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Growth factors in development: the role of TGF-β related polypeptide signalling molecules in embryogenesis

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SUMMARY

Embryonic induction, the process by which signals from one cell population influence the fate of another, plays an essential role in the development of all organisms so far studied. In many cases, the signalling molecules belong to large families of highly conserved proteins, originally identified as mammalian growth factors. The largest known family is related to Transforming Growth Factor-β (TGF-β) and currently consists of at least 24 different members. Genetic studies in *Drosophila* on the TGF-β related gene, *decapentaplegic* (*dpp*), reveal the existence of conserved mechanisms regulating both the expression of the protein during development and the way in which it interacts with other signalling molecules to generate pattern within embryonic tissues. Comparative studies on another TGF-β related gene, known as *Bone Morphogenetic Protein-4* (*BMP-4*), in *Xenopus* and mouse point to a conserved role in specifying posteroventral mesoderm during gastrulation. Analysis of other polypeptide signalling molecules during gastrulation suggests that their interaction in the generation of the overall body plan has also been conserved during vertebrate evolution.

Key words: TGF-β, embryonic induction, BMP, polypeptide signalling molecules

INTRODUCTION

An essential feature of embryogenesis is the process known as embryonic induction, by which signals produced by one cell population change the developmental fate of another. Inductive events may occur many times during the elaboration of an embryo, and involve several different levels of complexity. At one extreme, the inductive signal works over a relatively short distance and elicits a simple switch in the fate of the responding cells. At the other extreme, the signalling cells act as an organizing center, producing diffusible morphogens, which induce different responses in the target cells depending on their distance from the organizer and the concentration of signal to which they are exposed.

Over the past few years considerable progress has been made in identifying the signalling molecules mediating embryonic induction in both vertebrates and invertebrates. However, much less is known about their receptors and the downstream pathways eliciting cellular responses (for reviews see Smith, 1989; Slack, 1993, 1994). In many cases the signalling molecules belong to large, highly conserved families of proteins related to growth factors, such as fibroblast growth factor (FGF), epidermal growth factor (EGF), the Wnt gene products, and transforming growth factor-β (TGF-β). An attractive hypothesis is that the prototypic ancestral multicellular organism used a relatively small number of such signalling molecules, and associated receptors and signal transduction pathways, to co-ordinate embryogenesis. During evolution, these intercellular communication systems appear to have been conserved and elaborated upon to bring about increasingly complex morphogenetic processes.

THE TGF-β PROTEIN SUPERFAMILY: STRUCTURE, PROCESSING AND CLASSIFICATION INTO DIFFERENT SUBFAMILIES

All members of the TGF-β superfamily, of which there are currently at least 24, are synthesized as large prepro precursor molecules, which are cleaved at an RXXR site to release a C-terminal peptide of 110-140 amino acids (Fig. 1A). In most cases this region contains 7-9 cysteine residues, and studies on the crystal structure of the mature region of TGF-β2 have shown that one of these cysteines is involved in the intermolecular disulfide bonding associated with the formation of biologically active homo- or heterodimers (Fig. 1B; Daopin et al., 1992; Schlunegger and Grutter, 1992). Comparison of the crystal structure of TGF-β2 with that of three other growth factors/hormones, PDGF-BB (platelet-derived growth factor BB), NGF (nerve growth factor) and hCG (human chorionic gonadotrophin) has revealed a remarkable similarity in the three-dimensional structure of the proteins, with two pairs of antiparallel β strands and a conserved arrangement of intertwined disulfide bridges known as the 'cystine knot' (McDonald and Hendrickson, 1993; Lapthorn et al.,1994). Interestingly, all the proteins also form dimers, but the

