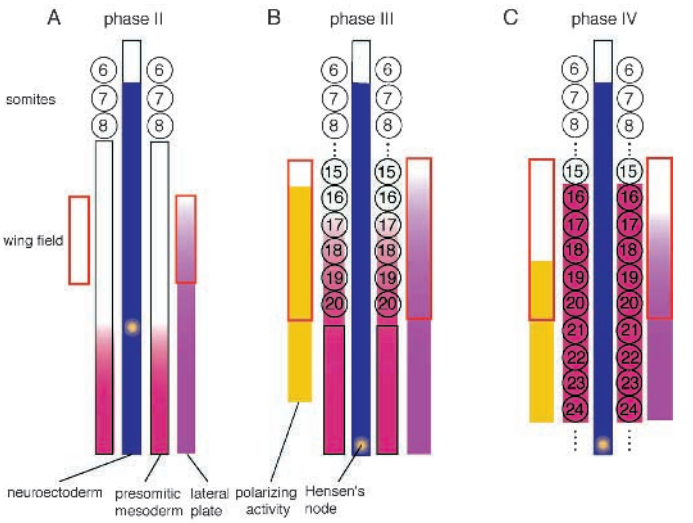


**Fig. 6.** Diagram illustrating the relationship between the *Hoxb-8* expression domain in lateral plate mesoderm (purple), the location of the wing field (red box) and the domain of polarizing activity (yellow). The expression domains of *Hoxb-8* in presomitic and somitic mesoderm (magenta) as well as in neural tube (violet) are illustrated. (A) Stage 9, beginning of phase II; (B) stage 13, beginning of phase III; (C) stage 16, beginning of phase IV. For details see Text. Number in circles represents the somite number.

### Retinoid receptor antagonists down-regulate *Hoxb-8* in lateral plate mesoderm

We have shown that blocking retinoid signal transduction prevents the formation of a ZPA. Because polarizing activity and *Hoxb-8* expression are associated (Figs 5 and 6 and Charité et al., 1994), we predict that retinoid receptor antagonists should not only abolish the ZPA but also the expression of *Hoxb-8* in the wing region. Anti-retinoids were locally applied to the prospective wing region (Fig. 1A) and, after 8-10 hours, embryos were analyzed for *Hoxb-8* mRNA. Treated embryos either lacked *Hoxb-8* transcripts ($n$=10) or showed a marked reduction ($n$=8) of expression in the treated wing primordium but not on the contralateral side (arrowhead in Fig. 7). Beads soaked in DMSO, the solvent for antagonists, had no effect on *Hoxb-8* expression (data not shown). Thus, blocking of the retinoid signaling pathway results either in a significant down-regulation or a loss of endogenous *Hoxb-8* expression paralleling the loss of polarizing activity of this tissue.

## DISCUSSION

Retinoid are required for normal limb development since an


**Fig. 7.** Retinoid receptor antagonists down-regulate *Hoxb-8* in lateral plate mesoderm. *Hoxb-8* expression in the lateral plate mesoderm of contralateral side is not affected by antagonists treatment (arrowhead). so 17, somite 17.

interruption of the retinoid signaling pathway with retinoid receptor antagonists prevents the formation of a normal limb pattern (Helms et al., 1996). Similarly, blocking RA synthesis in the prelimb bud stage using the dehydrogenase inhibitor disulfiram, blocks limb formation (Stratford et al., 1996). Effects of retinoid receptor antagonists are particularly striking when antagonists are provided to the wing region prior to bud formation, suggesting that retinoids operate early during limb development. This prompted us to search for factors that transduce the retinoid signal at this developmental stage. We suggest that Hoxb-8, a gene product previously associated with polarizing activity (Charité et al., 1994), is such a molecule. We show that *Hoxb-8* is rapidly induced by RA in the absence of protein synthesis, and that retinoid receptor antagonists abolish the expression of *Hoxb-8* in the lateral plate. Retinoid receptor antagonist treatment of the presumptive wing bud did not abolish wing bud initiation, and the expression of *fgf-8*, a mediator of limb bud initiation (Crossley et al., 1996; Vogel et al., 1996), was not affected. However, such buds lacked a functional ZPA and did not express *sonic hedgehog* and *bmp-2*, two signaling molecules implicated in limb patterning (Riddle et al., 1993; Duprez et al., 1996).

