INTRODUCTION

In the vertebrate limb, a complex network of signalling molecules mediates patterning and outgrowth. It is important to identify the components of signalling pathways, to understand how signals control pattern and growth along the proximo-distal, antero-posterior and dorso-ventral axes of the developing limb, and to determine how individual signalling pathways are coordinated. Pattern across the antero-posterior axis is specified by a group of cells in the posterior mesenchyme of the limb bud, called the polarizing region. Mirror-image digit duplications are produced when polarizing region cells are grafted to the anterior margin of the bud (Saunders and Gasseling, 1968; Tickle et al., 1975). Anterior application of retinoic acid mimics this effect on digit pattern (Tickle et al., 1982), and is believed to induce anterior mesenchyme cells to produce a cascade of polarizing signals (Wanek et al., 1991; Noji et al., 1991). Transcripts of the Sonic hedgehog (Shh) gene are expressed in the polarizing region and are activated in anterior mesenchyme by application of retinoic acid (Riddle et al., 1993). Moreover, both SHH-expressing cells and beads soaked in SHH induce digit duplications when grafted anteriorly (Riddle et al., 1993; López-Martínez et al., 1995). Transcripts of the bone morphogenetic protein-2 (Bmp-2) gene and of members of the HoxD complex of homeobox-containing genes are also expressed in posterior limb bud mesenchyme, and are activated in anterior mesenchyme by application of retinoic acid, polarizing region grafts or SHH (Francis et al., 1994; Izpisúa-Belmonte et al., 1991; Laufer et al., 1994).

The polarizing region and a region of undifferentiated, proliferating mesenchyme cells at the tip of the bud, known as the progress zone, are maintained by signals from the apical ectodermal ridge (reviewed by Tickle, 1995). Fibrobast growth factor (FGF) family members can substitute for ridge signals. FGF-4, expressed in the posterior part of the ridge, is probably the ridge signal which maintains the polarizing activity of the posterior mesenchyme in vivo (Niswander et al., 1993). FGF-8 and/or FGF-2 could be the ridge signal which maintains the undifferentiated cells of the progress zone (Crossley and Martin, 1995; Mahmood et al., 1995). A reciprocal signal from the mesenchyme maintains the ridge, but its identity is not known. Here, we investigate the position of BMP-2 in the polarizing region signalling pathway by expressing BMP-2 in the anterior part of the apical ectodermal ridge, followed later by ectopic expression of Hoxd-11 and Hoxd-13 in anterior mesenchyme. This suggests that BMP-2 is involved in regulating Fgf-4 and HoxD gene expression in the normal limb bud. Ectopically expressed hBMP-2 also induced duplication of digit 2 and bifurcation of digit 3, but could not produce the mirror-image digit duplications obtained with SHH-expressing cells. These results suggest that BMP-2 may be involved primarily in maintenance of the ridge, and in the link between patterning and outgrowth of the limb bud.

Key words: chick, limb, polarizing pathway, BMPx2, Fgf-4, Hoxd-13, Shh

SUMMARY

Bone morphogenetic protein-2 (BMP-2) has been implicated in the polarizing region signalling pathway, which specifies pattern across the antero-posterior of the developing vertebrate limb. Retinoic acid and Sonic Hedgehog (SHH) can act as polarizing signals; when applied anteriorly in the limb bud, they induce mirror-image digit duplications and ectopic Bmp-2 expression in anterior mesenchyme. In addition, the two signals can activate Fgf-4 expression in anterior ridge and HoxD expression in anterior mesenchyme. We tested the role of BMP-2 in this signalling cascade by ectopically expressing human BMP-2 (hBMP-2) at the anterior margin of the early wing bud using a replication defective retroviral vector, and found that ectopic expression of Fgf-4 was induced in the anterior part of the apical ectodermal ridge, followed later by ectopic expression of Hoxd-11 and Hoxd-13 in anterior mesenchyme. This suggests that BMP-2 is involved in regulating Fgf-4 and HoxD gene expression in the normal limb bud. Ectopically expressed hBMP-2 also induced duplication of digit 2 and bifurcation of digit 3, but could not produce the mirror-image digit duplications obtained with SHH-expressing cells. These results suggest that BMP-2 may be involved primarily in maintenance of the ridge, and in the link between patterning and outgrowth of the limb bud.

