STUDIES ON ACTIVIN RELATED PROTEINS AND ACTIVIN RECEPTORS IN EARLY *XENOPUS* EMBRYOS

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ABBREVIATIONS

AGPC	Acid Guanidinium Thiocianate-Phenol-Chloroform method
bp	base pair
BMP	Bone morphogenetic protein
DMEM	Dulbecco's modified Eagle's medium
EDF	Erythoid differentiation factor
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FSH	Follicle-stimulating hormone
MIF	Mesoderm-inducing factor
PDGF	Platelet-derived growth factor
PAGE	Polyacrylamide gel electrophoresis
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulfate
SSC	Standard saline sodium citrate
TGF-β	Transforming growth factor-β

CONTENTS

PREFACE

Over 70 years ago, Spemann and Mangold dramatically demonstrated that a small tissue explant from the dorsal blastopore lip of a newt gastrula could organize the formation of an entire secondary embryonic axis following its transplantation to an ectopic position in a similar host embryo [1]. The observation was interpreted to mean that embryonic cells altered their developmental pathways when brought in contact with different environments of donor cells. Developmental biologists named the phenomena "embryonic induction" [2]. Embryonic induction is an interaction between one (inducing) tissue and another (responding) tissue, as a result of which the responding tissue undergoes a change in its direction of differentiation. This is the most important mechanism in animal development leading to differences between cells and to the organization of cells into tissues and organs. One of the most intensively studied examples of embryonic induction in vertebrates is that of mesoderm induction in the frog *Xenopus*.

Early Amphibian Development

Unfertilized *Xenopus* eggs have easily distinguishable top and bottom poles that define the animal-vegetal axis. This axis in eggs corresponds to the anterior-posterior axis of the embryo. The top (animal hemisphere) is a dark brown because of pigment granules and the bottom (vegetal hemisphere) is a yolky yellow. After fertilization and a following period of rapid cleavage, the embryo becomes a blastula, resembling a hollow ball. The top of the embryo gives rise to cells that form the ectoderm that differentiates later into skin, neural and other tissues, whereas

the bottom of the embryo gives rise to endodermal derivatives, primarily the gut. Amphibian gastrulation begins with cell migrations at the dorsal side of the embryo. Cells moving up along the blastocoel roof form the mesoderm (Fig. 1). In the adult, the mesoderm finally organizes into tissues such as heart, kidney, bone, testis and ovary. Although the mechanism concerning the embryonic mesoderm induction has been extensively studied for the past century, most of the models have been revised over the past decade [2]. In recent years, it appears that the different developmental capacity which is necessary for the mesoderm induction is stored along the animal-vegetal axis in the cytoplasm of the egg [2]. In addition, the vegetal end of the egg produces a signal that is responsible for the formation or induction of the embryonic mesoderm. An intracellular signal that spreads from cells at the vegetal pole interacts with overlying cells in the middle region of the cleaving egg to specify the fate of the latter as mesoderm (see the top line in Figure 1). Such inducers and receptors are most probably in the form of different maternal mRNAs and/or proteins, because the embryo genome is not transcriptionally active until about eight hours after fertilization [3]. This is one hour after decisions about cell fates such as mesoderm induction have transpired.

Molecules involved in mesoderm induction

To assess the ability to induce mesoderm from endoderm, Nieuwkoop developed the animal cap assay in which a piece of ectoderm (*i.e.* animal cap) is cut out from a blastula-stage embryo and combined with vegetal tissue [4, 5, 6] (Fig. 2). Mesoderm tissues are induced by vegetal cells to the animal cap cells. Among the induced tissues, muscle is most easily identified because several molecular markers are available,

either as muscle specific-actin cDNA [7,8], or as antibodies [9,10]. This assay has recently been modified so that the animal cap cells are cultured in standard buffered salt solution with soluble factors instead of the intracellular signal that emanates from vegetal cells.

Inducing factors have been obtained from various sources over many years [2]. In 1987, Smith discovered such activity in the conditioned medium of *Xenopus laevis* XTC cells, which were generated from a metamorphosing tadpole [11]. Although the activities in this medium were highly effective in inducing mesoderm, it was not clear whether a similar activity is in the normal embryo.

On the other hand, a different approach to the identification of the inducer was taken by Melton *et al.* who isolated a cDNA designated by the Vg1 clone, that encodes the mRNA localized to the vegetal end [12,13]. The Vg1 mRNA encodes a 40 kDa protein similar to the human transforming growth factor-β (TGF-β). Although this gene initially appeared to be likely component of the mesoderm induction system, it has not so far shown any such activity.

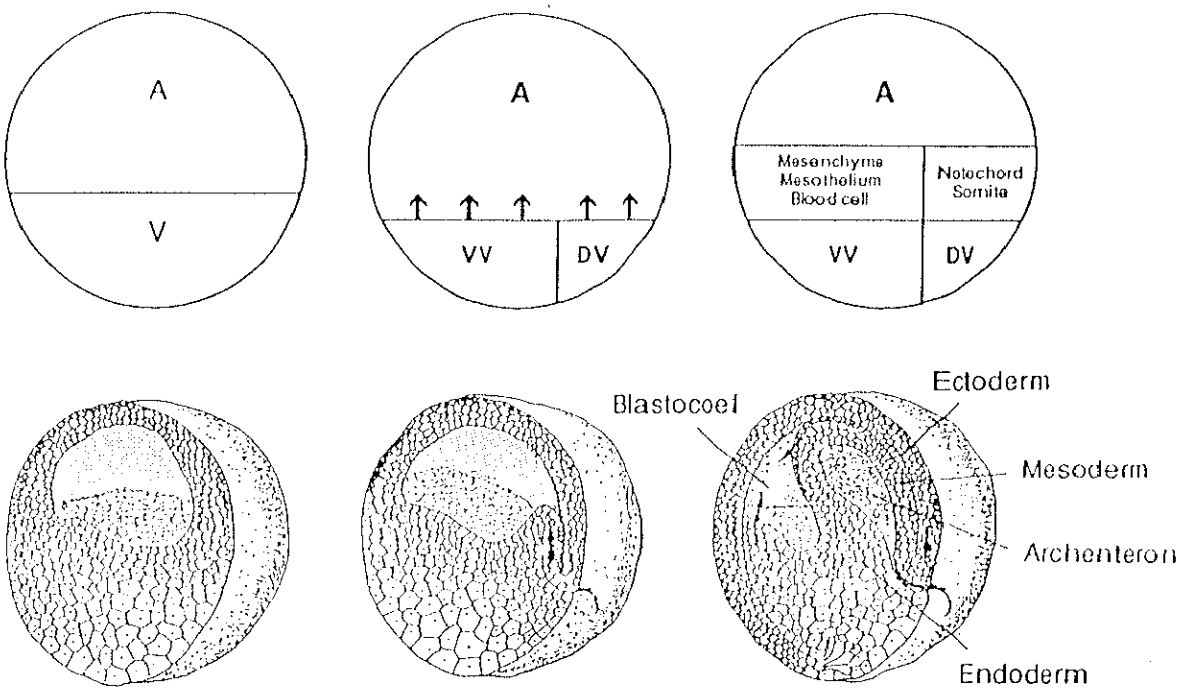

Figure 1. *Xenopus* early development and embryonic induction. Schematic representations in the top line show inductive interactions during early *Xenopus* development. At early blastula stages the *Xenopus* embryo can be considered to consist of two cell types: presumptive ectoderm in the animal hemisphere (A) and presumptive endoderm in the vegetal hemisphere (V). During mesoderm induction two signals are assumed to derive from each dorsal and ventral region of the vegetal hemisphere. The dorsal-vegetal(DV) signals induce dorsal mesoderm, such as the notochord and somite, while the ventral-vegetal signal (VV) induces blood, mesenchyme and mesothelium as ventral mesoderm. The direction of these inductive signals is designated by vertical arrows. Drawings in the bottom line show sections of the blastula and early gastrula embryos. Arrows show the direction of cell movements. [2]

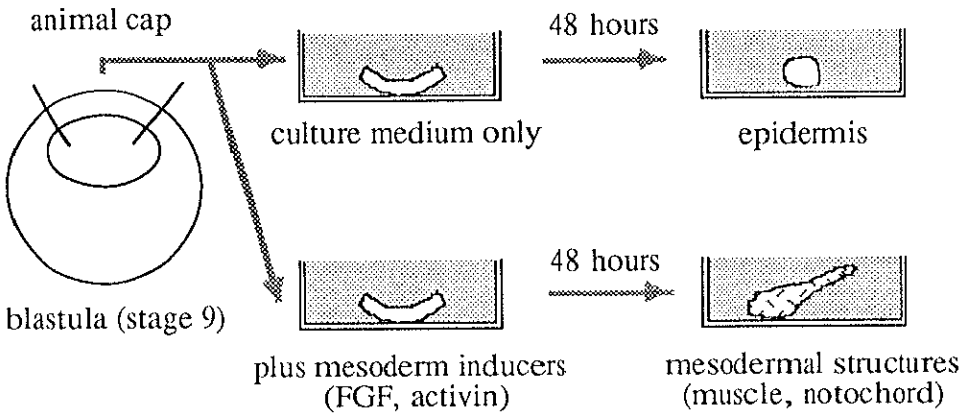

Figure 2. Animal cap assay [4, 5, 6].

Animal cap cells (presumptive ectoderm) isolated from blastula stage embryos are cultured in an amphibian salt solution without or with soluble factors. Animal caps in the absence of factors develop into atypical epidermis. When the cells were incubated with inducing factors, they gave rise to a variety of mesodermal tissues.

Growth factors and early development

The discovery of nerve growth factor [14, 15] and epidermal growth factor [16] has led to the identification of a wide array of factors that affect the growth of virtually all cell types. Such factors can not only act as positive and negative modulators of cell proliferation but also influence differentiation.

Several growth factors mimic various aspects of mesoderm induction. When explants of presumptive ectoderm (animal caps) of *Xenopus* embryos are exposed to basic FGF (bFGF), they differentiate into ventro-lateral mesodermal tissues [17], such as mesenchyme and muscle, and rarely into dorsal mesoderm, such as notocord. Both bFGF mRNA and protein are found in the embryo at specific times and at levels sufficient to induce mesoderm [18,19]. The mesoderm-inducing activity of FGF is synergistically enhanced by adding transforming growth factor-β1(TGF-β1) [18]. TGF-β2 was later shown to be capable of inducing mesoderm by itself [20]. Animal caps treated with TGF-β2 differentiated into dorsal axial mesoderm such as notocord, skeletal muscle and tissues with neural inductive capacity. However, the contribution of TGF-β2 to mesoderm induction remains unclear because TGF-β2 transcripts are not detectable in the embryos when mesoderm induction occurs.

Activin was first discovered because it stimulated the secretion of follicle-stimulating hormone (FSH) from anterior pituitary cells [21,22]. It was later demonstrated that activin is identical to erythroid differentiation factor(EDF) [23]. Many other biological activities have since been reported [24]. Since the amino acid sequence of activin is similar to that of TGF-β, activin showed to be a cell differentiation factor for various types of cells rather than a regulator of hormone secretion. In 1989, Asashima *et al.*

demonstrated that activin has potent mesoderm-inducing activity [25]. A variety of mesodermal tissue including muscle, notochord, blood cells and mesenchyme are induced by recombinant human activin in a concentration-dependent manner. The most important question was whether *Xenopus* activin is present in embryos when mesoderm is naturally induced.

To clarify the role(s) of peptide growth factors in embryonic inductions, I initially characterized not only activin but also activin-related gene products, such as bone morphogenetic proteins (BMPs) in early *Xenopus* embryos. In Chapter I, the isolation of five activin-related genes from a *Xenopus* liver genomic DNA library is described. One of these clones encoded a protein similar to mammalian bone morphogenetic protein (BMP), which was originally isolated from bone extract based upon its ectopic bone forming activity [26]. Temporal changes in the expression of these activin-related genes during *Xenopus* oogenesis and embryogenesis were also examined by Northern blot analysis. Chapter II describes the cloning of *Xenopus* BMP cDNAs from oocytes and the raising of a polyclonal antibody against *Xenopus* BMP-4 using a fusion protein with bacterial β-galactosidase as an antigen in order to investigate the molecular nature of BMP.

In order to reveal the physiological function of the ligand *in vivo*, it is essential to study its receptor. There are three molecular species of the activin receptor, namely types I, II, III, on a variety of mammalian cells [27, 28, 29]. Complementary DNA for the type II activin receptor has been cloned from mouse pituitary cells and encodes a serine/threonine (Ser/Thr) kinase in the cytoplasmic region [30]. In Chapter III, the isolation of 4 independent clones encoding the activin receptor from *Xenopus* oocyte cDNA library and the temporal changes in expression of the receptor genes

during early embryogenesis are described. One of them, XSTK2, encoded a receptor that lacks the carboxyl-terminal portion of cytoplasmic Ser/Thr kinase domain. In Chapter IV is described whether the truncated activin receptor can transmit the activin signal. Receptor mRNA was injected into early embryos and the function of the introduced receptor was evaluated not only by its morphological phenotype but also by the animal cap assay using explants from the injected embryos. Finally, based upon these results, I discuss the biological function of actvin-related proteins and the activin receptor during early embryogenesis.

Chapter I. Molecular cloning of *Xenopus* activin-related genes

SUMMARY

Activin, a member of the transforming growth factor-β (TGF-β) family, is a multi-functional factor that stimulates not only follicle-stimulating hormone (FSH) secretion but also erythrocyte differentiation. A human recombinant activin potently induces mesoderm at a concentration of 10 ng/ml and the resulting morphological change was similar to that induced by TGF-β. As an initial approach to the function of activin as an endogenous mesoderm-inducing factor, a *Xenopus* liver genomic DNA library was screened to identify the *Xenopus* homologue of mammalian activin gene, using a rat activin βA cDNA as a probe. Five independent genomic DNA clones, tentatively named Xar3, Xar4, Xar5, Xar9 and Xar14 were isolated. The deduced amino acid sequence of these genes all showed virtually perfect conservation of the distribution of cysteine residues, suggesting that these clones encoded activin-related proteins in amphibians. Northern blots showed that only the Xar14 gene, which encodes a protein similar to mammalian bone morphogenetic protein, is maternally transcribed and retained until the embryonic gasturula stage (stage 10-11).

