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Involvement of *Bone Morphogenetic Protein-4* (BMP-4) and *Vgr-1* in morphogenesis and neurogenesis in the mouse

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Summary

Bone Morphogenetic Protein-4 **(BMP-4) and** ***Vgr-1*** **are members of the TGF-β gene family most closely related to the *Drosophila Decapentaplegic* and *Xenopus Vg-1* genes. Members of this gene family have been implicated in diverse processes during embryogenesis including epithelial–mesenchymal interactions. Here, we use *in situ* hybridization to localize BMP-4 and Vgr-1 transcripts during murine development. BMP-4 mRNA is found in a variety of tissues. In the 8.5 days *p.c.* embryo, transcripts are localized to the mesoderm posterior to the last somite. Later gestation embryos show expression in developing limbs, the embryonic heart, the facial processes and condensed mesenchymal cells associated with early whisker follicle formation. In the developing central nervous system (CNS), BMP-4 expression is restricted to the floor of the diencephalon associated with pituitary development. In contrast, Vgr-1 transcripts are found along the anteroposterior axis of the CNS, in cells immediately adjacent to the floor plate and in the roof plate extending to the forebrain. Together, the data support the hypothesis that polypeptide growth factors of the TGF-β superfamily play key roles in the initial stages of neurogenesis and organogenesis during murine development.**

Key words: Bone Morphogenetic Protein 4, Vgr-1, TGF-β, *in situ* hybridization, organogenesis, neurogenesis, pituitary, central nervous system, limb bud, heart.

Introduction

Vertebrate embryogenesis involves a series of instructive interactions in which signalling molecules produced by one cell type influence the developmental fate and morphogenesis of another closely associated cell population. In this way, the diverse and complex tissues of the developing embryo are established and spatially organized to produce a functioning organism. Identifying these signalling molecules and understanding how they function is a primary goal in embryology. There is accumulating evidence that genes belonging to the Transforming Growth Factor-β (TGF-β) family are important regulators of many morphogenetic events during early vertebrate embryogenesis (for review see Whitman and Melton, 1989). For example, TGF-β1 in combination with basic fibroblast growth factor (Kimelman and Kirschner, 1987), TGF-β2 alone (Rosa *et al.* 1988) and activin A (XTC-MIF) (Asashima *et al.* 1990; Smith *et al.* 1990; van den Eijnden-Van Raaij *et al.* 1990) can induce mesodermal differentiation and specific gene expression in isolated *Xenopus* animal caps which would otherwise form ectoderm. Furthermore, studies localizing TGF-β1, -β2, and -β3 transcripts and/or protein have revealed temporal and spatial patterns of expression consistent with many roles for TGF-β-related genes in murine embryogenesis (Heine *et al.* 1987; Lehnert and Akhurst, 1988; Pelton *et al.* 1989, 1990). A group of the TGF-β gene family, whose members show greatest homology to the *Drosophila Decapentaplegic* (*dpp*) (Padgett *et al.* 1987) and *Xenopus Vg-1* (Weeks and Melton, 1988) genes includes the *Bone Morphogenetic Proteins* (BMP) 2, 3 (osteogenin), and 2b (now known as BMP-4) (Wozney *et al.* 1988; Luyten *et al.* 1989), as well as murine *Vgr-1* (Lyons *et al.* 1989*a*), *Osteogenic Protein 1* (Ozkaynak *et al.* 1990), and *GDF-1* (Lee, 1990). Recent studies have localized Vgr-1 RNA to mouse oocytes, suprabasal layers of keratinized epithelium and hypertrophic cartilage in developing bone (Lyons *et al.* 1989*b*). A different pattern of expression is seen for BMP-2, which is localized to the apical ectodermal ridge of limb buds, developing hair and whisker follicles, condensing, precartilagenous mesenchyme and the myogenic layer of the atrioventricular cushions of the developing heart, as well as other areas undergoing morphogenesis during murine development (Lyons *et al.* 1989*b*, 1990).

The original BMP-4 cDNA (formerly called BMP-2b) was isolated because of its homology to BMP-2 (Wozney *et al.* 1988). This cDNA encodes a protein whose carboxy terminus (TGF-β conserved region) shows 92% amino acid identity to the corresponding

region of BMP-2. The carboxy termini of BMP-2 and BMP-4 are more closely related to the corresponding region of *Drosophila* dpp protein than to any other known member of the TGF-β gene family.

In this study, we use *in situ* hybridization to survey expression of BMP-4 during murine embryogenesis. Like the other TGF-β-related genes, BMP-4 transcripts are localized to specific regions of the developing mouse that are undergoing morphogenesis. These include cells of newly formed mesoderm in the posterior primitive streak region of 8.5 days *p.c.* embryos, as well as cell types involved in craniofacial development, limb bud formation, cardiac development and neuroepithelium associated with pituitary development. In some of these tissues, it appears that the distribution of BMP-4 transcripts overlaps that of BMP-2. In other cases, the expression patterns of the two genes are different. In the central nervous system (CNS), the pattern of BMP-4 expression is also distinct from that of Vgr-1. We here extend previous findings of Vgr-1 expression by localizing transcripts in the roof plate and to neuroepithelium adjacent to the floor plate within the developing CNS.

Materials and methods

Postimplantation embryos

Nonpigmented embryos were collected from matings of ICR outbred females (Harlan Sprague-Dawley) with Swiss-Webster males (Taconic Farms). Noon on the day of vaginal plug was considered 0.5 days *post coitum* (*p.c.*). Embryos were fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for approximately 16 h and dehydrated through increasing concentrations of ethanol before embedding in paraffin wax.

Probe construction

A 1.6 kb murine BMP-4 cDNA was isolated from a 6.5 days *p.c.* mouse embryo library (kindly provided by David Weng, Johns Hopkins Medical School) using a partial murine BMP-4 cDNA clone previously described (Dickinson *et al.* 1990) and subcloned into pSP72 (Promega). To ensure no cross-reactivity with the closely related gene, BMP-2, the 3′ portion of the cDNA, encoding the TGF-β conserved region, was then eliminated by digestion with *Sma*I, and subsequent religation. However, the possibility exists that transcripts from two closely related BMP-4 genes are hybridizing to the antisense riboprobe. Dickinson *et al.* (1990) mapped BMP-4 sequences to two loci on chromosome 14 and the X chromosome, respectively, using Southern hybridization. They used a probe containing a substantial portion of the TGF-β conserved region, however, while we use a riboprobe construct made from the 5′ region of the BMP-4 cDNA. Under our stringent *in situ* hybridization conditions, this should substantially reduce any chances of cross-reactivity with another locus.

Our construct, containing approximately 1 kb of cDNA from the 5′ region of BMP-4 was linearized with either *Eco*RI (antisense) or *Bam*HI (sense) and an RNA probe was radiolabelled with [α-^{35}S]UTP to a specific activity of approximately 2×10^9 disints min^{-1} μg^{-1} RNA using either SP6 (antisense) or T7 (sense) RNA polymerase. For *in situ* analyses, the probes were reduced to an average size of 100 to 150 base pairs by limited alkaline hydrolysis (Cox *et al.* 1984).

The murine Vgr-1 probe was previously described (Lyons *et al.* 1989*b*).

In situ *hybridization*

Sections of 7 μm were cut from wax-embedded embryos and floated onto slides coated with either poly-L-lysine or 3-triethoxysilylpropylamine (Sigma). The slides were dried at 48–50°C overnight, dewaxed through xylene, rehydrated through decreasing concentrations of ethanol and refixed in 4% paraformaldehyde/PBS. The sections were then treated with 20 $\mu g\,ml^{-1}$ of Proteinase K (Sigma) in 50 mM Tris, 5 mM EDTA for 7.5 min, followed by refixation in 4% paraformaldehyde/PBS and acetylation with 25 mM acetic anhydride in 100 mM triethanolamine. Lastly, sections were dehydrated through ethanol and allowed to air dry for 30 min to 1 h. The slides were then hybridized under siliconized coverslips for 18 to 24 h at 55°C and 100% humidity in a solution containing 50% deionized formamide, 10% dextran sulfate, 1×Denhardt's solution, 300 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA, 10 mM Na_2HPO_4, 8 mM DTT, 200 $\mu g\,ml^{-1}$ tRNA, and radiolabeled probe at a final concentration of 2×10^4 cts min^{-1} μl^{-1}.