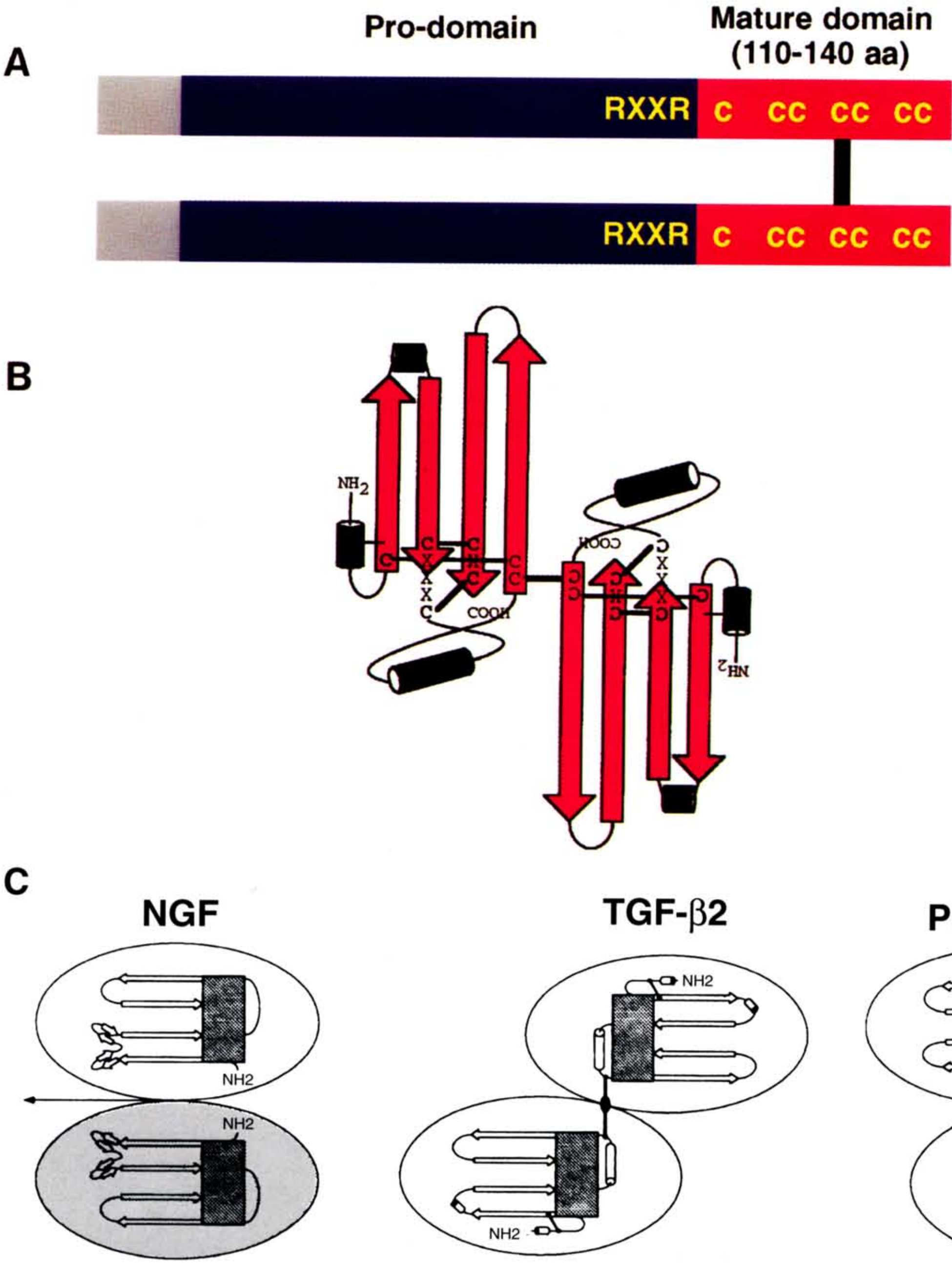

Fig. 1. Structural organization of TGF-β related proteins. (A) Proteins are synthesized as precursor molecules with a signal sequence (grey), a prodomain (blue) and a C-terminal mature region (red). A dibasic proteolytic cleavage site (RXXR) is invariably found near the junction of the pro and mature regions. In most members of the superfamily a single cysteine is involved in the formation of homo- or heterodimers (black line) In some members the spacing between this cysteine and the next is different, and in two known examples, Vgr-2 and GDF-9, this cysteine is absent (see text). (B) Crystal structure of TGF-β2 with the cystine knot motif. The two pairs of antiparallel β strands are shown in red. Interaction with the receptor is thought to involve the larger loop and alpha-helical region (Daopin et al., 1992; Schlunegger and Grutter, 1992). Figure adapted from Kingsley, 1994. (C) Different organization of the dimers of NGF, TGF-β2 and PDGF-BB. Taken from McDonald and Hendrickson, 1993.

protomers interact in very different ways. An intriguing possibility is that all cystine knot proteins have evolved from a common ancestral molecule that was monomeric (Fig. 1C; McDonald and Hendrickson, 1993) but convergent evolution of a very stable structure is also possible. Two TGF-β-related proteins have been identified that lack the cysteine involved in intermolecular disulfide bonding. These are Vgr-2/GDF-3 and GDF-9 (Jones et al., 1992b; McPherron and Lee, 1993). Recent preliminary studies have shown that Vgr-2 RNA injected into *Xenopus* embryos is able to elicit a strong biological response (C. M. J. unpublished results). This suggests that Vgr-2 protein can either act as a monomer or, more likely, form stable dimers through hydrophobic bonding of protomers in the absence of an intermolecular disulfide bridge.