INTRODUCTION

Peptide growth factors belonging to the fibroblast growth factor and transforming growth factor-β (TGF-β) families have been implicated in the induction of embryonic mesoderm in *Xenopus*[31]. When these growth factors of mammalian origin are applied to presumptive ectoderm fragments (animal cap explants) of the frog blastula, they induce mesoderm [17, 18, 20]. Members of FGF family can induce ventral mesoderm such as mesenchymal tissue, but rarely induce dorsal axial structures including notocord, neural tissues. Basic FGF is maternally expressed in the *Xenopus* oocyte. Members of TGF-β family also induce embryonic mesoderm in animal cap explants, but it is not known whether a TGF-β like factor is expressed in the early embryo.

Activin was first discovered for its ability to stimulate the secretion of follicle-stimulating hormone (FSH) from anterior pituitary cells [21, 22]. It was later demonstrated that activin is identical to erythroid differentiation factor(EDF) [23]. Since the amino acid sequence of activin is similar to that of TGF-β, it has been presumed that activin is a cell differentiation factor for various types of cells rather than a regulator of hormone secretion. Asashima *et al.* has demonstrated that activin has mesoderm-inducing activity [25]. A variety of mesodermal tissues including muscle, notochord, blood cells and mesenchyme were induced in the *Xenopus* presumptive ectoderm by a recombinant human activin in a concentration dependent manner. The most important question is whether *Xenopus* activin is present in embryos when mesoderm is naturally induced.

As an initial approach to the function of activin as an endogenous mesoderm-inducing factor, I isolated five activin related genes from a *Xenopus* genomic library. In this chapter, I show the amino acid sequence

of these proteins and the temporal expression pattern of their mRNAs in the oocyte and early embryos.

MATERIALS AND METHODS

Materials

All enzymes, including restriction endonucleases, were purchased from Toyobo, Boehringer Mannheim and Takara. The *in vitro* packaging kit was from Stratagene.

Preparation of genomic DNA and Southern blot analysis

Xenopus genomic DNA was isolated from the liver by the method of Maniatis [32]. Five micrograms of the genomic DNA was completely digested with *Eco*RI or *Hind*III. The DNA fragments were electrophoresed on a 1.2 % agarose gel and transferred in 6 X Standard saline sodium citrate(SSC) to a nitrocellulose membrane(Schleicher & Schuell) [33].

An *Eco*RI/*Hinc*II fragment (368 bp) of rat activin βA cDNA which corresponds to the segment from nucleotides 1047 to 1415 of pβA30 [34] was used as a probe. The fragment was labeled with [^{32}P]dCTP (Amersham) using a random labeling kit (Boheringer Mannheim) and hybridized on filters in 1 M NaCl / 0.2 % bovine serum albumin / 0.2 % Ficoll / 0.2 % polyvinylpyrrolidone / 50 mM Tris HCl, pH 7.4 / 20 mM EDTA / 0.1 % SDS containing yeast tRNA(0.2 mg/ml) at 60 °C for 15 hours. Filters were washed twice at 50 °C for 20 min with 1 X SSC / 0.1 % SDS.

Cloning and sequencing

A *Xenopus* genomic DNA library was constructed a partial *Sau*3AI digest of *Xenopus* genomic DNA cloned into Charon 28 [32]. The library was screened by the Benton and Davis method [35]. The hybridization proceeded essentially as described for Southern bloting with some

modifications. Duplicate filters were washed at 55 °C and 60 °C with 1 X SSC / 0.1 % SDS. After hybridization, positive clones were isolated and classified into groups according to their restriction enzyme maps. To investigate the nucleic acid sequence, the shorter DNA fragments hybridized with the probe from each group were subcloned into pUC19. Both strands of the DNA were sequenced by dideoxy chain-termination [36] using a sequencing reagent kit (Toyobo, Japan). The nucleotide sequence information directly transferred to a computer was analyzed using the GENETYX programs (SDC, Japan). A DNA fragment that covered the mature region of each predicted activin-related protein was used to detect the relevant transcripts in *Xenopus* ovary and early embryos. Finally one class of gene Xar 14 was found to be maternally expressed.

Northern blot analysis

Early embryos and ovaries were homogenized and total RNA was extracted as described by Chirgwin *et al.*[37]. Poly(A) RNA was purified on an oligo(dT) column (Pharmacia, Uppsala, Sweden). Poly (A) RNAs (10 μg) were denatured with 5 M glyoxal, electrophoresed in a 1 % agarose gel with 10 mM Sodium phosphate (pH 7.0), and transferred in 20 X SSC to a nitrocellulose membrane (Schleicher & Schuell) [38].

DNA fragments corresponding to the mature region from each clone were labeled with [^{32}P]dCTP (specific activity, 1X10^{9} cpm/μg) and used as a hybridization probe. The hybridization conditions were the same as those described for Southern blotting.

RESULTS AND DISCUSSION

That the human recombinant activin showed mesoderm-inducing activity on the animal caps of *Xenopus* embryos [25], led to the notion that activin or activin-related proteins play a role in the induction *in vivo*. To address the question of whether *Xenopus* activin or a similar molecule(s) is present in early embryos when mesoderm is induced, I analyzed *Xenopus laevis* genomic DNA by Southern blotting (Fig.3), using a probe from a DNA fragment corresponding to the entire mature region of rat activin βA. Detection of multiple hybridization signals implied that there are several activin-related genes in *Xenopus*.

Next, to investigate the structure of these *Xenopus* activin-related genes, I screened a *Xenopus* liver derived genomic DNA library by hybridization at low stringency with the rat activin probe. Among about $1x10^6$ individual recombinant phages, 50 positive clones were isolated and classified into five groups by restriction enzyme mapping. As shown in Fig. 4, one representative DNA clone was chosen from each group, *viz*. Xar3, Xar4, Xar5, Xar9 and Xar14 (Xar stands for '*Xenopus* activin-related').

DNA-sequencing analyses of these genes revealed that they all show a feature typical of the TGF-β family of proteins, which is a conserved distribution of seven of the nine cysteine residues at the C-terminal [39] (Fig. 5). Among them, Xar9 was found to encode a protein showing the highest similarity in amino acid sequences (87%) in the predicted mature region, to the human activin βA subunit (Fig. 4) [40]. The other four genes, Xar3, Xar4, Xar5 and Xar14, had significantly lower similarity in amino acid sequences (56, 53, 50 and 42%), respectively, to the human βA. The protein encoded by the Xar3 gene however, had the conserved core amino acid sequences seen in the activin β subunit, suggesting that the

protein product is a member of the activin family. A Xar 14 gene of a total length of 17 kb, encoded a protein that was almost identical to the human bone morphogenetic protein-2 (BMP-2) , that was originally isolated from bovine bone and is a cartilage and bone-forming protein [26,41]. In particular , the carboxyl-terminal region of the protein is highly conserved in human and frog, and there were only three amino acid substitutions when the predicted mature region of *Xenopus* protein was compared to its human counterpart. Xar4 and Xar5 genes encoded closely similar proteins, but they did not share any striking homology with the other TGF-β superfamily proteins.

Northern blots using five specific probes for each activin-related gene, have revealed that only mRNAs of the Xar14 gene, which is a counterpart of human BMP-2, were detected in the oocyte (Fig. 6). The Xar 9 gene encoding *Xenopus* activin βA was transcribed after stage 32 larva (tadpole) but not in unfertilized eggs and blastulae (stage 7). The mRNA of the other genes from Xar3, 4 and 5, were undetectable in early embryos, suggesting that they are pseudogenes which are not essentially transcribed.

In this chapter, I showed that there are several activin-related genes in *Xenopus*, which were cloned by hybridization at a low stringency using rat activin βA cDNA as a probe. One of them encoded a protein homologous to mammalian BMP. I performed Northern blots during early embryogenesis using cloned genes as probes. Activin mRNA was undetectable in oocytes but BMPs were maternally transcribed. Therefore, the question remains as to whether activin is a natural mesoderm-inducing factor in amphibians. It is possible that activin protein is present in oocytes due to translation from undetectable levels of mRNA or by the transport of

protein synthesized in other tissues such as the mechanism of vitellogenin, the egg yolk precursor protein [2, 42]. The other possibility also remained that maternally inherited BMP has inducing activity similar to that of activin. BMP-2 exhibits striking amino acid homology to the *Drosophila dpp* protein [43]. This protein plays an important role not only in dorso-ventral axis formation in early embryos, but also in the correct formation of the imaginal disks, which differentiate into the organs of the adult fly [44]. Finally, the structural similarity between BMP-2 and *dpp* protein supports the notion that vertebrate BMP of which the mRNA is present in *Xenopus* oocytes, controls early development as well as bone formation.

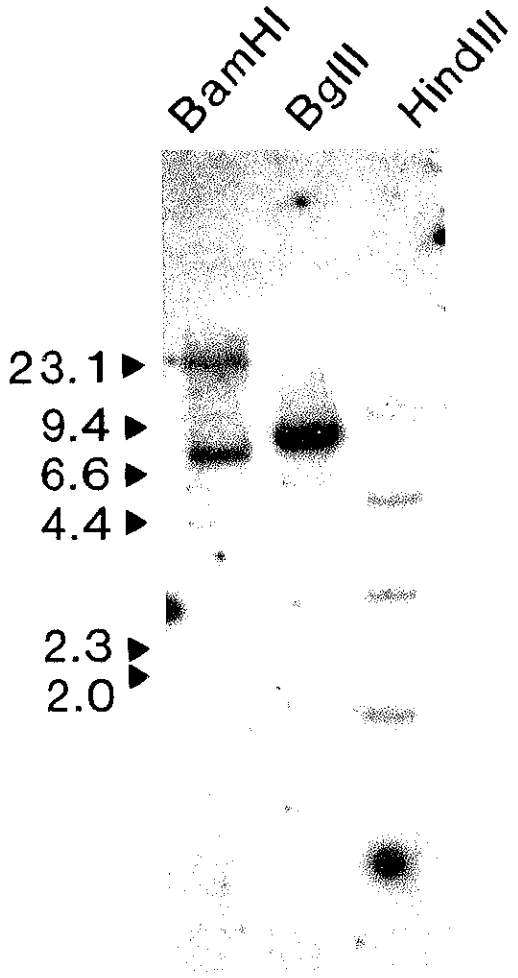

Figure 3. Southern blot analysis of *Xenopus* laevis genomic DNA with a rat activin cDNA as a probe. Ten micrograms of DNA was digested with *Bam*HI, *Bgl*II, or *Hind*III. The digests were electrophoresed on a 1 % agarose gel. Hybridization condition are described in Materials and Methods.

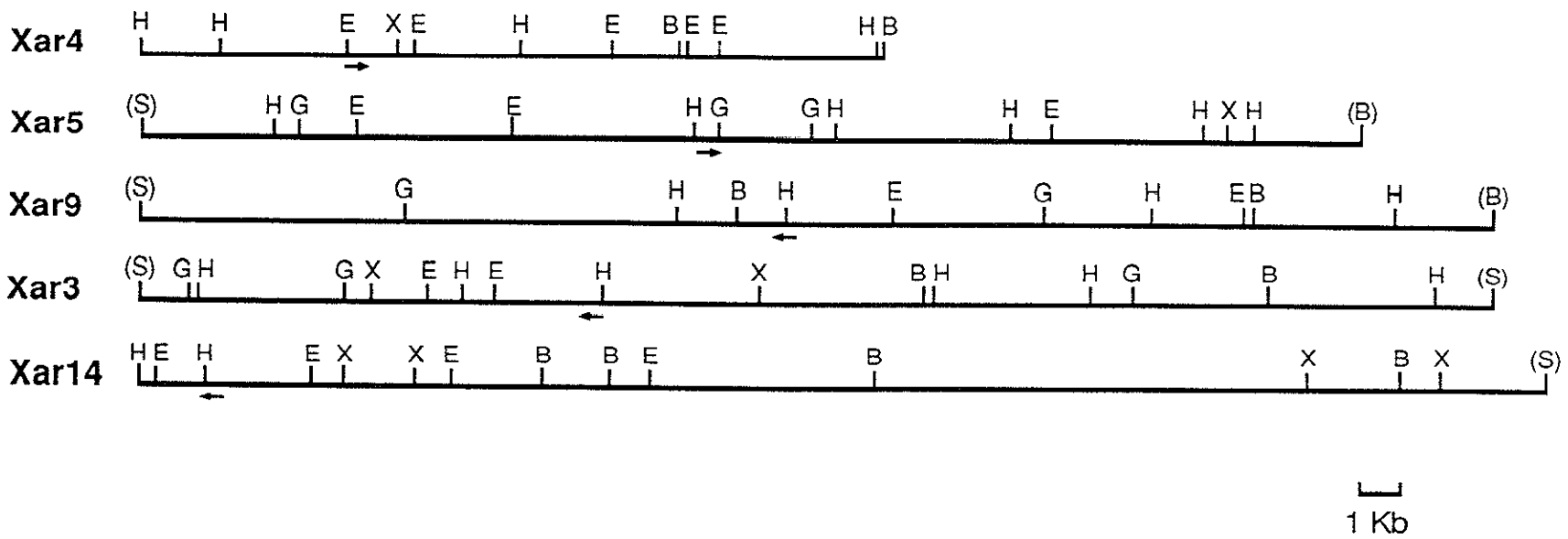

Figure 4. Restriction maps of five activin-related genes isolated from *Xenopus laevis*. The *Xenopus* liver genomic DNA was partially digested with *Sau*3AI and cloned into the phage vector, Charon 28. About 1 x 10^6 phages from the library were screened under low stringent conditions using mammalian activin cDNA as a probe. Arrow indicates the direction of translation and the sequenced region. Restriction enzymes were: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; G, *Bgl*II; X, *Xba*I; S, *Sau*3AI.