After hybridization, coverslips were removed in 5×SSC (1×SSC=0.15 M NaCl, 15 mM sodium citrate), 10 mM DTT at 50°C. The slides were then washed in a solution of 50% formamide, 2×SSC, 100 mM DTT at 60°C for 20 min, incubated at 37°C in a buffer of 0.5 M NaCl, 10 mM Tris, 5 mM EDTA containing RNAase A (10 $\mu g\,ml^{-1}$) for 30 min, and washed again at 60°C in the 50% formamide/SSC/DTT buffer. Sections were then rinsed through two successive washes each of 2×SSC and 0.1×SSC at 60°C followed by dehydration through ethanol containing 0.3 M ammonium acetate. After drying for at least 1 h, the slides were dipped in radiographic emulsion (Ilford K5) diluted 1:1 with 2% glycerol/water. Following 1–3 weeks exposure at 4°C, the slides were developed using Kodak D19 developer, and counterstained in 0.02% toluidine blue. Photographs were taken with Kodak Technical Pan film on a Zeiss Axiophot microscope using bright-field and dark-ground illumination.

Three-dimensional imaging

Three-dimensional and stereo images were reconstructed from serial sections using the BQ three-dimensional workstation and graphics display (BIOQUANT, R & M Biometrics, Nashville, TN).

Quantitation of hybridization in limb buds

To confirm an anterior–posterior gradient of BMP-4 hybridization in limb bud mesenchyme, hybridization grains over the mesenchyme, but excluding the apical ectodermal ridge, were counted on serial sections of limb buds from two separate embryos using software from Analytical Imaging Concepts, IM-Series (3000) Morphometric and Densitometric System, Micromeasure version 3.00 and a computer from American Leading Systems.

Results

Expression of BMP-4 in egg cylinder/primitive streak stage embryos

Although the murine BMP-4 cDNA was isolated from a 6.5 days *p.c.* embryo library, only one cDNA clone was obtained from an initial screening of 10^6 plaques (original complexity of the library was 5×10^6 plaque forming units (pfu), which was amplified once to 1×10^{10}

pfu ml^{-1}). *In vivo* expression was therefore expected to be at very low levels, and, by the sensitivity of our *in situ* hybridization protocol, localized expression could not be detected in either embryonic or extraembryonic tissues of 6.5 days *p.c.* (egg cylinder) embryos. By 7.5 days *p.c.* (primitive streak), localized expression is observed in the allantois and amnion (data not shown). Additionally, BMP-4 expression is seen in the maternal deciduum surrounding embryos at both of these stages (data not shown).

Expression of BMP-4 in early neurula stage embryos (8.5 days p.c.*)*

BMP-4 is expressed more extensively in 8.5 days *p.c.* embryos compared to earlier stages, as a screen of an 8.5 days *p.c.* cDNA library yielded 7 BMP-4 clones from 1×10^6 plaques (original complexity of the unamplified library was 2×10^6 pfu). By *in situ* hybridization, at the 8-somite stage, BMP-4 transcripts are localized to the mesoderm and definitive endoderm in the primitive streak region at the very posterior of the embryo and in the ventral mesoderm caudal to the last somite (Fig. 1). Transcripts are also present at this stage in the myoepicardium of the early heart (data not shown) and in the allantois and amnion (Fig. 1).

Expression of BMP-4 at 9.0 days p.c.

The overall expression pattern of BMP-4 is illustrated in three-dimensional reconstructions of serial sections of a 9.0 days *p.c.* embryo hybridized with the BMP-4 antisense riboprobe (Fig. 2). BMP-4 transcripts are localized to the neuroepithelium of the diencephalon that is destined to associate with Rathke's pouch and form the posterior pituitary gland (Figs 2 and 3), and to the posterior half of the otic vesicles (Fig. 2). Expression is also seen in the dorsal ectoderm of the first branchial pouch and the anterior half of the pocket between the frontonasal mass and the first branchial arch (Fig. 2). In the heart, high levels of expression are seen in the outer myocardial layer of the developing atrioventricular canal (Fig. 6A,B). In addition, several areas of expression are seen in ventral regions of the embryo. These include mesodermal tissues surrounding the developing gut and early lung bud (Fig. 2 and data not shown), and the somatopleure and splanchnopleure in the posterior of the embryo (Fig. 1). Finally, hybridization is seen in mesenchymal tissue of the flank in the vicinity of the forelimb bud (Fig. 2).

Expression of BMP-4 and Vgr-1 at 10.5 days p.c.

BMP-4 transcripts can now be localized in the floorplate of the diencephalon adjacent to Rathke's pouch (Fig. 3F), in the ectoderm within and around the developing nasal pits (Fig. 8B), in the distal ectoderm of the facial processes and in mesenchymal tissue around the gut (data not shown). Moreover, high levels of expression continue to be observed in the developing heart, but are now localized in the myocardial layer of the truncus arteriosus and not in the atrioventricular canal (Fig. 6C,D). Expression does not extend into the conus arteriosus.

In addition, BMP-4 transcripts are now found within the apical ectodermal ridge (AER) of the fore and hind limbs (Fig. 7). This is similar to the pattern reported for BMP-2 (Lyons *et al.* 1990). However, in contrast to the expression patterns reported for BMP-2 where the level of hybridization grains in the mesenchyme was not above background, BMP-4 transcripts are also localized in the mesenchyme of the limb bud, apparently in an anterior-to-posterior and distal-to-proximal gradient. The anteroposterior gradient was only revealed after reconstructing the limb buds from serial sections. The gradient was then confirmed by grain counting (see Material and Methods) and showed approximately 10-fold more grains/unit area in the mesenchyme in anterior sections compared to more posterior sections. Representative sections from different regions of a single limb bud are shown (Fig. 7).

In contrast to BMP-4 expression, which is widespread at this stage, Vgr-1 expression is much more restricted to regions within the developing central nervous system and heart. Vgr-1 transcripts are found along both the dorsal and ventral surfaces of the developing CNS (Figs 4 and 5). The anterior limit of expression in the roof plate extends to midway through the forebrain, extending laterally into the cerebral hemispheres. In contrast, ventral expression of Vgr-1 does not extend so far forward; its anterior limit seems to be aligned with the anterior limit of the notochord. The ventral expression is localized to cells immediately adjacent to the floor plate, while the floor plate itself is negative (Fig. 5F).

A weaker hybridization signal localizes Vgr-1 expression to the developing heart, showing transcripts restricted to the connection of the truncus arteriosus with the dorsal aorta and to mesenchymal cells of the atrioventricular cushions (Fig. 6H).

Expression of BMP-4 in late gestation embryos (11.5–17.5 days p.c.*)*

As gestation proceeds, BMP-4 continues to be expressed in many of the same organs, but, in general, transcripts are now localized in the underlying mesenchyme rather than in the epithelium. For example, in facial processes of an 11.5 days *p.c.* embryo, BMP-4 transcripts are no longer seen in the distal edge epithelium but are present in the underlying mesenchyme (data not shown). By 13.5 days *p.c.*, BMP-4 expression within the facial mesenchyme is particularly high in areas of condensing cells subjacent to epithelial bulges (Fig. 8 F and H). These mesenchymal condensations, in combination with the overlying epithelium, will form the whisker follicles (for description see Davidson and Hardy, 1952; Lyons *et al.* 1990).

