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Expression of *Hox-4* genes in the chick wing links pattern formation to the epithelial – mesenchymal interactions that mediate growth

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The relationship between the expression of *Hox-4* genes in the mesenchyme and the apical ectodermal ridge was investigated in both normal chick wing buds and wing buds treated with retinoic acid. Two conclusions emerge. One is that the activation of *Hox-4* domains and the elaboration of *Hox-4* gene expression patterns involve co-operation with a signal from the apical ridge. The second is that the domains of expression of 5′-located members of the complex correlate with the maintenance of the thickened ridge which is required for subsequent bud outgrowth.

Key words: apical ridge/epithelial – mesenchymal interaction/*Hox-4* genes/limb development/retinoic acid

Introduction

The products of genes of the HOX-4 complex are good candidates for encoding position across the antero-posterior axis of developing limbs. Cells at different positions across the antero-posterior axis of the limb bud express different combinations of *Hox-4* genes with genes in more 5′ positions in the cluster being expressed in progressively more posterior regions of the bud (Dollé *et al.*, 1989; Izpisúa-Belmonte *et al.*, 1991). Members of the HOX-4 gene complex are switched on in sequence according to their position in the cluster in a 3′ to 5′ direction as the bud forms. As the bud grows out, the pattern of overlapping domains is extended along the proximo-distal axis except for the domain of the most 5′ gene, *Hox-4.8*, which remains at the tip of the bud. In addition, expression becomes more intense as development proceeds so that, distally, expression is greater than proximally (Dollé *et al.*, 1989).

A signal from the polarizing region, a small group of mesenchyme cells at the posterior margin of the bud, appears to control patterning across the antero-posterior axis of the limb (Saunders and Gasseling, 1968; Tickle *et al.*, 1975). There is good evidence to suggest that retinoic acid is part of this signalling system (Thaller and Eichele, 1987; reviewed in Tickle and Brickell, 1991). When anterior cells are respecified to form posterior structures by either applying retinoic acid or grafting a polarizing region, a mirror-image pattern of *Hox-4* gene expression results which reflects the mirror-image duplication of the skeleton (Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991). The new domains are stable and their pattern during further bud outgrowth is similar to that of the normal domains.

A striking feature of the new domains of *Hox-4* expression induced by either retinoic acid or polarizing region grafts is that they are localized in distal mesenchyme at the tip of the wing bud (Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*, 1991). This contrasts with the more widespread activation of two other genes, *Hox-3.3* and RAR-β in anterior mesenchyme following retinoid treatment (Oliver *et al.*, 1990; Noji *et al.*, 1991). The localization of ectopic domains of 5′-located genes of the HOX-4 complex at the tip of the bud suggests that their activation requires an interaction between retinoic acid and/or another signal produced by polarizing region cells and a signal from the apical ectodermal ridge, the thickened epithelium at the tip of the bud. The apical ridge maintains a region of undifferentiated mesenchyme cells at the tip of the bud. This region is known as the progress zone (Summerbell *et al.*, 1973). When the apical ridge is surgically removed from limb buds, outgrowth is halted and truncated limbs develop (Saunders, 1948; Summerbell, 1974). Patterning along the proximo-distal axis is thus linked to bud outgrowth and the length of time cells spend in the progress zone appears to specify position along this axis (Summerbell *et al.*, 1973).

The interactions between the apical ridge and the mesenchyme appear to be reciprocal and cells in the progress zone interact in turn with the epithelium to maintain the thickening of the apical ridge (Zwilling, 1961). In normal limb development, outgrowth of the bud is at first symmetrical, but soon (after stage 20) becomes enhanced posteriorly in the region of the bud where the apical ridge is maintained. It is in this region of the bud that 5′-located members of the HOX-4 complex are expressed during further outgrowth. In buds in which anterior cells are re-specified to form posterior structures, 5′-located *Hox-4* genes are activated in the anterior cells; the apical ridge is now maintained over this part of the bud and the bud becomes broader (Lee and Tickle, 1985; Brickell and Tickle, 1989). These considerations suggest that expression of 'posterior' *Hox-4* genes in the mesenchyme may be part of the mechanism involved in ridge maintenance.

