Hox-4 gene expression in mouse/chicken heterospecific grafts of signalling regions to limb buds reveals similarities in patterning mechanisms

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Summary

The products of Hox-4 genes appear to encode position in developing vertebrate limbs. In chick embryos, a number of different signalling regions when grafted to wing buds lead to duplicated digit patterns. We grafted tissue from the equivalent regions in mouse embryos to chick wing buds and assayed expression of Hox-4 genes in both the mouse cells in the grafts and in the chick cells in the responding limb bud using species specific probes. Tissue from the mouse limb polarizing region and anterior primitive streak respecify anterior chick limb bud cells to give posterior structures and lead to activation of all the genes in the complex. Mouse neural tube and genital tubercle grafts, which give much less extensive changes in pattern, do not activate 5'-located Hox-4 genes. Analysis of expression of Hox-4 genes in mouse cells in the grafted signalling regions reveals no relationship between expression of these genes and strength of their signalling activity. Endogenous signals in the chick limb bud activate Hox-4 genes in grafts of mouse anterior limb cells when placed posteriorly and in grafts of mouse anterior primitive streak tissue. The activation of the same gene network by different signalling regions points to a similarity in patterning mechanisms along the axes of the vertebrate body.

Key words: homeobox gene expression, limb development, gastrulation, neural tube development, mouse/chicken grafts, pattern formation, Hox-4.

Introduction

Hox-4 genes are good candidates to encode position in vertebrate limbs. 5' members of the Hox-4 complex (Hox-4.4 to Hox-4.8) are expressed in developing limb buds in a series of overlapping domains with expression of the most 5' gene (Hox-4.8) being confined to the most posterior part of the bud (Dollé et al., 1989; Izpisúa-Belmonte et al., 1991a). When a limb polarizing region or retinoic acid is placed at the anterior margin of a chick wing, the adjacent cells are respecified to form posterior structures and the digit pattern is duplicated (Tickle et al., 1975, Tickle et al., 1982). This respecification results in mirror-image patterns of expression of Hox-4 genes that precede the development of mirror-image patterns of digits (Izpisúa-Belmonte et al., 1991a; Nohno et al., 1991). Other regions of embryos including Hensen’s node (Hornbruch and Wolpert, 1986; Wagner et al., 1990) and the floor plate of the neural tube (Wagner et al., 1990) in chicks and the genital tubercle in mice (Dollé et al., 1991a) also have polarizing activity and, when grafted to the anterior of chick wing buds, can lead to the formation of additional digits suggesting a universality in signalling mechanisms. The polarizing region is at the posterior margin of the limb bud (Saunders and Gasseling, 1968) and corresponds to a region where cells express the complete set of Hox-4 genes, including those located at the 5' end of the complex ('posterior' genes e.g. Hox-4.7, Hox-4.8). Mouse genital tubercle expresses only 5'-located Hox-4 genes (e.g. Hox-4.7, Hox-4.8; Dollé et al., 1991a) whereas primitive streak tissue and anterior neural tube do not express these 'posterior' genes (Dollé et al., 1991b). Here we graft mouse cells from all of these different regions into chick wing buds and use probes that specifically recognize transcripts of either mouse or chick Hox-4 genes. This provides a powerful way of analyzing gene expression in both responding and signalling cells. We find that polarizing activity does not require the expression of Hox-4 genes in the grafted tissue. In addition, the activation of Hox-4 genes in the wing bud tissue in response to the various grafts predicts the digit patterns that will result from such manipulations. The ability of different signalling regions to induce activation of the same gene network points to common signalling and response mechanisms in different regions of the embryo.
Materials and methods

Embryos from C57 black and tan or BALB/c mice provided the tissues for the grafts. The anterior part of the primitive streak was dissected from 8- to 8 1/2-day embryos (day of plug=day 0) which had mostly 0-6 pairs of somites. An equivalent region of the streak was also taken from two embryos that had 11 pairs of somites and was used to investigate the effects on digit pattern. The neural tube was taken from the anterior region (at the level 0-7 somites) of 8 1/2-day mouse embryos with 6-16 pairs of somites and the polarizing region and anterior mesenchyme was from either the forelimbs of 9-10 day embryos or the hindlimbs of 10 1/2-day embryos. The genital tubercles were taken from 12 1/2-day mouse embryos. The neural tube, the mesenchyme of the polarizing region or of the anterior part of the limb and the mesenchyme of the tip of the genital tubercle were isolated after an incubation with 2% trypsin at 4°C for half an hour to 1 hour to remove the notochord and somites, the limb epithelium and the genital tubercle epithelium, respectively. The length of neural tube provided three grafts and the complete tube was grafted because of its small size.