In 11.5 days *p.c.* embryos, hybridization of the BMP-4 probe is no longer seen in the floorplate of the diencephalon adjacent to Rathke's pouch (data not shown). However, expression persists in the developing ear, specifically in secretory/sensory epithelium of the inner ear, at least until 17.5 days *p.c.* (data not shown).

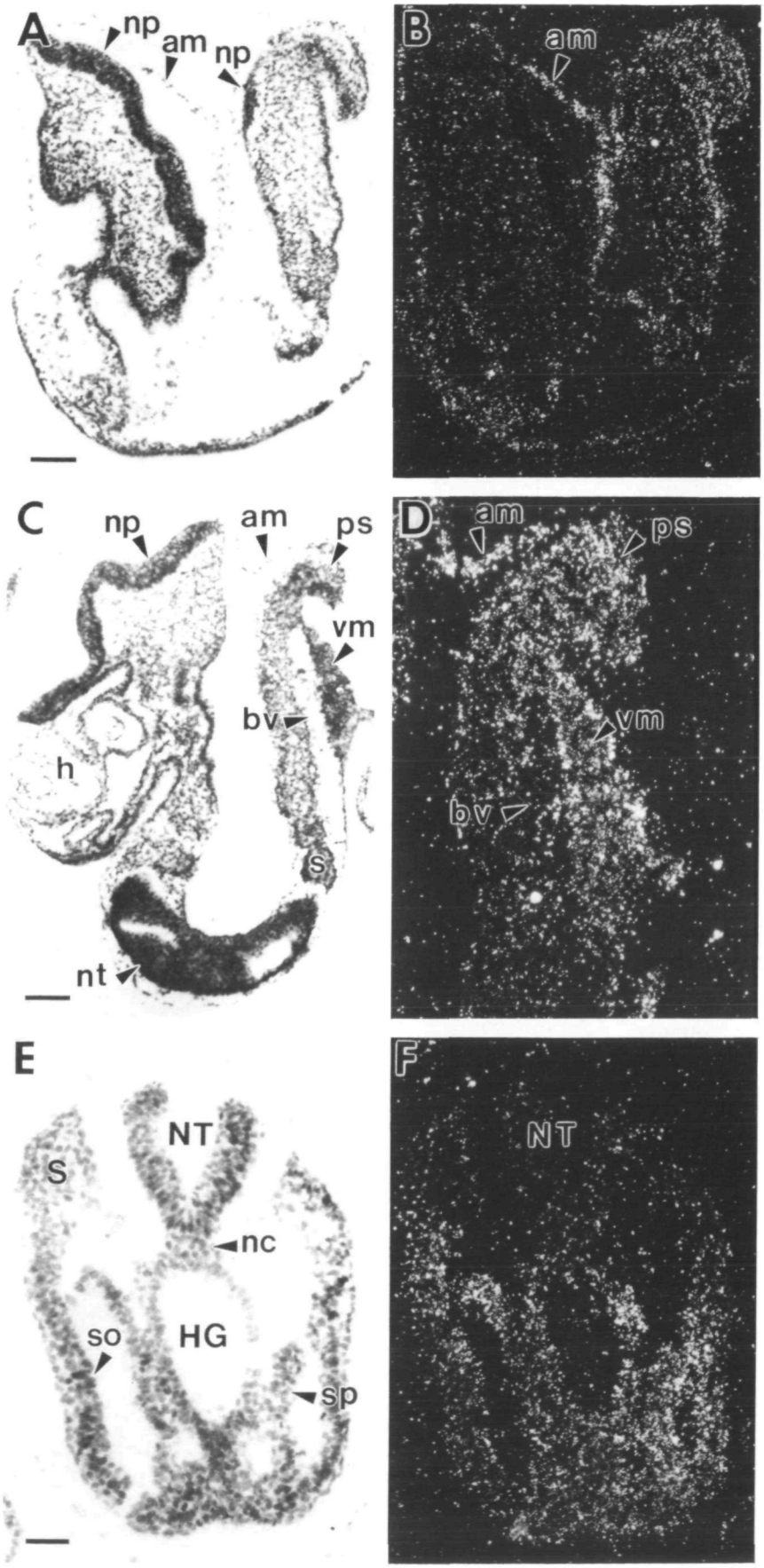
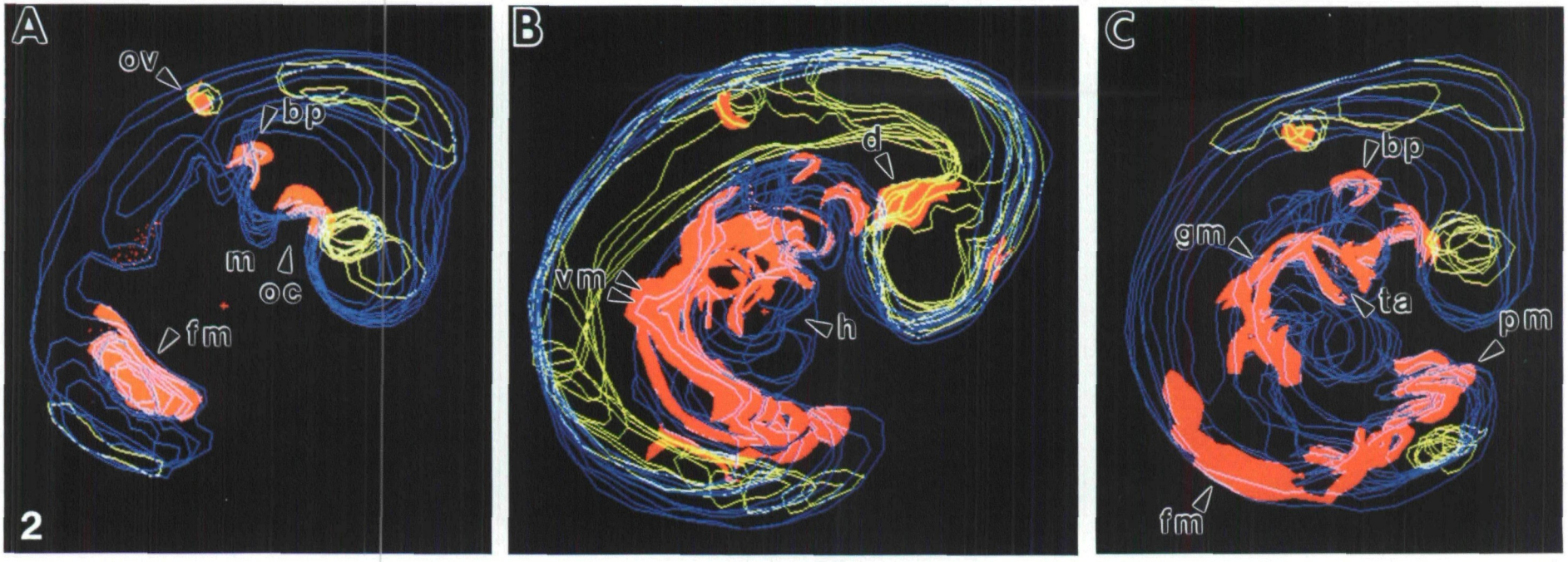


Fig. 1. Expression of BMP-4 in the posterior and ventral mesoderm of 8.5 and 9.0 days *p.c.* embryos. Bright-field (A,C) and dark-ground (B,D) photomicrographs of sections through an 8.5 days *p.c.*, 8-somite embryo. Reconstruction of serial sections showed that A is more lateral than C, and that the embryo had begun to turn. Other sections showed high levels of hybridization in the allantois. D is a higher magnification of the posterior region of C. Bright-field (E) and dark-ground (F) photomicrographs of a transverse section through the posterior of a 9.0 days *p.c.* embryo, showing expression in ventral and lateral mesoderm. am, amnion; bv, posterior blood vessel; h, heart; HG, hindgut; nc, notochord; np, neural plate; NT, neural tube; ps, posterior primitive streak; s, somite; so, somatopleure; sp, splanchnopleure; vm, ventral mesoderm. Bar=100 μm (A and C) or 50 μm (E).



