Bone morphogenetic proteins and a signalling pathway that controls patterning in the developing chick limb

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SUMMARY

We show here that bone morphogenetic protein 2 (BMP-2) is involved in patterning the developing chick limb. During early stages of limb development, mesenchymal expression of the Bmp-2 gene is restricted to the posterior part of the bud, in a domain that colocalizes with the polarizing region. The polarizing region is a group of cells at the posterior margin of the limb bud that can respecify the anteroposterior axis of the limb when grafted anteriorly and can activate expression of genes of the HoxD complex. We dissect possible roles of BMP-2 in the polarizing region signalling pathway by manipulating the developing wing bud. Retinoic acid application, which mimics the effects of polarizing region grafts, activates Bmp-2 gene expression in anterior cells. This shows that changes in anteroposterior pattern are correlated with changes in Bmp-2 expression. When polarizing region grafts are placed at the anterior margin of the wing bud, the grafts continue to express the Bmp-2 gene and also activate Bmp-2 expression in the adjacent anterior host mesenchyme. These data suggest that BMP-2 is part of the response pathway to the polarizing signal, rather than being the signal itself. In support of this, BMP-2 protein does not appear to have any detectable polarizing activity when applied to the wing bud. The pattern of Bmp-4 gene expression in the developing wing bud raises the possibility that BMP-2 and BMP-4 could act in concert. There is a close relationship, both temporal and spatial, between the activation of the Bmp-2 and Hoxd-13 genes in response to retinoic acid and polarizing region grafts, suggesting that expression of the two genes might be linked.

Key words: bone morphogenetic protein, Bmp-2, Bmp-4, Hoxd-13, polarizing region, retinoic acid, chick limb bud, limb development

INTRODUCTION

A fundamental problem in embryonic development is how patterns of differentiated cells are established. The complex pattern of skeletal elements found in the adult limb develops from a mass of undifferentiated mesenchymal cells in the limb bud. Patterning in the limb involves signalling between mesenchymal cells and between mesenchyme and epithelium, and some of the molecules involved in these signalling pathways have been identified. For example, certain retinoids and growth factors have been implicated as signalling molecules and a number of homeobox-containing genes have been found to respond to these signals (reviewed by Tabin, 1991; Tickle and Brickell, 1991). Here we present evidence that the bone morphogenetic protein BMP-2 is involved in a signalling pathway that controls patterning across the anteroposterior (a-p) axis of the developing limb.

The signalling pathways in the developing limb are complex and incompletely understood. Two regions in the limb bud are known to be important sources of signals that control the patterning of the developing limb. One region is the apical ectodermal ridge, which is required for the outgrowth of the limb and is involved in proximodistal specification. The other is the polarizing region, a group of cells in the posterior mesenchyme of the limb bud that specifies pattern across the a-p axis of the bud. Gifts of the polarizing region to the anterior mesenchyme result in the duplication of digits, such that anterior cells form posterior structures (Tickle et al., 1975). The effect of the polarizing region can be mimicked by the application of retinoic acid to the anterior margin of the limb bud (Tickle et al., 1982). Both polarizing region grafts and locally applied retinoic acid activate genes of the HoxD complex in anterior cells (Izpisúa-Belmonte et al., 1991; Nohno et al., 1991). However, this activation occurs at least 16 hours after treatment and the intervening steps are unknown. In addition, activation of HoxD genes requires cooperation with a signal from the apical ridge (Izpisúa-Belmonte et al., 1992a).

Transcripts of the Bmp-2 and Bmp-4 genes, which encode BMP-2 and BMP-4, have been found in developing mouse limb buds (Lyons et al., 1990; Jones et al., 1991). The BMPs were originally identified in extracts of bovine bone that could induce ectopic cartilage formation when implanted subcutaneously in rats. Biochemical analysis of this activity led to the discovery of seven evolutionarily conserved proteins, named BMP-1 to BMP-7 (reviewed by Rosen and Thies, 1992). BMP-2 to BMP-7 are structurally related to each other and are...
members of the TGF-β family, while BMP-1 has a completely different structure and may be involved in the proteolytic activation of the other BMPs. BMP-2 and BMP-4 are structurally more closely related to each other than to other BMPs, and have a high level of amino acid sequence identity to the product of the *Drosophila decapentaplegic* (dpp) gene. There is evidence that BMP-2, BMP-4 and DPP may be signalling molecules that regulate pattern formation and morphogenesis during embryonic development. For example, in *Drosophila*, DPP is thought to specify dorsal-ventral polarity in the embryo (Ferguson and Anderson, 1992) and to pattern the embryonic midgut in *Drosophila* (reviewed by Hoffman, 1991). In *Xenopus*, Bmp-4 mRNA can induce ventral mesoderm when injected into the animal hemisphere of fertilized embryos, and so BMP-4 is thought to be involved in mesodermal patterning (Dale et al., 1992; Jones et al., 1992). Recent descriptions of the distribution of Bmp gene transcripts in mouse embryos suggest that BMPs may play a role in limb development. To investigate this possibility, we isolated and characterized chicken Bmp-2 and Bmp-4 cDNA clones so that we could dissect the roles of the Bmp-2 and Bmp-4 genes, by carrying out experimental manipulations of developing chick wings. We show that the Bmp-2 gene is part of the response component of the patterning region signalling pathway and is closely related to expression of *HoxD* genes.