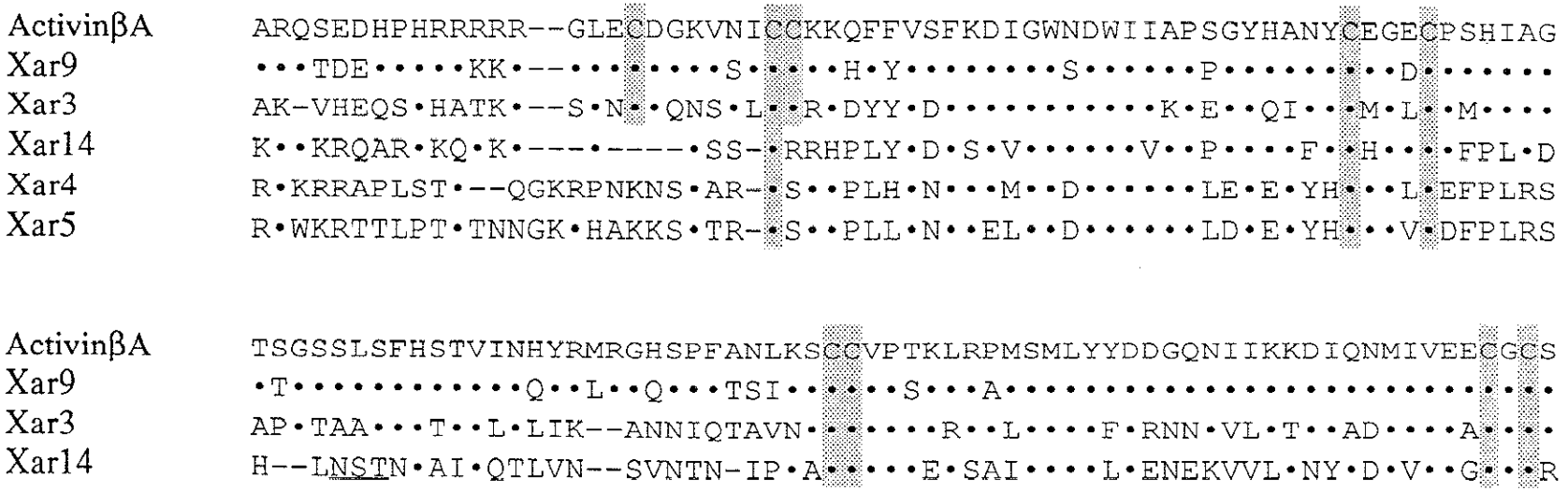

Figure 5. Amino acid sequences of *Xenopus* activin-related genes. Amino acid sequences are indicated by one letter symbols and amino acids identical to those of *Xenopus* activin (Xar9) are dotted.

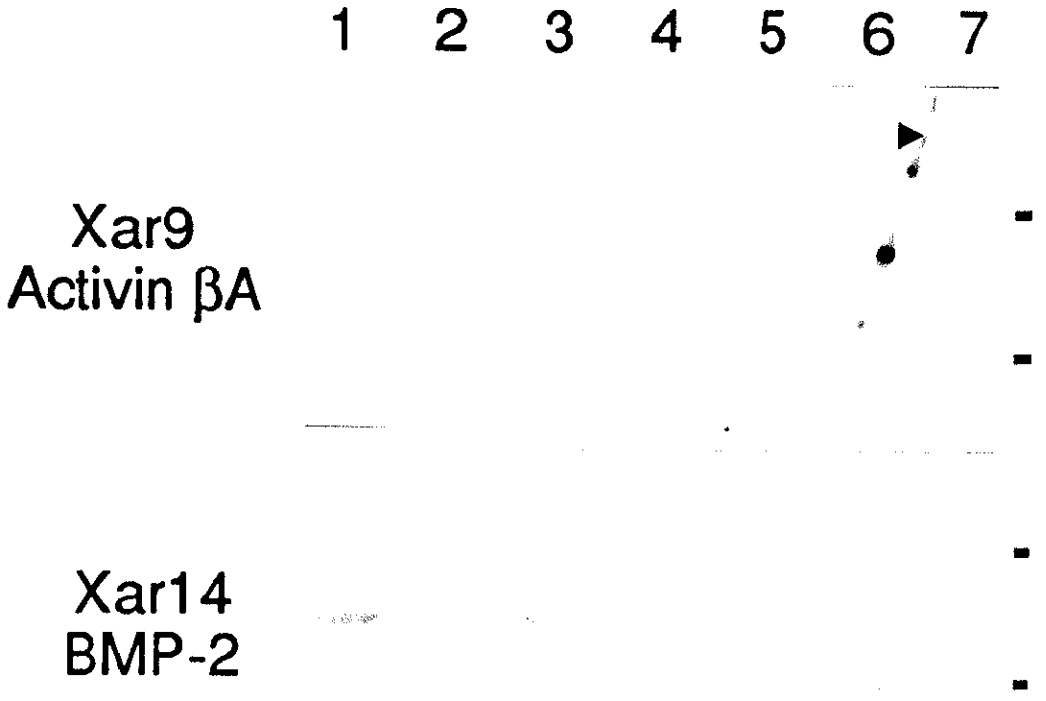

Figure 6. Northern blots of *Xenopus* activin and bone morphogenetic protein-2. Ten micrograms of poly $(A)^+$ RNA from staged embryos were hybridized with specific probes for activin (A) and BMP-2 (B). Lane 1, oocyte; lane 2, morula; lane 3, blastula; lane 4, gastrula; lane 5, neurula; lane 6, tailbud; lane 7, tadpole.

Chapter II. Molecular nature of *Xenopus* bone morphogenetic proteins in early embryos.

Section 1. Genes for bone morphogenetic proteins are differentially transcribed in early amphibian embryos

SUMMARY

As shown in Chapter I, I cloned five activin-related genes including a bone morphogenetic protein (BMP) gene, from a *Xenopus* genomic DNA library. In this section, first step in clarifying the molecular nature of BMP is described. I cloned the cDNAs for *Xenopus* BMP-2, BMP-4 and BMP-7 from an oocyte cDNA library. Northern blots revealed that these BMP genes are maternally transcribed and differentially regulated during early embryogenesis. An alkaline phosphatase-inducing assay using the recombinant BMP proteins showed that at least BMP-2 and -4 have activity similar to their mammalian counterparts.

INTRODUCTION

Several growth factors, including basic fibroblast growth factor (bFGF) [18, 19] and transforming growth factor-βs (TGF-βs) [45, 46], are implicated in the regulation of early *Xenopus* development. Asashima *et al.* have demonstrated that human recombinant activin has mesoderm inducing activity [25]. Subsequently, two groups [47, 48] independently isolated mesoderm-inducing factor (MIF) from the conditioned medium of the *Xenopus* cell line XTC, which was considered to be a major source of the activity. Activin (XTC-MIF) induces a variety of mesodermal tissues including the notochord depending on its concentration [49]. The potent mesoderm inducing activity and the concentration-dependent effect of activin thus postulate a gradient of activin protein in the early embryo that plays an important role in pattern formation during amphibian development.

After screening the cDNA library for the *Xenopus* activin gene, I isolated five independent clones which encode activin related-proteins including not only activin but also BMP. BMPs were originally identified in bone extracts by their ability to induce the formation of ectopic cartilage and bone following implantation in rats. However, the role of BMP in early development was not clarified. In this section, I report three *Xenopus* BMP cDNAs cloned from the library prepared from oocytes and the amino acid sequences of these amphibian BMPs. Further, I evaluated their biological activities by inducing alkaline phosphatase in mouse osteoblasts.

MATERIALS AND METHODS

Cloning and DNA Sequencing.

In Chapter I, I isolated a class of gene designated Xar 14 which encoded a protein almost identical to mammalian BMP-2. In order to investigate the structure and function of bone morphogenetic protein in early *Xenopus* embryos, I screened a *Xenopus* oocyte cDNA library screened using a *Pst*I/*Hind*III fragment (209 bp) of Xar14 as a probe. After screening 1.2 X 10^6 clones, four positive clones, Xbr7, 22, 23, and 41 were identified. The inserts of these Xar14-related gene clones were subcloned into pUC19 and sequenced.

Northern Blots of mRNA from Xenopus *Embryos.*

The *Xenopus laevis* embryos were staged according to Nieuwkoop and Faber [50]. Total RNA was extracted from the ovary and staged embryos and Northern blot analysis was performed as described in Chapter I. *Eco*RI/*Pst*I (484 bp), *Eco*RI/*Nco*I (308 bp) and *Eco*RI/*Eco*RV (549 bp) fragments corresponding to the 5' regions of Xbr7, Xbr23 and Xbr41, respectively showed no significant homology. These were used as the specific probes to prevent the cross-hybridization.

Expression of xBMP proteins in COS cells.

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM)(Sigma) supplemented with fetal bovine serum (FBS) (Boheringer). A full length cDNA of each BMP gene was cloned into the *Xho*I site of the mammalian expression vector pCDM8 (Invitrogen). The plasmid DNA was transfected into cells by the DEAE-dextran method [32] using the CellPhect transfection kit (Pharmacia). The cells were then cultured in DMEM/10% FBS. The next day, the medium was changed to αMEM with 10% FBS, and cultured for 3 days. The spent medium was

harvested for bioassay.

Bioassay.

Alkaline phosphatase (ALPase) activity was measured according to the method of Glay *et al.*[51] with minor modifications[52, 53]. Mouse osteoblastic MC3T3-E1 cells were incubated in 48-well multiplates ($2x10^4$ cells in 500 μl of α-MEM supplemented with 10% FBS per well) for 4 days when they reached confluence. The cells were washed once and incubated further in the conditioned media from COS cells (α-MEM) transfected with the expression vector pCDM8 containing the inserted xBMP cDNAs or not. After 48 hours, the cells were washed twice with PBS and added to 200 μl of 0.56 M 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, and 10 mM Na_2*p*-nitrophenyl phosphate. At the same time, cell-free wells containing 200 μl of α-MEM with various concentrations of *p*-nitrophenol were prepared. They were incubated at 37°C for 1 hour and added to 200 μl of NaOH. After 100 μl of each supernatant was transferred to 96-well multiplates, the absorbance of *p*-nitrophenyl phosphate was determined at 405 nm by an enzyme immunoassay plate reader (SLT, Salzburg, Austria). The standard curve was constructed from the absorbance values plotted against the nmol of *p*-nitrophenol produced per 10^4 cells incubated for 1 hour.

RESULTS

cDNA Cloning of Xarl4 (BMP-2)-related genes.

A *Xenopus laevis* oocyte cDNA library was screened using a fragment of the Xarl4 gene, corresponding to the region of the predicted mature protein as a probe. After screening 1.2 X 10^6 phages, four distinct clones, Xbr 7, 22, 23, and 41 (Xbr for *Xenopus* BMP-related) were isolated (Fig. 7A). Figure 7B shows the precursor structures predicted from the nucleotide sequences of the cloned cDNAs. Both Xbr7 and 22 encode *Xenopus* BMP-2 (xBMP-2) proteins of 398 amino acids but differ in the precursor region [54] (Fig. 8), which is probably attributable to the tetraploidization of *Xenopus laevis*[55]. Xbr23 and Xbr41 encode 401 and 426 amino acid proteins respectively, which are similar to *Xenopus* homologs of BMP-4 [26,56] and BMP-7 [57] or OP-1 [58]. The potential enzymic processing sites are conserved in the amphibian sequences and the mature regions of *Xenopus* BMP-2, BMP-4 and BMP-7 were predicted as 114, 114 and 144 amino acid peptides respectively, based upon the reported consensus amino acid sequence R-X-X-R and R-X-K-R [59, 60]. All the peptide structures exhibited the feature unique to TGF-β family proteins, namely a highly conserved distribution of Cys residues. The amino acid homology of xBMP-4 and xBMP-7 to the human counterparts is 98 and 93% respectively, in the predicted mature protein. There are potential N-linked glycosylation sites in both mature proteins. Although the structural similarity is extremely high between xBMP-2 and xBMP-4, the former has one and the latter has two such sites.

Expression of Xenopus *BMPs in oocytes and early embryos.*

The temporal expression of the *Xenopus* BMPs genes in oocytes and

early embryos was examined by Northern bloting using the respective specific probes. As shown in Figure 9 top row, xBMP-2 transcripts of 2.9 and 2.6 kb were detected in oocytes, stages 7 and 9 embryos (blastulae) but not after stage 11 (gastrulae). On the other hand, the mRNA from stage 11 gave the most intensive hybridization signal of 2.5 and 1.9 kb for xBMP-4 (Fig. 9, middle row). Maternal xBMP-4 transcripts were detected at a trace level and dramatically increased in abundance after stage 9. Both xBMP-2 and xBMP-4 transcripts are present in a diverse array of adult tissues including the ovary and testis [61], suggesting that a comparable level of the xBMP-4 expression after stage 24 is maintained throughout development. The 3.6 Kb and 1.8 Kb transcripts were detected with xBMP-7 probe (Figure 9, bottom row). At least the 1.8 Kb mRNA is maternally encoded and levels of both transcripts increase as development proceeds. The transcripts disappeared immediately after stage 11. As demonstrated here, transcription of these BMP genes is regulated independently despite their close structural similarity.

Biological Activities of xBMPs.

To investigate the function of BMP-2, BMP-4 and BMP-7, I initially expressed the proteins in mammalian cells and determined whether or not amphibian BMP-2, BMP-4 and BMP-7 have activities similar to their mammalian counterparts. The cDNA the xBMP-2, xBMP-4 and xBMP-7 insert that included the entire precursor protein were subcloned into pCDM8. These plasmid constructs and control pCDM8 were transfected into COS-7 monkey cells. After 3 days, the media were harvested and tested for the ALPase-inducing assay on MC3T3-E1 cells. As shown in Figure 10, at least xBMP-2 and xBMP-4 have significant ALPase-inducing

activity and xBMP-4 appears to be more potent than xBMP-2. The activity of xBMP-7 was not detectable in this assay. It is likely that xBMP-7 does not have ALPase-inducing activity, because there are no reports so far indicating that mammalian BMP-7 is active in the assay. Nevertheless, it is suggested that the amphibian BMPs have functions similar to those of mammals [62].

DISCUSSION

In this section, I showed that three types mRNAs encoding BMP-2, BMP-4 and BMP-7 are expressed in *Xenopus* oocytes and are differentially regulated in early embryos after fertilization. All three cDNAs were cloned by hybridization at low stringency using the *Xenopus* BMP-2 gene as a probe. As mammalian BMP-2 protein was originally purified based upon the ability to induce cartilage and bone formation *in vivo*[63, 64], both BMP-4 and BMP-7 (which is identical to OP-1[58]) genes were cloned by homology with BMP-2 [57].

As previously noted for human BMP-2, BMP-3 and BMP-4, *Xenopus* BMPs also exhibited significant amino acid sequence homology to a family of TGF-β proteins, especially the so-called DPP or DVR subfamily that includes the products of the *Drosophila decapentaplegic* (*dpp*) gene [43], *Xenopus* Vg1 [13], mouse Vgr-1 [65]. The putative mature protein xBMP-4 exhibits high amino acid homology (76% in the compared sequences) to the *Drosophila dpp* product which plays a role not only in the establishment of the dorsal-ventral specification during embryogenesis but also in the correct formation of the imaginal disks later in the process [44]. The close structural similarity between the xBMP-2 and -4, and DPP proteins thus supports the idea that xBMPs, whose mRNAs are maternally inherited, control early amphibian development. It has been shown by *in situ* hybridization that the BMP genes are developmentally regulated in mouse embryos [66, 67, 68]. I demonstrated that the amphibian BMPs have an activity similar to that of mammalian BMPs. The stimulation of cell differentiation must be involved in developmental regulation. I therefore propose that DPP protein or a similar protein(s) in both arthropods and vertebrates contribute in tandem with other factors, to cell differentiation as

a regulator of early development.