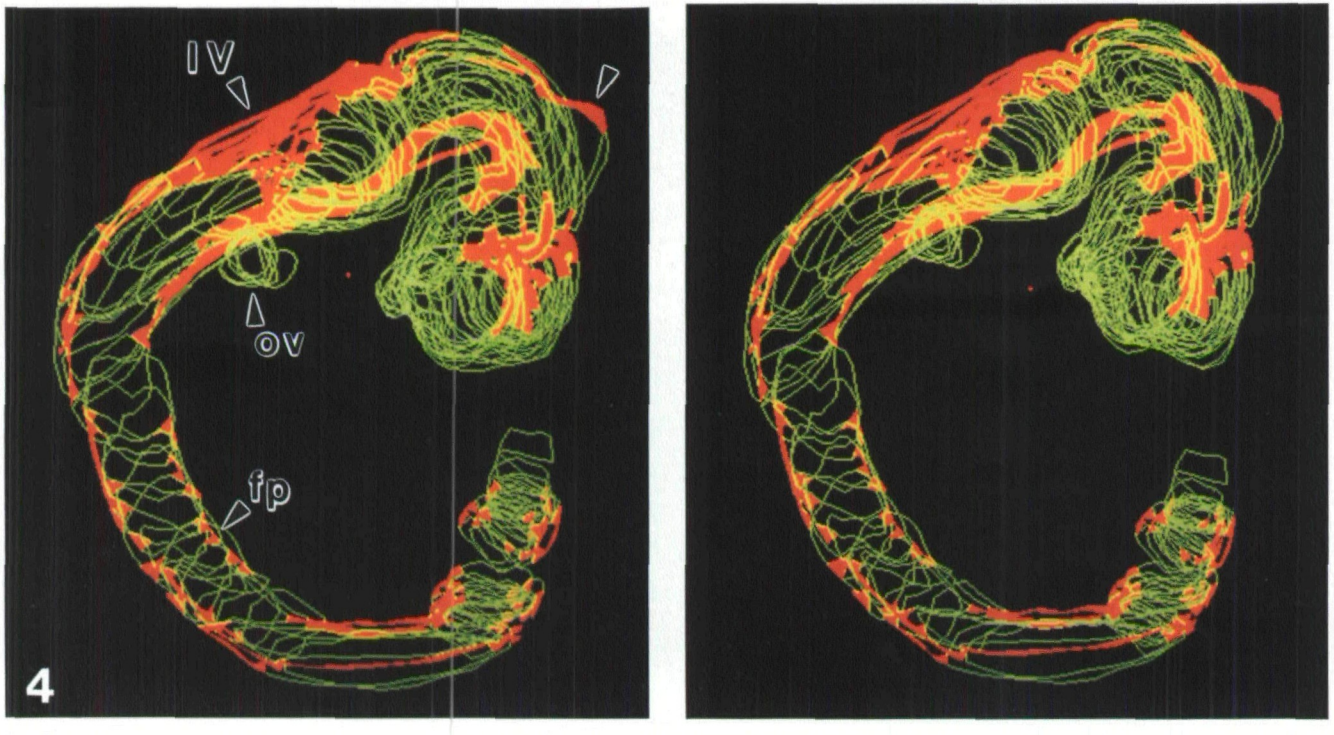


Fig. 2. BMP-4 expression in the 9.0 days *p.c.* mouse embryo. Computer-aided reconstruction of serial sections showing outer surface (blue), brain and spinal cord (yellow) and sites of BMP-4 expression (red). The white color is an artifact due to overlaying of blue and yellow. (A) Left hand one-third of reconstructed embryo. ov, otic vesicle; bp, first branchial pouch; m, mandibular arch; oc, oral cavity; fm, mesoderm in flank and forelimb bud. (B) Middle one-third of embryo. d, ventral diencephalon (see also Fig. 3B and D); vm, ventral mesoderm; h, heart. (C) Right-hand one-third of embryo. ta, truncus arteriosus; gm, mesoderm around foregut; pm, posterior ventral mesoderm.

Fig. 4. Vgr-1 expression in 10.5 days *p.c.* mouse CNS. Stereo images of a computer-aided reconstruction of serial sagittal sections showing brain and spinal cord (green) and sites of Vgr-1 expression in dorsal and ventral regions of the developing CNS (red). IV, roof of fourth ventricle; fp, cell adjacent to floorplate in spinal cord; ov, otic vesicle. Arrowhead: this section was artifactually distorted relative to the other sections.