**MATERIALS AND METHODS**

**Isolation of chicken Bmp-2 and Bmp-4 cDNA clones**  
Approximately 4×10^5 recombinant bacteriophage from a 10-day (stage-36) chick embryo cDNA library constructed in λgt11 (Clontech) were screened with a 1227 bp fragment prepared by PCR from the mouse Bmp-4 cDNA clone 1321 (Jones et al., 1991) and labelled with [α-32P]dCTP (New England Nuclear) by random priming (Feinberg and Vogelstein, 1984). The probe corresponded precisely to the mouse Bmp-4 coding region. Plaque lifts on to Hybond-N were performed according to the manufacturer's instructions and duplicate filters were hybridized overnight at 65°C in 6× SSC, 1% (w/v) SDS, 5× Denhardt's solution, 0.1 mg ml⁻¹ denatured sonicated herring testis DNA and 10⁶ cts minute⁻¹ ml⁻¹ of radiolabelled probe. The most stringent post-hybridization wash was for 30 minutes at 50°C in 2× SSC, 0.1% (w/v) SDS. Positive clones were picked, purified by rescreening under the same conditions, and their EcoRI inserts sub-cloned into plasmid vector pBluescript SK⁺ for further analysis.

The longest chicken Bmp-4 cDNA clone had an insert of 772 bp and was designated p6. To obtain the complete coding region of chicken BMP-4, polyadenylated RNA isolated from 10-day chick embryo limb was reverse transcribed and the cDNA product amplified by PCR using oligonucleotide primers with the sequences 5'-ATGATTCCTGGTAACCGAAT-3' (complementary to nucleotides 953-932) and 5'-ATGATTCCTGGTAACCGAAT-GCTG-3'. The latter sequence corresponds to the first 24 nucleotides of the cDNA (Dale et al., 1992), human (HSBMP2: EMBL Nucleotide Sequence Data Library) and mouse (Brigid Hogan, personal communication) BMP-4 coding regions. The PCR product (nucleotides 1-953) was cloned into pBluescript KS⁺ using a TA cloning system (Invitrogen), and designated p86. The longest chicken Bmp-2 cDNA clone had an insert of 1120 bp and was designated p5.

**Nucleotide sequencing**  
Dideoxy sequencing of double-stranded templates was performed using Sequenase Version 2.0 (US Biochemicals) according to the manufacturer's instructions. Oligonucleotides were synthesized in our laboratory. Nucleotide sequences were analyzed using the PC-GENE software package (Intelliigenetics).

**Construction of probes for northern hybridization and in situ hybridization**  
32P-labelled RNA probes for northern hybridization and 35S-labelled probes for in situ hybridization to tissue sections were synthesized as previously described (Devlin et al., 1988). Digoxigenin-labelled RNA probes for in situ hybridization to wholemount preparations were synthesized using a kit from Boehringer, according to the manufacturer's instructions.

Antisense RNA probes specific for chicken Bmp-4 transcripts were synthesized with T3 RNA polymerase, using *BamHI*-linearized p6.1 as a template (nucleotides 1-953). Sense RNA probes for use as controls were synthesized with T7 RNA polymerase, using *HindIII*-linearized p6.1 as a template.

To construct a template for the synthesis of Bmp-2-specific probes, nucleotides 1-797 of p5 were amplified by PCR. The PCR product was cloned into pBluescript SK⁺ using a TA cloning system (Invitrogen), and designated p5.1. Antisense RNA probes specific for chicken Bmp-2 transcripts were synthesized with T3 RNA polymerase, using *HindIII*-linearized p5.1 as a template and sense RNA control probes were synthesized with T7 RNA polymerase, using *BamHI*-linearized p5.1 as a template.

The Bmp-2- and Bmp-4-specific probes share approximately 57% nucleotide sequence identity and have never been observed to cross-hybridize under the hybridization and washing conditions used.