### *Hoxb-8* links retinoid signaling with polarizing activity and the establishment of the ZPA

This study provides evidence that *Hoxb-8* is directly regulated by retinoids. Retinoid receptor antagonists block *Hoxb-8* expression in the lateral plate mesoderm (Fig. 7). Additionally, exogenous RA rapidly induces *Hoxb-8* in wing bud mesenchyme (Fig. 3), and cultured limb bud cells turn on this gene in response to RA treatment in the presence of a protein synthesis inhibitor (Fig. 4). Although our studies have not attempted to identify a retinoid responsive element in *Hoxb-8*, the transcriptional regulation of several *Hox* genes by RA is well documented (see Hofmann and Eichele, 1993 for a review). Retinoid response elements have been identified e.g. in *Hoxa-1*, *Hoxb-1*, *Hoxd-4* (for review see Krumlauf, 1994) and *Hoxd-10/11* (Gérard et al., 1996). Endogenous RA is produced at the right time and location in the embryo to activate *Hoxb-8* in the lateral plate. We have previously shown that lateral plate tissue synthesizes RA at a high rate (Helms et al., 1996). Furthermore, transgenic reporters under control of a retinoid responsive element reveal the presence of RA in the lateral plate (Reynolds et al., 1991; Rossant et al., 1991; Balkan et al., 1992).

Ectopic expression of *Hoxb-8* in the mouse forelimb induces an ectopic ZPA. Based on this, it is proposed that *Hoxb-8* has a role in the establishment of the ZPA in the lateral plate (Charité et al., 1994). Our studies in the chick now show that polarizing activity is for the most part co-extensive with *Hoxb-8* expression. During stages 11 and 12, weak polarizing activity appears in tissue extending lateral of somite 14/15 to prospective somite 23/24 (Hornbruch and Wolpert, 1991). *Hoxb-8* is expressed in this region (Fig. 5D-F). After stage 13, polarizing activity in the lateral plate substantially increases (Hornbruch and Wolpert, 1991). Fig. 6B and C show that, during phases III and IV (stage 13-17), the rostral and caudal limits of polarizing activity and the expression of *Hoxb-8* essentially coincide within the resolution of the polarizing activity mapping data. Charité et al. (1994) have suggested that *Hoxb-8* expression is correlated with polarizing activity. Since there are no maps of polarizing activity for the mouse, this remained a conjecture that the present study affirms.

This association of polarizing activity with *Hoxb-8* expression, on the one hand, and the dependence of *Hoxb-8* transcription on retinoid signaling, on the other hand, suggests that *Hoxb-8* is a link between RA signaling and the establishment of polarizing activity. It is possible that *Hoxb-8* is not the only RA-regulated *Hox* gene that has this capacity. For example, *Hoxb-6* and *Hoxc-6* are also induced by RA (Oliver et al., 1990; Lu et al., 1997) and are expressed in the lateral plate in a pattern similar to that of *Hoxb-8* (DeRobertis, 1994; H.-C. Lu and G. Eichele unpublished data). It is well known that *Hox* genes can function in a combinatorial manner (Horan et al., 1995; Rancourt et al., 1995; Davis and Capecchi, 1996). Therefore, *Hoxb-8* may be only one of several *Hox* genes capable of specifying polarizing activity. This would imply that a loss of function in *Hoxb-8* alone might not affect limb patterning. In fact, the deletion of *Hoxb-6* gene has no effect on the limb pattern (Rancourt et al., 1995).