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The polarizing region and a region of undifferentiated, proliferating mesenchyme cells at the tip of the bud, known as the progress zone, are maintained by signals from the apical ectodermal ridge (reviewed by Tickle, 1995). Fibrobast growth factor (FGF) family members can substitute for ridge signals. FGF-4, expressed in the posterior part of the ridge, is probably the ridge signal which maintains the polarizing activity of the posterior mesenchyme in vivo (Niswander et al., 1993). FGF-8 and/or FGF-2 could be the ridge signal which maintains the undifferentiated cells of the progress zone (Crossley and Martin, 1995; Mahmood et al., 1995). A reciprocal signal from the mesenchyme maintains the ridge, but its identity is not known. Here, we investigate the position of BMP-2 in the polarizing region signalling pathway by expressing BMP-2 in the anterior part of the limb bud.

To understand these signalling pathways we need to work out how interactions of genes are organised, upstream and downstream of SHH, and how signalling pathways are integrated. It is known that FGF-4 from posterior ridge cooperates

Activation of Fgf-4 and HoxD gene expression by BMP-2 expressing cells in the developing chick limb

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with WNT-7a, a signal from dorsal ectoderm, to maintain Shh expression in the polarizing region (Niswander et al., 1994; Laufer et al., 1994; Yang and Niswander, 1995), and that SHH maintains Fgf-4 in posterior ridge expression via a feedback loop (Niswander et al., 1994; Laufer et al., 1994). SHH also induces expression of Bmp-2 and HoxD genes (Laufer et al., 1994), but the mechanism and significance of Bmp-2 induction is not known. It is striking that expression domains of Bmp-2, Hoxd-13 and Fgf-4 always appear to be closely linked to each other in the limb. For example, in normal chick limb buds, the mesenchymal expression domain of Bmp-2 is similar to that of Hoxd-13 (Francis et al., 1994) and these domains are close to the posterior ridge expression domain of Fgf-4 (Niswander et al., 1994). When anterior cells are respecified to form posterior structures, ectopic expression of Bmp-2 and Hoxd-13 is activated in similar regions of anterior mesenchyme (Izpisúa-Belmonte et al., 1991; Francis et al., 1994; Laufer et al., 1994), and ectopic expression of Fgf-4 is activated in the anterior ridge (Laufer et al., 1994; Niswander et al., 1994).

To further understand the link between Bmp-2, Fgf-4 and HoxD gene expression, we expressed BMP-2 ectopically in anterior mesenchyme of the early limb bud and monitored changes in expression of Fgf-4, Hoxd-11, Hoxd-13 and Shh. The results show that BMP-2 can activate ectopic expression of Fgf-4 in anterior ridge and of Hoxd-11 and Hoxd-13 in anterior mesenchyme. In addition, ectopically expressed BMP-2 induced duplication and bifurcation of digits.

**MATERIALS AND METHODS**

**Chick embryos**
Fertilized White Leghorn chicken eggs were obtained from Poyndon Farm, Waltham Cross, Hertfordshire, UK. Fertilized 0 line and C line chicken eggs were obtained from the BBRC Poultry Research Station, Compton, Berkshire, UK. Eggs were incubated at 38±1°C and staged according to the method of Hamburger and Hamilton (1951).

**Construction of a recombinant retroviral plasmid encoding human BMP-2**
The complete human BMP-2 (hBMP-2) coding region, and 40 nucleotides of 3′ non-coding region were excised from plasmid PS P65 (Wozney et al., 1988) by digestion with SalI, and inserted into the SalI site of adaptor plasmid Cla12 (Hughes et al., 1987). The inserted sequences were then excised by digestion with Clal and inserted into the Clal site of the replication defective retroviral vector pCRNCM (de la Pompa and Zeller, 1993). Clones containing hBMP-2 coding sequences in the sense (hBMP-2/pCRNCM) or antisense (Control/pCRNCM) orientation were selected, the latter for use as a negative control. The structure of hBMP-2/pCRNCM is shown in Fig. 2A.