Here we explore the cooperation between the signalling system across the antero-posterior axis and the epithelial – mesenchymal interactions between ridge and progress zone by applying retinoic acid to chick wing buds. To test whether the apical ridge is required for activation of *Hox-4* gene expression, we applied retinoic acid to the anterior margin of wing buds and surgically removed the apical ridge at a series of later times. We also removed the apical ridge from normal buds either at the time when *Hox-4* genes are being activated or later in development. We find that the presence of the apical ridge is required for progressive activation of genes in the HOX-4 complex in both manipulated and normal wing buds. Although activation of these genes is irreversible, further development of their pattern of expression is

dependent on continued signalling by the ridge. To investigate the relationship between *Hox-4* expression and subsequent maintenance of the apical ridge, we applied retinoic acid to different positions around the bud margin. When retinoic acid is applied at the apex or the posterior of the bud, the apical ridge flattens. At the apex, the flattening effect is widespread, and truncated limbs develop whereas with posterior application, the apical ridge persists more anteriorly so that outgrowth is shifted resulting in small wings with a normal digit pattern (Tickle *et al.*, 1985; Lee and Tickle, 1985). We find that expression of 5′-located members of the HOX-4 complex in these limbs is correlated with the maintenance of the apical ridge, thus suggesting a further link between patterning and growth of the developing limb.

Results

The effect of removing the apical ridge on Hox-4 gene activation by retinoic acid

When beads soaked in 0.1 mg/ml retinoic acid are implanted at the anterior margin of stage 18 chick wing buds, 5′ members of the HOX-4 complex are activated in discrete mesenchymal domains distal to the bead beneath the apical ridge (Izpisúa-Belmonte *et al.*, 1991). The genes are activated in sequence in a 3′ to 5′ direction just as they are in normal limb development, with ectopic expression of *Hox-4.6* appearing 20 h after retinoic acid application and *Hox-4.8* at 24 h (Table I).

To find out whether the apical ridge is required for induction of ectopic domains of *Hox-4* genes, beads soaked in retinoic acid were implanted at the anterior margin of wing buds, the apical ridge was removed either 2 h or 8 h later and the expression pattern of 5′-located *Hox-4* genes was examined at 30 h. Removal of the ridge will prevent additional digits forming because outgrowth of the bud will be inhibited and distal structures do not develop. Nevertheless it is possible that respecification of cells by retinoic acid could occur in the absence of the apical ridge and this should lead to activation of *Hox-4* genes even though subsequent development of the additional digits cannot proceed. Removal of an extensive length of ridge including that over the anterior margin of the bud at 2 h prevents the formation of additional digits (Table I). In such wing buds, no ectopic domains of *Hox-4.7* and *Hox-4.8* are induced (Figure 1). In addition, expression of *Hox-4.8* in the normal posterior domain does not increase in intensity whereas expression has become stronger in the contralateral wing bud. Similar results are also obtained when extensive regions of the ridge are removed at 8 h. No additional digits develop in the operated wings and there is no activation of 5′-located *Hox-4* genes (Table I; Figure 2). In the presence of the ridge, *Hox-4.6* is activated by retinoic acid at 20 h whereas the most 5′-located gene in the cluster, *Hox-4.8* is not activated until 24 h (Izpisúa-Belmonte *et al.*, 1991). Therefore to find out whether the apical ridge is required during the progressive activation of successive genes in the cluster, beads soaked in retinoic acid were implanted and the anterior part of the ridge was removed at either 20 h or 24 h. These ridge removals lead to wings with no additional digits (Table I). When the anterior ridge is removed at 20 h, there has been no activation of either *Hox-4.6* or *Hox-4.8* even when the buds are examined at 38 h. In contrast, when the anterior ridge is removed at 24 h, then, at 30 h, an ectopic domain of *Hox-4.7* has been activated but there is no detectable activation of *Hox-4.8*. Therefore the apical ridge is required for the progressive activation of 5′-located genes in the HOX-4 cluster (Table I). In early wing buds, extensive regions of the apical ridge must be removed in order to prevent additional digits forming. When just the anterior part of the ridge is removed at either 2 h or 8 h, additional digits can develop but these appear to be disconnected from the hand plate (Figure 2). In such wing buds, new ectopic domains of 5′-located genes are established by 30 h and correlate with the subsequent development of 'posterior' digits. When just the posterior part of the ridge is removed at 2 h, an inverted pattern of the three wing digits develops from respecified anterior cells (Figure 2). This pattern is similar to that of wings that develop following retinoic acid application and removal of the posterior half of the bud (Eichele, 1989). At 30 h, ectopic domains of expression of 5′-located *Hox-4* genes have been established in the anterior part of the bud whereas expression in the normal domains in the posterior has not increased in intensity and now expression appears weaker than in the contralateral wing bud (Figure 2).

Hox-4 gene expression following removal of the apical ridge at different stages of development of wing buds

The results obtained above suggest a role for the apical ridge in establishing and maintaining *Hox-4* expression. To

Table I.