What is the relationship between *shh* and *Hoxb-8* expression? It has been proposed that *shh* is expressed in those *Hoxb-8*-positive cells that are in proximity to the AER and thus receive a FGF signal (Charité et al., 1994; Tabin, 1995). While the general idea of AER proximity is correct, expression data suggest a more complex regulation. During stages 16 and 17, *Hoxb-8* expression in the lateral plate is very strong and extends up to somite level 17/18 (Fig. 6C). *shh* transcripts are not found at stage 16 (Crossley et al., 1996) but, by stage 17, a small number of *shh*-positive cells are detected in the posteriormost wing bud mesenchyme, opposite the boundary somites 19/20. By stage 18, about 5 hours later, the population of *shh*-expressing cells has considerably expanded, but now *Hoxb-8* transcripts have disappeared. Apparently, there seems to be only a brief temporal overlap between *Hoxb-8* and *shh* expression and furthermore, only a subset of *Hoxb-8*-positive cells will eventually express *shh*. Thus there are mechanisms limiting *shh* expression to a subset of *Hoxb-8-positive* cells. The expression of *shh* may depend on the simultaneous action of several *Hox* genes (Hox code), or *Hoxb-8* function could require locally expressed co-activators (Pöpperl et al., 1995). Another hypothetical mechanism to limit *shh* expression would be a localized release of a growth factor by the posteriormost AER cells. Recent work from our laboratory suggests the existence of a RA-induced ectodermal factor that is required for the formation of a ZPA (Helms et al., 1996).

### Evolutionary conservation of the *Hoxb-8* expression pattern

The dynamic behavior of *Hoxb-8* expression in the lateral plate of chick and mouse is very similar (this study; Deschamps and Wijgerde, 1993; Charité et al., 1994). In the mouse, forelimbs arise between somites 7 and 11; in the chick, the wings arise between somites 15 and 20 (see Burke et al., 1995 for a discussion of axial position of vertebrate limbs). In a 9-somite mouse embryo, the anterior boundary of *Hoxb-8* expression in lateral plate is at somite level 9 (Charité et al., 1994). In a 13-somite mouse embryo, this boundary has retracted to somites 10/11. In the chick, there is an analogous caudal retraction (Fig. 6). First, the anterior expression boundary resides opposite somite 15/16 then it repositions to opposite of somite 17/18. The anterior shifting of the caudal expression boundary of *Hoxb-8* is also very similar between chick and mouse. Thus, within the resolution of the expression data, the establishment of *Hoxb-8* expression boundaries in the lateral plate is conserved between chick and mouse, despite the fact that the two species have their forelimbs at different axial levels.

Fate maps in the chick define limb-forming regions well before limb buds appear (Chaube, 1959). The present study shows that the anterior boundary of *Hoxb-8* expression in the lateral plate resides within the prospective wing region from the time such a region can be delineated (Fig. 5, green dots; Fig. 6, red box). Additional evidence for a role of *Hox* genes in limb positioning comes from comparisons of *Hox* gene expression patterns in different species (see above and e.g. Burke et al., 1995). Furthermore, mutations in *Hoxb-5*, *d-10* and *d-11* genes can shift limb position (Rancourt et al., 1995; Gérard et al., 1996). *Hoxd-10* and 11 are regulated by retinoids (Gérard et al., 1996) and *Hoxb-5* is inducible by RA in the limb bud (G.E. unpublished data). Of note, local treatment of the wing-forming region with disulfiram, a RA synthesis blocker, can result in a posterior displacement of the wing (Stratford et al., 1996). We suggest that absence of RA in the wing region prevents the expression of *Hox* genes in the wing field, but since the blocking agent acts locally, the expression of these *Hox* genes are not affected more posteriorly, and thus the wing arises at a more posterior site. The application of retinoid receptor antagonists to very early chick embryos may further clarify this interpretation. Taken together, it is possible that retinoids are not only involved in the establishment of polarizing activity but are also mediate, through genes such as *Hoxb-8*, the specification of limb position.

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