**Production of BMP-2-expressing cell lines**
Infectious hBMP-2/pCRNCM and Control/pCRNCM viruses were produced in the quail packaging cell line Q2bn (Stoker and Bissel, 1988). Q2bn cells were grown in 40% (v/v) Dulbecco’s modified Eagle’s medium (DMEM), 40% (v/v) Ham’s F12 medium, 8% (v/v) fetal calf serum, 2% (v/v) chicken serum and were transfected with 10 μg of hBMP-2/pCRNCM or Control/pCRNCM DNA using Lipofectin as described by the manufacturer (Gibco/BRL). Stable transfectants were selected using 800 μg ml−1 G418 and cloned as described by de la Pompa and Zeller (1993). The titre of virus particles produced by each selected clone was determined as described by de la Pompa and Zeller (1993). The hBMP-2/pCRNCM clone that produced the highest titres of retrovirus particles (approximately 10^5 ml^{-1}) were chosen for further work.

**Micromass culture of limb bud mesenchyme**
Whole limb buds were dissected from stage 21/22 White Leghorn chick embryos. The ectoderm was removed and high density micromass cultures were prepared from mesenchymal cells as described by Ahrens et al. (1977). Cells were plated at 2×10^7 ml^{-1} in Ham’s F12 medium containing 10% (v/v) fetal calf serum and cultured overnight. The cultures were then supplemented with supernatant from hBMP-2/pCRNCM- or Control/pCRNCM-transfected Q2bn cells. After two more days of incubation, the micromass cultures were fixed in 2% (w/v) paraformaldehyde for 15 minutes and stained for cartilage with Alcian blue.

**Grafting of BMP-2-expressing cells into chick limb buds**
Spherical cell aggregates were prepared as described by de la Pompa and Zeller (1993). hBMP-2/pCRNCM- or Control/pCRNCM-transfected Q2bn cells were grown to approximately 90% confluence in 90 mm culture dishes and were trypsinized, seeded into 90 mm bacteriological Petri dishes and incubated overnight in 40% (v/v) DMEM, 40% (v/v) Ham’s F12 medium, 8% (v/v) fetal calf serum, 2% (v/v) chicken serum, without G418. The cells formed small spherical aggregates of different sizes which were then selected under the microscope. Unless otherwise stated, aggregates with a diameter of approximately 100 μm, containing 1,600±100 cells, were grafted into stage 19-20 White Leghorn or C line chick wing buds. In some experiments large aggregates with a diameter of approximately 400 μm, containing 12,000±300 cells, were used. In other experiments, cells were pelleted by centrifugation as described by Riddle et al. (1993) and then grafted. For examination of skeletal pattern, embryos were harvested on embryonic day 10, fixed in 5% (w/v) trichloroacetic acid overnight, stained for cartilage with alcian green and cleared in methyl salicylate (Tickle et al., 1982). Other embryos were harvested 24, 40 and 48 hours after grafting, fixed overnight in 4% (w/v) paraformaldehyde and processed for whole mount in situ hybridization as previously described (Francis et al., 1994).

**Grafting of beads soaked in FGF-4 to the anterior margin of chick wing buds**
Beads were soaked in FGF-4 as described by Niswander et al. (1993) and grafted to the anterior margin of stage 19-20 chick wing buds.

**In situ hybridization to whole mounts**
In situ hybridization of digoxigenin-labelled RNA probes to whole mounts was performed as previously described (Francis et al., 1994). Probes specific for chicken Bmp-2 (Francis et al., 1994), human Bmp-2 (Wozney et al., 1988), chicken Hoxd-11 and Hoxd-13 (Izpisúa-Belmonte et al., 1991), chicken Fgf-4 (Niswander et al., 1994) or chicken Shh and Bmp-7 (Francis-West et al., 1995) transcripts were prepared as described by the authors cited.