It is assumed, without a great deal of supporting evidence, that all TGF-β precursor molecules are proteolytically cleaved in vivo to release biologically active C-terminal protein. This proteolysis is thought to involve both the RXXR sequence adjacent to the mature region and possibly the degradation of the propeptide that, at least in the case of TGF-β1-3, can form a latent complex with the C-terminal region. However, remarkably little is known about the precise in vivo mechanisms of dimer formation, cleavage of the C-terminal region and further processing, and how these different steps may be regulated particularly during embryonic development. Recent studies with *Xenopus* Vg-1 suggest that under some circumstances post-translational modification is a step at which important regulation can be exerted (Dale et al., 1993;

Thomsen and Melton,1993). Initial experiments in which Vg-1 RNA was injected into *Xenopus* eggs failed to show any biological response. Although synthesis of full length, monomeric protein could be detected with an antibody, no proteolytic cleavage and dimer formation occurs. By contrast, mesoderm induction is seen if the embryo is tricked into processing mature Vg-1 by injecting RNA encoding a chimeric protein, in which the pro region of BMP-2 or BMP-4 (including or not including the cleavage site) is joined to the mature region of Vg-1. This raises the possibility that in the unfertilized egg the Vg-1 protein is blocked from processing or dimer formation or that a Vg-1-specific processing protease is inactive. According to this model, after cortical rotation there is local activation of Vg-1 modification in the dorsal-vegetal blastomere, releasing a small amount of mature protein which then induces the Nieuwkoop center (Thomsen and Melton, 1994).

At present the factors involved in producing active Vg-1 have not been characterised. However, genetic studies in *Drosophila* have suggested that the product of the *tolloid* gene is involved in the activation of DPP, the TGF-β related protein encoded by the *decapentaplegic* gene. The sequence of the tolloid protein is closely related to that of mammalian BMP-1 (Bone Morphogenetic Protein-1), a protein that copurified with the TGF-β related BMPs from demineralized bone (Wozney et al., 1988; Shimell et al., 1991) Both tolloid and BMP-1 have an N-terminal domain related to the astacin family of metalloendopeptidases, as well as CUB and EGF repeats, and proteins related to tolloid have also been found in sea urchins (Hwang et al.,1994). Mutational analysis of *Drosophila* tolloid suggests that the protein does not directly activate DPP but may form a multiprotein complex with it and work indirectly by activating another accessory protein(s) in the processing pathway (Finelli et al., 1994; Childs and O'Connor, 1994). The story is likely to be complicated, however, since tolloid related proteins have recently been found in *Drosophila* (R. Padgett and M. O'Connor, personal communication) suggesting that a gene family may exist. Moreover, it is unclear whether tolloid interacts only with DPP or with other TGF-β related proteins in *Drosophila* such as 60A and screw (Childs and O'Connor, 1994).

Recently, both *Xenopus* and mouse genes closely related to human BMP-1 have been cloned and their expression patterns studied (Maeno et al., 1993; Fukagawa et al., 1994). The mouse protein differs from both human and *Xenopus* BMP-1 in having additional CUB and EGF repeats, making it more similar to tolloid. BMP-1 transcripts are found in the mouse embryo from 7.5 days p.c. on, but are distributed at low levels throughout the mesoderm rather than being localized to a few cell types. However, the gene is expressed at high levels in the floor plate of the spinal cord and midbrain/hindbrain from about 9.5 days where it is regulated directly or indirectly by the transcription factor, HNF-3β (Sasaki and Hogan, 1994). At present, the significance of the localized expression of BMP-1 in the floor-plate is unclear. If BMP-1 is playing a role in the activation of a TGF-β related protein one might expect the genes encoding the protease and the substrate to be co-expressed, as seen for *tolloid* and *dpp* in *Drosophila*. However, although several TGF-β related genes are expressed locally in the developing spinal cord and brain (for example, dorsalin in the chick embryo roof plate, as shown by Basler et al., 1993), none has so far been found to be expressed specifically in the floor plate. Further elucidation of the role of putative processing enzymes like tolloid/BMP-1 must await gene knock-out studies in the mouse and the identification of more members of what appears to be a family of evolutionarily conserved proteins.

