Apical ridge dependent and independent mesodermal domains of *GHox-7* and *GHox-8* expression in chick limb buds

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Summary

The homeobox-containing genes *GHox-7* and *GHox-8* have been proposed to play fundamental roles in limb development. The expression of *GHox-8*, by the apical ridge cells, and *GHox-7*, in the subridge mesoderm, suggests the involvement of these two genes in limb outgrowth and proximo-distal pattern formation. A straightforward way to test this is to remove the apical ridge. Here we report the relationship between the mesodermal expression of *GHox-7* and *GHox-8* and the apical ectodermal ridge in the chick limb bud. The data from ridge removal experiments indicate that there are at least two domains of *GHox-7* expression in the apical limb bud mesoderm. The posterior subridge *GHox-7* domain in the progress zone requires the influence of the apical ridge for continued expression, while the anterior *GHox-7* domain continues expression after ridge removal. Posterior subridge mesoderm is exquisitely sensitive to the loss of the ridge in that *GHox-7* expression by these cells is reduced in only two hours and undetectable by three hours after ridge removal. It would appear that one of the ways progress zone cells respond to the apical ridge signal is by expressing *GHox-7*. The loss of ridge influence whether by growth at the apex or by ridge removal is followed by an unusually rapid decline in detectable *GHox-7* transcripts. Maintenance of *GHox-8* expression by the anterior mesoderm appears to be independent of the presence of the apical ridge. Finally our data indicate that neither *GHox-7* nor *GHox-8* transcripts need be detectable in the cells that are induced to die after apical ridge removal.

Key words: apical ectodermal ridge, homeobox-containing genes, *GHox-7*, *GHox-8*, limb development, epithelial-mesenchymal interactions.

Introduction

The chick limb bud consists of an ectodermal jacket and a core of mesenchymal cells. At the tip of the limb bud is a pseudostratified columnar epithelium, the apical ectodermal ridge, which is required for the elongation of the bud (Saunders, 1948; Summerbell, 1974; Rowe and Fallon, 1982; Fallon et al., 1991). The mesenchymal region that underlies the ridge remains in an undifferentiated condition and is called the progress zone or apical zone (Summerbell et al., 1973). Rowe et al. (1982) showed not only that the ridge is required for proximo-distal elongation but also that it is indispensable for subridge mesoderm survival during stages 18-22. Therefore the apical ridge plays a crucial role in limb development.

Recently there has been a great deal of interest in possible roles for homeobox-containing genes in the development of the limb pattern (reviewed in Tabin, 1991). For example, *GHox-7* and *GHox-8* have been shown to be expressed in patterns that correlate with limb epithelial-mesenchymal interactions. *GHox-8* is highly expressed by the apical ridge cells (Robert et al., 1991; Coelho et al., 1991a; Yokouchi et al., 1991). *GHox-7* is expressed at low levels by the apical ridge cells (Robert et al., 1991; Suzuki et al., 1991; M. A. Ros, unpublished data). The expression of these two genes distinguishes the apical ridge cells on a molecular level from the dorsal and ventral ectoderm.

At stages 16-18, when the limb starts development *GHox-7* is expressed over the whole mesoderm (Robert et al., 1989, 1991) while *GHox-8* is expressed by the mesoderm of the anterior border of the limb bud (Robert et al., 1991; Yokouchi et al., 1991). During stage 18 of development, the domains of expression of these genes become limited; specifically, *GHox-7* transcripts are abundant in the progress zone where they are expressed in an asymmetric pattern with the deepest levels located anteriorly (Robert et al., 1991; Suzuki et al., 1991; Coelho et al., 1992a). *GHox-8* overlaps the anterior domain of expression of *GHox-7* but...
does not extend into the posterior subridge region. Both genes are also expressed in regions corresponding with zones of programmed cell death such as the posterior necrotic zone and the interdigital mesenchyme (Hill et al., 1989; Robert et al., 1991; Coelho et al., 1991a, 1992a).

The expression of GHox-8 in the apical ridge and GHox-7 in the underlying mesoderm suggests a role for these genes in the apical ridge mediated outgrowth and proximodistal axis specification. This proposal has been strengthened by the in situ hybridization studies of the expression of these genes in the chick limb mutant limbless (see Robert et al., 1991; Coelho et al., 1991b, 1992c). Similarly in situ hybridization studies after experimental manipulation (see Yokouchi et al., 1991; Coelho et al., 1992b) and with the mutants diplopodia-5 and talpid2 (see Krabbenhoft and Fallon, 1992; Coelho et al., 1992b,c) indicate a possible role for GHox-7 and GHox-8 in the specification of anterior positional identity in limb development.