**Immunohistochemistry**
Cloned cell lines that had been stably transfected with hBMP-2/pCRNCM or Control/pCRNCM were grown to 70% confluence and fixed with 2% (w/v) paraformaldehyde at room temperature for 15 minutes. Immunohistochemistry was performed using the Vectastain kit (Vector Labs), following the manufacturer’s instructions. The primary antibody, a monoclonal antibody against human BMP-2 (Genetics Institute, Cambridge MA), was used at a dilution of 1/300 in PBS.

**RESULTS**

**Expression of Shh, Bmp-2, Fgf-4 and Hoxd-13 genes in stage 20 chick limb buds**
To compare the endogenous Bmp-2 expression domain with...
BMP-2 and limb development

Bmp-2 expression was found in posterior mesenchyme and throughout the apical ridge (Fig. 1A; Francis et al., 1994). The Shh domain lay within the mesenchymal Bmp-2 domain (Fig. 1B; Laufer et al., 1994), which in turn lay within the Hoxd-13 domain (Fig. 1C; Francis et al., 1994; Laufer et al., 1994). Fgf-4 was expressed in posterior ridge (Fig. 1D; Niswander et al., 1994), in a region overlying the Hoxd-13 domain and the distal part of the mesenchymal Bmp-2 domain, but lying distal and anterior to the Shh domain (Fig. 1A, 1D). The positions of these domains are summarised in Fig. 1E.

At stage 26, Shh (see Fig. 5C) and Bmp-2 (Francis et al., 1994) transcripts remained localised to posterior mesenchyme of the bud, with the Shh domain lying wholly within the Bmp-2 domain. Hoxd-13 transcripts were no longer restricted to posterior mesenchyme, but were found in mesenchyme right across the distal tip of the bud (see Fig. 5A). Fgf-4 expression was found in the apical ridge, with levels being high in posterior ridge and fading anteriorly (see Fig. 4D). The positions of these domains are summarised in Fig. 1F.

Ectopic expression of human BMP-2 in chick wing buds

The human BMP-2 (hBMP-2) coding region was inserted into the replication defective retroviral vector pCRNCM, yielding plasmids hBMP-2/pCRNCM and Control/pCRNCM (Fig. 2A), which were stably transfected into Q2bn cells. Immunocytochemistry with a monoclonal antibody against hBMP-2 showed that hBMP-2/pCRNCM-transfected cells synthesized hBMP-2 (Fig. 2C), while Control/pCRNCM-transfected cells did not (Fig. 2B). In addition, supernatant from hBMP-2/pCRNCM-transfected cells stimulated a marked increase in cartilage formation when added to micromass cultures of stage 21/22 chick wing bud mesenchyme cells, compared to supernatant from Control/pCRNCM-transfected Q2bn cells (data not shown). This shows that hBMP-2/pCRNCM-transfected cells secrete hBMP-2 protein that is biologically active on chicken cells (Roark and Greer, 1994).

Aggregates of hBMP-2/pCRNCM-transfected cells were grafted to the anterior margin of stage 19/20 White Leghorn wing buds. The distribution of virus was monitored by in situ hybridisation to whole mount preparations, using a species-specific probe for hBmp-2 transcripts that hybridizes to viral...
genomic RNA transcribed from the viral LTR and to hBmp-2 mRNA transcribed from the CMV promoter (Fig. 2A). Transcripts were restricted to the anterior margin of the bud (Fig. 3), in both grafted cells and immediately adjacent host mesenchyme, at both 24 hours (n=6, Fig. 3A) and 48 hours (n=5, Fig. 3B) after grafting. This indicates that hBMP-2/pCRNCM acts as expected for a replication-defective retrovirus and is transcribed locally at the anterior of the bud, although the levels of hBMP-2 produced by different grafts may vary.

**Effects of anterior BMP-2 expression on gene expression in wing buds**

Anterior expression of BMP-2 induced expression of Fgf-4 in anterior apical ectodermal ridge and of Hoxd-11 and Hoxd-13 in anterior wing bud mesenchyme. Twenty-four hours after grafting hBMP-2/pCRNCM-transfected Q2bn cells to the anterior margin of the bud, strong ectopic expression of Fgf-4 was visible in the anterior ridge (Fig. 4A; Table 1), whereas in normal wing buds (Fig. 4A), and in buds grafted with Control/pCRNCM-transfected cells (Fig. 4B; n=5), the domain of Fgf-4 expression faded anteriorly. In contrast, the grafts had no effect on Hoxd-13 expression at this time (Table 1).