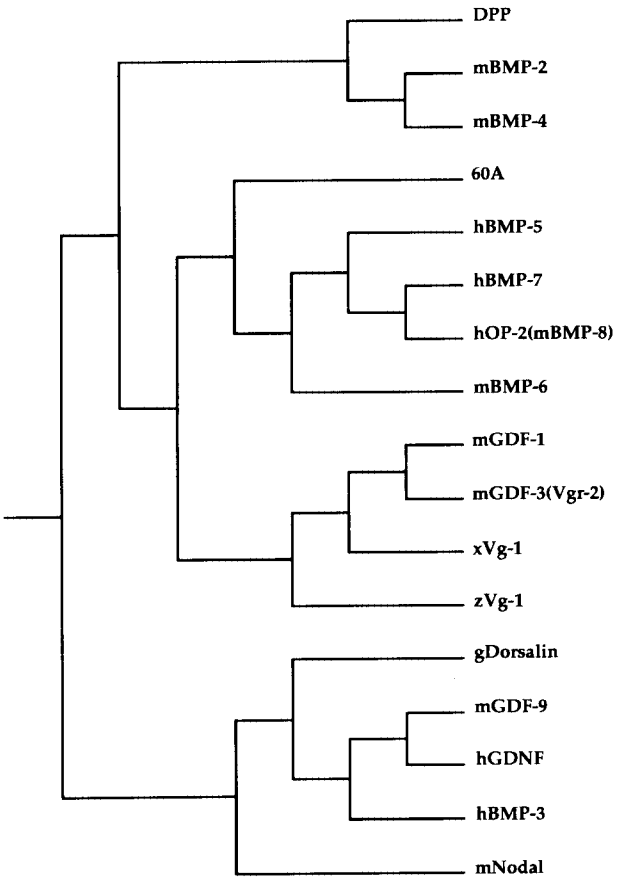

Fig. 2. Possible phylogenetic relationships between members of the DVR (decapentaplegic-Vg-related) subfamily of signalling molecules. This analysis is based on comparison of C-terminal amino acid sequences using PAUP 3.1 (Swofford and Berlocher, 1987) m, mouse; h, human; x, *Xenopus*; z, zebrafish; g, *Gallus* (chick); OP, osteogenic protein; GDF, growth and differentiation factor; Vgr, Vg related.

Comparison of the C-terminal mature regions of TGF-β superfamily proteins suggests that the members can be classified into a number of different subgroups (for recent review see Kingsley, 1994). By far the largest of these is the so-called DVR (Decapentaplegic-Vg-related) subfamily. The evolutionary relationship of members of this subfamily is shown in Fig. 2. Although preliminary, and almost certainly incomplete, this figure suggests that the family has evolved from a few ancestral proteins present before the divergence of vertebrates and invertebrates. In some cases the amino acid sequences of related proteins from insects and mammals are so similar that they can substitute for each other functionally. For example, both *Drosophila* DPP and 60A induce bone when injected subcutaneously into rat skin (Sampath et al., 1993), and Padgett et al. (1993) showed that the C-terminal region of human BMP-4 can substitute for that of DPP in the rescue of mutants in dorsoventral patterning of the embryo.

RECEPTORS

The nomenclature for the receptors of TGF-β-like ligands is based on that originally developed for the cell associated proteins which bind TGF-β itself (for review see Artisano et al., 1992; Massague, 1992). Thus, the type III receptor is a high molecular mass membrane-associated proteoglycan known as betaglycan (Wang et al., 1991), while the type I and II receptors are transmembrane proteins of much smaller size (53 and 70-85×10^3 M_r, respectively). The initial cloning of the *C. elegans daf-1* receptor (Georgi et al., 1990) and the type II receptors for TGF-β and activin (Mathews and Vale, 1991; Lin et al., 1992) finally opened up studies on the signal transduction pathways activated by TGF-β-like molecules. These studies revealed that the type II receptors belong to a novel family of transmembrane serine/threonine kinases. It was then shown that type I receptors also belong to a closely related family of serine/threonine kinases (Attisano et al., 1993; Estevez et al., 1993) and that type I and type II receptors form heteromeric complexes which bind different ligands and regulate different intracellular responses. Stated very simply, it appears at present that type I receptors are somewhat promiscuous and can bind different ligands depending on the more limited, but not totally inflexible, ligand specificity of the type II receptor with which they associate. However, while type II receptors are responsible for initiating certain intracellular signalling pathways, they are inactive in this process without associating with a type I receptor. Clearly, there is a great deal more to be learnt about the functional significance of combinatorial associations of type I and type II receptors and about the role they play in determining the different response of embryonic cells to TGF-β related ligands. Meanwhile, it is likely that the results of experiments in which a dominant-negative type II receptor is overexpressed in embryos (see, for example, Hemmati-Brivanlou and Melton, 1992) will have to be interpreted with caution since the truncated receptor protein may interact with more than one kind of type I receptor and so alter the response of cells to more than one ligand.

GENETIC ANALYSIS OF *decapentaplegic (dpp)* FUNCTION IN *DROSOPHILA* PROVIDES CLUES FOR MECHANISM OF ACTION OF RELATED GENES IN VERTEBRATE EMBRYOS

One of the best studied TGF-β related genes is *decapentaplegic* (*dpp*) in *Drosophila*. Genetic analysis has shown that it is required for cell-cell interaction during several different morphogenetic processes. These include dorsal-ventral patterning of the blastoderm embryo, proximal-distal patterning of the imaginal discs, and midgut morphogenesis. Each example provides an important paradigm for understanding the equally diverse roles of TGF-β related genes in vertebrate embryos. Indeed, as more is learnt about the role of TGF-β related genes in vertebrate embryogenesis, the factors that regulate their expression and the downstream genes that they control, a unifying concept may emerge based on the evolutionary conservation of not just a protein structure but of a whole signalling pathway.