The purpose of the present work is to analyze the expression of GHox-7 and GHox-8 in the limb bud after apical ectodermal ridge removal in an attempt to extend our knowledge about the developmental significance of the expression of these genes. This approach has the advantage of allowing us to study the kinetics of the possible modification of the expression of these genes in the absence of the apical ectodermal ridge.

Materials and methods

Fertilized White Leghorn eggs were incubated at 38°C for 2.5-3 days. They were opened and the embryos staged (Hamburger and Hamilton, 1951). At each of the stages 20-22, the apical ectodermal ridge was removed from the right wing bud with a tungsten needle and the eggs were sealed with Scotch Magic Transparent Tape and returned to the incubator. Great care was taken to ensure that the apical epithelium was removed from anterior body wall to posterior body wall. The average time that each egg was out of the incubator during the experimental manipulation was about 13 minutes. The embryos were allowed to develop for 1, 2, 3, 6, 8, 12, 24 and 48 hours and then fixed in Bouin's solution on ice, embedded and sectioned at 6 μm. Some embryos were allowed to develop until day 10 of incubation. The right wing development of these embryos coincided with the data for stage 20-22 published in Summerbell (1974).

In order to have the contralateral nonoperated limb as control, portions of the trunk including both wing buds were collected. Tissues were processed for in situ hybridization according to the protocol described by Mallein-Gerin et al. (1988), except that the sections were treated with 10 μg/ml of proteinase K. The chicken GHox-7 probe was a 332 base pair fragment located 3' to the homeobox and consisted primarily of untranslated sequence (Coelho et al., 1992a). The chicken GHox-8 probe was a 331 base pair fragment from the 3' untranslated region of the GHox-8 cDNA (Coelho et al., 1991a). The cDNA probes were labeled with [35S]dCTP (NEB) using a random-primed DNA labeling kit (Boehringer Mannheim).

In situ hybridization was performed for GHox-7 and GHox-8 in neighboring serial sections (12 to 18 μm apart) of each embryo. At least three limb buds of each period of ridge removal were studied. In order to ascertain the three-dimensional distribution of the gene products in the limb buds, frontal, cross and longitudinal sections of the experimental and contralateral left control limb bud were analyzed. We found that longitudinal sections affecting both experimental and contralateral control limbs (i.e. cross sections referring to the whole embryo) were the most useful because they allowed us to easily evaluate whether the removal of the ridge was properly done and also the contralateral limb was visible as a control of normal expression. Due to the asymmetric distribution of GHox-7 transcripts in the normal limb bud across the anterior posterior axis, extraordinary care was taken during embedding and sectioning to make sure that the level of both limbs was the same along the anteroposterior axis so that proper comparison could be made. In order to confirm that the surgery did not affect the underlying mesoderm mechanically, in situ hybridization with both probes was performed on tissue embedded immediately after the operation. In these cases, the expression of both genes in the denuded mesoderm was completely normal (data not shown).

Results

GHox-7 and GHox-8 expression after ridge removal

During stages 20 to 22, the GHox-7 domain of expression extends in an asymmetric arc from the anterior border of the limb to the cells in the subridge region. Surgical removal of the apical ectodermal ridge during this period results in two distinct responses in the subapical mesoderm where GHox-7 is expressed. The subridge mesoderm of approximately the posterior two-thirds of the limb bud shows severely reduced expression of GHox-7, while the expression of this gene at the anterior border of the limb continues. The reduction in GHox-7 expression after removal of the ridge takes place in a very rapid and specific way. As shown in Fig. 1A, one hour after the removal of the apical ridge GHox-7 transcripts are conspicuous in both experimental and contralateral control limbs, although the experimental limb may have reduced expression. However, as soon as two hours after the operation the level of expression of GHox-7 in the distal posterior mesoderm of the operated limb is clearly reduced compared to the control limb bud (Fig. 1B). By three hours after the excision of the ridge (Fig. 1C), GHox-7 transcripts are undetectable in the distal posterior mesoderm while expression is maintained in the nonoperated control limb bud. The fact that the subridge mesodermal region is still actively transcribing other genes like β-actin (Fig. 1D) indicates that the disappearance of GHox-7 transcripts is a specific phenomenon.