After 40 or 48 hours, ectopic expression of Fgf-4 was still clearly visible in the anterior ridge of wing buds grafted with hBMP-2/pCRNCM-transfected cells, but was barely detectable in posterior ridge (Fig. 4C; Table 1). In normal wing buds, Fgf-4 expression was restricted to posterior ridge (Fig. 4D; Table 1). At this time, ectopic expression of Hoxd-13 (Fig. 5A; Table 1) and Hoxd-11 (Fig. 5B; Table 1) was also induced by grafts of hBMP-2/pCRNCM-transfected cells, whereas grafts of Control/pCRNCM-transfected cells had no effect on Hoxd-13 or Hoxd-11 expression (n=3). Ectopic Hoxd-11 and Hoxd-13 expression was found in discrete domains in anterior mesenchyme immediately adjacent and distal to the graft. In some cases, where grafts were placed closer to the tip of the bud than as shown in Fig. 5A, the ectopic Hoxd-13 domain merged with the anterior margin of the endogenous Hoxd-13 domain (n=2; data not shown).

Since BMP-2 induced ectopic Fgf-4 expression after 24 hours and ectopic Hoxd-13 expression after 40 hours, it is possible that FGF-4 mediates induction of Hoxd-13 by BMP-2. However, when we grafted beads soaked in FGF-4 to the anterior margin of the bud, we found no evidence of Hoxd-13 activation (n=4), suggesting that BMP-2 acts together with FGF-4 to induce Hoxd-13 in anterior distal mesenchyme.

Previous work has shown that SHH induces Hoxd-13 in anterior mesenchyme and Fgf-4 in anterior ridge. To determine whether ectopic expression of BMP-2 induces Shh expression, we grafted hBMP-2/pCRNCM-transfected Q2bn cells to the anterior margin of the bud and analyzed Shh expression. Shh transcripts were not detectable in grafted cells or in adjacent wing bud mesenchyme, at either 24 or 48 hours after grafting (Fig. 5C; Table 1), and neither were chicken Bmp-2 or Bmp-7 transcripts (data not shown).

**Effects of anterior BMP-2 expression on digit pattern**

Grafting of hBMP-2/pCRNCM-transfected cells to the anterior margin of the wing bud led to a change in bud shape, which was visible 40 and 48 hours after grafting and involved anterior extension of the bud (compare Fig. 4C and D; Fig. 5). We therefore examined skeletons of wings that developed 7 days after grafting. In 28 out of 45 cases (62%), extra digital elements were seen (Fig. 6; Table 2). In 14 of these cases there was an additional digit 2 (Fig. 6A), and 13 cases there was bifurcation of digit 3 and loss of digit 2 (Fig. 6B). In one case there was bifurcation of digit 3 without loss of digit 2 (Fig. 6C). Bifurcation of digit 3 was more often observed when grafts were positioned closer to the tip of the bud, rather than closer to the body wall.

To see whether increasing the number of cells in the graft would increase the extent of digit duplication, we grafted 400

### Table 1. Activation of ectopic gene expression following grafting of hBMP-2/pCRNCM transfected Q2bn cells to the anterior margin of the wing bud

<table>
<thead>
<tr>
<th>Gene transcripts</th>
<th>24 hours</th>
<th>40 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoxd-11</td>
<td>n.d.</td>
<td>n.d.</td>
<td>+ (3/4)</td>
</tr>
<tr>
<td>Hoxd-13</td>
<td>− (6/6)</td>
<td>+ (1/3)</td>
<td>+ (3/5)</td>
</tr>
<tr>
<td>Shh</td>
<td>− (2/2)</td>
<td>n.d.</td>
<td>− (6/6)</td>
</tr>
<tr>
<td>Fgf-4</td>
<td>+ (3/5)</td>
<td>+ (1/2)</td>
<td>+ (3/5)</td>
</tr>
</tbody>
</table>

Numbers in brackets, number of cases giving the result out of number of embryos examined. +, activation of ectopic expression; −, no detectable expression; n.d., not determined.