To test for polarizing activity and to investigate the effects on Hox-4 gene expression, the grafts were placed at the anterior margin of wing buds of chick embryos at stage 18 or 20 (Hamilton and Hamburger stages). In most cases, the mouse limb tissues were grafted at stage 18 so that the results were directly comparable to the implantation of beads soaked in retinoic acid that were carried out previously (Izpisua-Belmonte et al., 1991a). The apical ectodermal ridge was cut away from the mesenchyme to form a loop to hold the graft in place (Fig. 1). In a short series of experiments, grafts of anterior limb mesenchyme were made under a loop of apical ridge lifted along the posterior margin of wing buds at stage 18. The embryos were then reincubated at 38°C. At a series of time points between 16 and 72 hours, embryos were removed from the egg and placed in PBS. They were then pinned out in 4% paraformaldehyde and fixed overnight (for a maximum of 24 hours). The torsos with attached wing buds were dehydrated and then embedded in wax for sectioning and in situ hybridisation. In each series of experiments, some embryos were allowed to continue to develop for up to 6 days after grafting and then the wings were fixed in either 5% TCA (trichloroacetic acid) or formal saline and then stained with either alcian green or alcian blue to show the skeletal pattern. Selected wings were then embedded in wax, sectioned and stained with Biebrich Scarlet to identify the mouse cells of the graft. In a few cases, wings with grafts of neural tube or primitive streak tissues were fixed at 48 hours in half-strength Karnovsky's fixative, embedded in Araldite and 2 μm sections cut and stained with toluidine blue.

In situ hybridizations were performed as previously described (Dollé and Duboule, 1989) with 35S-labelled riboprobes specific for either mouse (Dollé et al., 1988; Izpisúa-Belmonte et al., 1991b) or chicken (Izpisúa-Belmonte et al., 1991a) Hox-4 genes.

Results

The effects of grafts on limb pattern

The mouse polarizing region and genital tubercle are known to produce digit duplications in chick wing buds (Tickle et al., 1976; Dollé et al., 1991a). Grafts of mouse embryonic tissue taken from the anterior part of the primitive streak and from the neural tube, which will contain the presumptive floor plate, also have polarizing activity (Table 1). The chick wing has three digits, an anterior digit 2, a middle digit 3 and a posterior digit 4. The additional digits specified by grafts to the anterior margin is a measure of their polarizing activity, with an additional digit 2 being formed in response to a weaker signal than an additional digit 4.

Of the mouse tissues used as grafts, limb polarizing region gives extensive duplications which often include additional digits 3 and 4 (Table 1, Fig. 1). Anterior primitive streak tissue taken from 8-day embryos also has strong polarizing activity and gives additional digit 3s in many cases (Table 1, Fig. 1) whereas neural tube from 8 1/2-day embryos and the tip of the genital tubercle have weaker polarizing activities and can specify only an additional digit 2.

Expression of Hox-4 genes in chick limb bud cells in response to grafts of mouse signalling regions

Grafts of mouse polarizing region and anterior primitive streak tissue that give additional posterior digits, activate the most 5'-located members of the HOX-4 complex, including the last one, Hox-4.8, in anterior chick cells (Table 1). The spatial pattern of Hox-4 transcript domains in the limb buds correlates strictly with the subsequent digit pattern. For example, in a wing bud to which anterior primitive streak had been grafted, the new domains of expression of 5'-located members of the Hox-4 complex are not at the anterior margin but displaced more posteriorly towards the apex where the graft had ended up (Fig. 1). This fits a
Hox-4 expression in signalling and responding cells