Although GHox-7 expression is undetectable in the subridge posterior mesoderm three hours after ridge removal, it is maintained at the anterior border of the limb bud. Fig. 2A shows the loss of GHox-7 expression in the posterior part of the operated limb bud (stage 20 three hours after ridge removal) compared to the contralateral side. However, if the section is through the anterior-most part of the limb, GHox-7 transcripts are detectable in the operated limb (Fig. 2C) although the apical ridge was removed over this region. GHox-7 levels of expression in the anterior distal mesoderm appear slightly reduced which is consistent with our working hypothesis that there are two populations of GHox-7 expressing cells in this region (see Discussion).

During stages 20 to 22, the normal domain of expression of GHox-8 is located at the anterior border of the wing bud where it is in part coexpressed with the anterior domain of
expression of \textit{GHox-7}. In contrast to the above described behavior of \textit{GHox-7} transcripts after ridge removal, \textit{GHox-8} localization and expression remain unaffected three hours after the experimental manipulation. When the section passed through the anterior limb bud (Fig. 2D), both experimental and contralateral control limbs exhibit similar levels of expression of \textit{GHox-8} transcripts as detected by in situ hybridization. As shown in Fig. 2B, a longitudinal section through the posterior level of a wing bud three hours after ridge removal at stage 20 exhibits no detectable levels of \textit{GHox-8} expression in the mesoderm. Note the high expression of \textit{GHox-8} in the ridge of the left limb bud and the absence of ridge in the experimental (right) limb bud. Indeed the expression of \textit{GHox-8} in the ridge is so specific that the hybridization with \textit{GHox-8} probe easily detects any ridge cell left after the operation.

The continued presence of \textit{GHox-7} transcripts in the cells at the anterior border of the limb bud when cells at the most distal tip have ceased expression could simply reflect different rates of response to ridge removal between distinct
populations of mesenchymal cells with different locations. It is possible that the expression of GHox-8, although depending on ridge presence, would take longer to be affected by ridge removal than GHox-7. It has been suggested that GHox-8 transcripts could be more stable than those of GHox-7 (Robert et al., 1991). To test this, we

Fig. 2. GHox-7 and GHox-8 expression three hours after ridge removal. (A,B) Adjacent transverse sections through the posterior region of the wing bud. (C,D) Adjacent transverse sections through the anterior part of the wing bud. (A,C) Sections were hybridized with GHox-7 probe; (B,D) Sections were hybridized with GHox-8 probe. Arrowhead: apical ectodermal ridge; nt, neural tube. Bar, 200 µm.
Ridge removal: \textit{GHox-7 and GHox-8} expression

extended our in situ studies for longer periods after ridge removal. Fig. 3 shows frontal sections of a limb bud whose ridge was excised at stage 22, fixed 12 hours later and hybridized with the \textit{GHox-7} and \textit{GHox-8} probes. Expression of \textit{GHox-7} (Fig. 3B) at the anterior border is clearly visible 12 hours after surgery. The possibility that \textit{GHox-8} expression is independent of ridge activity is supported by the observation that its expression continues 12 hours after ridge removal (Fig. 3C). \textit{GHox-7} and \textit{GHox-8} transcripts remain present at the anterior proximal border of the limb bud 48 hours after surgery (data not shown).

To emphasize further the specificity of ridge action upon \textit{GHox-7} expression, the following experiment was carried out as a control. Dorsal ectoderm of wing buds from stage 20 to 22 was removed up to but without touching the apical ridge. In this case, the expression of \textit{GHox-7} remains unaffected in the mesenchymal cells under the ectoderm denuded area as can be seen in Fig. 4B. The expression of \textit{GHox-8} also remains unaffected after dorsal ectodermal removal (data not shown).

Cell death after ridge removal

There is a period during limb development when the presence of a ridge not only is necessary for distal outgrowth to occur but also is required for survival of subridge mesodermal cells (Rowe et al., 1982). Removal of the ridge from stage 18 to 20 results in the induction of patterned cell death beginning with the anterior mesoderm distal cells. In our present experiments, cell death was observed and the cells that died had stopped expressing detectable levels of \textit{GHox-7} either at the time of death or several hours earlier. The anterior mesodermal cells were the first to die at about three hours after ridge removal. This cell death occurred in the anterior limb bud region where the expression of \textit{GHox-7} and \textit{GHox-8} was maintained. At about five hours after ridge removal, cells had died more posteriorly. This cell death was at the interface between the region where \textit{GHox-7} and \textit{GHox-8} are coexpressed and where \textit{GHox-7} is expressed alone. At about 8 hours, cell death was seen where \textit{GHox-7} had been expressed alone. As already noted \textit{GHox-8} was never detectable in the posterior cells that died after ridge removal.