During dorsal-ventral patterning of the *Drosophila* blastoderm embryo *dpp* is transcribed in the dorsal half of the embryo, but is repressed ventrally by the action of the *dorsal* gene product (Irish and Gelbart, 1987; Ferguson and Anderson, 1992; Wharton et al., 1993). Homozygous null mutants of *dpp* show complete ventralization of the embryo, while heterozygotes and hypomorphic mutants show graded deletions of dorsal structures and expansion of the ventral domain. By contrast, adding extra copies of the *dpp* gene, either to wild-type or to mutant embryos, increases the number of amnioserosal cells, which normally differentiate from the most dorsal ectoderm. These observations are all consistent with a model in which a gradient of DPP activity determines cell fate along the dorsal-ventral axis. In other words, in dorsal-ventral patterning, DPP acts as a morphogen. The crucial question is how this activity gradient is established. Among the many possibilities are the following: differential translation of a uniformly distributed mRNA, differential proteolytic processing, dimer formation or binding of accessory proteins that modify the activity of DPP, and graded synthesis of other TGF-β related protein(s) that can form heterodimers with DPP, which are more or less active than homodimers and/or elicit different responses in target cells. The recent identification of a putative type I DPP receptor should also throw new light on the way in which the protein regulates early development (Xie et al., 1994).

Midgut morphogenesis is a second process in the *Drosophila* embryo where *dpp* is required. In many ways it provides a model for understanding the role of TGF-β proteins in epithelial/mesenchymal interactions in vertebrates, since DPP is synthesized by the visceral mesodermal cells and then interacts locally with adjacent epithelial cells of the embryonic gut to influence their differentiation (Immergluck et al., 1990; Panganiban et al., 1990; Reuter et al., 1990). Recent studies have shown that in the mesoderm the *dpp* gene is activated directly by the product of the HOM gene *Ultrabithorax* (*Ubx*), which binds to multiple sites in a 5′ upstream regulatory region. By contrast, abdominal-A (abd-A) inhibits *dpp* expression in the mesoderm by interfering with this Ubx binding (Hursh et al., 1993; Masucci and Hoffmann, 1993; Capovilla et al., 1994). In the endoderm, binding of DPP protein leads to the activation of the HOM gene, *labial*, via a specific response element in the 5′ upstream region of the gene (Tremml and Bienz, 1992). These studies clearly show that *dpp* acts during development both upstream and downstream of homeotic genes, and, as we shall see, this has become an important paradigm for understanding the role of TGF-β related proteins in tissue patterning in higher organisms.

In the leg imaginal disc of *Drosophila*, *dpp* has been shown by genetic analysis to play a role in establishing the proximal-distal axis, and different mutations in *dpp* progressively reduce distal elements. Paradoxically, *dpp* is not expressed along the future P-D axis of the disc, nor in the distal tip, but in a stripe, immediately adjacent to, and just anterior of, the dorsal posterior compartment, which expresses engrailed (en) (Raftery et al., 1991). An important discovery is that *dpp* expression is maintained in this boundary by the short range signalling molecule, hedgehog (hh), produced by the posterior cells (Basler and Struhl, 1994). Another signalling molecule, Wingless (wg), a member of the Wnt family, is expressed in a similar stripe to dpp, but in the ventral half of the disc. Recent evidence suggests a model in which the expression domain of *dpp* can be reconciled with its patterning function in the leg disc. According to this model, local interaction of DPP and wg, at the intersection of the A-P and D-V axes, induces focal expression of the

homeobox gene, *aristaless* (*al*) and this establishes an organizing center promoting P-D growth and patterning (Campbell et al., 1993; French and Daniels, 1994). This model, if correct, shows how the interaction of at least two polypeptide signalling molecules, wg and dpp, can set up pattern in an epithelial layer of cells. The recent discovery of a family of hh related proteins in vertebrates (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993; Roelink et al.,1994; Smith, 1994) raises the possibility that they regulate the expression patterns of *dpp*-related genes such as *BMP-2* and *BMP-4*, which are described below.

ESTABLISHING THE VERTEBRATE BODY PLAN: THE ROLE OF TGF-β RELATED SIGNALLING MOLECULES IN *XENOPUS* MESODERM INDUCTION AND SPECIFICATION

Mesoderm formation and patterning in the *Xenopus* embryo is perhaps the best understood example of embryonic induction in vertebrates. To date, several different TGF-β related proteins have been implicated in the overall process, namely Vg-1, activin (β_A and/or β_B), BMP-2 and BMP-4, and two nodal-related proteins, xNR-1 and xNR-2 (Slack, 1993, 1994; Smith,1989; C. M. J,. unpublished results). These are thought to work in conjunction with other polypeptide signalling molecules, including FGFs and Wnts. The complex sequence of interactions starts around the 32-64 cell stage when signals from the vegetal hemisphere induce two types of mesoderm - dorsoanterior and ventroposterior - in the overlying cells of the equatorial zone. Further regionalization of the mesoderm occurs during late blastula and gastrula stages, after which the basic body plan is established.

