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Source: Zoological Science, 12(3) : 355-358

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.12.355

ZOOLOGICAL SCIENCE 12: 355–358 (1995)

[RAPID COMMUNICATION]

Expression of BMP-2,4 Genes during Early Development in *Xenopus*

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ABSTRACT—The transforming growth factor-β (TGF-β) family has been implicated in the regulation of cell differentiation during early development. Bone morphogenetic proteins (BMPs) are thought to play particularly important roles in the formation of the dorso-ventral axis of *Xenopus laevis*. We found by means of *in situ* hybridization that BMP transcripts were localized during early *Xenopus* embryogenesis. BMP-4 transcripts were localized first to neural crest cells, presumptive heart mesoderm and around the yolk plug at the neurula stage and to the eye, auditory vesicles, the heart and mesenchymal cells of fin at the tailbud stage. In sharp contrast, BMP-2 transcripts were strictly localized to olfactory placodes at the tailbud stage. These results showed temporal and spatial regulation of the genes and thus support the notion that BMP proteins are important for directing cell growth and differentiation during organogenesis in the amphibian embryo.

INTRODUCTION

Vertebrate embryogenesis involves a series of interactions between various cells and tissues. These interactions are critical to the constitution of the body plan. There is growing evidence that peptide growth factors are involved in these cell to cell interactions. BMPs, which are members of the TGF-β superfamily, play important roles in the induction of embryonic mesoderm in *Xenopus*. A BMP-4 mRNA injection into dorsal blastomeres causes ventralization of the embryo [1, 7]. These results indicate that the exogenous factors interact with endogenous receptors and trigger cell differentiation. Overexpression of the mutant BMP receptor that lacks a putative serine/threonine kinase domain in ventral blastomeres, results in transformation to dorsal mesoderm and a secondary body axis by respecification to the dorsal mesoderm [4, 10, 18]. In addition, BMP-4 is mRNA expressed in the ventral marginal zone of the gastrula stage [3]. Furthermore, BMP-2/4 immunoreactivity in oocytes and early embryos has been reported [13, 16]. These data suggest that the specification of the dorsal ventral axis of *Xenopus* embryo is controlled by endogenous BMPs in the embryo.

In contrast to extensive studies on the role of BMPs in early *Xenopus* induction during axis formation, little information is available regarding organogenesis. The distribution of BMP transcripts in the myocardium of the early mouse embryonic heart, limb buds, central nervous system, whisker follicles, tooth buds and other organs, suggests that BMP-2 and -4 act as signaling molecules during murine development. These results imply that BMPs play essential roles in epithelial-mesenchymal interactions [6, 9]. However, the role of BMPs during organogenesis in the *Xenopus* embryo has not been fully established.

Here we examined localization of BMP-2,-4 transcripts expressed during the development of *Xenopus*, by in situ hybridization.

Accepted March 7, 1995
Received November 24, 1994

MATERIALS AND METHODS

Embryos

Xenopus laevis embryos were obtained by natural mating. The jelly coat was removed with 2.5% cysteine-HCl pH 7.6. Embryos were staged according to Nieuwkoop and Faber [11], then fixed for 2 hr in MEMFA (0.1 M MOPS pH 7.4, 2 mM EGTA, 1 mM $MgSO_4$ and 3.7% formaldehyde) or 4% paraformaldehyde/phosphate-buffered saline (PBS).

Probe construction

Digoxigenin-labeled single strand RNA was prepared using a DIG RNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions.

A 1.8 kb *Xenopus* BMP-2 cDNA subcloned into pSP64 was linearized with *Eco*RI and transcribed with SP6 RNA polymerase to generate an antisense probe. A 1.3 kb *Xenopus* BMP-4 cDNA subcloned into pCDM8 was linearized with *Bst*EII and transcribed

with T7 RNA polymerase to generate an antisense probe. Both probes were reduced to about 200 bp by limited alkaline hydrolysis.