Time at which apical ridge was removed after retinoid application	*Hox-4* genes activated in anterior cells at 30 h			Digit pattern at 6 days
	Hox-4.6	*Hox-4.7*	*Hox-4.8*	
2 h	nd	–	–	4, 4 (1[b]) truncations (3)
8 h	nd	–	–	3, truncation (1) truncation (2)
20 h	–[a]	nd	–	34 (1) 4 (5)
24 h	+	+	–	34 (1) 2234 (1)

nd, not determined.
[a]In this case the pattern of *Hox-4* gene expression in anterior cells was monitored at 38 h.
[b]Number in brackets = number of cases; a comma indicates an extensive gap.

examine this in normal bud development, the entire apical ridge was removed from wing buds at stage 17/18 and at stage 20. At stage 17/18, the progressive activation of successive members of the complex is taking place whereas by stage 20, the posteriorly centered pattern of *Hox-4* gene expression has been established in the well formed bud (Izpisúa-Belmonte *et al.*, 1991). Removal of the ridge at both stages leads to a marked reduction in outgrowth within 24 h and ultimately truncated wings develop that lack distal structures. Removal of the ridge at stage 17/18 gave three wings truncated at the humerus and three wings with only posterior structures (ulna and digit 4) whereas removal of the ridge at stage 20 gave three wings truncated at the wrist, one wing truncated at the elbow and one wing with reduced digits.

26 h after removal of the apical ridge at stage 17/18, a small *Hox-4.7* domain could be detected. There was no expression of *Hox-4.8* in the operated wing bud although this is clearly present in the left wing bud that serves as a control. In another embryo examined at 44 h, there is no expression of either *Hox-4.7* or *Hox-4.8* in the thickened flank which would have developed into the wing bud (Figure 3).

In wing buds in which the ridge was removed at stage 20, the expression of *Hox-4.7* and *Hox-4.8*, 24–30 h later, appears less intense than in the contralateral wing buds and the expansion of the domains has been clearly inhibited. This is most readily appreciated by inspection of the domain for *Hox-4.8* (Figure 3). In the normal bud, on the right, there is strong expression of *Hox-4.8* distally and a proximal region which does not express this gene. In contrast, in the operated wing bud, the domain of expression of *Hox-4.8* has not increased in intensity and the proximal region in which cells do not express *Hox-4.8* is absent. It is as though the pattern of expression of *Hox-4* genes is 'frozen' when the apical ridge is removed.

Spatial pattern of Hox-4 activation in relation to the apical ridge

Application of retinoic acid to different positions along the rim of the limb bud has various effects on pattern and these are coupled with alterations in the extent of the apical ridge (Lee and Tickle, 1985) which lead to different forms of bud outgrowth. To examine the relationship between the position of domains of *Hox-4* genes and the maintenance of the apical ridge, we explored the pattern of *Hox-4* expression when