A detailed analysis of the experimental evidence in favor of the current model of *Xenopus* mesoderm induction and patterning is beyond the scope of this article (for most recent review, see Beddington and Smith, 1993; Slack, 1994). Very briefly, the most favored candidates for inducers of the ventroposterior mesoderm phenotype in the equatorial zone are Vg-1, Wnt-11, FGFs, BMPs and activin. In fact, it seems most likely that they act in a 'cascade', with Vg-1 and Wnt-11 acting as primary mesoderm inducers and FGF, BMPs and activin behaving as secondary factors. Vg-1 may also initiate the formation of the dorsovegetal Nieuwkoop centre. This, in turn, induces the Spemann organizer which releases factors responsible for dorsalizing the mesoderm. Current candidates for potent dorsalizing factors are noggin, nodal-related proteins and activin. Again, these factors may act in combination or in a cascade.

Most simple models for mesoderm patterning in *Xenopus* propose that ventroposterior mesoderm is a kind of 'default' phenotype, assumed by the marginal zone mesoderm furthest removed from the dorsalizing influence of the Spemann organizer. However, studies on the effect of misexpressing BMP-4 have suggested an alternative hypothesis in which BMP-4 (alone, or in combination with Wnt-8) is an active ventralizing factor counteracting or attenuating the dorsalizing factors produced by the organizer (Dale et al., 1992; Jones et al., 1992a). One problem with this model is that although maternal BMP-4 mRNA is present in the *Xenopus* blastula prior to mesoderm induction, it is not localized to any specific region. It is therefore necessary to invoke some differential activation of the mRNA or protein. However, studies have shown that BMP-4 autoinduces its own expression (Jones et al., 1992a), so that a small local production of active protein in the ventral vegetal hemisphere could subsequently be translated into activation of gene transcription in the posterior mesoderm of the marginal zone.

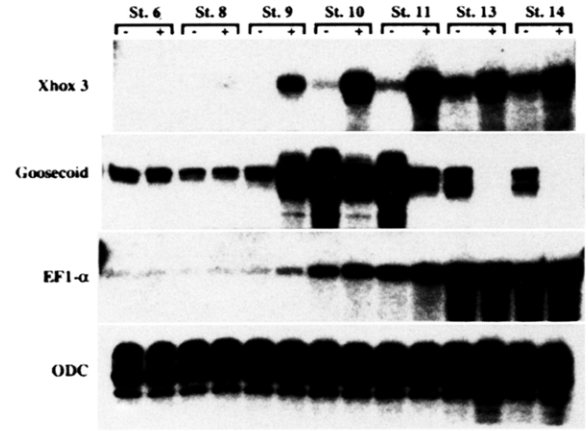

Fig. 3. Effect of BMP-4 on *goosecoid* and *Xhox3* expression in *Xenopus* embryos. BMP-4 RNA was injected into one-cell embryos (0.5-1.0 ng/embryo) as described by Jones et al. (1992a) and incubation continued through different stages. RNAse protection was carried out using approximately three embryo equivalents of RNA for each sample. *Goosecoid* expression is detected maternally (Stages 6 and 8) and through early gastrula (Stage 10). Subsequently, *goosecoid* transcripts rapidly decline in injected embryos (+) compared with controls (−) and become undetectable by the end of gastrulation. In contrast, *Xhox 3* transcipts accumulate to much higher levels in the same BMP-4-injected embryos compared with controls. The precocious expression of *Xhox 3* could mediate the ventralizing effect of BMP-4 during gastrula stages, resulting in the down regulation of goosecoid expression. Accumulation of EF-1α transcripts marks the beginning of zygotic transcription, and ODC serves as a loading control for all lanes.

Studies on the effect of injecting BMP-4 RNA into the fertilized *Xenopus* egg show that, while the injected embryos become ventralized, they apparently develop normally to the early gastrula stage, complete with the formation of a dorsal blastopore lip. This suggests that BMP-4 ventralizes mesoderm during gastrulation, in addition to any possible earlier effects on ventroposterior mesoderm induction. In support of this hypothesis it has recently been shown that *goosecoid*, a gene first expressed in the organizer region of the *Xenopus* embryo (Cho et al., 1991) is expressed at normal (or even slightly higher) levels in BMP-4-injected embryos at the initiation of gastrulation, but is then rapidly down-regulated during mid-gastrula stages (Fig. 3). Furthermore, in the same experiment, transcripts for *Xhox 3*, a homeobox containing gene previously shown to be preferentially expressed in posterior regions (Ruiz i Altaba and Melton, 1989a,b), accumulate to much higher levels in BMP-4-injected embryos than in control embryos of the same stage (Fig. 3). These data support the idea that the dorsal lip that forms in BMP-4-injected embryos initially has properties of a normal organizer, but that signals induced by BMP-4, possibly through regulation of the homeobox gene, *Xhox 3*, subsequently block differentiation of dorsal mesoderm. Further support for this hypothesis comes from experiments using a DNA construct in which the regulatory elements of the cytoskeletal actin gene drive expression of BMP-4 only after the initiation of zygotic transcription (C. M.