Discussion

The data reported here demonstrate that removal of the apical ectodermal ridge from the chick limb bud affects the expression of mesodermal \textit{GHox-7} in different ways. The mesoderm beneath the posterior apical ridge loses the expression of \textit{GHox-7} within three hours. However, the mesoderm beneath the anterior ridge maintains \textit{GHox-7} expression after ridge removal. These anterior cells are also expressing \textit{GHox-8} and the expression of this gene is also continued.

Previous data showing the spatial and temporal pattern of expression of \textit{GHox-7} and \textit{GHox-8} in normal limb development (Coelho et al., 1991a, 1992a; Robert et al., 1991; Yokouchi et al., 1991; Suzuki et al., 1991; Nohno et al., 1992) along with the study of the expression of these genes in chick limb mutants (Coelho et al., 1991b, 1992b; Robert

Fig. 3. Expression of \textit{GHox-7} and \textit{GHox-8} twelve hours after the removal of the ridge at stage 22. Phase (A) and corresponding dark-field micrographs of neighboring frontal sections hybridized with \textit{GHox-7} (B) and \textit{GHox-8} (C) probe. The anterior (a) and posterior (p) borders of the limb bud are indicated. The posterior part of the limb in C shows some non-specific hybridization. Bar, 200 µm.
et al., 1991; Krabbenhoft and Fallon, 1992) and some experimental manipulations (Yokouchi et al., 1991; Coelho et al., 1992b) have suggested a role for the apical ectodermal ridge in the maintenance of mesodermal G\textit{Hox}-7 and G\textit{Hox}-8 expression. A straightforward way of approaching this problem is the purpose of the present work analyzing the pattern of expression of G\textit{Hox}-7 and G\textit{Hox}-8 after removal of the apical ectodermal ridge.

Our data indicate that, after initial limb budding, there are at least two domains of expression of G\textit{Hox}-7 in the limb bud mesoderm. First, there is a domain of expression of G\textit{Hox}-7 immediately subjacent to the posterior ridge, in the so-called progress zone. Second, there is a domain of expression located at the anterior margin of the limb which remains after ridge removal. It appears that the expression of G\textit{Hox}-7 by a given cell can be under the control of different factors depending on its location. This also could imply that the expression of G\textit{Hox}-7 by a cell could have different developmental significance depending on factors such as previous history of the cell, different combinatorial sets of genes expressed at the same time and so forth.

The observation that G\textit{Hox}-7 transcripts are undetectable in the cells in the region under posterior ridge influence three hours after its removal strongly supports involvement of G\textit{Hox}-7 in the mesenchymal-ectodermal interactions that are taking place at this level (Coelho et al., 1991b; Robert et al., 1991). A signal emanating from the apical ectodermal ridge is thought to maintain the subridge mesoderm in an undifferentiated state and this allows the proximo-distal determination of the limb parts (Summerbell et al., 1973). It seems likely that one of the ways that the mesodermal cells in the progress zone respond to the ridge signal is by expressing G\textit{Hox}-7. The loss of ridge signal is rapidly followed by the cessation of expression of G\textit{Hox}-7 by the posterior responding mesoderm cells. The fact that the decrease in the expression of the gene is so rapid suggests that the signal emanating from the ridge is continuously produced, constantly required and has a very short half life. It also suggests that the half life of the G\textit{Hox}-7 transcripts is likely very short. Another possible explanation of the rapid disappearance of G\textit{Hox}-7 transcripts following ridge removal could be a dramatic decrease in the half life of its mRNA following ridge removal. Wang and Sassoon (1991) have recently reported that expression of \textit{Hox}-7.1, the mouse homologue of G\textit{Hox}-7, is not detectable three to six hours after mouse limb bud cells are dissociated and grown in tissue culture. Our data also fit well with the observations of Davidson et al. (1991) who showed activation of \textit{Hox}-7.1 in mouse proximal limb bud mesoderm five hours after it was grafted beneath the chick ridge. These observations