Antisense RNA probes specific for mouse Bmp-2 transcripts were synthesized as described by Lyons et al. (1989). Antisense RNA probes specific for chicken *HoxD-13* transcripts were synthesized as described previously (Izpisúa-Belmonte et al., 1991).

**Chick embryos**  
Fertilized chicken eggs were obtained from Poyndon Farm, Waltham Cross, Herts, and were incubated at 38±1°C. Embryos were staged according to Hamilton (1951), dissected into fresh PBS and processed for RNA isolation or in situ hybridization as described below.

**Retinoic acid treatment of chick wing buds**  
AG1-X2 beads soaked in a solution of all-trans retinoic acid (0.1 mg ml⁻¹ or 1 mg ml⁻¹) in dimethyl sulphoxide were implanted at the anterior margin of the wing bud of stage-19/20 embryos as previously described (Tickle et al., 1985). At a series of times between 16 and 48 hours later, the beads were removed and the embryos pinned and fixed in 4% (w/v) paraformaldehyde. To confirm the efficacy of the retinoic acid treatment of chick wing buds

**Grafting of mouse polarizing region to chick wing buds**  
The forelimb buds were removed from 10.5-day-old C57BL/H-α mouse embryos and trypsinized at 4°C for 30-60 minutes to remove the epithelium (Szabo, 1955). The polarizing region, a cube of mesenchyme from the posterior margin of the bud, was dissected out and grafted to the anterior margin of stage-19/20 chick embryo wing buds, as described previously (Izpisúa-Belmonte et al., 1992b). The position of the graft was determined by staining tissue sections with carboxyfluorescein.

**Application of BMP-2 protein to chick wing buds**  
Recombinant human BMP-2 protein was supplied by Dr Elizabeth Wang, Genetics Institute, Cambridge, Massachusetts, at a concentration of 2 mg ml⁻¹ in aqueous solution containing 0.5 M arginine, 0.01
Bmp-2 and Bmp-4 expression in the chick limb bud

M histidine, pH 6.5. Where necessary, this stock was diluted in 0.02 M sodium acetate, pH 5.0, containing 0.2% (w/v) BSA. The protein was active, as judged by its ability to enhance cartilage production in cultures of chick wing bud mesenchyme cells (P. Francis and A. Page, unpublished data). Heparin-acrylic beads (200-250 μm, H5263, Sigma) were soaked in 2 ml of BMP-2 solution (at concentrations of 2 μg ml⁻¹ to 2 mg ml⁻¹) for at least 1 hour at room temperature. Control beads were soaked in aqueous solution containing 0.5 M arginine, 0.01 M histidine, pH 6.5. Beads were placed into slits cut in the anterior margins of the wing bud, and the wings were allowed to develop for a further 6 days and the wings were then stained with alcian green to determine the skeletal pattern.

RNA preparation and northern analysis

Total RNA was extracted from fresh tissue by the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) and polyadenylated mRNA was selected by two passages through an oligo(dT) cellulose column. Polyadenylated mRNA (1 μg per track) was size fractionated on 1% (w/v) agarose Mops-formaldehyde gels and blotted onto Genescreen Plus nylon membranes (Dupont). Membranes were hybridized with 32P-labelled antisense RNA probes specific for Bmp-2 and Bmp-4 transcripts, prepared as described above. Hybridization and washing conditions were as described previously (Rowe et al., 1991). The sizes of Bmp-2 and Bmp-4 mRNAs were estimated by reference to RNA size markers (Boehringer) run in adjacent tracks.

In situ hybridization to tissue sections

Embyros were fixed in 4% (w/v) paraformaldehyde, processed and embedded in wax as described by Wilkinson (1993).

RESULTS

Isolation of chicken Bmp-2 and Bmp-4 cDNA clones

Screening of a chick embryo cDNA library at low stringency with a mouse Bmp-4 cDNA probe resulted in the isolation of six clones. These were sequenced and identified as chicken Bmp-2 and Bmp-4 cDNA clones by comparing the predicted amino acid sequences of the proteins that they would encode with those of human BMP-2 and BMP-4 (Wozney et al., 1988). The chicken Bmp-4 cDNA sequence has been

In situ hybridization to whole-mount preparations

Embyros were processed, hybridized, washed and developed as described by Wilkinson (1993).
deposited in the EMBL Nucleotide Sequence Data Library. There is approximately 84.5% identity between the predicted amino acid sequences of chicken and human pre-pro-BMP-4 (Fig. 1A). The carboxyl-terminal domain of chicken BMP-4, which would be predicted to form mature dimers, shares approximately 95% amino acid identity with that of both human and mouse BMP-4 (Fig. 1A).