### Table 2. Phenotypes of the wings grafted at stage 19/20 with Q2bn transfected cells with hBMP-2/pCRNCM or Control/pCRNCM

<table>
<thead>
<tr>
<th>Virus</th>
<th>Position of the graft</th>
<th>Size of the graft (µm)</th>
<th>Number of cases</th>
<th>Digit pattern</th>
<th>Other pattern abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td>hBmp-2/pCRNCM</td>
<td>Anterior</td>
<td>100</td>
<td>45</td>
<td>17 14 14* 0</td>
<td>−</td>
</tr>
<tr>
<td>hBmp-2/pCRNCM</td>
<td>Anterior</td>
<td>400</td>
<td>10</td>
<td>5 5 0 0</td>
<td>−</td>
</tr>
<tr>
<td>Control/pCRNCM</td>
<td>Anterior</td>
<td>100</td>
<td>15</td>
<td>15 15 15 15</td>
<td>−</td>
</tr>
<tr>
<td>hBmp-2/pCRNCM</td>
<td>Apex</td>
<td>100</td>
<td>13</td>
<td>3 1 3 0</td>
<td>Truncations† (6)</td>
</tr>
<tr>
<td>Control/pCRNCM</td>
<td>Apex</td>
<td>100</td>
<td>10</td>
<td>10 10 10 10</td>
<td>−</td>
</tr>
<tr>
<td>hBmp-2/pCRNCM</td>
<td>Posterior</td>
<td>100</td>
<td>11</td>
<td>1 0 0 0</td>
<td>Truncations‡ (10)</td>
</tr>
<tr>
<td>Control/pCRNCM</td>
<td>Posterior</td>
<td>100</td>
<td>4</td>
<td>4 0 0 0</td>
<td>−</td>
</tr>
</tbody>
</table>

*In 13 of these cases, there was no digit 2 (Fig. 6B). In one case, digit 2 was present (Fig. 6C).
†Truncated limbs always contained the proximal parts of the digits.
‡Truncated limbs contained only a part of the humerus (n=2), the humerus and part of radius/ulna (n=2), or the humerus, radius and ulna and part of the digits (n=6).
μm aggregates which contained approximately seven times as many cells as the 100 μm aggregates. In five out of ten cases, the larger grafts induced duplication of digit 2 similar to that seen with smaller grafts (Table 2). Similar grafts of Control/pCRNCM-transfected cells had no effect on digit development (n=3). We also grafted pellets of cells obtained by centrifugation of sub-confluent cells (Riddle et al., 1993), in case leaving cells to form aggregates led to loss of viability and activity. Again, we saw only formation of an extra digit 2, in 2 out of 6 cases.

When grafts were made to the apex or posterior margin of the wing bud, the wings were frequently truncated (Table 2). Grafts of Control/pCRNCM-transfected cells to the anterior margin, apex or posterior margin of the wing bud had no effect on digit pattern (Table 2).

The effects of hBMP-2/pCRNCM-transfected cells on gene expression and expression pattern could be mediated solely by hBMP-2 secreted by the grafted cells, or could require infection of wing bud mesenchyme cells adjacent to the graft. To distinguish between these possibilities, we grafted hBMP-2/pCRNCM-transfected cells to the anterior margin of C line chick embryo wing buds, which cannot be infected by subgroup A hBMP-2/pCRNCM virus, and monitored digit pattern as a convenient indicator of the activity of grafted cells. Duplication of digit 2 was seen in 4 out of 9 cases, demonstrating that hBMP-2 produced by the grafted cells is sufficient to induce an additional digit 2.