- PRIMITIVE STREAK
- NEURAL TUBE
- LIMB POLARIZING REGION
- GENITAL TUBERCLE
- ANTERIOR MESENCHYME

1. chicken
2. mouse
3. chicken
4. mouse
5. chicken

-4.7*/4.8
-4.6*/4.7*
Table 1. HOX-4 expression in grafted and host tissues and the final pattern of host wing digits

<table>
<thead>
<tr>
<th>Tissue grafted</th>
<th>Time at which expression analyzed (hours)</th>
<th>Expression in GRAFT</th>
<th>Expression in HOST</th>
<th>Digit pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-4.5</td>
<td>-4.6</td>
<td>-4.7</td>
<td>-4.8</td>
</tr>
<tr>
<td>(A) Mouse tissues placed anteriorly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>17</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Primitive streak</td>
<td>24</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tip of anterior notochord</td>
<td>48 (2)</td>
<td>+ (+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Neural tube</td>
<td>24 (2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Polarizing region</td>
<td>24 (2)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Genital tubercle</td>
<td>48</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>72 (3)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>(B) Mouse tissues placed posteriorly</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior limb</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>20</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>24</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>41</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Anterior primitive streak</td>
<td>24 (2)</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Number in brackets = number of cases where n>1. Blip is small outgrowth containing cartilage but not sufficiently well-developed to be classified as additional digit.

subsequent digit pattern such as 23234, which frequently results.

With both polarizing region and primitive streak cells, expression of genes such as Hox-4.7 and Hox-4.8 is induced in chick cells distal to the graft to produce discrete new domains beneath the apical ridge, which are similar to the ectopic domains produced by beads soaked in retinoic acid (Izpisúa-Belmonte et al., 1991a). The new domains are induced between 24 and 41 hours after grafting. At 24 hours, there is no visible activation of the extreme 'posterior' genes with mouse polarizing region grafts whereas Hox-4.5 to Hox-4.7 are already switched on with streak tissue grafts. To gain further information about the time course of activation of Hox-4 genes by signalling tissues, we also carried out grafts with chick polarizing region cells which more readily heal into the wing bud than the mouse tissue. At 24 hours, the graft could still be distinguished from the host bud and Hox-4.7 was now clearly activated in the responding tissue (Fig. 2).

In contrast to the complete activation of the HOX-4 complex with grafts of polarizing region and anterior primitive streak tissue, genes such as Hox-4.8, Hox-4.7 and Hox-4.6 are not generally expressed in the responding tissue following grafts of neural tube and genital tubercle that lead to the formation of an additional 2. In only one limb (1/5 cases) with a neural tube graft, small ectopic domains of Hox-4.6 and Hox-4.7 could just be detected (Fig. 1; Table 1). With genital tubercle grafts, there is no distinguishable change in Hox-4 gene expression domains in the chick wing bud even after 72 hours (Fig. 1; Table 1). A small number of grafts were also made with tissue from the very anterior part of 8 day embryos which includes the tip of the anterior notochord and neural folds. None of these specified additional digits or had any effect on Hox-4 gene expression in the wing.

Expression of mouse Hox-4 genes in grafted signalling regions

Both the mouse polarizing region and genital tubercle express strongly 5'-members of the HOX-4 complex and this expression pattern was maintained when the tissue was grafted to the anterior margin of the limb bud. Hox-4.6, Hox-4.7 and Hox-4.8 are expressed in mouse polarizing region cells for at least 41 hours after grafting. At 41 hours the tissue is still near the tip of the wing bud and Hox-4.8 is expressed throughout the graft. The graft is now almost entirely surrounded by anterior cells in which expression of 5' genes have been activated. Later on, when the cartilage of the extra digits begins to differentiate, the grafted mouse cells are found in soft tissues at the base of the digits and along the anterior margin of the hand-plate (Fig. 3A). Hox-4 gene expression is also stable in transplanted genital tubercles and the graft continues to express strongly 5'
genes even after 72 hours despite the absence of expression of 5' genes in the adjacent wing mesenchyme (Fig. 1).

The anterior primitive streak and neural tube grafts were taken from parts of the mouse embryo that do not express the 'posterior' genes of the complex. Rather unexpectedly, we found that these genes are subsequently activated in the grafts of the primitive streak although this occurs later than the time at which the same genes are also activated in the responding chick tissue (Fig. 1; Table 1).