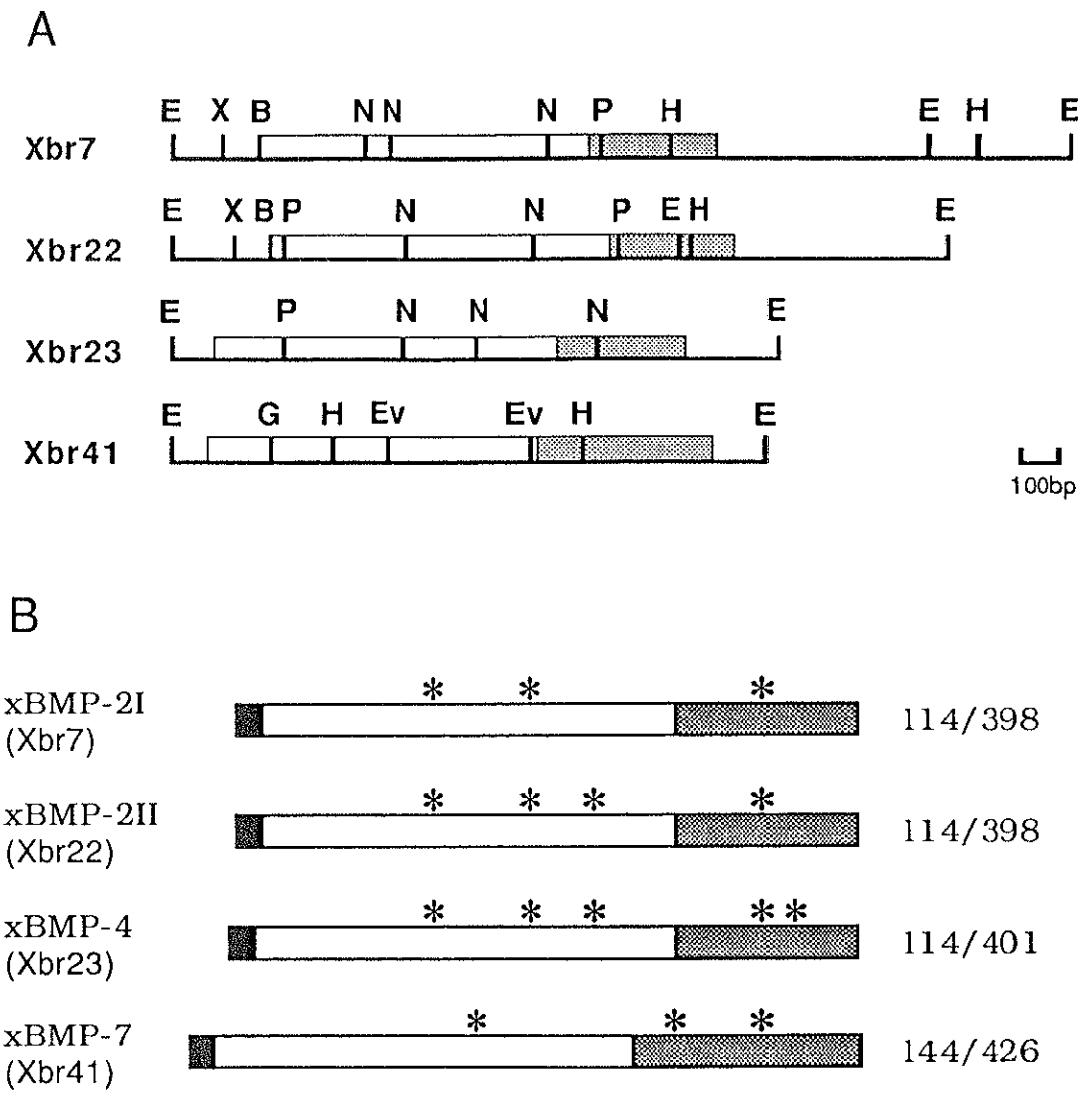

Figure 7. Structure of *Xenopus* BMP cDNAs and deduced precursor proteins. (A) Restriction map of isolated cDNA clones. Open boxes represent the translated region of the cDNAs. Letters denote restriction enzymes: B, *Bam*HI; E, *Eco*RI; Ev, *Eco*RV; G, *Bgl*II; H, *Hind*III; N, *Nco*I; P, *Pst*I; and X, *Xba*I. (B) Schematic representation shows the precursor structure of the proteins deduced from four cDNAs including the predicted leader sequence (closed box), propeptide (open box), and the mature region (hatched box). Potential N-linked glycosylation sites are indicated by asterisks.

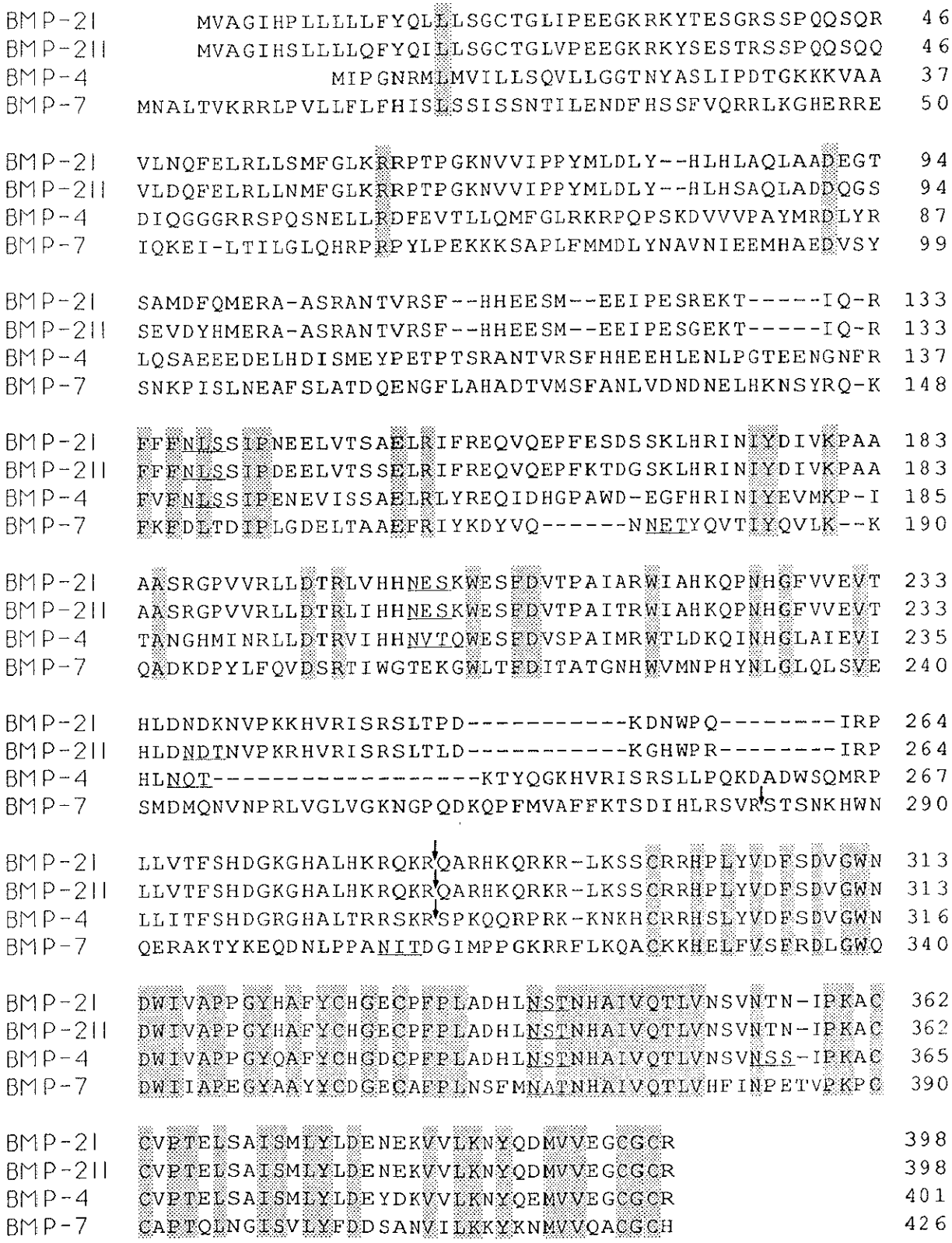

Figure 8. Alignment of *Xenopus* BMP-2I, BMP-2II, BMP-4, and BMP-7 amino acid sequences. The alignment was generated by the GENETYX computer program (SDC, Japan). The locations of the proposed amino termini of the mature region are indicated with arrows. Potential N-linked glycosylation sites are indicated by underlines.

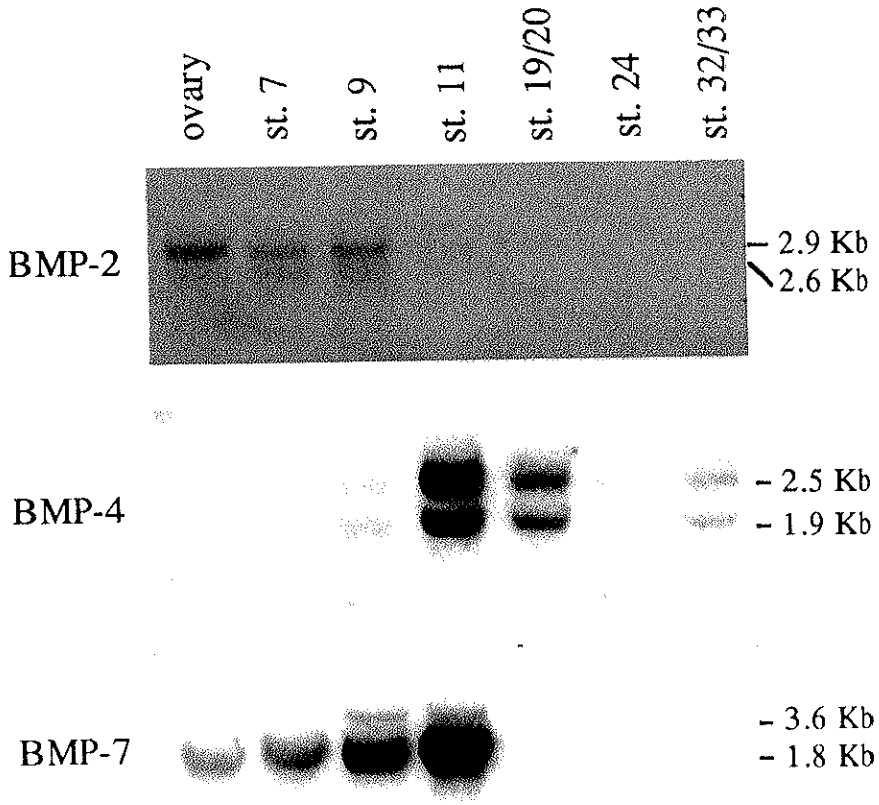

Figure 9. Northern blots of xBMP mRNAs from staged *Xenopus* embryos. Embryos were staged and mRNAs were purified as previously described(12). The specific probes for xBMP-2, xBMP-4, and xBMP-7 were prepared from a DNA fragment corresponding to the propeptide region of the respective cDNA to prevent cross-hybridization.

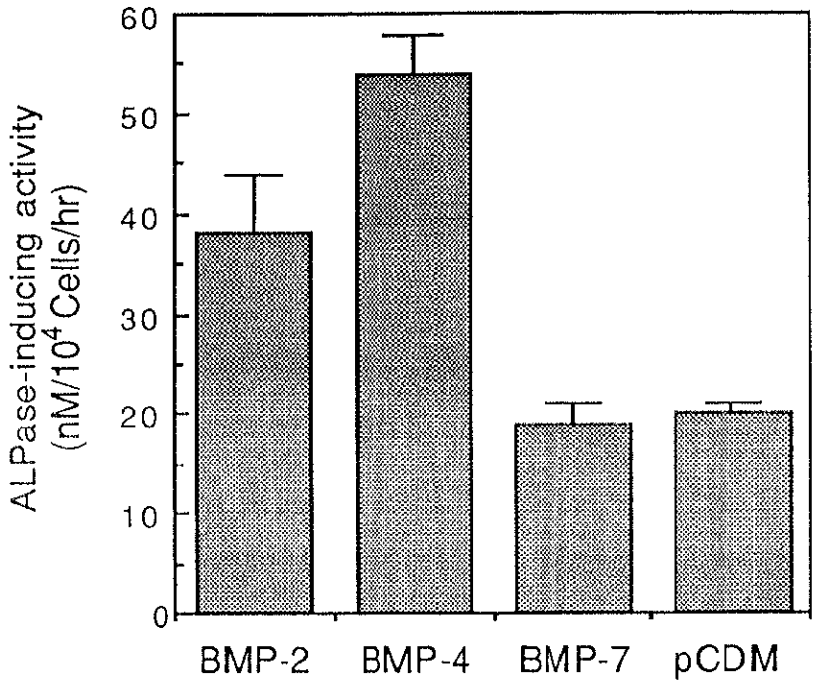

Figure 10. ALPase-inducing activity of recombinant *Xenopus* BMPs. MC3T3-E1 cells were cultured for 3 days in conditioned medium from COS-7 cells transfected with *Xenopus* BMP cDNAs and pCDM8 as a control. ALPase-inducing activity was determined as described in Materials and Methods. Results are means ± SD (n=3).

Section 2. Immunodetection of *Xenopus* bone morphogenetic protein-4 in early embryos.

SUMMARY

Specific antibodies to *Xenopus laevis* bone morphogenetic protein-4 (xBMP-4) were raised by immunizing rabbits with a fusion protein of bacterial β-galactosidase and xBMP-4. The antibodies were used to detect xBMPs expressed in mammalian cells by Western blotting. The antibodies specifically recognized xBMP-4 and did not cross-react with either xBMP-2 or xBMP-7, which are similar to xBMP-4. In addition, the antibodies recognized the dimeric active form of xBMP-4 whereas other antibodies, which were raised with a synthetic peptide identical to xBMP-2, recognized the reduced form only. The antibodies detected an immunoreactive 27 kDa protein in extracts of developing *Xenopus* embryos from the oocyte to the tail bud embryo. The xBMP-4 peptide appeared to be monomeric in structure because the molecular mass did not shift upon reduction of disulfide bond(s).