**DISCUSSION**

**BMP-2 and signalling in the limb bud**

Signalling by the polarizing region requires cooperation with signalling by the apical ectodermal ridge. Previous work has shown that both retinoic acid and SHH can act as polarizing signals and cooperate with FGF-4, a ridge signal, to induce Bmp-2 and HoxD gene expression in anterior mesenchyme. SHH-expressing cells can induce Fgf-4 expression in anterior ridge (Laufer et al., 1994). Our current results show that BMP-2-expressing cells also induce Fgf-4 expression in anterior ridge, suggesting that BMP-2 may mediate the induction of Fgf-4 expression by SHH (Fig. 7). Consistent with this, the endogenous expression domain of Fgf-4 is more closely related spatially to the mesenchymal Bmp-2 domain, than to that of Shh (Fig. 1). Fgf-4 expression in posterior ridge was weaker in manipulated wing buds than in normal contralateral buds, but the reason for this is unclear. A similar expression pattern is seen in Rim4 and Strong’s Luxoid mice, which have partial mirror image digit duplications and a domain of Fgf-4 expression that extends more anteriorly than normal, but fades posteriorly (Masuya et al., 1995; Chan et al., 1995).

Bmp-2 transcripts are normally present throughout the ridge, as well as in posterior mesenchyme (Francis et al., 1994). If BMP-2 activates Fgf-4, why is Fgf-4 expression normally restricted to posterior ridge? One possibility is that BMP-2 forms different species of dimers in ridge and mesenchyme, and that only the mesenchymal species regulate Fgf-4. Another possibility is that the concentration of BMP-2 produced by ridge alone is insufficient to activate Fgf-4, and that this activation requires additional BMP-2 produced by posterior mesenchyme. Niswander and Martin (1993) showed that BMP-2 inhibits FGF-4-induced outgrowth of mouse limb buds from which apical ridge has been removed. This may relate to our finding that grafting of hBMP-2/pCRNCM-transfected cells to the distal tip of the bud, immediately beneath the ridge, resulted in limb truncations.

Hoxd-11, Hoxd-12 and Hoxd-13 are activated in two distinct phases during normal chick limb bud outgrowth (Morgan and Tabin, 1994; Laufer et al., 1994). First, Hoxd-11, Hoxd-12 and Hoxd-13 are activated in response to SHH, in a nested set of domains in posterior mesenchyme. Then, the three genes become expressed in approximately the same region of distal mesenchyme, whilst posterior expression fades. It has been shown previously that retinoic acid, polarizing region grafts and SHH-expressing cells activate Bmp-2 and HoxD expression anteriorly within 24 hours (Izpisúa-Belmonte et al., 1991; Riddle et al., 1993; Francis et al., 1994; Laufer et al., 1994), with the HoxD genes being activated in nested domains corresponding to the early phase of posterior HoxD expression (Izpisúa-Belmonte et al., 1991). In contrast, we have shown here that BMP-2 requires 40 hours to activate Hoxd-13 anteriorly. This suggests that BMP-2 plays no part in regulating the early phase of HoxD expression but, rather, may be involved in regulating the later phase (Fig. 7). Most interestingly, Luo et al. (1995) have recently shown that limb buds of Bmp-7 null mice have a normal Shh domain and a posterior Hoxd-13 domain, but that Hoxd-13 expression was much reduced in the distal domain. We have previously shown that Bmp-7 is co-expressed with Bmp-2 in normal limb buds and is activated anteriorly by retinoic acid (Francis-West et al., 1995). Taken together, these data suggest that BMP-2 and BMP-7 may work together to control distal HoxD gene expression in late buds (Fig. 7).