Whole mount in situ hybridization

Whole mount *in situ* hybridizations were performed essentially as described Harland [5]. All solutions used in the hybridization and pretreatment were treated with 0.02% DEPC (diethylpyrocarbonate) and then autoclaved. All glassware was baked at 180°C for over 3 hr to inactivate RNase. Embryos fixed in MEMFA were dehydrated in methanol. After rehydration, proteinase K digestion, acetylation and refixation, embryos were hybridized for 16 hr at 60°C with the labeled probes, digested with RNase A and RNase T1, then washed. For the chromogenic reaction, embryos were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim, 1:2000 dilution), and reacted with a solution (nitro blue tetrazolium chloride; NBT 340 ng/ml, 5-bromo-4-chloro-3-indolyl phosphate; BCIP 175 ng/ml). After 30 to 90 min, the reaction was stopped with MEMFA. Embryos which were hybridized with the antisense BMP-2 probes were embedded in tissue-tek then sectioned using a cryostat.

In situ hybridization

Fixed embryos were dehydrated by exposure to increasing concentrations of ethanol and embedded in paraplast. Sections of 6–8 μm were cut and floated onto slides coated with 3-triethoxysilylpropylamine (Merck).

Hybridization and detection were performed using the Nucleic Acid Detection Kit (Boehringer Mannheim) according to the manufacturer's instructions with minor modifications. Sections were deparafinized, hydrated, refixed with fresh 4% paraformaldehyde in 0.1 M PB (phosphate buffer) for 15 min, washed with 0.1 M PB for 1 min, and digested with proteinase K (3 μg/ml) for 5 min. They were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0 for 10 min. Subsequently, they were rinsed in 0.1 M PB for 1 min, dehydrated with 70, 80, 90 and 100% ethanol, and air dried. The hybridization solution contained 50% deionized formamide, 10 mM Tris-HCl pH 7.6, 200 μg/ml yeast tRNA, 1×Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and about 0.5 μg/ml of probe. Hybridization solution was placed on each section, which was then covered with parafilm and incubated at 50°C for 14–16 hr in a moisture chamber. Thereafter, the parafilm was removed and the slides were washed in 5×SSC at 50°C, followed by 50% formamide, 2×SSC at 50°C for 30 min. After rinsing in 1×TES (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.5 M NaCl) for 15 min at 37°C for 30 min, the slides were digested with RNase A (20 μg/ml in 1×TES) at 37°C for 30 min, then rinsed again in 1×TES at 37°C for 10 min. The slides were incubated with 2×SSC for 20 min and with 0.2×SSC for 20 min twice at 50°C. They were rinsed with 1×TES containing 1.5% (W/V) skim milk powder for 1 hr at room temperature, followed by 1×TES then alkaline phosphatase-conjugated anti-digoxigenin antibody (1:500 dilution) overnight at 4°C. These after, they were twice with 1×TES for 15 min rinsed with 1×TSM(100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) briefly and reacted with color solution (NBT 340 ng/ml; BCIP 175 ng/ml in 1×TSM) overnight at room temperature. The reaction was stopped with 10 mM Tris-HCl pH 8.0, 1 mM EDTA, and the sections were mounted using crystalmount (Cosmo Bio).

RESULTS AND DISCUSSION

Complementary DNA sequences of BMP-2 and -4 are very similar and encode proteins that differ in 21 amino acids in their predicted mature regions as 114 amino acid peptides (81.6%). BMP-2 transcripts can be detected in oocytes and blastulae but not in gastrulae. On the other hand, maternal BMP-4 mRNA transcripts are detected at a low level and dramatically increased in abundance after blastulae [12]. These results suggest that these BMP genes are independently regulated in spite of their close structural similarity. Regional differences of BMP-2 and -4 gene expression during early development were also revealed by *in situ* hybridization (Figs. 1 and 2). Although significant staining was not observed at stage 23 and 45 embryos (data not shown), intense staining of BMP-2 mRNA was exclusively localized to a pair of nasal placodes at stage 34 (Fig. 1A). In horizontal sections, BMP-2 transcripts were detected in mesenchymal cells (Fig. 1B). Morphologically, the nasal pit formed from the nasal placode derived from the sensory layer of the stage 23 embryo, gradually invaginates after stage 31 [8]. The BMP-2 expression coincides with the moment when the cells that form nasal pit mobilize and reach the telencephalon and differentiate into the olfactory epithelium. At stage 45, when these nasal pit extends toward the throat and the cell strands become more massive, BMP-2 expression becomes undetectable. These results suggest that BMP-2 is involved in the formation of sensory neuroepithelium.