INTRODUCTION

Bone morphogenetic protein (BMPs) are polypeptides that were isolated from bone extracts based upon their ability to induce ectopic cartilage and bone formation *in vivo*[63]. The BMP family consists of 7 members, namely BMP-1 to BMP-7. Except for BMP-1, they are structurally related not only to each other, but also to the transforming growth factor-β (TGF-β) family of proteins[69]. In Chapter II Section 1, the cloning of *Xenopus* homologues of mammalian BMP genes is described and I showed that their transcripts are present in early embryos and that their expression level is temporally regulated. However, the role of embryonic BMPs is not clear in contrast to that of the defined function of BMPs in bone induction in adult animals. One clue to the function of embryonic BMP could be provided by primary structures of BMPs, especially of BMP-2 and BMP-4, which are similar to the *Drosophila dpp* gene product that is essential for the specification of the dorso-ventral axis of the fly embryo [44]. To determine the precise physiological significance of embryonic BMPs in vertebrates, it is essential to characterize the BMP proteins in early embryos. Ueno *et al.* have shown that most of the immunoreactive xBMP-2 protein recognized by anti-xBMP-2 peptide antibodies(Ab383) in *Xenopus* embryos is not dimerized [70] whereas BMPs produced in mammalian cells or derived from adult bone are normally detected as homodimers[71]. In this section, I show that xBMP-4 is a monomeric form in early embryos, using xBMP-4 specific antibodies which were raised against an xBMP-4 fusion protein.

MATERIALS AND METHODS

Materials

Peroxidase-conjugated goat anti-rabbit IgG was purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA) and Protein A was from Pharmacia (Uppsala, Sweden).

Production and immunization of BMP fusion protein

To generate the antigen, I fused the xBMP genes in frame to the *lac Z* gene of *Escherichia coli*, which encodes β-galactosidase. A 300 bp *Hpa*II-*Xho*II fragment encoding the mature region of xBMP-4 was ligated with *Bam*HI linker, subcloned into the *Bam*HI site of pUEX2(Amersham, England) and used to transform *E. coli* DH1. The transformed cells were cultured for 2 hours at 30 °C, then further cultured for 2 hours at 42 °C to express the fusion protein. The bacteria were harvested by centrifugation at 4000 x g for 10 minutes, then inclusion bodies were purified and the proteins solubilized by standard methods [72] with minor modifications [73]. The proteins were separated by preparative 6 % SDS-PAGE and the protein bands were visualized by incubating the gel with cold 0.25 M KCl. A major protein band migrating at 130 kDa was excised from the gel and pulverized. A piece of the gel slice equivalent to about 100-200 μg of fusion protein was mixed with PBS and emulsified with an equal volume of complete Freund's adjuvant (Detroit, WI, USA). The adjuvant was subcutaneously injected at multiple sites on the backs of three New Zealand White rabbits weighing about 2.5 kg. Several weeks later, the rabbits were boosted at two weeks intervals with a subcutaneous injection of 200 μg of the fusion protein in Freund's incomplete adjuvant.

In order to confirm that the antibodies were directed against the xBMP-4 sequence, another xBMP fusion protein linked to the T7 gene 10 was also

prepared [74]. A *Eco*RI to *Pst*I fragment derived from the vector pUEX2/ xBMP-4 was subcloned into the *Eco*RI and *Nsi*I site of expression vector pGEMEX-1 (Promega, WI, USA), and transformed *E. coli* JM109/DE3. The T7 gene 10/xBMP-4 fusion protein was produced as described (Promega's instruction).

Antibody purification

The antiserum was purified as described [75] with some modifications. The antiserum was depleted of anti-β-galactosidase and anti-bacterial protein antibodies by incubation with Affigel-15(BioRad, Richmond, CA) which was coupled with proteins isolated from bacteria expressing β-galactosidase according to the manufacturer's instructions. The bound fraction was also eluted and used in Western blotting as a negative control. The flow-through fraction which included the anti-xBMP-4 antibody, was further affinity-purified by matrix coupled with the β-galactosidase/xBMP-4 fusion protein. The finally purified antibody was designated Ab97 and used to probe the xBMP-4 protein in embryos.

Preparation of embryo extracts and Western blotting

The extracts from various staged *Xenopus* embryos were prepared by ultracentrifugation at 10,000 x g for 1 hour [76]. About 200 μl of supernatant was mixed with an equal volume of 2 x SDS sample buffer [125 mM TrisHCl(pH 6.8) / 20 % Glycerol / 4 % SDS / 0.04 % Bromophenol blue] containing 0.1 M DTT, then separated on a 15 % SDS-polyacrylamide gel. After electrophoresis, proteins were transfered to a PVDF membrane (Millipore, MA, USA) using a semi-dry blotting apparatus (Milliblot SDE, Millipore). The membrane was blocked with 5 % nonfat dry milk (NFDM) in Tris-bufferd saline(TBS) and reacted with the purified antibodies. The Ab97 antibody was diluted 25-fold with dilution buffer [150 mM NaCl / 1%

NP-40 / 0.5 % Sodium deoxycholate/ 0.1 % SDS / 50 mM TrisHCl (pH7.4) / 1 % NFDM) at 4°C overnight. The filter was then washed twice with TBS containing Tween 20 and once with TBS, then reacted with peroxidase-conjugated anti-rabbit antibody for 6 hours at 4 °C. After washing with TBS as above, proteins were visualized by the addition of 0.05 % 4-Chloro-1-naphthol and H_2O_2.

Expression of xBMPs in mammalian cells

COS cells were transfected with xBMP-2, xBMP-4 and xBMP-7 cDNAs that encode the entire precursor proteins as described in Chapter II, Section 1. Recombinant xBMP-4 was also produced by CHO cells [77].

RESULTS AND DISCUSSION

To analyze xBMP-4 protein in *Xenopus* early development, I raised a polyclonal antibody against a β-galactosidase/xBMP-4 fusion protein, which containing the carboxyl-terminal 100 amino acids of the xBMP-4 protein(Fig. 11). This antibody recognized both the β-galactosidase/xBMP-4 fusion protein (data not shown) and the T7 gene 10/xBMP-4 fusion protein on Western blots, suggesting that the antibody interacts with the common xBMP-4 protein region of these proteins.

To examine the specificity of Ab97, the antibody was reacted with three distinct recombinant BMPs, xBMP-2, xBMP-4 and xBMP-7 which were expressed in COS cells (Fig. 12A). The Ab97 antibody detected xBMP-4 as a 20 kDa protein (lane3) under reducing conditions and showed no cross-reactivity with either xBMP-2 or xBMP-7 (lanes 2 and 4). The presence of alkaline phosphatase-inducing activity in the medium (see Chapter II, Section 1) excluded the possibility that xBMP-2 was not secreted in the test medium. The Ab97 antibody recognized neither T7 gene 10/BMP-2 nor T7 gene 10/xBMP-7 fusion proteins(data not shown). Comparison of the amino acid sequence of xBMP-4 revealed a rather low (56%) homology with that xBMP-7, but a high degree of sequence identity (94%) with xBMP-2 (Fig. 11). The antibody is unlikely to recognize xBMP-7 which is more diverged than xBMP-2. These results showed that the Ab97 antibody is specific for xBMP-4. It is noteworthy that the Ab97 antibody has overcome the disadvantage of the anti-xBMP-2 peptide antibodies(Ab383) which recognizes both xBMP-2 and xBMP-4 equally [70]. When the Ab 97 antibody was used to detect another recombinant xBMP-4 in the medium from CHO cells transfected with an xBMP-4 cDNA encoding the entire precursor structure, it detected a 40 kDa protein under

non-reducing conditions (Fig.12B, lane1). The molecular mass was shifted to about 20 kDa when 0.1M DTT was added to the medium (Fig. 12B, lane2), suggesting that the xBMP-4 peptide expressed in mammalian cells forms a homodimer before it is secreted. In addition, the data shows that the precursor of xBMP-4, which consists of about 400 amino acids as deduced from the cDNA structure (see Chapter II Section 1) is properly processed to yield a 20 kDa mature peptide. The estimated molecular mass is similar to that of the mammalian BMP-4 expressed in human 293 cells [78]. An additional immunoreactive protein with a molecular mass of about 80 kDa was also detected under non-reducing conditions (Fig. 12B, lane1). This protein probably represents a heterodimer consisting of a 60 kDa precursor and a 20 kDa mature xBMP-4 peptide because a 60 kDa species was obtained under reducing condition (Fig. 12B, lane2). These results also demonstrate that Ab97 detects the dimeric form of xBMP-4, while the other antibody Ab383 recognize the monomeric protein exclusively [70].

In Section1, the presence of xBMP-4 mRNA in oocytes and early embryonic stages is described. To investigate the molecular nature of the embryonic xBMP-4, extracts from developing embryos were separated on 15 % SDS-PAGE and analyzed by Western blotting using the Ab 97 antibody. As shown in Figure 13, Ab 97 detected a 27 kDa protein under reducing conditions in the extracts of embryos at the early developmental stages before that of the tail bud (stage 24). The immuno-staining was specific because the anti-β-galactosidase antibody (see Materials and Methods) did not react with the 27 kDa protein (data not shown). In Section 1, I showed that the level of xBMP-4 transcript varies during early embryonic development. The transcript is present in the unfertilized oocytes at a low level, then it increases to the highest level at stage 11

followed by a gradual decline.

The mass of xBMP-4 in the extract was not shifted to a higher molecular mass under non-reducing conditions (data not shown), suggesting that the major immunoreactive 27 kDa protein is a monomer. The estimated molecular mass is slightly higher than that of the secreted form of xBMP-4 shown in Figure 12A and B. This is in agreement with the finding that the molecular mass (30 kDa) of the immunoreactive xBMP-2 detected by Ab383 in *Xenopus* embryos is higher than that of the human BMP-2 secreted form (18 kDa for monomer) expressed in CHO cells [77]. The 30 kDa xBMP-2 is processed into 18 kDa by acidification of the extract [70]. However, this was not the case for xBMP-4 and the molecular mass was not reduced by acidification for several hours with 1M acetic acid(data not shown). This discrepancy probably caused the specific amino acid sequence in xBMP-2 which is cleaved under acidic conditions.

The presence of monomeric peptides of xBMP-4 implies that there is a specific mechanism in early embryos which regulates the activity of BMPs by dimerization through disulfide bridge(s).

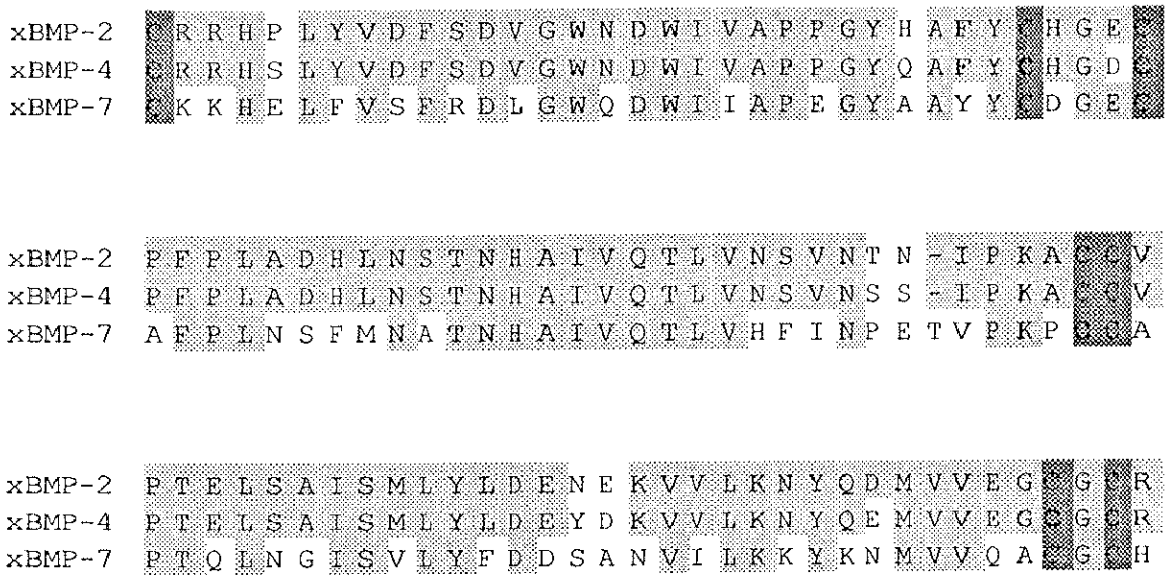

Figure 11. Amino acid sequence comaparison of xBMP-2, xBMP-4 and xBMP-7. Arrow indicates the region of xBMP-4 protein that was fused to β-galactosidase. Conserved amino acid residues are half-tone shaded. Cysteine residues are darkly shaded.

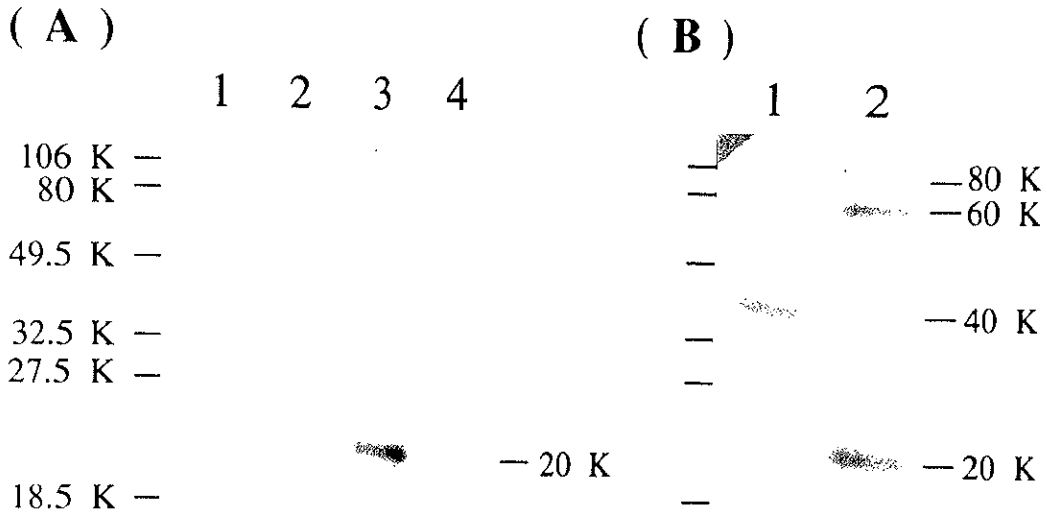

Figure 12. Western blots of recombinant xBMPs probed with the Ab97 antibody. (A) 2.5 ml of conditioned medium from COS cells transfected with the control expression vector pCDM8 (lane 1); the vectors pCDM8/xBMP-2 (lane 2); pCDM8/xBMP-4 (lane 3) and pCDM8/xBMP-7 (lane 4). (B) Conditioned medium from CHO cells (1 ml) transfected with pSVD(X)/xBMP-4 analyzed under non-reducing (lane 1) and reducing (lane 2) conditions. Proteins were separated by 15% SDS-PAGE. The relative molecular mass is indicated on the left.