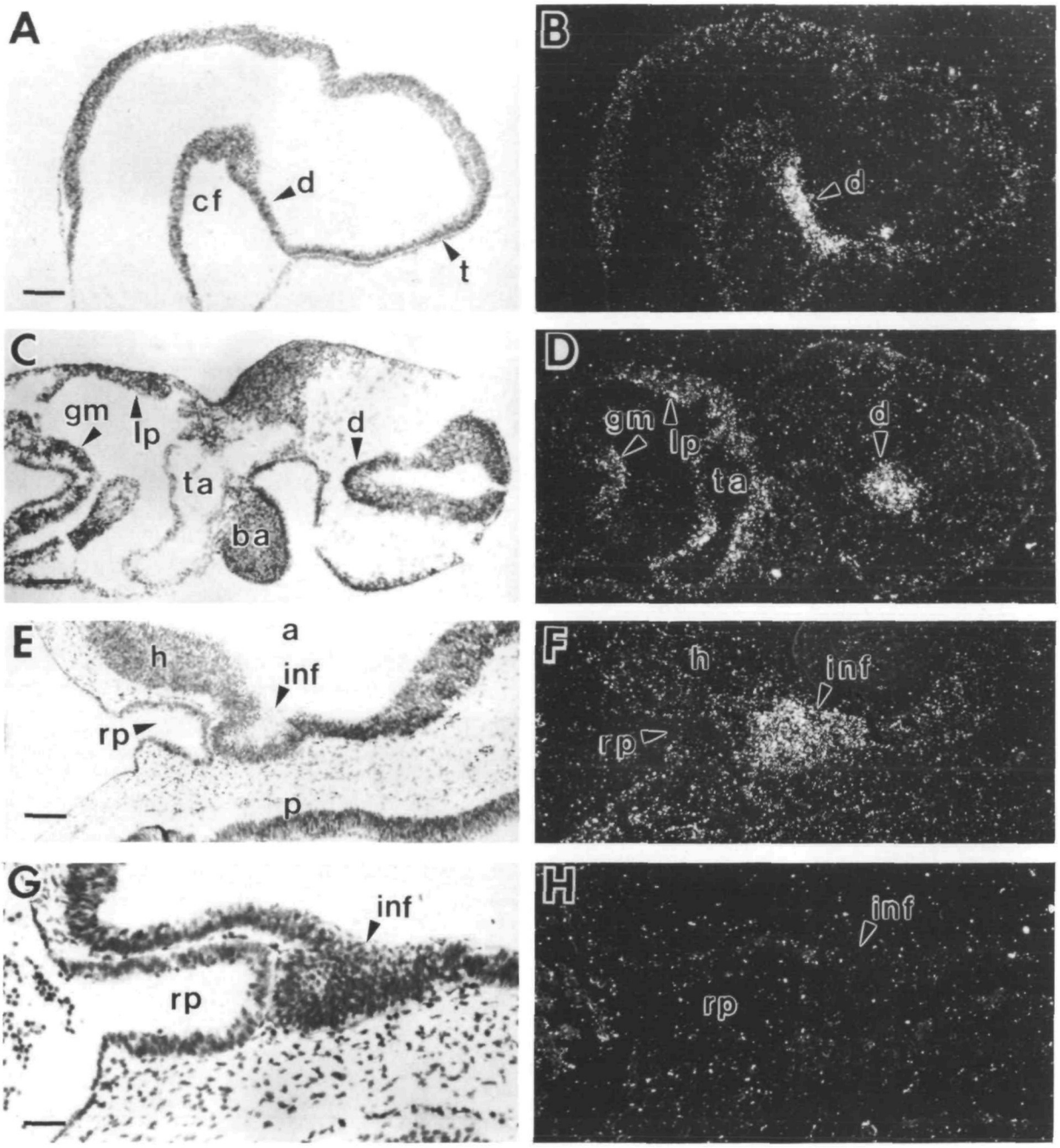


Fig. 3. Expression of BMP-4 in the 9.0 and 10.5 days *p.c.* brain. Bright-field (A,C) and dark-ground (B,D) photomicrographs of sagittal (A,B) and cranial (C,D) sections of 9.0 days *p.c.* embryos hybridized to BMP-4 antisense probe. Hybridization is seen in the ventral diencephalon (B and D) as well as the truncus arteriosus, mesoderm surrounding the gut, and in lateral plate mesoderm (D). d, ventral diencephalon; t, telencephalon; cf, cranial flexure; ba, branchial arch; ta, truncus arteriosus; gm, mesoderm around gut; lp, lateral plate mesoderm. Bright-field (E,G) and dark-ground (F,H) photomicrographs of sagittal sections of 10.5 days *p.c.* embryos hybridized to antisense (E,F) and sense (G,H) BMP-4 probes. A BMP-4-specific signal is seen in the infundibulum (F). rp, Rathke's pouch; inf, infundibulum; h, hypothalamus; a, anterior; p, posterior. Bar=100 μm (A,C,E) or 50 μm (G).

Discussion

The spatial and temporal patterns of BMP-4 expression reported here suggest that the protein is a mediator of inductive tissue interactions required for the establishment of several different organ systems, including heart, pituitary gland, limbs, craniofacial processes, whisker follicles and gut. In all of these systems, there is good experimental evidence that interactions take place between different cell populations, usually between epithelial and mesenchymal cell layers (for review see Wessels, 1977). Moreover, expression patterns of BMP-4 allow comparisons to the known functions of *dpp* in *Drosophila*. *dpp* is required for correct dorsal–ventral

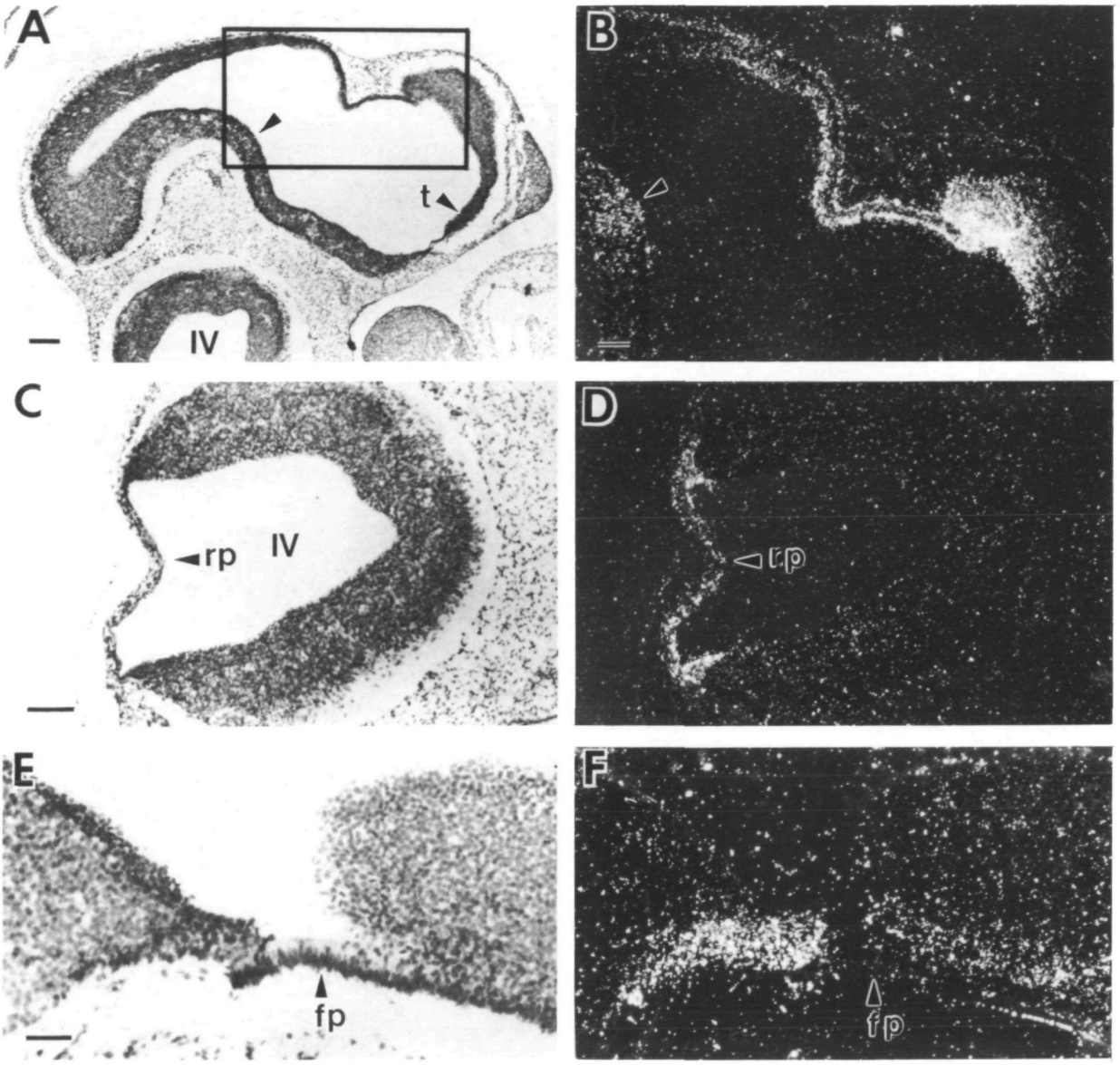


Fig. 5. Expression of Vgr-1 in the 10.5 days *p.c.* CNS. Bright-field (A,C,E) and dark-ground (B,D,F) photomicrographs of selected sections from the embryo shown in Fig. 4. The boxed area in A is enlarged in B. Hybridization of the Vgr-1 antisense probe at the level of the cerebral hemispheres (B), in the roof plate (D), and in cells adjacent to the floor plate (F) is shown. The section in E/F is oblique, due to curvature of the spinal cord. IV, fourth ventricle; rp, roof of IV ventricle; t, telencephalon; arrowhead, ventral wall at mesencephalon/diencephalon junction; fp, floor plate cells in hindbrain/spinal cord region. Bar=100 μm (A,C,E) or 50 μm (B).

patterning in the embryo, and is also expressed in the imaginal discs associated with establishment of the proximal–distal axis of the appendages and in the midgut mesoderm associated with differentiation of specific segments (Padgett *et al.* 1987; St Johnston and Gelbart *et al.* 1987; Bryant, 1988; Gelbart, 1989; Immergluck *et al.* 1990). The localization of BMP-4 transcripts in ventral regions of early embryos, in limb buds and in gut mesoderm suggests that the gene may perform similar patterning functions in the mammal.