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig4}
\caption{The effect of dorsal ectodermal removal on G\textit{Hox}-7 expression. Phase (A) and dark-field micrograph (B) of a transverse section through the middle wing bud level hybridized with G\textit{Hox}-7 probe. The dorsal ectoderm on the right side was removed at stage 20 and the embryo fixed three hours later. The region from which the ectoderm was removed is marked by arrowheads. Note that the apical ectodermal ridge is intact (arrow). Red blood cells appear refractile in dark-field micrographs. n, notochord. Bar, 200 \textmu m.}
\end{figure}
taken together indicate that limb mesoderm is exquisitely sensitive to signals from the apical ridge and part of this sensitivity is the regulation of \textit{GHox-7} transcription and possibly degradation. They also show that the ridge signal is highly conserved throughout evolution.

The rapid loss of detectable \textit{GHox-7} transcripts after ridge removal leads to a reasonable explanation for the observed relatively sharp boundary in the transition between the subridge progress zone mesoderm and the proximal mesoderm. That is, with normal growth, cells in the proximal progress zone regularly are removed from ridge influence by increasing distance from the ridge. The relatively sharp boundary of \textit{GHox-7} transcripts at the transition, very likely is due to a very rapid loss of these transcripts similar to what we have observed after ridge removal viz., within three hours. There are likely other molecular correlates in this transitional zone.

\textit{GHox-8} expression however remains after ridge removal. Two previous reports indicate a relationship of experimental \textit{GHox-8} induction by apical ridge stimulation. Robert et al. (1991) reported the induction of \textit{GHox-8} in \textit{eudiplopo-dia} phenocopy limbs. Interestingly, they also reported that \textit{GHox-8} was maintained in \textit{limbless} embryos until the time of regression (i.e. during stage 21; see also Coelho et al., 1991a). The other report is the work of Davidson et al. (1991) where the expression of \textit{Hox-8.1} transcripts was turned on at low levels in proximal mouse limb bud cells grafted under a chick ridge. From these observations, it would appear that \textit{GHox-8} can be induced in proximal limb mesoderm. Our data and the \textit{limbless} observations cited above indicate that the ridge is not necessary to maintain \textit{GHox-8} expression. However, the presence of an apical ridge by itself seems not enough to bring about \textit{GHox-8} expression by the anterior mesoderm since the \textit{diplopodia-5} (Coelho et al., 1992b) and \textit{talpid} 5 observations (Krabbenhoft and Fallon, 1992; Coelho et al., 1992b), which have an extensive thickened ridge, do not express detectable levels of \textit{GHox-8}. It is noteworthy that the apical ridge induces high level expression of \textit{GHox-7}, but only low expression of \textit{GHox-8}, in limb mesenchymal cells in vitro, whereas ectoderm from the anterior border of the limb bud induces high expression of both \textit{GHox-7} and \textit{GHox-8} (Coelho, Upholt and Kosher, unpublished data). It has therefore, been suggested that ectoderm from the anterior border of the limb bud may be involved in maintaining the anterior mesodermal domains of \textit{GHox-7} and \textit{GHox-8} expression. It has also been reported that the ridge is necessary for the activation but not maintenance of the expression of genes in the \textit{Hox-4} complex (Izpisua-Belmonte et al., 1992).

There appears to be a correlation between zones of programmed cell death in the limb bud mesoderm and the expression of \textit{GHox-7} and \textit{GHox-8} (Hill et al., 1989, Robert et al., 1991a, 1992a). Whether or not there is a causal relationship between these events (see Krabbenhoft and Fallon, 1992 and Coelho et al., 1992c) will require further experimentation. We note that induced cell death after ridge removal need not be, and likely is not, accompanied by the same molecular events that regulate programmed cell death. It is apparent that the induced cell death after ridge removal can occur in the area of cells expressing both \textit{GHox-7} and \textit{GHox-8} and in areas of cells expressing neither \textit{GHox-7} nor \textit{GHox-8} (having lost detectable \textit{GHox-7} transcripts). It is clear that cells in the region which was normally expressing \textit{GHox-7} along with \textit{GHox-8} die after ridge removal. In fact, these die first and are likely most sensitive to ridge removal. All the cells that die after ridge removal had been in areas naturally expressing \textit{GHox-7}. However, specific \textit{GHox-7} or \textit{GHox-8} synthesis never appear to occur as a prelude to the cell death induced by ridge removal.

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