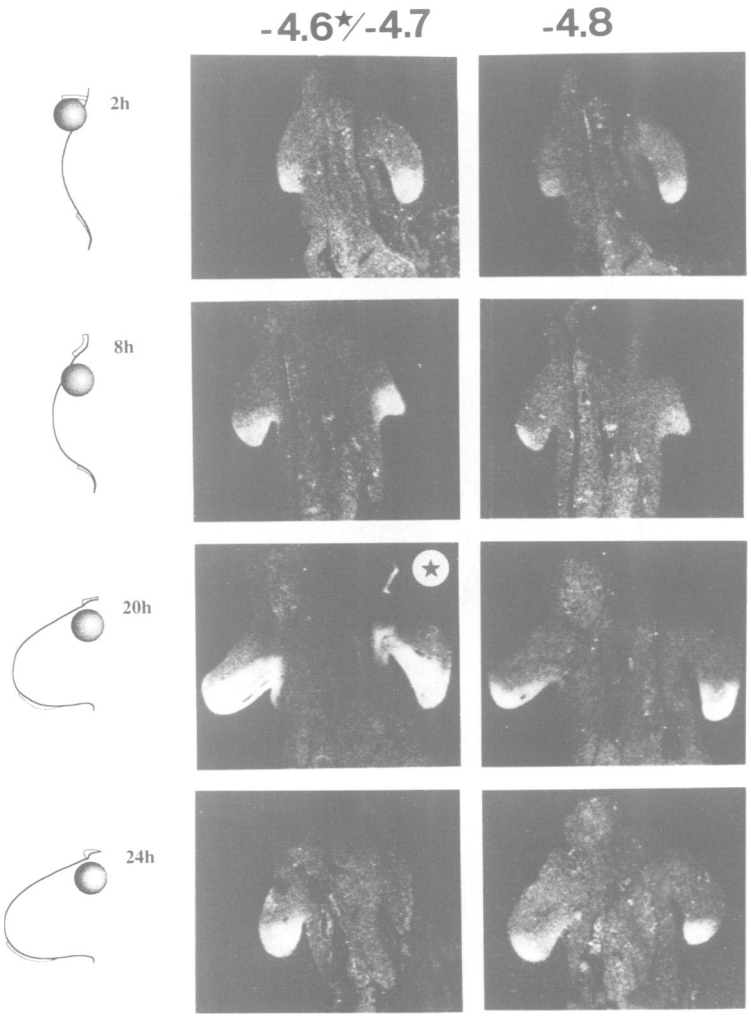

Fig. 1. *Hox-4* gene expression 30 h after implantation of a bead soaked in 0.1 mg/ml retinoic acid at the anterior margin of stage 18 chick wing buds and subsequent removal of the apical ridge at 2, 8 and 24 h later as shown diagrammatically on the left. The chick wing buds in which the ridge was removed 20 h after implantation of the bead were fixed at 38 h. In the panels of dark-field photographs showing *in situ* hybridization, the operated wing bud is on the left in each case, and the transcript distribution is for the particular *Hox-4* gene indicated at the top of each column.

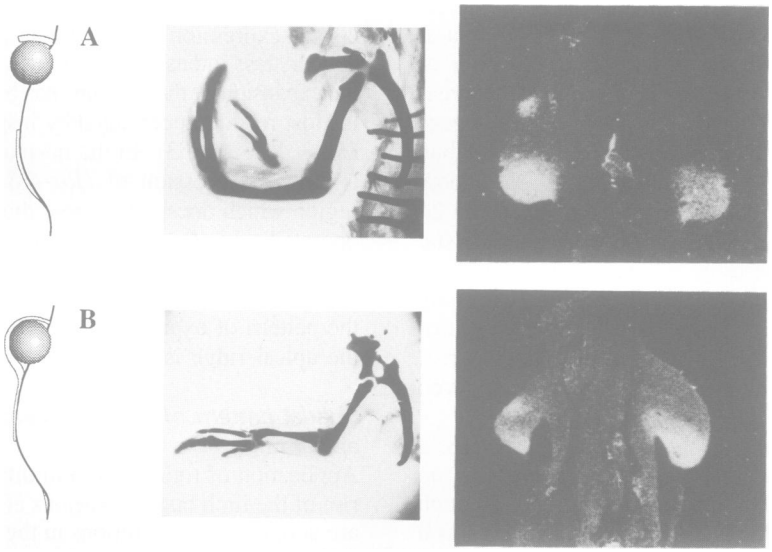

Fig. 2. Effects of removing part of the apical ridge soon after treating wing buds at stage 18 with retinoic acid on limb pattern and *Hox-4* gene expression. A bead soaked in 0.1 mg/ml retinoic acid was implanted at the anterior margin of chick wing buds. 2 h after the bead was implanted, either (**A**) the anterior or (**B**) the posterior part of the apical ectodermal ridge was removed. The pattern of cartilage differentiation that results at 6 days after such operations is shown in whole mounts. In the wing illustrated as a result of (A), the digit pattern is 32,34 and the additional digits are not connected with the normal digits. The wing in (B) has developed a set of digits in reversed polarity and the single forearm element appears to be an ulna. Dark-field photographs show the pattern of transcripts for *Hox-4.7*.in the wing buds at 30 h after bead implantation.