BMP-4 in newly established mesoderm

The very low levels of BMP-4 expression in egg cylinder stage embryos (as judged by screening of a 6.5 days *p.c.* embryo cDNA library and by *in situ* hybridization) seem to exclude a role for the gene product in the initial induction of mesoderm. Localized expression of BMP-4 is first observed in the allantois and amnion of gastrula-stage embryos (7.5 days *p.c.*) and in ventral regions of newly formed posterior mesoderm and definitive endoderm in the early neurula (Fig. 1) i.e. *after* the initial induction of mesoderm from the epiblast. Moreover, while BMP-4 transcripts are localized in mesoderm caudal to the last somite at 8.5 days *p.c.* (Fig. 1), expression extends more anteriorly, but remains in ventral and lateral regions, in 9.0 days *p.c.*

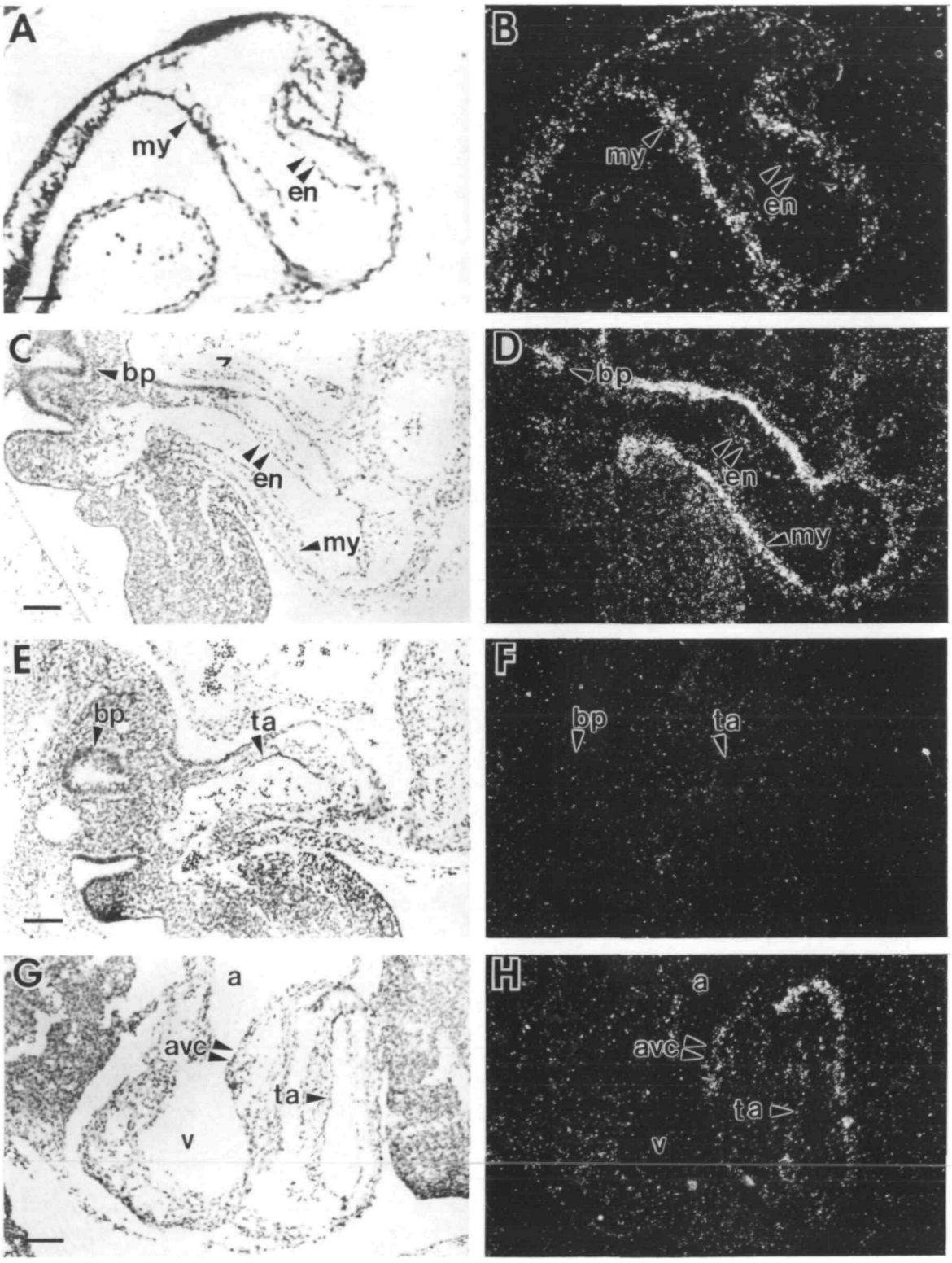


Fig. 6. Expression of BMP-4 and Vgr-1 in the developing heart. Bright-field (A,C,E) and dark-ground (B,D,F) photomicrographs of sagittal sections of 9.0 days *p.c.* (A,B) and 10.5 days *p.c.* (C,D,E,F) embryos hybridized with antisense (A,B,C,D) and sense (E,F) BMP-4 probes. Hybridization is seen in the myocardial layer of the developing atrioventricular canal (A,B) or truncus arteriosus (C,D). my, myocardial layer; en, endocardial layer; bp, branchial pouch. Bright-field (G) and dark-ground (H) photomicrographs of sagittal sections of a 10.5 days *p.c.* embryo hybridized with antisense Vgr-1 probe, showing expression in the truncus arteriosus at the junction of the dorsal aorta and in the atrioventricular cushions. avc, endocardial layer of the atrioventricular cushions; v, ventricle; a, atrium; ta, truncus arteriosus. Bar=50 μm (A) or 100 μm (C,E,G).