The chicken Bmp-2 cDNA nucleotide sequence has been deposited in the EMBL Nucleotide Sequence Data Library. Chicken pre-pro-BMP-2 is predicted to share approximately 80% amino acid identity with both human and mouse BMP-2 in the region of overlap (Fig. 1B). The carboxyl-terminal domain of chicken BMP-2 shares approximately 97% amino acid identity with that of both human and mouse BMP-2 (Fig. 1B).

**Northern analysis**

Chicken Bmp-2 and Bmp-4 mRNAs were analyzed by hybridization of specific probes to northern blots of polyadenylated mRNA isolated from 10-day chick embryo limbs. The Bmp-4-specific probe hybridized to a single transcript of approximately 1.9 kb (Fig. 2, track 1), while the Bmp-2-specific probe hybridized to two transcripts, of approximately 2.6 and 3.2 kb (Fig. 2, track 2). Genomic Southern blotting and the characterization of chicken Bmp-2 genomic clones demonstrated that there is a single Bmp-2 gene within the chicken genome (Forbes-Robertson, Francis and Brickell, data not shown).

**Distribution of Bmp-4 transcripts in the developing chick limb**

Bmp-4 transcripts were detectable throughout the pre-wing mesenchyme of stage-16 and -16/17 embryos and in the overlying ectoderm (Fig. 3A). At stage 18, transcripts were found throughout the limb bud mesenchyme and in the apical ectodermal ridge (data not shown). In the apical ridge, Bmp-4 transcripts remained detectable until stage 26, as the bud elongated (Figs 3G, 4A). In the mesenchyme, however, Bmp-4 expression became progressively restricted between stages 19 and 24. High levels of Bmp-4 transcripts were found in two regions, one at the anterior margin of the limb bud and one at the posterior margin (Figs 3D, 4A). Lower levels of Bmp-4 transcripts were found in the mesenchyme directly beneath the apical ridge (Figs 3D, 4A). At stages 25 and 26, mesenchymal expression of Bmp-4 in the wing bud was found predominantly at the anterior margin although a small posterior zone of low expression persisted (Figs 3G, 4D). In the leg bud at these stages, however, Bmp-4 transcripts were still found at approximately equal levels in both posterior and anterior mesenchyme (data not shown). Anterior expression in the leg bud was found in two regions.

![Fig. 3. Distribution of Bmp-4 (A,D,G,J), Bmp-2 (B,E,H,K) and Hoxd-13 (C,F,I,L) transcripts in adjacent longitudinal sections of wing buds at stage 16/17 (A,B,C), stage 22 (D,E,F), stage 26 (G,H,I) and stage 28 (J,K,L). The anterior margin of each bud is uppermost. Autoradiographic signal is visible as white grains under dark-field illumination. Scale bars, 250 mm.](image)
on either side of the indentation that marks the presumptive knee. At stages 28 and 30, in both wing and leg, Bmp-4 transcripts were found in the necrotic zone between the developing digits and in the mesenchyme surrounding chondrogenic regions corresponding to the long bones (Figs 3J, 4F). As development of the digits proceeded, interdigital expression became confined to mesenchyme immediately surrounding chondrogenic regions (data not shown).

**Distribution of Bmp-2 transcripts in the developing chick limb**

In contrast to Bmp-4, Bmp-2 transcripts could not be detected in the pre-wing flank mesenchyme at stage 16. Bmp-2 transcripts were first detectable in the pre-wing flank mesenchyme at stage 16/17 and were restricted to the posterior part of the mesenchyme (Fig. 3B). Bmp-2 transcripts were also detectable in the overlying ectoderm at this stage (Fig. 3B). Between stages 17 and 26, Bmp-2 transcripts were still clearly restricted to the posterior mesenchyme and were also detectable in the apical ridge (Figs 3E, 4B,C). Expression of Bmp-2 in the apical ridge continued until stage 26 (data not shown). As the bud grew out, between stages 25 and 26, the posterior domain of expression remained distal, so that strongest expression was always found near the tip of the bud (Figs 3H, 4E). At stage 25 and 26, an additional weak domain of expression was detectable in the anterior proximal part of the limb bud (Figs 3H, 4E). Between stages 26 and 28, the anterior domain of expression became progressively stronger and larger, so that by stage 28, Bmp-2 transcripts could be detected all along the anterior margin of the limb except in the distal hand plate (Fig. 3K). At stage 28, Bmp-2 transcripts were also found interdigitally and around regions of cartilage differentiation (Fig. 3K). A similar distribution was found in the leg at stage 30 (Fig. 4G).