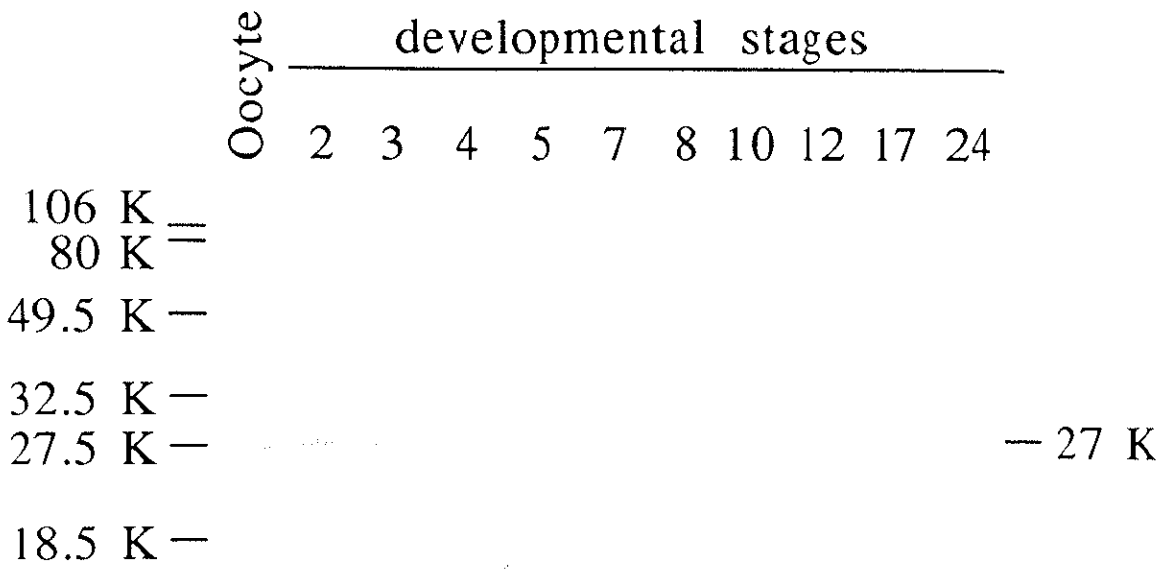

Figure 13. Detection of xBMP-4 protein during embryonic development. Embryo stages were classified according to Nieuwkoop and Faber *et al.* [50]. Embryo extract from each developmental stage (10 µl) was separated by 15% SDS-PAGE. Relative molecular mass is indicated on the left.

Chapter III. Multiple genes for *Xenopus* activin receptor are expressed during early embryogenesis.

SUMMARY

Four distinct cDNAs for the activin receptors designated XSTK2, 3, 8 and 9, were cloned from a *Xenopus laevis* cDNA library. The protein structures deduced from the cDNAs have shown that they all have a putative extracellular ligand-binding domain, a single transmembrane domain and cytoplasmic Ser/Thr kinase domain except that XSTK2 is almost identical to the XSTK3 gene but it lacks the carboxyl-terminal portion of the kinase motif. Northern blots showed that all the transcripts are maternally inherited. The levels of XSTK2, 3 and 8 transcripts appeared to fluctuate during early development, while those for XSTK9 remain constant.

INTRODUCTION

Activin, a member of transforming growth factor-β (TGF-β) family, was originally purified from gonadal fluids and defined as a stimulator of follicle-stimulating hormone (FSH) secretion from anterior pituitary cells [21, 22]. Activin was later found to be identical to erythroid differentiation factor (EDF) which stimulates the differentiation of F5-5 friend leukemia cells into hemoglobin synthesizing cells [23]. It now understood that the functions of activin are not limited to hormonal regulation but are closely correlated with cell differentiation [24]. As described in Chapter I, I revealed the structure of the *Xenopus laevis* activin gene whose mRNA is in tailbud stage embryos. Another group [79] have also reported that mammalian activin is a potent mesoderm-inducing factor and by means of an RNase protection assay, showed that its mRNA is transcribed from the late blastula (stage 9). These findings have prompted many developmental biologists to identify endogenous activin or an activin-like factor in early *Xenopus* embryos [80]. In 1991, Asashima *et al.* identified an activin-like activity [81] in early *Xenopus* embryos. Subsequently, it was speculated that a receptor(s) must also be present because the presumptive ectoderm (animal cap) responds to mammalian activin and induces remarkable changes in morphology as well as activation of the muscle specific α-actin gene. Although the presence of activin receptors on a variety of mammalian cells has been reported [24, 27, 28], it was only recently, that the mouse activin receptor gene was isolated and found to encode a serine/threonine (Ser/Thr) kinase [30].

In this Chapter, I report the cloning of a family of *Xenopus* activin receptor genes and their temporal expression in early embryos.

MATERIALS AND METHODS

Complementary DNA for mouse activin receptor [30] was cloned by reverse transcription of mRNA from mouse AtT20 cells followed by PCR using the specific primers, 5'-TAGCTAGCGAGAACTTCC-3' and 5'-TAGGAGCTCCAGTTCAGA-3'. The amplified cDNA was digested with *Kpn*I to prepare probes for the cytoplasmic Ser/Thr kinase and extracellular domains. About 1 X 10^6 recombinant phages of the *Xenopus* cDNA library of stage 5-6 embryos [82](a gift from Dr. K. Cho) were first screened with the probe for Ser/Thr kinase domain under the conditions described in Chapter I. All DNA's were sequenced by means of dideoxy chain termination using a Sequenase kit (USB, USA), and [α-^{32}P] dCTP (Amersham, U.K.). Sets of nested deletions were prepared with Exonuclease III and Mung Bean nuclease and both strands of the DNA were sequenced. RNA was prepared from *Xenopus* embryos and Northern blots were performed as described in Chapter I.

RESULTS AND DISCUSSION

Specific primers designed according to the nucleotide sequence of the mouse activin receptor [30] enabled me to obtain by PCR, an approximately 1.6 kb mouse cDNA which covered the entire translated region of the receptor. Southern hybridization that used two probes, one for kinase domain and the other for the extracellular domain suggested that there are multiple genes which are related to the activin receptor (Fig. 14). The *Xenopus* cDNA library was first-screened by hybridization with the probe for the kinase domain. After screening 1 X 10^6 phages, 21 positive clones were isolated. Subsequently, they were classified into 4 groups based upon restriction enzyme mapping (Fig. 15) and the intensity of the hybridization signals with the probe for the extracellular domain. One representative cDNA clone was chosen from each group (XSTK2, 3, 8, and 9) for nucleotide sequence analysis. The intensity of the hybridization signal was XSTK9 > 8 > 3 > 2. The amino acid sequence deduced from each cDNA structure showed that XSTK 9, 8, 3, and 2 encode proteins consisting of 512, 510, 510 and 386 amino acids, that have a putative extracellular ligand-binding domain, a transmembrane domain (underlined) and a Ser/Thr kinase domain (indicated by arrows in Fig. 16). A gene for a truncated form of the receptor was also cloned. It was found that although XSTK 2 and 3 are extremely similar, the former lacks the carboxyl-terminal half of the kinase domain, due to an interruption by a stop codon. It is not clear at present whether or not it was generated by alternative splicing. Nevertheless, the novel receptor structure impaired at the kinase domain suggests that it plays a role as a loss-of-function regulator of activin effects. It was thus intriguing to determine whether or not the function of the kinase is indeed lost. The positions of the 10 cysteine residues in the

putative ligand-binding domain shaded in Figure 16 are perfectly conserved among the four *Xenopus* and mammalian receptors [30]. This implies that these cysteine residues are essential to maintain the conformation of the binding site for activin. Two potential *N*-glycosylation sites in the extracellular domain are also highly conserved (double underlined in Fig. 16). An another remarkable conservation of primary structure was found in the Ser/Thr kinase domain, especially in subdomains VIB and VIII (wavy underlined) [83]. Kinase activity has recently been confirmed in the TGF-β receptor [84], which is structurally related to the activin receptor. The homology of functional domains of *Xenopus* activin receptors to the mouse receptor is schematically indicated in Figure 17. Among the four receptors, XSTK9 has the highest homology to the mouse activin receptor [30] and is identical to XAR5 [85]. The extracellular domain of XSTK2/3 and 8 were most similar to the receptor reported by Attisano et al. [86].

To examine how activin receptor genes are regulated in embryogenesis, poly(A)$^+$ RNA was purified from oocytes and embryos, and analyzed by Northern blotting. The results show that there are several transcripts of different sizes for each gene, that all transcripts are maternally encoded and that the levels are sustained during development (Fig. 18, A-C). It is noted, however, that the level of XSTK 2/3 and XSTK8 transcripts appears to fluctuate somewhat during embryogenesis.

The intention of this study was to correlate the function of the activin receptor with early induction events observed in *Xenopus* embryos. If activin is an endogenous mesoderm-inducing factor, its receptor should also be present when mesoderm induction occurs. In addition, experiments using animal caps have indicated that the competence for activin or the endoderm factor is acquired after stage 8, and lost after stage 11 [49].

Northern blots showed that mRNAs for all receptors are maternally present in unfertilized eggs and remain throughout the embryogenesis, supporting the hypothesis that the activin receptor is involved in mesoderm induction. However, the rather stable expression of these receptor genes does not explain the loss of competence after stage 11. Possible explanations are that synthesis of a functional activin receptor is regulated at the translational or post-translational level, or that an inhibitor such as an activin-binding protein, follistatin [87] or phosphatase controls activin action in the *Xenopus* embryo.

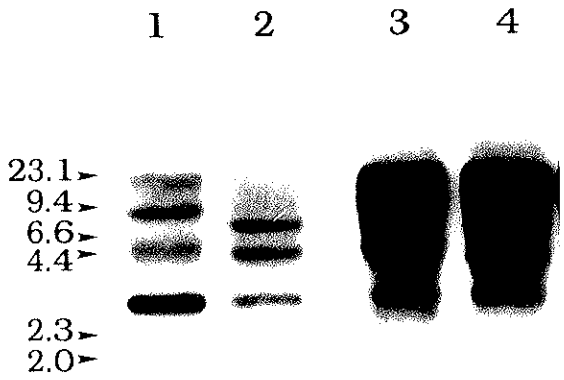

Figure 14. Southern blots of *Xenopus* genomic DNA using the mouse activin receptor cDNA as a probe. Ten micrograms of genomic DNA was digested with *Eco*R I (lane1 and 3) and *Hind*III (lane 2 and 4). Lanes 1 and 2 were hybridized with the probe for the extracellular domain, lanes 3 and 4 for the Ser/Thr kinase domain.