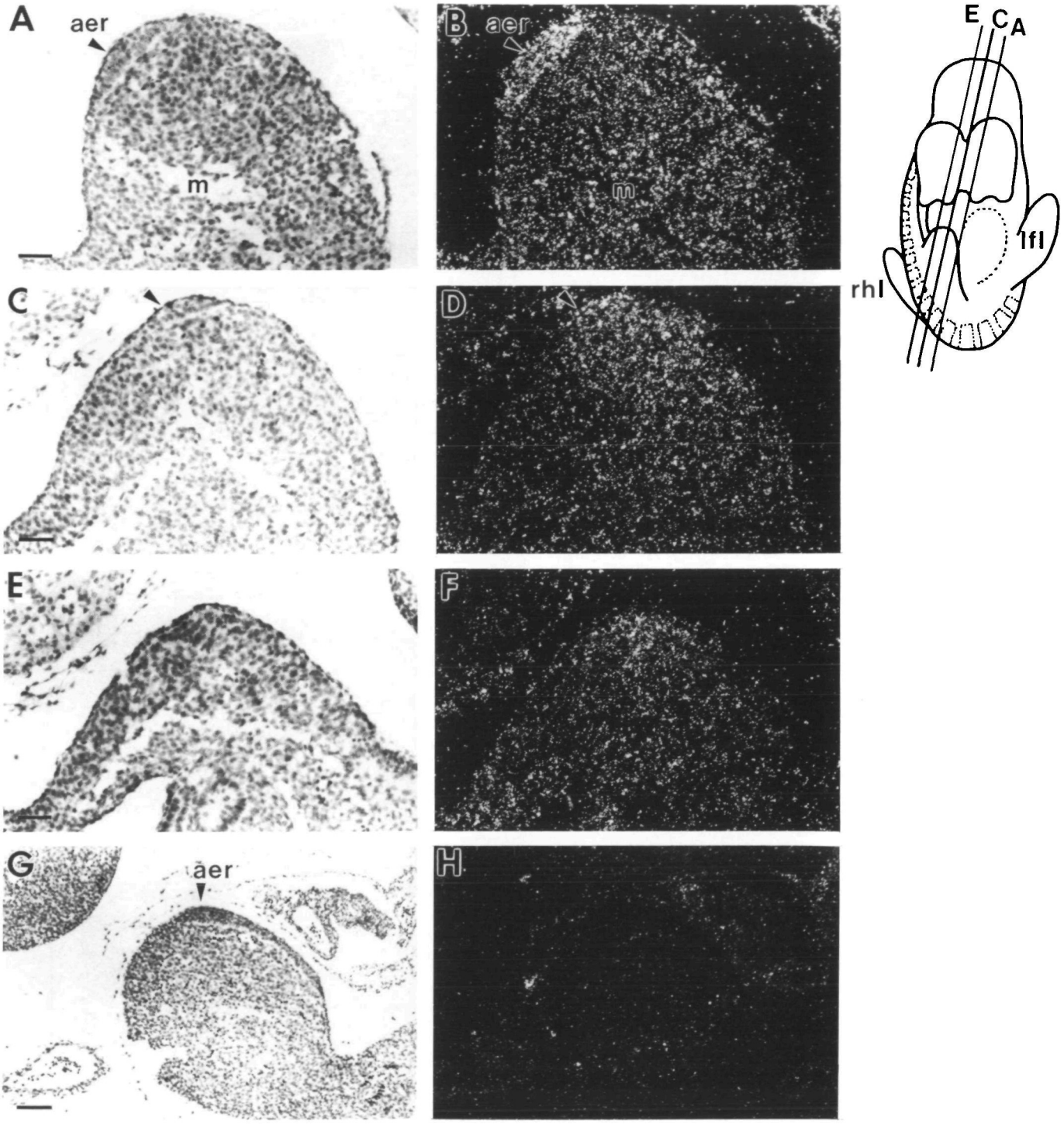


Fig. 7. Expression of BMP-4 in the 10.5 days *p.c.* limb bud. Bright-field (A,C,E,G) and dark-ground (B,D,F,H) photomicrographs of sections through the limb bud, showing BMP-4 expression in the AER (B and D) as well as in the mesenchyme. Reconstruction of serial sections and grain counting (see Materials and methods) revealed an anteroposterior and distal–proximal gradient of hybridization in the mesenchyme. The position of the sections is shown in the inset; section A is more anterior than section C, which is more anterior than section E. Sections A–E are hybridized with the antisense BMP-4 probe, while section G is hybridized with the sense BMP-4 probe. aer, apical ectodermal ridge; m, mesenchyme; rhl, right hindlimb bud; lfl, left forelimb bud. Bar=50 μm (A,C,E) or 100 μm (G).

embryos (Figs 1 and 2). This extension of BMP-4 expression to more anterior regions again suggests that the gene is not involved in the initial specification of mesoderm. This is because mesoderm is formed in an anterior–posterior direction, while BMP-4 expression is first apparent in the more posterior regions of

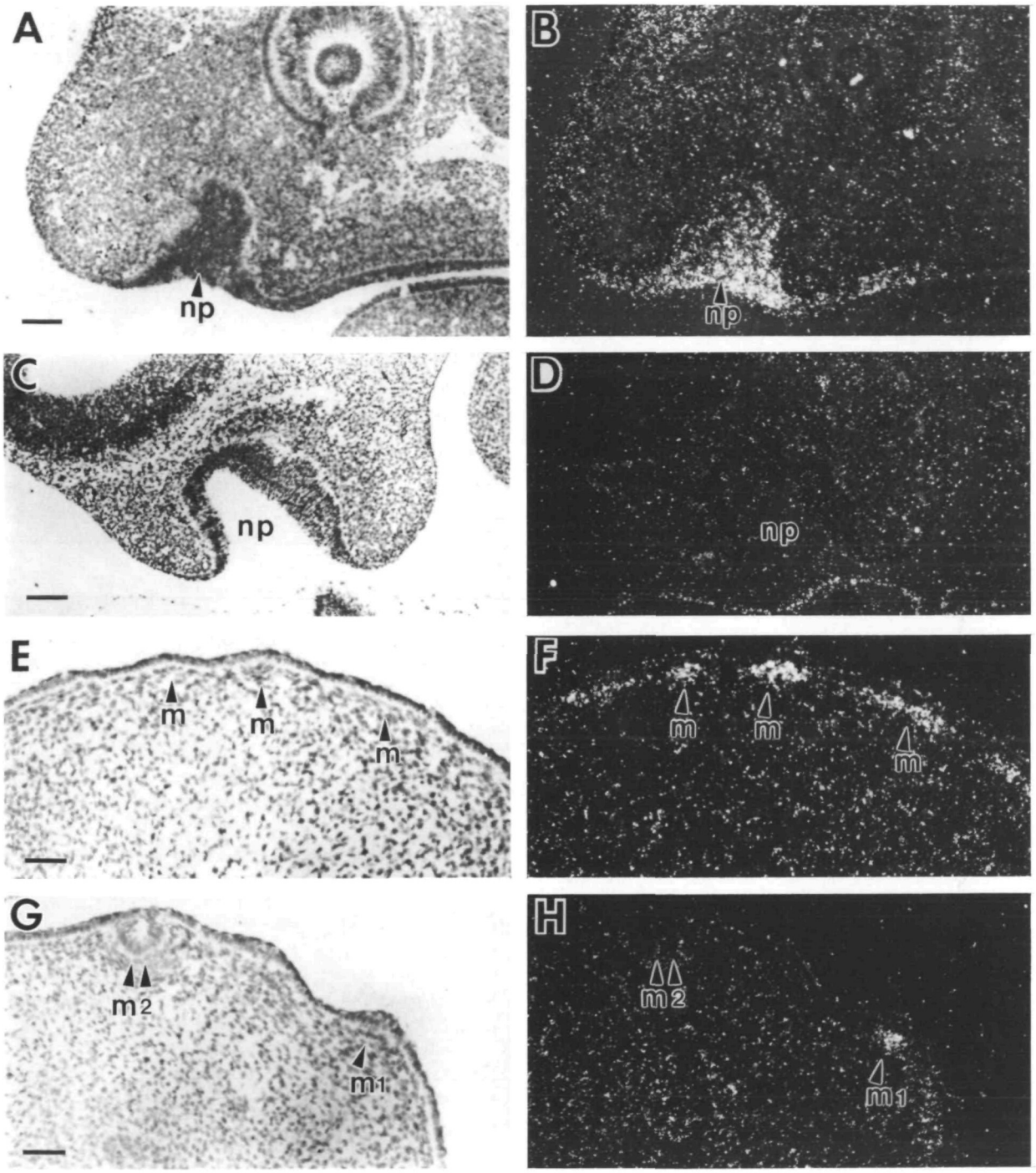


Fig. 8. Expression of BMP-4 in facial regions. Bright-field (A,C,E,G) and dark-ground (B,D,F,H) photomicrographs of sagittal sections of 10.5 days *p.c.* (A,C) and 13.5 days *p.c.* (E,G) embryos hybridized with antisense (A,E,G) and sense (C) BMP-4 probes. Expression is seen in the epithelium of the developing nasal pits (B) and in mesenchymal condensations involved in whisker follicle formation (F and H). np, nasal pit; m, mesodermal condensations of whisker follicle primordia prior to appearance of ectodermal placodes; m1, mesodermal condensations of stage 1 whisker follicles (see Lyons *et al.* 1990); m2, mesodermal condensation of stage 2 whisker follicle. Bar=100 μm (A and C) or 50 μm (E and G).

mesoderm, and spreads anteriorly until most of the ventral mesoderm exhibits expression.

BMP-4 in pituitary development

The pituitary gland has a dual origin. The anterior and intermediate lobes arise from Rathke's pouch, an invagination of oral ectoderm originating from an anterior placode (Couly and Le Douarin, 1985). In contrast, the posterior lobe has its origin in the basal neuroectoderm of the diencephalon which forms an

outpocket known as the infundibulum. By *in situ* analysis, BMP-4 RNA is transiently expressed specifically in the neuroepithelium of the diencephalon that makes contact with Rathke's pouch and gives rise to the posterior lobe of the pituitary (Fig. 3F). The BMP-4 protein may therefore act in an autocrine fashion to affect the proliferation and/or differentiation of the neural tissue from which the neurohypophysial cell lineage is established. Alternatively, BMP-4 could function in a paracrine fashion to influence the growth and differentiation of adjacent cells in Rathke's pouch. There is good evidence that the differentiation of at least the intermediate lobe of the pituitary requires interactions between Rathke's pouch and the infundibular wall (for review and references see Etkin, 1967; Hayes and Loh, 1990). Moreover, experiments with a coculture system have established that the basal diencephalon produces diffusible factor(s) that influence the proliferation and size of Rathke's pouch (Daikoku *et al.* 1982). BMP-4 could well be one such factor.