**Comparison of the distribution and time of appearance of Bmp-2 and Hoxd-13 transcripts in wing buds**

The expression domain of Bmp-2 in limb bud mesenchyme is similar to that of Hoxd-13, a gene that responds to a signal from the polarizing region (Izpisúa-Belmonte et al., 1991). Both Bmp-2 and Hoxd-13 are expressed posteriorly, with maximum expression towards the distal tip of the limb. In order to compare the expression domains of the two genes in detail, we examined the distribution of Bmp-2 and Hoxd-13 transcripts in adjacent sections.

Examination of sections through stage-18 wing buds showed that the domains of Hoxd-13 and Bmp-2 expression overlapped considerably at the posterior margin of the limb bud (Fig. 5). The Hoxd-13 domain extended more anteriorly than the Bmp-2 domain (Fig. 5A,B) and the Bmp-2 domain extended more ventrally than the Hoxd-13 domain (Fig. 5C,D). A similar relationship between the two domains was maintained through subsequent stages of bud outgrowth (Fig. 5E,F and H,I). The positions of maximal Bmp-2 and Hoxd-13 expression in these domains did not coincide. The highest levels of Hoxd-13 transcripts were found at the very tip of the limb bud, under the apical ridge, whilst the highest levels of Bmp-2 transcripts were found slightly more proximally, just behind the tip (Fig. 3E,F,H,I). By stage 28, both genes were expressed distally, around developing digits (Fig. 3K,L).

The similarity between the domains of Bmp-2 and Hoxd-13 expression in posterior limb bud mesenchyme suggests that the two genes might be regulated by the same factors. Alterna-
tively, BMP-2 could activate Hoxd-13 gene expression or vice versa. To address this question, we determined the order in which Hoxd-13 and Bmp-2 transcripts were first detectable in the wing bud by examining adjacent sections of wing buds from stage-16, -16/17 and -17 embryos. Bmp-2 transcripts were not found in pre-wing mesenchyme at stage 16, but were present in all three stage-16/17 embryos examined (Fig. 3B). In contrast, Hoxd-13 transcripts were not detectable in any of the three stage-16/17 embryos examined (Fig. 3C). Hoxd-13 transcripts were first detectable in a stage-17 embryo, in the same region as Bmp-2 transcripts (data not shown).

**Activation of Bmp-2 gene expression by retinoic acid**

The expression domain of Bmp-2 in the posterior mesenchyme of the limb bud has a similar location to that of the polarizing region (Maccabe et al., 1973; Honig and Summerbell, 1985). This suggests that BMP-2 might be involved in the polarizing region signalling pathway. Retinoic acid can mimic the action of the polarizing region. When it is applied to the anterior margin of a limb bud, expression of the HoxD gene complex is activated in anterior cells and posterior cells give rise to posterior structures (Izpisúa-Belmonte et al., 1991). We examined whether application of retinoic acid to the anterior margin of a limb bud could activate Bmp-2 expression anteriorly and, if so, how this activation compared with that of Hoxd-13.

Treatment with retinoic acid resulted in the appearance of ectopic domains of both Bmp-2 and Hoxd-13 transcripts in the anterior mesenchyme of the bud (Fig. 6A,B,D,E; Table 1). In both cases, ectopic domains were detectable 24 and 48 hours after treatment but not at 16 or 20 hours. The ectopic domains were located distal to the retinoic-acid-soaked bead, beneath the apical ridge at the tip of the bud (Fig. 6A,B,D,E). At 24 hours, the ectopic Hoxd-13 domain was located distally within the ectopic Bmp-2 domain (Fig. 6A,B). By 48 hours, the ectopic Bmp-2 and Hoxd-13 domains were larger and partially overlapping, with the Hoxd-13 domain extending more distally and the Bmp-2 domain extending more proximally, closer to the bead (Fig. 6D,E). The ectopic Hoxd-13 domain also extended further towards the posterior part of the bud than the ectopic Bmp-2 domain (Fig. 6D,E). The spatial relationship between the overlapping ectopic Bmp-2 and Hoxd-13 domains was similar to that between the normal posterior Bmp-2 and Hoxd-13 domains. The relationship was maintained when retinoic acid was applied to the posterior margin of the limb bud (Fig. 6F,G), with the posterior Bmp-2 and Hoxd-13 domains both being shifted anteriorly.