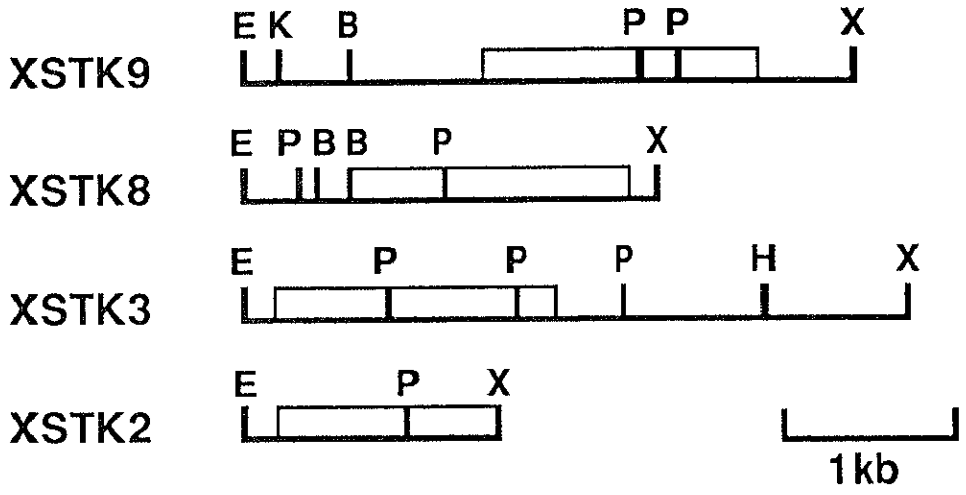

Figure 15. Restriction map of isolated cDNA clones. Open box represents the translated region of the cDNAs. Letters denote restriction enzymes: B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; X, *Xho*I.

```
XSTK9                                MGAATKLAFAVFLIFCSSGAILGRLETKECIYYNANWEK  39
XSTK8                                MGASVALTFLLLLATFRAGSGHDEVETRECIYYNANWEL  39
XSTK3                                MGAAVPLTLALLLATFRAGSGHDEVETRECIYYNANWEL  39
XSTK2  MLRLLPESSLLLLRAADPRGNMGAAVPLTLALLLATFRAGSGHDEVETRECIYYNANWEL  60

XSTK9  DKTNSNGTEPCYGDNDKRKHCFATWKNTSGSIEIVKQGCWLDDVNCYNKNECIEKKESPD  99
XSTK8  EKTNQSGVESCEGEKDKRLHCYASWRNNSGFIELVKKGCWLDDFNCYDRQECIAKEENPQ  99
XSTK3  EKTNQSGVESCEGEKDKRLHCYASWRNNSGFIELVKKGCWLDDFNCYDRQECIAKEENPQ  99
XSTK2  EKTNQSGVESCEGEKDKRLHCYASWRNNSGFIELVKKGCWLDDFNCYDRQECIAKEENPQ 120

XSTK9  VFFCCCEGNACNERFYHSPEMEVTQPTSNPVPKPPLFNTLLYSLVPIIVVAVIVLFLFWM 159
XSTK8  VFFCCCEGNYCNKKFTHLPEVETFDPKPQP--SASVLNILIYSLLPIVGLSMAILLAFWM 157
XSTK3  VFFCCCEGNYCNKKFTHLPEVETFDPKPQP--MPSVLNILIYSLLPIAGLSMVILLAFWM 157
XSTK2  VFFCCCEGNYCNKKFTHLPEVETFDPKPQP--MPSVLNILIYSLLPIAGLSMVILLAFWM 178

XSTK9  YRHHKLGYPPELVPTQDPGPPPPSPLLGLKPLQLLEVKARGRFGCVWKAQLLNETVAVKI 219
XSTK8  YRHRKPPY-GHVEINEDPGLPPPSPLVGLKPLQLLEIKARGRFGCVWKARLLNEYVAVKI 216
XSTK3  YRHRKPPY-GHVDLNEDPGPTPPSPMVGLKPLQLLEIKARGRFGCVWKARLLNEYVAVKI 216
XSTK2  YRHRKPPY-GHVDLNEDPGPSPPSPMVGLKPLQLLEIKARGRFGCVWKARLLNEYVAVKI 237

XSTK9  FPIQDKLSWQNEYEIYSLPGMKHENILHFIGAEKRGTNLDTDLWLITTFHEKGSLTDFLK 279
XSTK8  FPVQDKQSWQCEKEIFTTPGMKHENLLEFIAAEKRGSNLEMELWLITAFHDKGSLTDYLK 276
XSTK3  FPVQDKQSWQCEKEIFNTPGMKHENLLEFIAAEKRGSNLEMELWLITAFHDKGSLTDYLK 276
XSTK2  FPVQDKQSWQCEKEIFNTPGMKHENLLEFIAAEKRGSNLEMELWLITAFHDKGSLTDYLK 297

XSTK9  ANIVSWNELCHIAETMARGLSYLHEDIPGLR-DGHKPAVAHRDIKSKNVLLKNNLTACIA 338
XSTK8  GNLVSWNELCHITETMARGLAYLHEDVPRCKGEGHKPAIAHRDFKSKNVLLRNDLTAILA 336
XSTK3  GNLVNWNELCHITETMARGLSYLHEDVPRCKGEGHKPAIAHRDFKSKNVLIRNDLTAILA 336
XSTK2  GNLVNWNELCHITETMARGLSYLHEDVPRCKGEGHKPAIAHRDFKSKNVLIRNDLTAILA 357

XSTK9  DFGLALKFEAGKSAGDTHGQVGTRRYMAPEVLEGAINFQRDAFLRIDMYAFGLVLWELAS 398
XSTK8  DFGLAVRFEPGKPPGDTHGQVGTRRYMAPEVLEGAINFQRDSFLRIDMYAMGLVLWEIVS 396
XSTK3  DFGLAVRFEPGKPPGDTHGQVGTRRYMAPEVLEGAINFQRDSFLRIDMYAMGLVLWEIVS 396
XSTK2  DFGLAVRFEPGKPPGDTHGQVITCAAINL                                 386

XSTK9  RCTAADGPVDEYMLPFEEEAGQHPSLEDMQEVVVHKKKRPILRECWQKHAGMAMLCETIE 458
XSTK8  RCTAADGPVDEYLLPFEEEIGQHPSLEDLQEVVVHKKIRPVFKDHWLKHPGLAQLCVTIE 456
XSTK3  RCTAADGPVDEYLLPFEEEIGQHPSLEDLQEVVVHKKMRPVFKDHWLKHPGLAQLCVTIE 456

XSTK9  ECWDHDAEARLSAGCVEERIIQMQKLTNIITTEDIVTVVTMVTNVDFPPKESSL         512
XSTK8  ECWDHDAEARLSAGCVEERISQIRKSVNGTTSDCLVSIVTSVTNVDLPPKESSI         510
XSTK3  ECWDHDAEARLSAGCVEERISQIRKSVNGTTSDCLVSIVTSVTNVDLPPKESSI         510
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Figure 16. Alignment of *Xenopus* activin receptor amino acid sequences. Potential *N*-linked glycosylation sites are indicated by double underlines, the transmembrane domain by a single underline, the Ser/Thr kinase domain by two arrows, and subdomains VIB and VIII of the kinase by wavy underlines. The 10 conserved cysteine residues in extracellular domain are shaded.

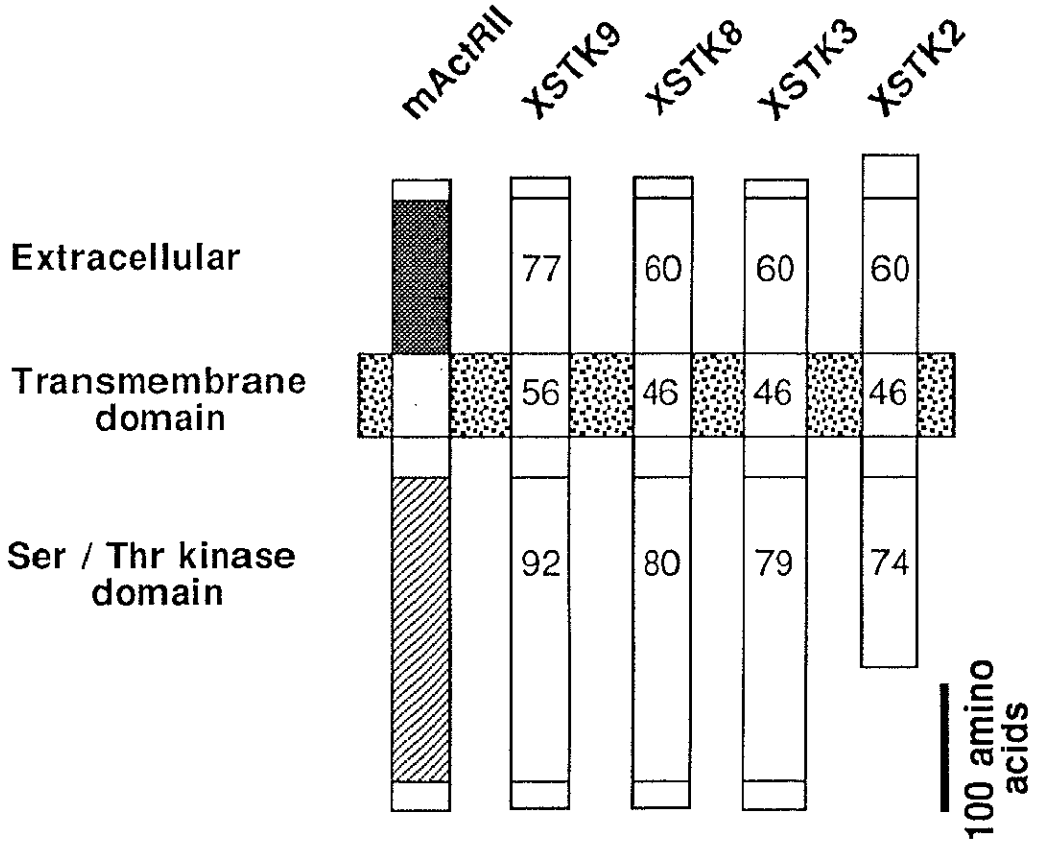


Figure 17. Structural comparison of *Xenopus* and mouse activin receptors. The numbers represent the percentage of amino acid identity to the mouse activin receptor (mActRII) [9].

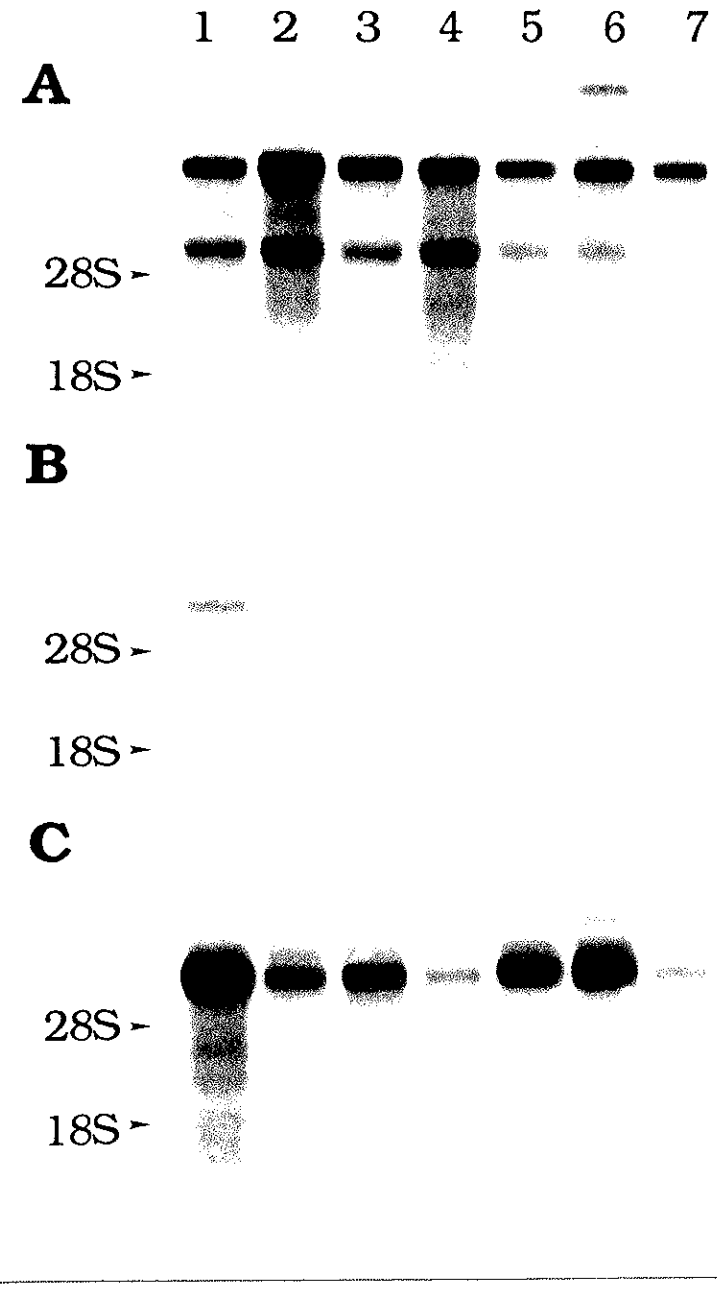


Figure 18. Northern blot analysis of *Xenopus* activin receptor mRNAs during early embryogenesis. Ten micrograms of poly $(A)^+$ RNA from staged embryos were hybridized with probes specific for XSTK9 (A), XSTK8 (B) and XSTK2/3 (C). Lane 1, oocyte; lane 2, molula; lane 3, blastula; lane 4, gastrula; lane 5, neurula; lane 6, tailbud; lane 7, tadpole.

Chapter IV. A carboxyl-terminal truncated version of the activin receptor mediates activin signals in early *Xenopus* embryos.

SUMMARY

The function of a carboxyl-terminal truncated version of the *Xenopus* activin receptor, encoded by XSTK2 cloned in Chapter III, was investigated in early embryos. The transcript corresponding to the truncated receptor gene was detected throughout embryonic development, although the temporal expression pattern differed from that of the intact receptor. Injection of XSTK2 mRNA into early embryos resulted in the formation of a duplicated body axis. Mesoderm induction as evaluated by the activation of the α-actin gene in presumptive ectoderm (animal cap) treated with exogenous activin was significantly enhanced by injecting XSTK2 mRNA. These results suggest that the truncated receptor can transmit the activin signal to the same extent as the native receptor.

INTRODUCTION

Activin, a member of the TGF-β superfamily, functions not only as a regulator of hormone secretion [21, 22] but also as a regulator of differentiation of a variety of cell types [23, 88, 89]. It has been demonstrated by affinity cross-linking, that there are three molecular species of the activin receptor in murine erythroleukemia F5-5 cells and these have been designated as types I (42 kDa), II (51 kDa) and III (151 kDa) [28]. The structure of a mouse activin receptor has been investigated by gene cloning [30]. Based upon the molecular weight predicted from the cDNA, the receptor was classified as being type II. Furthermore, the type II receptor for TGF-β1 has also been cloned [84]. All the type II receptors characterized to date, appear to possess protein Ser/Thr kinases in their cytoplasmic domains [90]. Similar activin receptor geness have been identified from amphibian source and these are involved in morphogenesis during early development [85, 91].

In Chapter III, I showed that there are at least four genes that encode activin receptor-like proteins in *Xenopus laevis*. One gene encoded a receptor protein which lacks the carboxyl-terminal part of the Ser/Thr kinase located distal to domain VIII. I reasoned that the truncated receptor might represent a protein which plays a negative regulatory role in activin signaling. This study was performed to determine whether or not the truncated activin receptor could correctly transmit the activin signal. Here I report that the truncated receptor is functional and causes duplication of the partial body axis as well as enhancement of mesoderm induction to the same extent as the intact receptor.

MATERIALS AND METHODS

Reverse transcription-PCR (RT-PCR)

RT-PCR was performed as described [92, 93] to distinguish transcripts of the XSTK2 gene from those of the XSTK3 and XSTK8 genes whose nucleotide sequences are highly similar to XSTK2 in the N-terminal region. The sequences of the various oligonucleotide primers were as follows. The upper strand primer sequence, 5'-GAAACAATGGCTCGTGGGC-3', is located in a region in common to the XSTK2, XSTK3 and XSTK8, genes whereas the lower strand primer sequences, 5'-TCGCTGCACAAGTGATT ACC-3' and 5'-CTCTAGAACCTCAGGAGCC-3', were chosen from regions specific to the XSTK2 and XSTK3/8 genes respectively. The PCR conditions were as follows: denaturation at 94°C for 30 second, annealing at 60°C for 30 second and extension at 72°C for 60 second. A total number of 30 reaction cycles proceeded in the presence of [α-^{32}P] dCTP (Amersham). Template cDNA was synthesized with the use of MMLV reverse transcriptase (BRL) from total RNA purified from staged embryos by the AGPC method [94]. The PCR products were separated on 0.2 mm-thick polyacrylamide gels, which were then dried for autoradiography. In order to quantify the muscle-specific α-actin mRNA, I reverse transcribed total RNA isolated from animal cap explants and performed PCR as previously described by Rupp et al. [95].

In vitro *transcription and translation*

Capped synthetic RNAs were generated as described [96]. The inserts of the *Xenopus* activin receptor clones XSTK2 (*Sac*II/*Xho*I insert of 0.7 Kb) and XSTK8 (*Bam*HI/*Xho*I insert of 1.5Kb) were subcloned into a pSP73polyA vector which was constructed by inserting an 83 bp *Eco*RI /

*Hin*dIII fragment containing a polyA tract and the multiple cloning site region from pSP64poly(A) (Promega) into pSP73. The pSP73poly(A)-based constructs were transcribed with T7 RNA polymerase *in vitro*. Synthetic RNAs were translated in the rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S] methionine (Amersham) and analyzed by SDS-PAGE.

Injection of mRNAs into embryos

Receptor mRNAs were microinjected essentially as described by Yuge et al.[97], to study phenotypic alterations. Briefly, mRNAs were injected into the ventral blastomeres of 4-cell embryos in Steinberg's solution [58 mM NaCl / 0.67 mM KCl / 0.34 mM Ca(NO3)2 / 0.83 mM MgSO4 / 4.6 mM TrisHCl (pH7.4) / 1 mg/ml kanamycin] after removing the vitelline membrane (see Materials and Methods in Chapter I). The embryos were then allowed to develop in the same solution for several days during the experimental period. For the mesoderm-inducing assay, injections were performed according to the method of Moon *et al.* [98] in 5 % Ficoll.