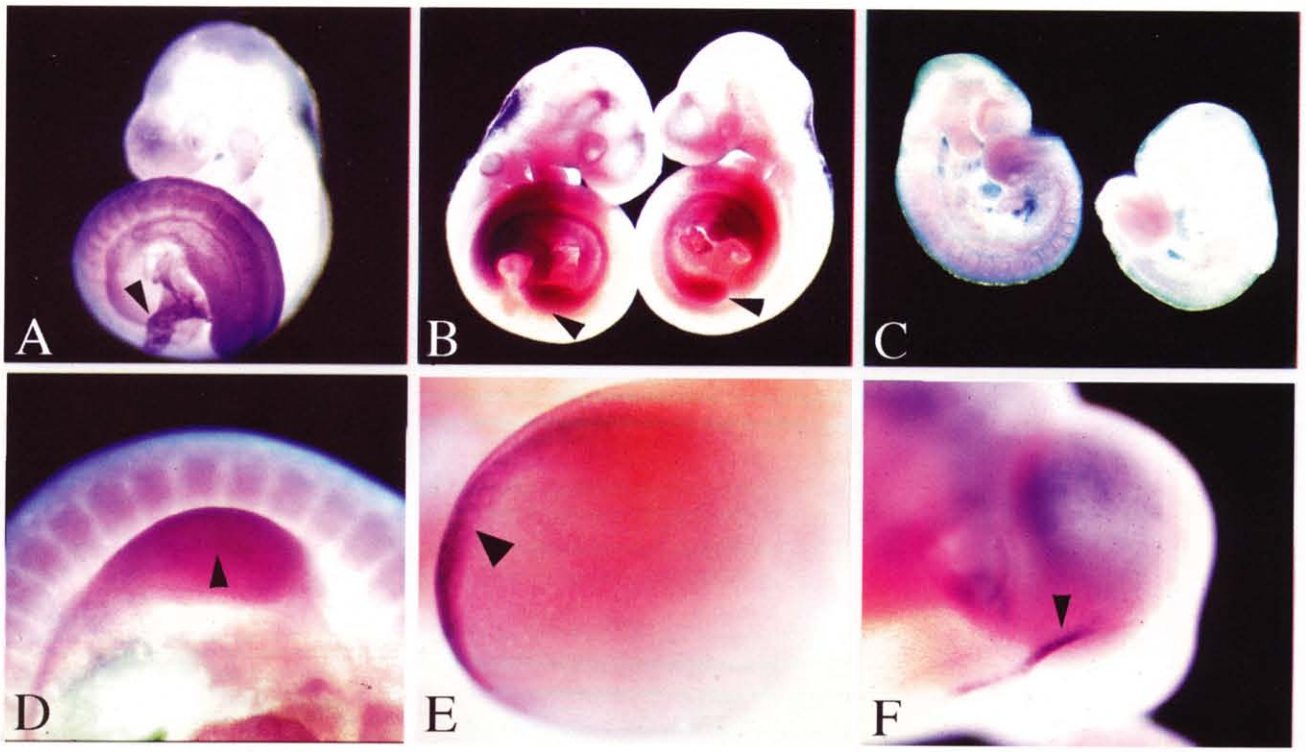

Fig. 4. Localization of transcripts for BMP-4 in 9.0-10.5 day p.c. mouse embryos, as revealed by whole-mount in situ hybridization. (A) 9.0-day embryo showing strong expression in the posterior mesoderm and around the umbilical vessels (arrowhead). (B) Two 9.5-day embryos, showing expression in the posterior mesoderm and in the mesenchyme of the forelimb bud (arrowheads). (C) Control embryos hybridized with sense strand probe. (D) High power magnification of the limb shown in B, with expression throughout the mesenchyme. (E) By 10.5 days strong BMP-4 expression is seen in the apical ectodermal ridge of the forelimb bud (arrowhead). (F) In the 10.5-day embryo BMP-4 transcripts are also seen in the ectoderm of the nasal pits derived from the first branchial arch (arrow). For probe construction, see Jones et al. (1991). The approximately 1 kb insert contains 360 bp of 5′ noncoding sequence, followed by coding sequence up to the beginning of the conserved region. It contains no sequences coding for the mature region. The whole-mount in situ hybridization was carried out essentially as described by Sasaki and Hogan, (1993).

J., unpublished observations). In this case, BMP-4 has the same ventralizing effect, and *Xhox 3* transcripts accumulate with the same kinetics, as when BMP-4 RNA is injected into the fertilized egg. The conclusion from these different studies is that BMP-4 elicits its ventralizing effect on mesoderm during gastrulation. As we shall see, this conclusion is compatible with the proposed role for BMP-4 during late gastrulation in the mouse embryo and suggests that this function of BMP-4 has been conserved during vertebrate evolution.