On the other hand, BMP-4 transcripts were detected throughout development, including stages 23 and 34 (Fig. 2). The distribution of BMP-4 transcripts was further examined in serial sections by sectioning whole-mount embryos of stage 23 and 34 that had been hybridized *in situ* with the same probes (Fig. 2C, D, E, F, H and I). At stage 23, a BMP-4 signal was observed in the blastpore region (Fig. 2B). An enlarged view shows that BMP-4 is expressed in the circumblastporal colla (Fig. 2F).

In the stage 23 embryo, BMP-4 transcripts were found along the neural tube (Fig. 2A and B). An enlarged view shows that BMP-4 is expressed in the mesenchymal cells above the roof plate along the central nervous system (Fig. 2E). At stage 34, BMP-4 transcripts were localized to the deep epithelial layer and mesenchyme of the fin (Fig. 2G). The BMP-4 positive cells along the neural tube may be neural crest cells. Vagal and truncal neural crest cells move along three defined routes. These results suggested that BMP-4 positive cells of the peripheral fin and somite at stage 34 were derived from truncal neural crest cells. Taken together, BMP-4 may be concerned with epithelial-mesenchymal interaction.

In the stage 23 embryo, BMP-4 transcripts were also found in the presumptive heart region (arrowheads in Fig. 2B), then localized to the myocardium and pericardium at stage 34 (arrowhead in Fig. 2G). Figure 2C shows the BMP-4 expression in the presumptive heart (arrowheads in Fig. 2C). *Xenopus* BMP-4 gene was expressed in the heart mesoderm at the late neurula stage. In *Xenopus*, the presumptive heart region is determined during the late gastrula stage and it is believed to be induced by a signal elaborated

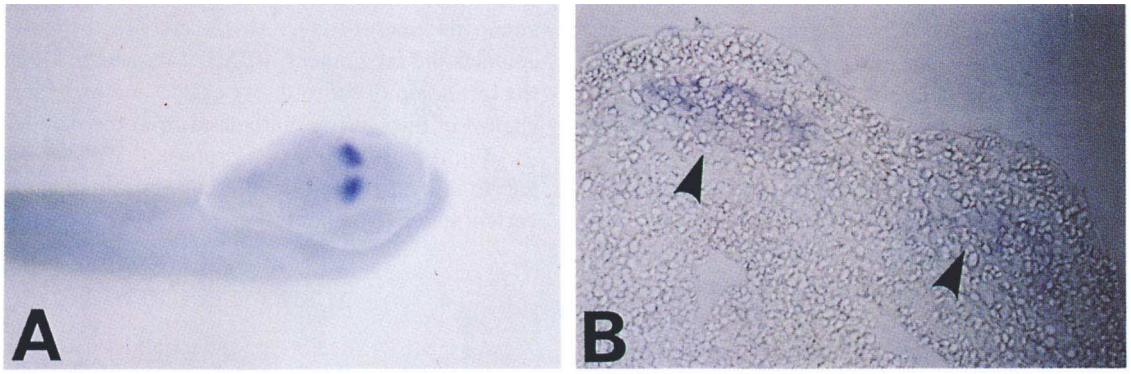

FIG. 1. Localization of BMP-2 transcripts during early development using whole-mount *in situ* hybridization. (A) The antisense BMP-2 probe hybridized to a stage 34 embryo. BMP-2 transcripts are localized to the nasal pit. (B) Horizontal section of nasal pit. BMP-2 mRNA is localized in mesenchymal cells(arrow).