Vgr-1 in the developing CNS

In contrast to the restricted expression of BMP-4, Vgr-1 transcripts are present in a more extensive, but still localized, pattern along the developing CNS. Previous reports describe Vgr-1 expression in the pyramidal layer of the hippocampus at 16.5 days *p.c.* persisting through adulthood (Lyons *et al.* 1989*b*). We now extend those findings by localizing Vgr-1 transcripts in the early neural tube (10.5 days *p.c.*) to the roof plate and to groups of cells adjacent to the floor plate (Figs 4 and 5).

We are presently unable to state whether *Vgr-1* is expressed prior to neural tube closure, but comparisons can be drawn between expression patterns of Vgr-1 in later stages and those reported for another presumed signalling molecule, *int-1* (*wnt-1*) (Wilkinson *et al.* 1987). The pattern of *int-1* expression is similar to the *Vgr-1* expression that we report in the roof plate of the neural tube. However, int-1 expression extends anteriorly only to the level of the cerebral hemispheres, while Vgr-1 expression has an anterior boundary midway through the forebrain and extends laterally into the cerebral hemispheres. Vgr-1 transcripts are also found in the ventral regions of the developing neural tube, immediately adjacent (lateral) to the floor plate (Fig. 5F), and represent the first molecular marker for these groups of cells.

BMP-4 and other TGF-β-related genes in heart and blood vessel morphogenesis

Recent evidence suggests that members of the TGF-β family are involved in the morphogenesis of the heart, particularly in the epithelial–mesenchymal transformation and subsequent cell migration that occurs during the formation of cushion tissue from the primitive endocardium (Akhurst *et al.* 1990; Potts and Runyan, 1989; Lyons *et al.* 1990). BMP-2 expression is seen in the myocardial layers of both the atrioventricular (AV) canal and truncus arteriosus (TA) at 9.5 days but subsequently persists only in the AV region (Lyons *et al.* 1990). In contrast, BMP-4 expression appears first in the myocardial layer of the AV canal but by 10.5 days is only in the TA. Taken together, these results suggest that early in development BMP-2 and BMP-4 cooperate to influence the differentiation of the AV cushions, but later in development BMP-4 alone mediates some process unique to the truncus arteriosus.

Extending previous descriptions of Vgr-1 expression during mouse development (Lyons *et al.* 1989*a*,*b*), we present evidence here that Vgr-1 may also be involved in heart development. A low hybridization signal for Vgr-1 is seen in the mesenchyme of the atrioventricular cushions, drawing comparisons to expression patterns seen for other TGF-β related molecules (Lyons *et al.* 1989*b*; Akhurst *et al.* 1990). We also detect very restricted expression in the truncus arteriosus in the region of contact with the dorsal aorta (Fig. 6). Therefore, it seems that several members of the TGF-β family, including TGF-β1 (Akhurst *et al.* 1990), BMP-2 (Lyons *et al.* 1990), BMP-4 and Vgr-1 have distinct expression patterns within the developing heart, indicating that each may play a unique role in cardiac morphogenesis.

BMP-4 and patterning in the early limb bud

Previous work from this laboratory has shown that BMP-2 transcripts are expressed in the thickened ventral ectoderm of the limb bud at 9.5 days *p.c.* and in the AER at 10.5 days p.c., suggesting that the protein plays a role in the patterning of the developing limb (Lyons *et al.* 1990). In this paper, we show that BMP-4 is expressed both in the AER at 10.5 days *p.c.* and in the mesenchyme, apparently in an anterior–posterior and distal–proximal gradient (Fig. 7). The possibility therefore exists that BMP-2 and BMP-4 cooperate to influence early limb development. Moreover, heterodimers of BMP-2 and BMP-4 could be formed within the AER. TGF-β-related growth factors have been shown to have clearly different activities when acting as a homodimer or heterodimer. For example, inhibin β homodimers selectively stimulate FSH release in the pituitary gland, while inhibin β/α heterodimers inhibit such secretion (Hsueh *et al.* 1987). BMP-4 could therefore have differing effects if it is a homodimer in the mesenchyme or complexed with BMP-2 in the AER.

The fact that BMP-4 is apparently expressed in a gradient in the mesenchyme invites comparison with the gradients detected for homeobox-containing genes such as members of the Hox 5 cluster (Dollé *et al.* 1989*a*) and Xlhbox 1/Hox 3.3 (Oliver *et al.* 1988). These results raise the possibility that BMP-4 is part of a cascade of polypeptide signalling molecules, homeobox and retinoic acid receptor (Dollé *et al.* 1989*b*) genes involved in establishing pattern within the developing limb. Support for complex networks of interaction between such molecules comes from recent observations that the homeobox-containing gene, *Ubx*, activates *dpp* expression, with *dpp* in turn regulating *wg* and *lab* in the *Drosophila* midgut (Immergluck *et al.* 1990) and that XTC-MIF (activin A) induces a

characteristic level of *Xhox 3* expression in *Xenopus* animal caps (Ruiz i Altaba and Melton, 1989).

Confirmation of such interactions during limb development must await the localization of active BMP proteins and their cellular receptors within the limb bud, as well as the effects of the gene products on the developing cells. Nevertheless, it is now clear that along with retinoic acid and homeobox-containing genes, polypeptide growth factors must be considered as having an integral part in patterning within the developing limb.

BMP-4 and other TGF-β molecules in whisker follicle development

Hair and whisker follicle development involves a sequence of reciprocal interactions between the epidermis and underlying dermis (for description and references see Davidson and Hardy, 1952). Previous work from this laboratory had localized different TGF-β related gene transcripts to specific cell populations in the developing whisker follicles, with BMP-2 appearing first, in the ectodermal placodes at stage 1 (Lyons *et al.* 1990). Here we show that BMP-4 expression occurs even earlier, in the underlying mesenchymal condensations preceding stage 1 follicles (Fig. 8F and H). It appears that BMP-4 expression in the mesenchyme is transient, since no hybridization signal is found in the mesenchyme of stage 2 follicles (m2 in Fig. 8G). From this localization pattern, BMP-4 could be acting as a signalling molecule, instructing the overlying ectoderm. Consistent with this view, mesenchymal condensations can induce hair follicle formation when transplanted under previously hairless epithelium (Kollar, 1970).

BMP-4 and craniofacial morphogenesis

The expression of BMP-4 in facial regions of the early mouse embryo (Figs 2, 6C and D, 8A and B) suggests a role for the gene in craniofacial morphogenesis. Expression in the early otic vesicle (Fig. 2) also raises the possibility that BMP-4 is involved in development of the inner ear. This is supported by the observation that later in development BMP-4 transcripts are found in the sensory/structural epithelium of the inner ear (data not shown). Additionally, in contrast to expression patterns reported for BMP-2, BMP-4 transcripts are not detectable in developing tooth buds (data not shown).

Concluding Remarks

From the data reported here, it is clear that BMP-4 expression is often coordinated in a temporal and spatial manner with expression of other members of the TGF-β gene family, particularly the closely related gene, BMP-2. While the full significance of these patterns must await studies with the purified proteins and localization of the cellular receptors for each molecule, they nevertheless strongly suggest that members of the TGF-β family function in inductive tissue interactions during the establishment of several specialized organ systems within the developing embryo.

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