When a retinoic-acid-soaked bead was placed at the anterior margin of the bud, Bmp-4 transcripts were also found around the bead (Fig. 6C). Unlike the ectopic Bmp-2 domain, which was distal to the bead, Bmp-4 transcripts were present both proximal and distal to the head (Fig. 6C). The anterior domain of Bmp-4 expression overlapped with the ectopic anterior domain of Bmp-2 expression, with the Bmp-4 domain extending more proximally. Since there is an anterior domain of Bmp-4 expression in normal limb buds (Figs 6C, 3D, 4A,D), it was not clear whether the levels of Bmp-4 transcripts had changed in response to retinoic acid treatment.

**Activation of Bmp-2 and Hoxd-13 gene expression by polarizing region grafts**

The activation of Bmp-2 expression by retinoic acid shows that this gene is part of the polarizing region signalling pathway. However, since it has been suggested that retinoic acid acts by inducing a new polarizing region, it is not clear whether BMP-2 is itself a polarizing molecule or whether it acts further downstream. To investigate these questions we examined Bmp-2 expression in wing buds following grafting of a polarizing region to the anterior margin. Such grafts induce digit duplications (Tickle et al., 1976) and activate expression of genes of the HoxD complex anteriorly (Ispizúa-Belmonte et al., 1992). However, they do not induce polarizing activity in adjacent host mesenchymal cells (Smith, 1979). Thus, if BMP-2 is the polarizing signal, Bmp-2 expression should be maintained in the graft but not activated in adjacent host mesenchyme cells. We used mouse, rather than chick, polarizing region tissue so that species-specific probes could be used to
We isolated and characterized mouse Bmp-2 transcripts in the graft from ectopic chicken Bmp-2 transcripts in the host.

Mouse polarizing region grafts continued to express Bmp-2 after grafting to the anterior margin of stage-19/20 chick embryo wing buds (Fig. 7C,F; Table 2). In addition, the polarizing region grafts induced anterior host mesenchyme cells to express Bmp-2 (Fig. 7A,D; Table 2). Chicken Bmp-2 transcripts were found in cells adjacent to the graft, beneath the apical ridge (Fig. 7A,D). We compared the timing of this induction with that of Hoxd-13 by hybridizing adjacent sections with probes for Bmp-2 and Hoxd-13 transcripts. Ectopic expression of both genes was detectable by 30 hours after grafting, at which time the ectopic domain of Hoxd-13 expression was restricted distally within that of Bmp-2 expression (Fig. 7A,B; Table 2). By 42 hours after grafting, the Hoxd-13 and Bmp-2 domains were larger than at 30 hours and partially overlapped (Fig. 7D,E; Table 2). The Bmp-2 domain extended more proximally around the graft while the Hoxd-13 domain extended more distally. In addition, the Hoxd-13 domain extended further towards the posterior part of the bud (Fig. 7D,E). This sequence of events is similar to that seen following local application of retinoic acid to the anterior margin of the bud (Fig. 6).

Application of Bmp-2 protein to chick wing buds

The effects of polarizing region grafts on Bmp-2 expression suggest that Bmp-2 is not itself a polarizing molecule. To test this directly, beads were soaked in recombinant human BMP-2 protein at concentrations of 0.2 mg ml\(^{-1}\) (n=5), 1 mg ml\(^{-1}\) (n=2) or 2 mg ml\(^{-1}\) (n=4) and were placed at the anterior margin of chick wing buds. In all 11 cases the pattern of digits that developed was normal and no additional digits formed. This confirms that Bmp-2 protein alone does not have polarizing activity. However, application of Bmp-2 protein to the anterior margin of wing buds did result in defects in the shoulder girdle (11/11 cases) and radius (5/11 cases). Beads soaked in buffer alone (n=4) or in recombinant human BMP-2 protein at very low concentrations (2 \(\mu\)g ml\(^{-1}\), n=1; 20 \(\mu\)g ml\(^{-1}\), n=2) gave no shoulder or digit abnormalities. These data indicate that active BMP-2 protein was released from the grafted beads. Thus, although BMP-2 did not polarize the wing, it did affect the development of proximal structures.

DISCUSSION

We isolated and characterized chicken Bmp-2 and Bmp-4 cDNA clones and determined the distribution of Bmp-2 and Bmp-4 transcripts in the developing chick limb. Both genes are expressed in the apical ectodermal ridge and in discrete, and hitherto unsuspected, mesenchymal domains. Whereas Bmp-4 is expressed both anteriorly and posteriorly in the mesenchyme with some weak expression beneath the apical ridge, Bmp-2 expression is confined to the posterior mesenchyme during early limb development. This posterior domain of Bmp-2 expression colocalizes with the polarizing region and overlaps with the domain of Hoxd-13 expression. Application of retinoic acid or grafting of a mouse polarizing region to the anterior margin of chick wing buds activates ectopic expression of Bmp-2 in anterior cells.