Since grafts of BMP-2-expressing cells activated Fgf-4 before Hoxd-13, it is possible that FGF-4 itself is responsible for late activation of Hoxd-13. However, FGF-4-soaked beads did not activate Hoxd-13, suggesting that BMP-2 and FGF-4 may work together to induce HoxD expression (Fig. 7). A similar result is obtained when FGF-4 is applied in the absence of the apical ridge (Laufer et al., 1994; Niswander et al., 1994). This close relationship between Bmp-2, Fgf-4 and HoxD expression is seen in early talpid3 (ta3) mutant limb buds, where posterior HoxD genes and Bmp-2 are expressed uniformly throughout distal limb bud mesenchyme, and Fgf-4 is expressed throughout the apical ridge, instead of being posteriorly restricted as in normal limb buds (Francis-West et al., 1995). Recombination experiments have shown that the defect in ta3 limb patterning lies with mesenchyme and not with ridge (Ede, 1980). One possibility is that anarchic expression of BMP-2 in ta3 mesenchyme induces FGF-4 expression throughout the ridge, and that BMP-2 and FGF-4 then induce Hoxd-13 expression throughout distal mesenchyme. All of this occurs independently of SHH, which remains restricted to posterior mesenchyme in ta3 buds, as in normal buds (Francis-West et al., 1995). Some similar features are also found in limb buds that develop from reaggregated anterior leg bud mesenchyme (Hardy et al., 1995), where Bmp-2 and Hoxd-13 expression are found together, and the apical ridge is maintained in the absence of Shh expression.

While the simplest explanation of these data is that hBMP-2 produced by grafted cells is responsible for inducing Fgf-4 and HoxD expression in host buds, it is possible that another
factor produced specifically by hBMP-2/pCRNCM-transfected Q2bn cells, and perhaps induced by hBMP-2, is responsible. Our results show that this putative factor is not likely to be BMP-7 or SHH.

**Induction of digit duplication and bifurcation by BMP-2**

Local mis-expression of BMP-2 at the anterior margin of the chick wing bud induced an additional digit 2 or bifurcation of digit 3, but never resulted in mirror image digit duplications. Previous work with BMP-2-soaked beads also failed to give digit duplications (Francis et al., 1994). Therefore BMP-2 alone does not appear to mediate the activity of SHH, which induces mirror image digit duplications (Riddle et al., 1993), even though BMP-2 expression is induced by SHH-expressing cells (Lauffer et al., 1994). It seems unlikely that any other BMP homodimers or heterodimers would give mirror image duplications since grafts of anterior mesenchyme from ta 3 mutant limb buds induce only an additional anterior digit, even though they express Bmp-2, Bmp-4 and Bmp-7 (Francis-West et al., 1995). This contrasts with development of the *Drosophila* wing imaginal disc, where patterning by *Hedgehog*, which is the *Drosophila* homologue of *Shh*, appears to be mediated entirely through activation of Dpp, which is the homologue of BMP-2 and BMP-4 (Heberlein et al., 1993; Basler and Struhl, 1994; Felsenfeld and Kennison, 1995).

Induction of extra digital elements by hBMP-2 could result from its activation of FGF-4 and/or HoxD expression. This seems more likely than the possibility that hBMP-2 produced by grafted cells has a late chondrogenic effect, since grafted cells remain in the proximal part of the bud during outgrowth, far from the site of digit formation. FGF-4 expression in anterior ridge might stimulate sufficient growth in underlying mesenchyme.
and, presumably, (Riley et al., 1993). Alternatively, induction of anterior wing bud induced the appearance of an extra digit 2 elements. In previous work, ectopic expression of FGF-2 in terning. rather reflect the role of BMP-2 in linking growth and pattering. The changes in the positional identity of wing bud cells, but is present in C. 2, 3, 4 = digits 2, 3 and 4; b3 = bifurcated digit 3.

Fig. 6. Effects on digit pattern of grafting hBmp-2/pCRNCM-infected Q2bn cells to the anterior margin of stage 19/20 wing buds. Whole mount preparatons were stained with alcian green on embryonic day 10. Three grafted wings (A,B,C) and one normal contralateral wing from the same embryo as C (D) are shown. In A there is a duplication of digit 2. In B and C the distal phalanges of digit 3 are forked. Digit 2 is missing from B, but is present in C. 2, 3, 4 = digits 2, 3 and 4; b3 = bifurcated digit 3.

Fig. 7. Proposed model of molecular interactions in the polarizing region signalling pathway. FGF-4 and WNT-7a maintain SHH expression in posterior mesenchyme. SHH activates early posterior HoxD expression, and induces expression of BMP-2. BMP-2 maintains FGF-4 expression in the ridge and, in concert with FGF-4, activates late HoxD gene expression in distal mesenchyme.

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REFERENCES


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