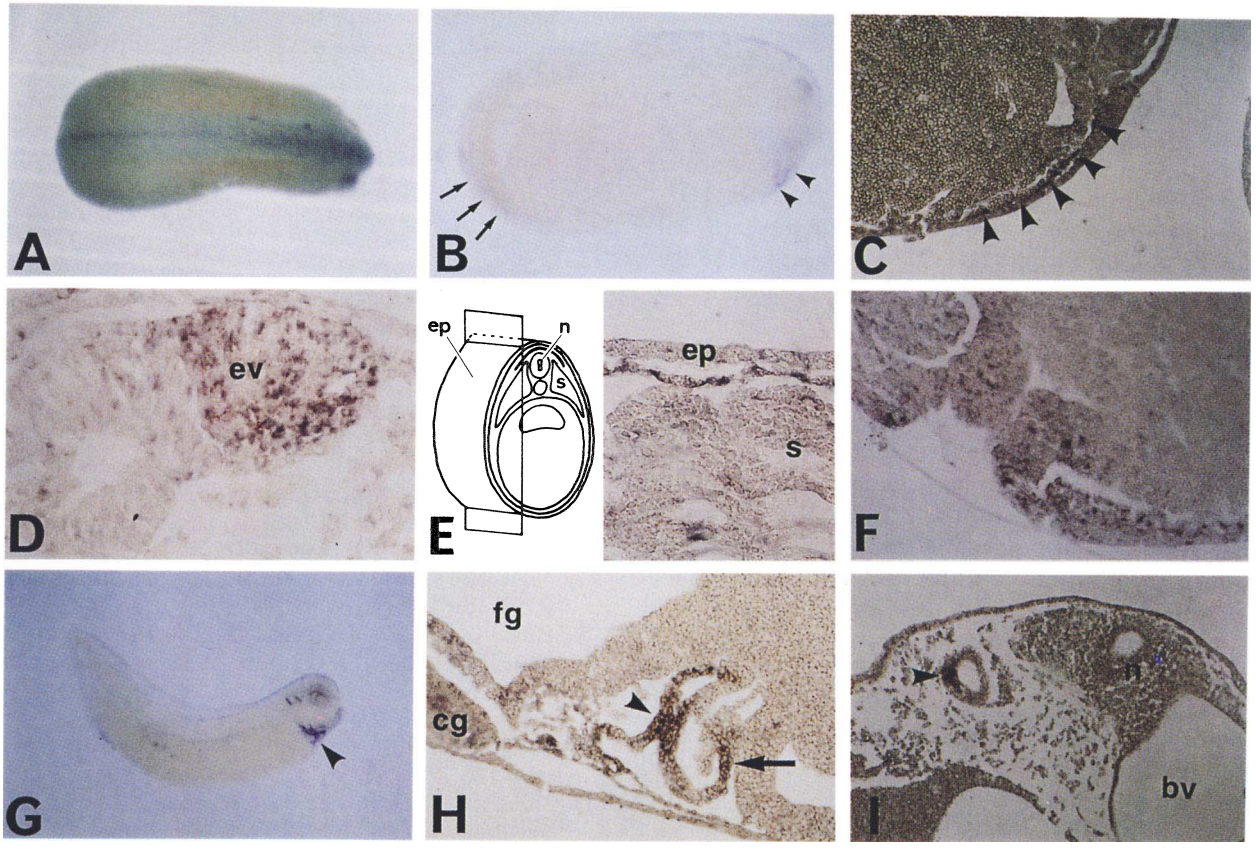

FIG. 2. Regional expression of BMP-4 transcripts in the late neurula and tailbud stage. (A) In the dorsal view, BMP-4 transcripts are located in the epidermis cells along the neural tube. (B) Lateral view, BMP-4 transcripts are localized in the eye vesicle, presumptive heart region (arrowhead) and around the yolk plug region (arrow). A sagittal section of a late neurula stage embryo(C, D, E, F). (C) Close-up of the heart region . BMP-4 transcripts are expressed in the presumptive heart mesoderm region (arrowheads). (D) Enlarged eye vesicle. BMP-4 transcripts are localized in eye vesicle, but not detected in the prosencephalon. (E) Close-up of the dorsal region. BMP-4 mRNA is localized to neural crest cells. (F) Close-up of the blastpore regions. There is a BMP-4 signal in the circumblastporal collar. (G) In the lateral view, antisense BMP-4 probes are hybridized to a stage 34 embryo. BMP-4 transcripts are distributed among the eye vesicle, the auditory vesicle, the heart region (arrow), peripheral cells of the somite and the mesenchyme of the fin. (H) Close-up of the heart of a tailbud stage embryo. BMP-4 transcripts are localized to the myocardium and pericardium. The arrowhead indicates the presumptive outflow tract region and the arrow indicates the presumptive atrioventricular canal. (I) Close-up of the auditory vesicle of a tailbud stage embryo. BMP-4 transcripts are localized in the auditory vesicle cells(arrowhead). bv, brain ventricle; cg, cement grand; ep, epidermis; ev, eye vesicle; fg, foregut; n, neural tube; s, somite.

from the endoderm [15]. The results presented here indicated that BMP-4 expression is not actively involved in the induction event, because the BMP-4 transcript becomes abundant after the heart determination. Rather, it may contribute to the heart formation that occurs after the determination.

At stage 34, BMP-4 localization in the heart was distinct and high levels of expression were seen in the presumptive myocardium of the outflow tract and atrioventricular regions (arrowhead and arrow in Fig. 2H). In the mouse, BMP-4 mRNAs are localized to heart, particularly to the endocardium. Thus, the biological function of BMP-4 in the heart appears to be same between mouse and *Xenopus*. As the development progressed to the embryo, the transcript localized to myocardium and pericardium. The myocardium where BMP-4 transcript