The data presented here identify Bmp-2 as a component of the polarizing region signalling pathway. This is the first report implicating a growth factor in a-p patterning in the limb bud. The Bmp-2 gene is expressed by mesenchyme cells in the posterior part of the limb bud, where the polarizing region has been mapped (Maccabe et al., 1973; Honig and Summerbell, 1985). grafts of polarizing region cells, which respecify cell position, contain Bmp-2 transcripts. In addition, Bmp-2 expression is activated in anterior mesenchyme following either local application of retinoic acid or grafting of a mouse polarizing region to the anterior margin of the bud. Finally, the domain of Bmp-2 expression overlaps substantially with that of Hoxd-13.
How might BMP-2 operate in the polarizing region signalling pathway? Retinoic acid is a good candidate for the polarizing region signal. However, an alternative view is that locally applied retinoic acid induces a new polarizing region that then signals by means of another, unidentified, molecule (Wanek et al., 1991). BMP-2 could be such a molecule. However, anterior cells exposed to retinoic acid acquire some polarizing region activity after 15 hours (Wanek et al., 1991), but activation of Bmp-2 expression in anterior mesenchyme is not detectable until 24 hours after retinoic acid treatment. In addition, Bmp-2 expression is activated by polarizing region grafts, but Smith (1979) showed that polarizing region grafts do not appear to induce polarizing activity in adjacent host tissue. Moreover, Bmp-2 expression is not detectable in the pre-wing mesenchyme of stage-16 embryos, which has polarizing activity (Maccabe et al., 1973; Honig and Summerbell, 1985; Hornbruch and Wolpert, 1991). It therefore appears that BMP-2 may be a downstream component of the polarizing region signalling pathway rather than being the polarizing region signal itself. As would be predicted, application of recombinant BMP-2 protein to the anterior margin of chick wing buds does not induce digit duplications. However, application of the protein does result in shoulder girdle abnormalities. Interestingly, shoulder girdle abnormalities also result when either retinoic acid or polarizing region grafts are placed at the anterior margin of the wing bud (Oliver et al., 1990).

Genes of the HoxD complex are also activated in response to either retinoic acid or polarizing region grafts. This suggests that the Bmp-2 and HoxD complex genes could be connected components of the response to signals that re-specify position in the limb bud. Indeed, the temporal and spatial relationship between the activation of Bmp-2 and Hoxd-13 expression in manipulated buds is most striking. The two genes are activated in anterior mesenchyme, over a similar time period, in response to retinoic acid or polarizing region grafts. Following both types of manipulation, Hoxd-13 transcripts are first detected in a small domain located within the Bmp-2 domain, and then spread to form a domain that overlaps with and spreads beyond the Bmp-2 domain. This suggests that cells might activate Hoxd-13 shortly after they have activated Bmp-2 and raises the possibility that BMP-2 is involved in the activation of Hoxd-13. Limited diffusion of BMP-2, such as occurs for Drosophila DPP (Panganiban et al., 1990), could account for the finding that the Hoxd-13 domain becomes slightly larger than the Bmp-2 domain. In normal buds, Bmp-2 transcripts are detectable before Hoxd-13 transcripts. This also suggests that BMP-2 could be involved in activating Hoxd-13 in normal limb development.

Table 1. Activation of ectopic Bmp-2 and Hoxd-13 gene expression following application of retinoic acid to the anterior margin of the wing bud

<table>
<thead>
<tr>
<th>Retinoic acid concentration (ng ml⁻¹)</th>
<th>Time after treatment (hours)*</th>
<th>Number of cases</th>
<th>Ectopic domain of transcripts</th>
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<tbody>
<tr>
<td></td>
<td>Bmp-2</td>
<td>Hoxd-13</td>
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<td>0.1</td>
<td>16</td>
<td>1</td>
<td>n.d.</td>
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<td>4</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
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<td>2</td>
<td>+</td>
<td>+†</td>
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<tr>
<td>48</td>
<td>2</td>
<td>+</td>
<td>+</td>
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<tr>
<td>24</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>48</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

n.d., not done

*At the 16- and 20-hour time points, every second section through the thickness of the bud was probed to ensure that ectopic domains would not be missed.

†The ectopic Hoxd-13 domain was very small.