At 48 hours, cells in grafts of mouse anterior primitive streak express 'posterior' genes (Fig. 1). Araldite sections show that the primitive streak has developed into neural tissue but has not formed a well-organized tube and, in addition, there are large cavities lined with simple cuboidal epithelium (Fig. 4A,B). In this particular graft, a row of rosette-like structures that resemble somites are also present. In a second specimen, a small 'bud' of limb tissue capped with a thickened apical ridge has grown out anterior to the graft which consists of neurectoderm (Fig. 4C,D).

At 48 hours, grafts of mouse neural tube do not express 'posterior' Hox-4 genes (Fig. 1). Araldite sections show that the neural tube has closed and maintained its form (Fig. 5A,B). Mitoses in nuclei near the lumen indicate that the characteristic interkinetic migration occurs. Cells have also migrated from the
graft to form spinal ganglia. Fig. 5C,D shows outgrowth of the host bud adjacent to the graft but no thickened epithelium could be detected.

Expression of Hox-4 genes in grafts of mouse cells in response to endogenous signals in the chick limb bud

Anterior tissue from a mouse limb bud was grafted to the posterior margin of a chick wing bud to monitor the response of cells to the normal polarizing region. In the 1/9 wings that were left to develop for a further 6 days, a small structure developed at the posterior of the handplate, which appears to be a mouse digit. In most of the other wings, there is a normal pattern of digits (Table 1) and the mouse tissue appears to integrate into the limb resulting in only slight disturbances to the form of the cartilage elements. The mouse cells are typically found distally in soft tissues extending from the wrist region and running between digits 3 and 4 (Fig. 3 C,D). 5'-located Hox-4 genes are rapidly activated in the grafted mouse cells. Although at 10 hours, none of the 5'-located genes are expressed, at 16 hours, all members of the complex are now expressed in the graft (Table 1). It is also striking that the pattern of mouse Hox-4 gene expression in grafts of anterior cells is in register with the pattern of expression of chick Hox-4 genes in the host wing bud. When the graft is totally enclosed within a host transcript domain, the cells in the graft express the same combination of mouse genes as the host tissue throughout. When the graft straddles a host transcript domain boundary, the appropriate cognate mouse Hox-4 genes are expressed in each part of the graft to match the pattern of chick Hox-4 genes in the surrounding host tissue.

The expression of Hox-4 genes in tissue grafted posteriorly appears to provide an assay for the responsiveness of cells to endogenous signals in the limb bud. Therefore, tissue from the anterior primitive streak of mouse embryos was grafted to this position. At 24 hours, Hox-4.6 is activated in the grafted streak tissue. The activation of this gene therefore occurs more rapidly than in grafts placed at the anterior of the bud.

Fig. 4. Anterior primitive streak from mouse embryos with 3-6 somites grafted into chick wing buds. Buds fixed 48 hours after grafting mouse tissues at the anterior margin of stage 20 buds. (A) Low-power view of graft at anterior of wing bud. (B) High-power of part of A showing neural tissue (n), cavity lined with cuboidal epithelium (C) and rosette-like structures below (arrowed). (C) Low-power view of a second graft. (D) High-power view of limb outgrowth anterior to graft showing thickened ridge-like epithelium.
Discussion

Mouse tissue from a number of different regions of the embryo has polarizing activity. Anterior primitive streak and neural tube of mice lead to changes in chick wing pattern and thus behave similarly to the same tissues from chick embryos. This reinforces the idea that the signals in both mouse and chick development have a common basis. However, a comparison of the effectiveness in respecifying anterior cells to form posterior structures between chick and mouse tissues shows that mouse tissues generally have weaker activity.

The pattern of Hox-4 gene expression in chick limb buds induced by different signalling regions correlates with the pattern of digits that will develop and provides further support for the idea that Hox-4 genes encode position in the limb. When cells are respecified to form posterior structures such as digits 3 and 4, 5'-located members of the complex (the 'posterior' genes) are expressed locally. An additional digit 2 can apparently form in the absence of activation of these genes. It may be that an additional digit 2 can form almost by default if the bud widens sufficiently to allow an extra digit to be fitted into the handplate (Wolpert and Stein, 1984).