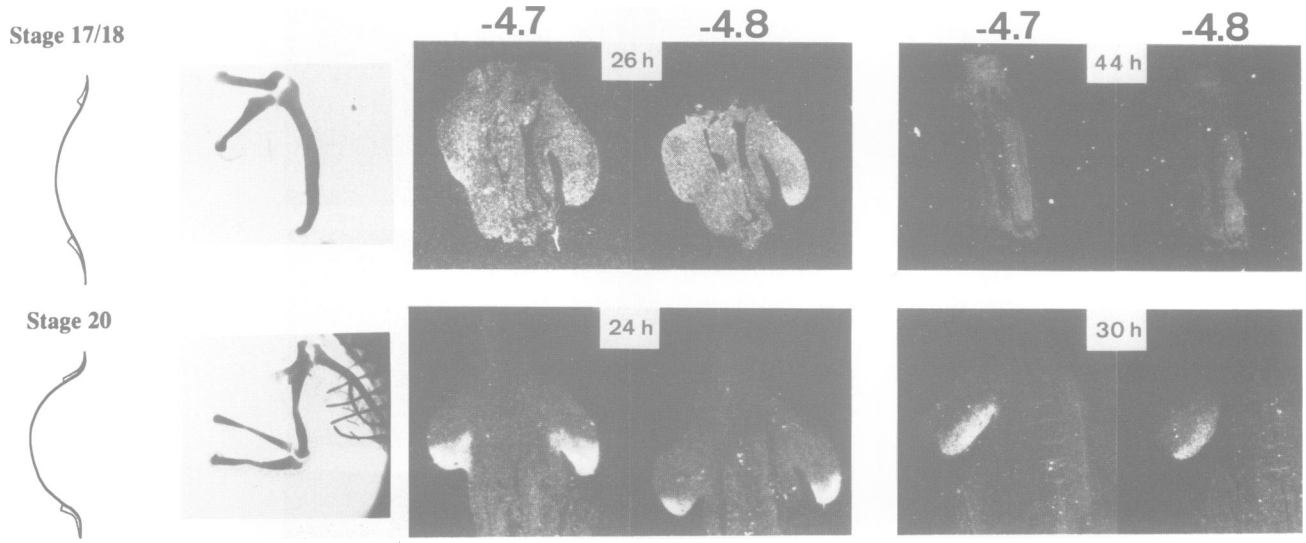

Fig. 3. The effects of removing the apical ridge from stage 17/18 and stage 20 wing buds on limb pattern and *Hox-4* gene expression in the buds. The diagrams on the left show the operation. Whole mount photographs of chick wings show typical truncations; at stage 17/18, only part of the humerus is present, whereas following ridge removal at stage 20, the wing is truncated at the wrist. The dark-field photographs show the pattern of expression of particular *Hox-4* genes soon after the operation as indicated. Note that the operated bud in the first two panels is on the left. In the last two panels, only the operated side of the embryo is shown.

beads soaked in 0.1 mg/ml retinoic acid were placed in different positions around the rim of the wing bud (Figure 4). Application of retinoic acid just anterior to the bud apex results in duplicated patterns of digits, commonly 4334, which lack digits 2 (Figure 4A) and the bud is slightly broader. At 24 h, activation of 5′-located *Hox-4* genes could be detected. Ectopic domains of *Hox-4.7* occur distal to the bead beneath the apical ridge and may extend both anteriorly and posteriorly (Figure 4A). The new domains are continuous with the normal posterior domain so that the entire tip of the bud over which the apical ridge is maintained expresses *Hox-4.7* (Figure 4A). In a wing bud examined at 30 h after retinoid treatment, the ectopic domain of *Hox-4.7* is a smaller discrete patch posterior to the bead but still confined to distal mesenchyme (Figure 4A). When beads soaked in 0.1 − 1 mg/ml retinoic acid are placed at the bud apex, the mostly severely affected wings are truncated and the apical ridge flattens (Figure 4B, see also Tickle *et al.*, 1989). At 24 h, the distribution of transcripts of 5′-located *Hox-4* genes correlates with the maintenance of the ridge. In buds with either the lower concentrations of retinoic acid or where the bead has ended up slightly anterior to the apex, outgrowth still occurs posteriorly and transcripts of *Hox-4.7* could be detected just distal to the bead and are continuous