Fig. 7. Distribution of chicken Bmp-2 (A,D), chicken Hoxd-13 (B,E) and mouse Bmp-2 (C,F) transcripts in adjacent sections of wing buds 30 hours (A,B,C) or 42 hours (D,E,F) after a mouse polarizing region was grafted to the anterior margin of the bud. Autoradiographic signal is visible as white grains under dark-field illumination. Ectopic domains of Bmp-2 and Hoxd-13 transcripts in anterior mesenchyme of treated buds are indicated with arrows. Scale bars, 250 μm.
The expression of HoxD complex genes appears to be related spatially to the maintenance of the apical ridge in the wing bud (Izpisúa-Belmonte et al., 1992a). Therefore, another attractive possibility is that BMP-2 is part of the mechanism that links patterning across the a-p axis with bud outgrowth. When anterior cells are re-specified to form posterior structures in response to retinoic acid or a polarizing region graft, the apical ridge is maintained anteriorly by a signal from the mesenchyme (Tickle et al., 1989; Oliver et al., 1990). If BMP-2 is such a signal from mesenchyme to epithelium, it would resemble DPP, which is synthesized by mesoderm and activates expression of the homeotic gene, labial, in neighbouring endoderm (Panganiban et al., 1990). In mouse limb buds, Fgf-4 gene expression is restricted to the posterior part of the apical ridge (Niswander and Martin, 1992). It is possible that BMP-2, synthesized by posterior mesenchymal cells beneath the apical ridge, could regulate Fgf-4 gene expression.

BMP-2 could function in the polarizing region signalling pathway in concert with BMP-4. The Bmp-2 domain overlaps with that of Bmp-4 in the posterior mesenchyme of the normal limb bud. In the anterior mesenchyme of manipulated buds, the ectopic Bmp-2 domain overlaps with a Bmp-4 domain. Members of the TGF-β family, including BMPs, have been shown to form both homodimers and heterodimers (Sampath et al., 1990). BMP-2 and BMP-4 might therefore act as homodimers and/or heterodimers in the limb bud. This introduces further complexity because homodimers and heterodimers can have opposing actions, as in the case of the inhibins, which are also members of the TGF-β family (Hsueh et al., 1987). However, the distribution of Bmp-2 and Bmp-4 transcripts may not accurately reflect the distribution of active protein, since there is the possibility of further control at the levels of translation, secretion and proteolytic activation after secretion (reviewed by Wozney, 1989).

The distribution of Bmp-2 and Bmp-4 transcripts in the developing limb suggests that BMP-2 and BMP-4 could have at least two other distinct functions in limb development. Both genes are expressed in the apical ridge. Signals from the apical ridge are responsible for maintaining a zone of undifferentiated mesenchymal cells at the tip of the limb bud and BMP-2 and BMP-4 could be involved in this, as previously suggested in the mouse (Lyons et al., 1990; Jones et al., 1991; Niswander and Martin, 1993). Finally, both genes are expressed in chondrogenic regions in the later bud, suggesting a role in cartilage differentiation and morphogenesis (Lyons et al., 1989).

### Table 2. Activation of ectopic Bmp-2 and Hoxd-13 gene expression following grafting of mouse polarizing region to the anterior margin of the wing bud

<table>
<thead>
<tr>
<th>Time after grafting (hours)</th>
<th>Number of cases</th>
<th>Mouse Bmp-2 transcripts in graft</th>
<th>Ectopic domain of transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bmp-2</td>
<td>Hoxd-13</td>
</tr>
<tr>
<td>30</td>
<td>2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>42</td>
<td>2</td>
<td>+</td>
<td>+†</td>
</tr>
</tbody>
</table>

*In one embryo, the ectopic Hoxd-13 domain was very small compared with the ectopic Bmp-2 domain. In the other embryo, the ectopic Hoxd-13 domain was confined distally within the ectopic Bmp-2 domain.

†In both embryos, the Hoxd-13 domain extended more distally and posteriorly than the Bmp-2 domain. In one embryo, the Bmp-2 domain extended more proximally than the Hoxd-13 domain.

It is very interesting that members of the TGF-β family, including BMPs, are emerging as common components in a range of signalling systems in developing embryos. For example, in *Drosophila*, DPP appears to specify position along the dorsoventral axis of the embryo (Ferguson and Anderson, 1992), whilst in *Xenopus*, the activins, BMP-4 and Vg1 are thought to act together to specify dorsal and ventral mesoderm (Smith et al., 1989; Dale et al., 1992; Jones et al., 1992; Thomsen and Melton, 1993). Here we suggest that BMP-2 is involved in the polarizing region signalling pathway that specifies position across the a-p axis of the vertebrate limb bud.

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