ESTABLISHING THE VERTEBRATE BODY PLAN: BMP-4 EXPRESSION IN THE EARLY MOUSE EMBRYO

BMP-4 is one of the first DVR genes to be expressed in the mouse embryo. cDNAs were isolated from a 6.5-day p.c. cDNA library (Jones et al., 1991) but whole-mount in situ hybridization does not detect transcripts until around 7.5 days, at low levels in the posterior primitive streak, allantois and amnion. By 8.5 days, higher levels are present in the posterior of the embryo, specifically in the mesoderm of the primitive streak and around the hindgut and in the ventral mesoderm caudal to the last somite (Jones et al., 1991 and data not shown). At approximately this time BMP-4 also begins to be

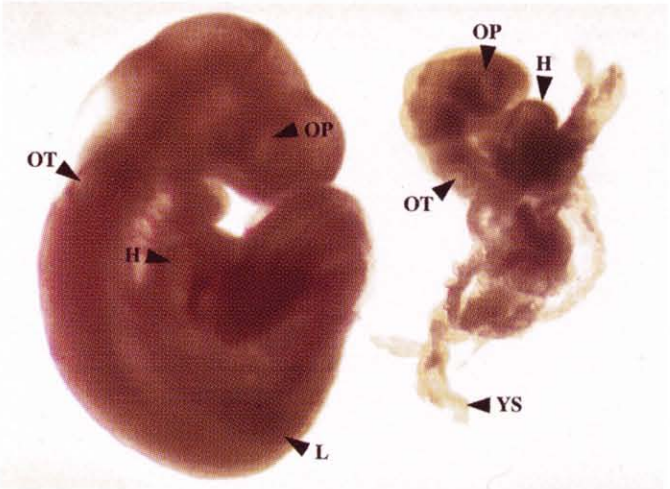

Fig. 5. Morphology of a homozygous null BMP-4 mouse embryo. Male and female (129 × C57BL/6) F_2 mice, heterozygous for a null mutation in the *BMP-4* gene, were mated and embryos collected at 9.5 days and genotyped by analysis of DNA from extraembryonic tissues. Shown here is a heterozygous embryo (left) and a homozygous mutant littermate (right). Note the disorganized posterior region of the homozygous embryo OT, otic vesicle; OP, optic pit; H, heart; YS, yolk sac.

expressed in the myocardium of the heart. At 9.0-9.5 days, whole-mount in situ hybridization clearly shows BMP-4 transcripts in the posterior and around the umbilical vessels (see Fig. 4). Sectioning these embryos reveals that posterior transcripts are localized in the somatopleure and splanchnopleure mesoderm. Expression is also detected in the mesenchyme of the limb bud and in the neuroepithelium of the diencephalon, in the ectoderm of the branchial arches, and in the myocardium of the heart (Fig. 4 and Jones et al., 1991). As the limbs develop, strong expression is seen in the apical ectodermal ridge, and transcripts in the mesenchyme become localized to the posterior, before shifting anteriorly. Some details of this limb bud expression are shown in Fig. 4 and are discussed fully elsewhere (Francis et al., 1994).

In summary, high levels of BMP-4 expression are first seen in the mouse embryo in the posterior primitive streak, and in ventral mesoderm around the posterior gut and umbilical blood vessels, and in the body wall (i.e. in the splachnopleure and somatopleure). This pattern of expression is consistent with BMP-4 playing a role in the specificiation of posterior and ventral mesoderm, as hypothesised from experiments described earlier with *Xenopus*. Later, expression of BMP-4 is seen in a variety of tissues undergoing mesenchymal/epithelial interactions, and models have been proposed by us (Jones et al., 1991; Lyons et al., 1991; Jones et al., 1992a) and by others (Francis et al., 1994; Vainio et al., 1993) in which BMP-4 and the closely related protein, BMP-2, play key roles in mediating the intercellular signalling involved.

In order to explore the role of BMP-4 in vivo, a null mutation has been introduced into the mouse gene by homologous recombination in embryonic stem cells (M. B., unpublished results). The targeting construct was designed to eliminate most of the first coding exon, and therefore most of the pro region of the protein, and to introduce a stop codon into all three reading frames of the second coding exon. Heterozygous mice appear normal, but homozygous embryos die between about 7.5 and 10.5 days p.c., with a rather variable phenotype, which probably depends upon genetic background. A 9.5-day p.c. homozygous embryo is shown in Fig. 5 along with a heterozygous littermate. It is retarded in overall growth, but anterior structures such as fore-, mid- and hind-brain, optic and otic vesicles, and heart are all present. However, the regions of the embryo posterior to the heart are disorganized. The abnormalities also include the extraembryonic mesoderm of the yolk sac, which shows fewer blood islands than normal and the mesoderm does not adhere closely to the endoderm. It is possible that some of the growth retardation of the homozygotes is due to anaemia resulting from a deficiency of blood cells and abnormalities of the blood vessels connecting the yolk sac with the embryo. In addition, there appears to be a general deficiency of posterior structures and splanchnopleure mesoderm. Further analysis is underway of both the abnormal phenotype of homozygous mutant embryos, and the effect of genetic background on the penetrance of the null mutation. However, while preliminary, these findings support the idea that BMP-4 is required for the normal differentiation of posterior and ventral mesoderm of the mouse embryo, and suggests that this function has been conserved during vertebrate evolution.

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