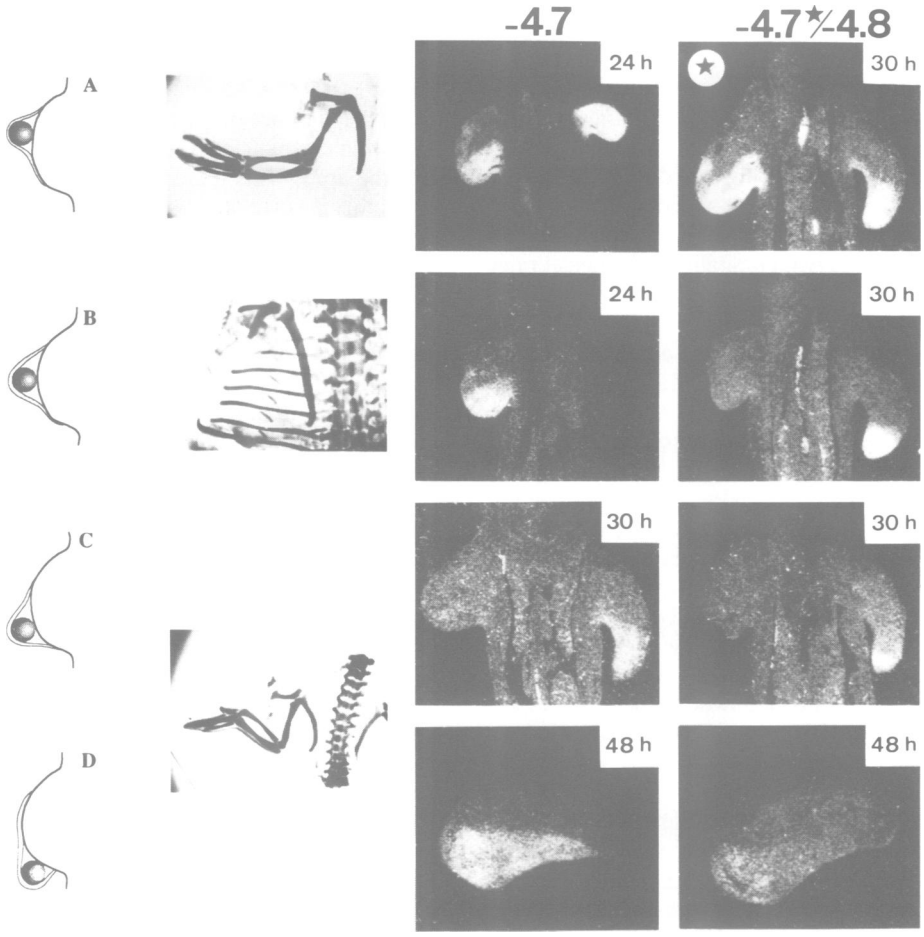

Fig. 4. The effects of applying retinoic acid to different positions in chick wing buds on limb pattern and *Hox-4* gene expression. **A–D** are diagrams showing how beads soaked in 0.1 mg/ml retinoic acid were implanted at different positions in stage 20 wing buds: A, just anterior to the apex; B, at the apex; C, just posterior to the apex; D, posterior. The whole mount photographs show wing patterns that typically result in: A, wing with mirror-image pattern 4334; B, truncated wing with only a very short length of humerus; C and D, small wing with normal pattern that commonly results especially with application posteriorly. Dark-field photographs show expression pattern of particular *Hox-4* genes at different times after application of retinoic acid, as indicated. The treated wing bud is always on the left. The bottom four panels show buds in which beads soaked in retinoic acid were implanted posteriorly. At 48 h, only treated buds are illustrated.

with the transcripts that are present in the normal posterior domain (Figure 4B). In another bud, in which outgrowth was totally inhibited, the expression of *Hox-4.4* and *Hox-4.6* is normal, but the signal for *Hox-4.8* is extremely weak. At 30 h, although the domain of *Hox-4.7* still appears to be enlarged distally, transcripts of *Hox-4.8* are much less abundant both distal to the bead and in the most posterior part of the bud where this gene is usually strongly expressed (Figure 4B). Beads soaked in 0.1 mg/ml retinoic acid placed posteriorly adjacent to the normal domains of 5′-located *Hox-4* genes can also lead to truncated wings but more commonly small wings with normal digit patterns develop (Figure 4C and D). These small wings result because of a delay in development while outgrowth is shifted due to the anterior displacement of the apical ridge (Lee and Tickle, 1985). At 30 h, the expression of *Hox-4.7* and *Hox-4.8* in the posterior part of the bud adjacent to the bead is much weaker than normal (Figure 4C). However, at 48 h, when outgrowth has clearly recommenced from more anterior parts of the bud, a normal nested set of 'posterior' domains is re-established at the new bud margin under the apical ridge (Figure 4D).

Discussion

Cooperation between retinoic acid and a signal from the apical ectodermal ridge in activation of Hox-4 genes

Retinoic acid appears to cooperate with a signal from the apical ectodermal ridge to induce activation of 5′-located genes of the HOX-4 complex in manipulated chick wing buds. This conclusion emerges from the absence of induction of ectopic domains when the apical ectodermal ridge is removed. The new domains of *Hox-4* genes induced by application of retinoic acid are always located in distal mesenchyme under the ridge. The precise localization of the domains can be envisaged as being determined by the overlap in the distribution of two diffusible factors, retinoic acid diffusing from the carrier bead and a signal diffusing from the apical ridge. This can account, for example, for the spatial pattern of expression of *Hox-4* genes when retinoic acid is applied posteriorly.