was detected is the region where an epithelial-mesenchymal transformation from endothelial cells into mesenchymal cells occurs in later heart development (arrowhead and arrow in Fig. 2H). During the mouse heart formation, gene activation of TGF-βs 1, 2 and 3 is temporally and spatially regulated [2]. *In situ* hybridization has shown that the expression of TGF-β2 regionally overlaps with that of BMP-4, especially in the outflow tract and atrioventricular regions. Although *in vitro* studies demonstrated that TGF-β1 and 2 can transform endothelial cells into mesenchymal cells, it is not clear whether the TGF-βs themselves are the inducing signal [14]. And BMP-4 may function either as the signal or cooperate with TGF-β1 and 2.

Vainio *et al.* [19] have revealed that BMP-4 may be a morphogen during tooth development in the mouse embryo and that BMP-4 induced the transcriptional factors, *Msx-1, Msx-2* and *Egr-1*. On the other hand, *Xhox -7.1*, that is the homologue of mouse *Msx-1* gene, is localized to neural crest cells and the cardiogenic region at stage 21 of *Xenopus* embryo [17]. This may be induced in response to BMP-4 in *Xenopus*. These results suggested that the BMP-4 signal may be important for the migration of neural crest cells and heart morphogenesis in the *Xenopus* embryo through the gene regulation of such transcription factors.

In addition, BMP-4 mRNA was detected during eye development. The BMP-4 transcript was localized to the eye vesicle(Fig. 2D and G), suggesting that BMP-4 is also involved in the formation of these tissues.

At stage 34, the anterior and posterior part of the auditory vesicle was positively stained (arrowhead in Fig. 2I). The auditory vesicle is formed in a similar way to the nasal pit. At this time, the ear vesicle becomes thinner and distinct, indicating the beginning of the formation of sensory epithelium. The BMP-4 transcript was localized to the area where the sensory layer that gives rise to auditory vesicle contacts the integument. BMP-4 may induce signals during the formation of the auditory organ, and may be related to a change in the sensory epithelium from the ectoderm.

From these results, we propose that these BMPs are of importance in cell differentiation and organogenesis during vertebrate development.

ACKNOWLEDGMENTS

We thank Dr. Ken W. Y. Cho for his technical advice.

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