The ability of both polarizing region and anterior primitive streak to produce activation of the same Hox-4 genes in wing buds points to a striking uniformity in the response mechanism. This similar activation of Hox-4 genes in the chick limb by tissues from different parts of the embryo could be brought about in different ways. The polarizing region and primitive streak could produce different signals that independently activate 5' members of the complex (e.g. Hox-4.7 or Hox-4.8). A second possibility is that the signalling regions all produce the same signal. The final possibility is that the signalling regions produce different signals that form part of a common pathway. Retinoic acid activates expression of Hox-4 genes in anterior limb bud cells and is a good candidate for the signal produced by the polarizing region (Thaller and Eichele, 1987). Cells of the floor plate of the neural tube can produce retinoic acid (Wagner et al., 1990) but it is not known whether the primitive streak has this property. The primitive streak, which, like the polarizing region, activates 5' genes in the wing bud, could act in a common pathway

Fig. 5. Neural tube from mouse embryos with 3-6 somites grafted to chick wing buds. (A) Low-power view of graft showing neural tube and ganglia (g). High-power view of part of neural tube graft showing cells in mitosis at luminal surface. (C) Low-power view of a second graft. (D) High-power view of epithelium of the chick limb anterior to graft. No ridge-like structure was found.
either leading to the generation of retinoic acid or bypassing this step.

We compared the time course of activation of Hox-4 genes in anterior limb cells by grafts of signalling regions with activation brought about by retinoic acid application. With mouse tissues, the timing of induction of expression is somewhat variable and it is not clear why activation of Hox-4 genes occurs after a shorter time interval with mouse anterior primitive streak than with mouse polarizing region. However, our results suggest that signalling cells may act more rapidly than retinoic acid in inducing Hox genes since Hox-4.6 is activated before 16 hours by chick polarizing region grafts whereas with retinoic acid, expression of this gene is not detected before 20 hours (Izpisua-Belmonte et al., 1991a). In addition, activation of all the Hox-4 genes occurs in anterior limb cells before 16 hours when these cells are placed posteriorly next to polarizing region tissue. The more rapid action of polarizing cells suggests that they may produce a second factor that potentiates or cooperates with retinoic acid in ectopic activation of Hox-4 genes. It is also possible that retinoic acid may induce signalling cells (Wanek et al., 1991; Noji et al., 1991).

The expression of 5' members of the HOX-4 complex in signalling tissue is not correlated with its polarizing activity. Although the genital tubercle and the polarizing region both express these genes and this expression is maintained in the grafts, they differ considerably in their ability to specify additional digits and activate Hox-4 genes in the limb. The relatively small effect of the genital tubercle is difficult to reconcile with a model where disparities in Hox-4 gene expression would drive local interactions that intercalate cells expressing the intervening members of the complex. The maintenance of expression of the mouse Hox-4 genes in genital tubercle grafts, in the absence of activation of the corresponding chicken genes in the adjacent wing tissue, shows that, once these genes have been activated, their expression is independent of local conditions and appears to involve a ratchet-like mechanism. This contrasts with the lability of expression of two other homeobox genes, Hox-7.1 and Hox-8.1 which appear to be involved in epithelial-mesenchymal interactions that lead to limb bud outgrowth (Davidson et al., 1991).

Anterior primitive streak tissues used for grafts do not express the 5'-located members of the complex (e.g. Hox-4.8 to Hox-4.4) but nevertheless induce expression of these genes in the limb bud tissue. It is interesting that, later on, transcripts encoded by the mouse Hox-4.6 to Hox-4.8 genes can be detected in the grafted cells. In addition, when anterior primitive streak tissue is placed posteriorly next to the polarizing region of the bud, there is also activation of 5'-located genes in the graft and this activation is more rapid than in grafts placed anteriorly. This suggests that the cells of anterior primitive streak, even though they will give rise to the anterior part of the embryo which does not express Hox-4 genes, are still able to respond if exposed to appropriate signals. The activation of Hox-4 genes by different signalling regions and in different tissues reinforces the idea that similar signalling and response mechanisms lead to specification of position along different axes of the body.

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References


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