Analysis of the time course of digit induction by retinoic acid application shows that the first 12–14 h of exposure appears to be a priming phase during which no irreversible

changes in pattern occur. In the second phase, the 'duplication' phase, from 14 to 20 h, additional digits are induced sequentially (Eichele *et al.*, 1985). It is during this second phase that *Hox-4* genes are activated, also in sequence working in a 3′ to 5′ direction along the gene cluster (Ipzisúa-Belmonte *et al.*, 1991). Removal of the ridge either soon after retinoid application or during the duplication phase prevents activation of 5′-located *Hox-4* genes. These results show that the apical ridge is not only required during the priming phase. This leaves two possibilities: the apical ridge may be required continuously together with retinoic acid throughout both priming and duplication phases, or the apical ridge signal may be important only in the duplication phase.

Once *Hox-4* genes have been activated, their expression appears to be relatively stable. In wings to which retinoic acid has been applied at the anterior margin, removal of the retinoid source (Izpisúa-Belmonte *et al.*, 1991) or removal of the ridge does not switch off expression of *Hox-4* genes, such as *Hox-4.6*, that have already been activated. This stability of expression is also found when cells expressing *Hox-4* genes are transplanted to new positions (Izpisúa-Belmonte,J.-C., Brown,J.M., Duboule,D. and Tickle,C., in preparation). For example, polarizing region cells still express 5′-located *Hox*-4 genes when transplanted to the anterior margin of the wing bud where these genes are normally not expressed. However, high concentrations of retinoic acid can inhibit expression of these genes not only *in vivo* (see also Izpisúa-Belmonte *et al.*, 1991) but also *in vitro* (Simeone *et al.*, 1991). This suggests an explanation for the decrease in expression of *Hox-4.8* when retinoic acid is applied posteriorly where the endogenous concentration of retinoic acid is high (Thaller and Eichele, 1987).

The ridge appears to be required for activation of the *Hox-4* genes during normal wing bud development. Removal of the ridge at stage 17/18 prevents the subsequent expression of 5′-located *Hox-4* genes. This activation in the normal bud could also involve retinoic acid generated by the polarizing region (Thaller and Eichele, 1987). However, it is also possible that some other signal produced by the polarizing region acts in concert with the signal from the ridge. Recently it has been suggested that retinoic acid application to wing buds leads to duplication of digit patterns by first converting anterior cells into polarizing region cells (Wanek *et al.*, 1991; Noji *et al.*, 1991). The experiments on normal wing buds also show that expression of *Hox-4* genes continues in the absence of the ridge, although, as outgrowth is inhibited, the distalward movement of the *Hox-4.8* domain does not occur and the expression of the 'posterior' genes does not become more intense.

Is expression of Hox-4 genes related to apical ridge maintenance?

Our results suggest a relationship between expression of 5′-located *Hox-4* genes and the maintenance of the overlying ridge. 5′-located *Hox-4* genes are consistently expressed in regions of the bud where outgrowth is occurring under the influence of the apical ridge. For example, when *Hox-4* genes are expressed in a mirror-image pattern, the apical ridge lengthens and this leads to a broader bud outgrowth. In addition, when the nested pattern of *Hox-4* domains is displaced anteriorly by posterior application of retinoic acid, the apical ridge is similarly displaced and outgrowth is shifted anteriorly. It is also interesting that in limb buds of the *talpid*^{3} poydactylous mutant, expression of *Hox-4.8* extends all the way across the antero-posterior axis and this correlates with the greatly lengthened apical ridge that is maintained around the rim of the very broad bud (Izpisúa-Belmonte *et al.*, 1992). The relationship between *Hox-4* expression and the presence of the ridge is further demonstrated in wings in which retinoic acid, particularly at high doses, is applied to the apex. This treatment inhibits expression of *Hox-4.8* and this is correlated with the flattening of the apical ridge. The idea that maintenance of the ridge is in some way connected with the pattern of *Hox-4* gene expression in the mesenchyme is also consistent with the results of recombination experiments between tissues of retinoid-treated and normal wing buds (Tickle *et al.*, 1989; Oliver *et al.*, 1990). The development of these recombinations shows that it is the mesenchyme that has been irreversibly changed by retinoid treatment whereas, in contrast, any changes in the ridge are reversible.

Molecular basis of signalling and response pathways governing interactions in the limb

Our results suggest that expression of *Hox-4* genes is integrated with signalling pathways between the apical ridge and underlying mesenchyme that govern bud outgrowth. There are a number of candidates for molecules involved in these epithelial–mesenchymal interactions in vertebrate limb buds. Apical ridge cells transcribe a number of different genes encoding growth factors, the bone morphogenetic proteins, BMP-2A (Lyons *et al.*, 1990) and BMP-4 (Jones *et al.*, 1991) and a member of the fibroblast growth factor family, FGF-4 (Niswander and Martin, in preparation), in addition to *wnt-5* (Gavin *et al.*, 1990) and the homeobox genes, *Hox-8.1* (Coelho *et al.*, 1991) and, at low levels, *Hox-7.1* (D.R.Davidson and J.M.Brown, unpublished data). The ridge cells also express the product of the *ld* gene (Zeller *et al.*, 1989; Trumpp *et al.*, 1991). Mesenchyme cells at the tip of the limb transcribe the genes for BMP-4, Wnt-5 and AP-2 (Mitchell *et al.*, 1991) and a number of homeobox genes in addition to the HOX-4 complex, including *Hox-7.1* and *Hox-8.1*. Products of the *ld* gene are also expressed in the mesenchyme at an early stage in bud development (Trumpp *et al.*, 1991). At present, the significance of these expression patterns is largely unknown although the control of expression of the homeobox genes *Hox-7.1* and *Hox-8.1* in mesenchyme at the tip of the limb bud appears to involve a signal from the apical ridge (Davidson *et al.*, 1991; Robert *et al.*, 1991). There may also be other important molecules that have not yet been identified. In view of these uncertainties, it is not yet possible to identify those molecules that cooperate with *Hox-4* expression in the mesenchyme. In early *Xenopus* embryos, basic FGF and retinoic acid act synergistically to bring about changes in homeobox gene expression (Cho and De Robertis, 1990) and therefore, by analogy, FGF could be a candidate for the signal from the ridge that is required for *Hox-4* activation. However, the expression of FGF-4 in the mouse apical ridge (Niswander and Martin, in preparation) occurs at a time when *Hox-4* genes have already been activated (Dollé *et al.*, 1989).

Hox-4 genes are clearly involved in the cellular response to positional signals in the developing limb. It has been suggested that their primary role is to encode positional values of cells across the antero-posterior axis of the limb and the patterns of *Hox-4* gene expression following experimental manipulation of chick wing buds are consistent with this idea (Izpisúa-Belmonte *et al.*, 1991; Nohno *et al.*,

1991). Based on the expression patterns of *Hox-4* genes, as successive structures along the proximo-distal axis are laid down, it appears that qualitative combinations of *Hox-4* genes could specify position across the antero-posterior at all levels (Izpisúa-Belmonte *et al.*, 1991; Yokouchi *et al.*, 1991), although there are also quantitative differences in expression that could be important (Dollé *et al.*, 1989). According to this view, the cooperation of a signal from the apical ridge in activating *Hox-4* genes would assign position in the initial bud; we then suggest that the consequent driving of bud outgrowth as a result of activation of these genes would ensure the elaboration of the expression pattern to provide positional information at successive levels along the limb.

The signal from the apical ridge could act directly with retinoic acid and/or a downstream signal to activate *Hox-4* genes in the new cells generated as the bud grows. Another possibility is that the signal from the apical ridge acts more indirectly and simply maintains the proliferation of cells which is required for *Hox-4* gene activation by positional signals. In this context, the signal from the ridge could be part of a mechanism 'opening' the *Hox-4* genes thus allowing their successive expression.

Materials and methods

Manipulation of chick wing buds

Beads soaked in 0.1 mg/ml or 1 mg/ml retinoic acid (Sigma lot numbers 98F 0778 and 40H 0313) were implanted into the right wing buds of chick embryos at stage 17/18 or stage 20 (Hamburger–Hamilton stages; for details see Tickle *et al.*, 1985). In the first series of experiments (Figure 1), beads were placed at the anterior margin of stage 17/18 wing buds. Two, eight, 20 or 24 hours later, the apical ridge was cut away from the mesenchyme using sharpened needles as indicated in Figures 1 and 2. The buds were all then fixed 30 or 38 h after the start of retinoid treatment in 4% paraformaldehyde, embedded and sectioned for analysis of *Hox-4* expression patterns (see below). In the second series of experiments, the entire apical ridge was cut away from wing buds at either stage 17/18 or stage 20 and the buds were fixed 25–44 h later. In the third series of experiments (Figure 4), beads were placed at different positions around the wing bud margin at stage 20. The buds were then fixed at 24, 30 or 48 h.

For all these experiments, a small number of embryos was also left to develop for 6 days so that the final wing pattern could be ascertained in whole mounts stained with Alcian Green. In addition, in the series of experiments in which the apical ridge was removed following application of retinoic acid, some embryos in the same series were treated with retinoic acid and then allowed to develop to 6 days without any further operation to serve as controls for the effectiveness of retinoid treatment.

In situ hybridization

Hybridizations were performed as previously described (Dollé and Duboule, 1989) with ^{35}S-labelled riboprobes for chicken *Hox-4* genes (Izpisúa-Belmonte *et al.*, 1991).

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