Animal cap assay

The animal cap assay using embryos injected with activin receptor mRNAs was carried out as described [25]. Injected embryos were allowed to develop into stage 9 blastulae, then the presumptive ectoderms were dissected with tungsten needles. These explants were cultured in Steinberg's solution containing various concentrations of human recombinant activin (supplied by Drs H. Shibai and Y. Eto) and 0.2 % bovine serum albumin for 1 day at 20 °C. After incubation, the explants were stored at -80 °C until RNA extraction. The response of the caps to activin was assessed by

quantifing muscle specific α-actin mRNA with a RT-PCR assay described above.

RESULTS AND DISCUSSION

As showed in Chapter III, extensive screening of a *Xenopus* embryo cDNA library with mouse activin receptor cDNA [30] identified several activin receptor clones. One of the clones designated XSTK2, encoded a protein highly similar to that of the intact receptors encoded by XSTK3 and XSTK8 but which lacked the carboxyl-terminal part of the Ser/Thr kinase region located distal to domain VIII (Fig. 19A). The nucleotide sequence of XSTK2 contained a polyadenylation signal, AATAAA, followed by a polyA tract in the 3' untranslated region consistent with the fact that the XSTK2 gene is transcribed. In Chapter III, I showed by Northern bloting, that the XSTK2 and XSTK3 genes are transcribed in early embryos (Fig. 18, B and C). However, I was unable to distinguish transcripts of XSTK2 from those of XSTK3 and XSTK8, since the probe was chosen from a region common to the three receptor genes. In order to distinguish transcripts of XSTK2 from those of closely related intact receptor genes, oligonucleotide primer sets, were designed taht were specific to XSTK2 or XSTK 3/8 (Fig. 19B). Using the respective primer sets, the transcript levels during early development were examined by RT-PCR. Figure 20 shows an autoradiogram of the PCR products from XSTK2 and XSTK3/8. These products were of the expected size, namely an XSTK2 product of 219 bp and XSTK3/8 products of 242 bp. It is evident that all genes are transcribed during early development. The levels of the XSTK2 transcript appeared to increase as development proceeded, whereas that of the intact receptor (XSTK3/8) appeared to fluctuate during early embryogenesis (Fig. 20), thus suggesting that the gene for the truncated receptor is regulated independently of the intact receptor gene. The temporal expression profile of XSTK3/8 was consistent with the results obtained by Northern blotting

(see Fig. 18, B and C in Chapter III).

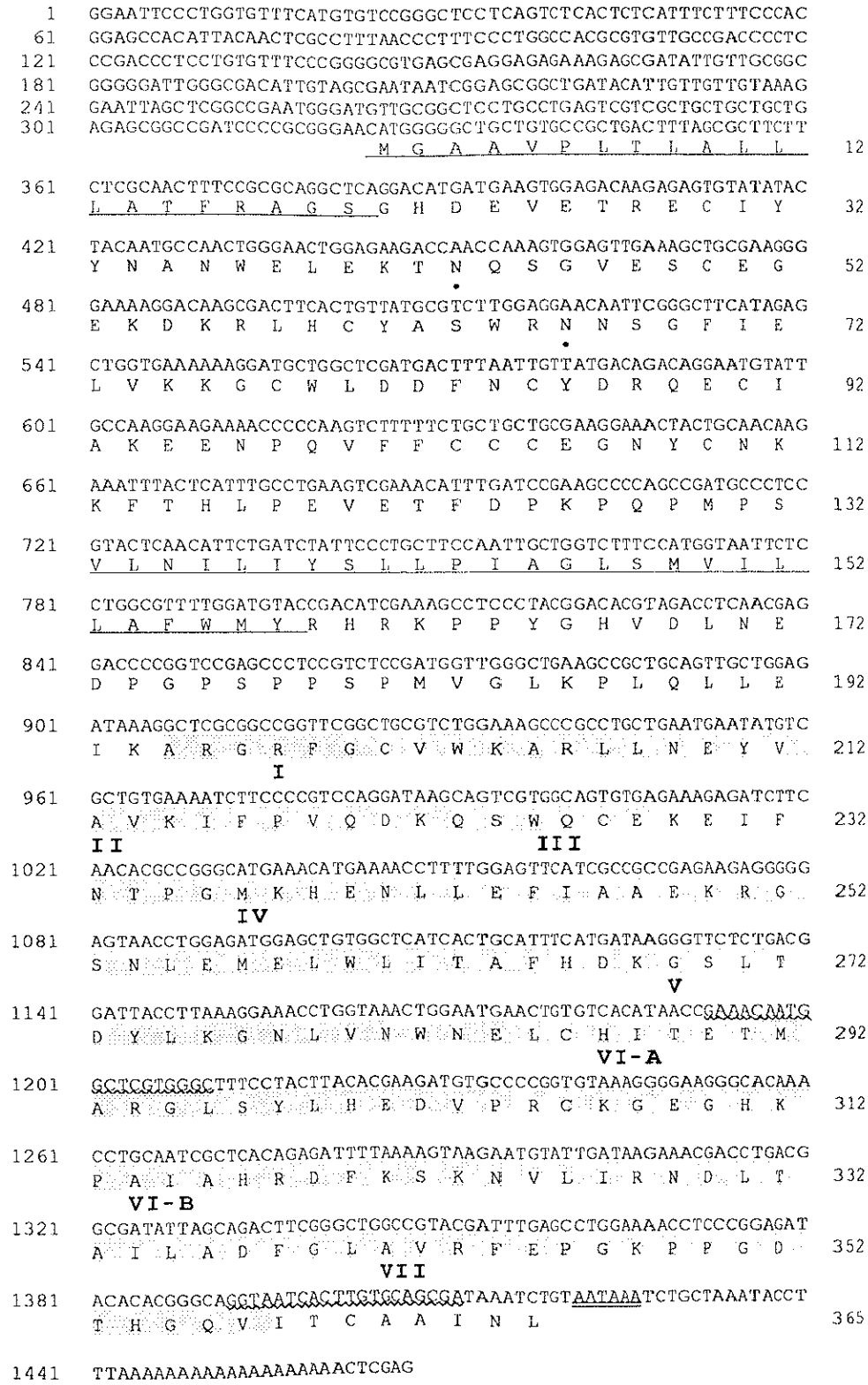
It was reasoned that the truncated receptor can bind activin, since a mouse activin receptor with an even larger carboxyl-terminal deletion binds activin in a similar fashion to the intact receptor [30]. Therefore, I speculated that the truncated receptor encoded by XSTK2 could bind activin but could not transmit activin signals because of the impaired kinase domain. To examine this hypothesis I studied the effect of overexpression of both the truncated and intact receptor proteins. I injected XSTK2 mRNA into early embryos since it has been previously demonstrated that injection of intact activin receptor mRNA results in duplication of the body axis [85, 91] and an increased level of responsiveness of animal cap explants to activin [91]. To determine the molecular mass of the receptor proteins that would be translated by the injected mRNAs, I first translated the mRNAs in the rabbit reticulocyte lysate system (Fig. 21). The addition of XSTK2 mRNA to the lysate resulted in the synthesis of a protein with an apparent molecular mass of 46 kDa (Fig. 21, lane 2) which is close to the molecular weight predicted from the cDNA structure as shown in Figure 19. On the other hand, the molecular mass of the protein translated from the intact receptor gene, XSTK8, was estimated at 64 kDa (Fig. 21, lane 1) representing the deletion of about 120 amino acids at the C-terminal end of XSTK2. Figure 22 shows the typical phenotypic defects of embryos which were injected with various receptor mRNAs. The results of this experiment were surprising in that overexpression of the carboxyl-terminal truncated receptor led to significant alterations in normal development, with the production of a duplicated body axis in a similar fashion to the effects demonstrated with intact activin receptors [85, 91] (Fig. 22B). The frequency of the malformation induced by XSTK2 overexpression was

about 13 % which is comparable to that produced by injection with the intact receptor XSTK8, used in the present study (Table 1) and to that of xActRIIB as reported by Mathews *et al.*[91]. In contrast to the effect observed with *Xwnt-8*, a gene which induces complete body axis formation [99, 100], the additional body axis formed by injected of XSTK2 mRNA is incomplete (Fig 22, A and B) and occurs partially along the antero-posterior axis. It is of note that the bifurcation produced by the truncated receptor occurs exclusively in the posterior region which includes the tail, whereas overexpression of the intact receptor often results in duplication of the anterior region.

Next, I examined the effect of overexpression of the truncated receptor on the competence of animal cap cells to response to exogenous activin. The animal cap of the blastula can respond to activin and thereby differentiates into mesodermal derivatives [25, 79]. This differentiation is associated with the activation of mesodermal marker genes such as *MyoD* [95] and the muscle specific α-actin gene [8]. When animal caps isolated from embryos injected with XSTK2 or XSTK8 mRNAs were tested for α-actin induction, they all demonstrated a significant increase of α-actin mRNA as compared with animal caps from control embryos (Fig. 23). This induction was increased about 3-fold by overexpression of the intact receptor and 5-fold by the truncated receptor. These results suggest that truncation of the carboxyl-terminus causes an increase in the intrinsic activity of the receptor. This increase in activity may be explained by the deletion of a regulatory region, such as an autophosphorylation site(s), which may attenuate receptor kinase activity. This is consistent with observations relating to the c-*erb*B-2 gene, in that the lack of an autophosphorylation site located close to the carboxyl-terminus enhances the

kinase and transforming activities of ErbB-2 [101].

A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61 GGAGCCACATTACAACTCGCCTTTAACCCTTTCCCTGGCCACGCGTGTTGCCGACCCCTC
121 CCGACCCTCCTGTGTTTCCCGGGGCGTGAGCGAGGAGAGAAAGAGCGATATTGTTGCGGC
181 GGGGGATTGGGCGACATTGTAGCGAATAATCGGAGCGGCTGATACATTGTTGTTGTAAAG
241 GAATTAGCTCGGCCGAATGGGATGTTGCGGCTCCTGCCTGAGTCGTCGCTGCTGCTGCTG
301 AGAGCGGCCGATCCCCGCGGGAACATGGGGGCTGCTGTGCCGCTGACTTTAGCGCTTCTT
M G A A V P L T L A L L 12
361 CTCGCAACTTTCCGCGCAGGCTCAGGACATGATGAAGTGGAGACAAGAGAGTGTATATAC
L A T F R A G S G H D E V E T R E C I Y 32
421 TACAATGCCAACTGGGAACTGGAGAAGACCAACCAAAGTGGAGTTGAAAGCTGCGAAGGG
Y N A N W E L E K T N Q S G V E S C E G 52
481 GAAAAGGACAAGCGACTTCACTGTTATGCGTCTTGGAGGAACAATTCGGGCTTCATAGAG
E K D K R L H C Y A S W R N N S G F I E 72
541 CTGGTGAAAAAAGGATGCTGGCTCGATGACTTTAATTGTTATGACAGACAGGAATGTATT
L V K K G C W L D D F N C Y D R Q E C I 92
601 GCCAAGGAAGAAAACCCCCAAGTCTTTTTCTGCTGCTGCGAAGGAAACTACTGCAACAAG
A K E E N P Q V F F C C C E G N Y C N K 112
661 AAATTTACTCATTTGCCTGAAGTCGAAACATTTGATCCGAAGCCCCAGCCGATGCCCTCC
K F T H L P E V E T F D P K P Q P M P S 132
721 GTACTCAACATTCTGATCTATTCCCTGCTTCCAATTGCTGGTCTTTCCATGGTAATTCTC
V L N I L I Y S L L P I A G L S M V I L 152
781 CTGGCGTTTTGGATGTACCGACATCGAAAGCCTCCCTACGGACACGTAGACCTCAACGAG
L A F W M Y R H R K P P Y G H V D L N E 172
841 GACCCCGGTCCGAGCCCTCCGTCTCCGATGGTTGGGCTGAAGCCGCTGCAGTTGCTGGAG
D P G P S P P S P M V G L K P L Q L L E 192
901 ATAAAGGCTCGCGGCCGGTTCGGCTGCGTCTGGAAAGCCCGCCTGCTGAATGAATATGTC
I K A R G R F G C V W K A R L L N E Y V 212
I
961 GCTGTGAAAATCTTCCCCGTCCAGGATAAGCAGTCGTGGCAGTGTGAGAAAGAGATCTTC
A V K I F P V Q D K Q S W Q C E K E I F 232
II III
1021 AACACGCCGGGCATGAAACATGAAAACCTTTTGGAGTTCATCGCCGCCGAGAAGAGGGGG
N T P G M K H E N L L E F I A A E K R G 252
IV
1081 AGTAACCTGGAGATGGAGCTGTGGCTCATCACTGCATTTCATGATAAGGGTTCTCTGACG
S N L E M E L W L I T A F H D K G S L T 272
V
1141 GATTACCTTAAAGGAAACCTGGTAAACTGGAATGAACTGTGTCACATAACCGAAACAATG
D Y L K G N L V N W N E L C H I T E T M 292
VI-A
1201 GCTCGTGGGCTTTCCTACTTACACGAAGATGTGCCCCGGTGTAAAGGGGAAGGGCACAAA
A R G L S Y L H E D V P R C K G E G H K 312
1261 CCTGCAATCGCTCACAGAGATTTTAAAAGTAAGAATGTATTGATAAGAAACGACCTGACG
P A I A H R D F K S K N V L I R N D L T 332
VI-B
1321 GCGATATTAGCAGACTTCGGGCTGGCCGTACGATTTGAGCCTGGAAAACCTCCCGGAGAT
A I L A D F G L A V R F E P G K P P G D 352
VII
1381 ACACACGGGCAGGTAATCACTTGTGCAGCGATAAATCTGTAATAAATCTGCTAAATACCT
T H G Q V I T C A A I N L 365
1441 TTAAAAAAAAAAAAAAAAAAACTCGAG

(Figure 19A)

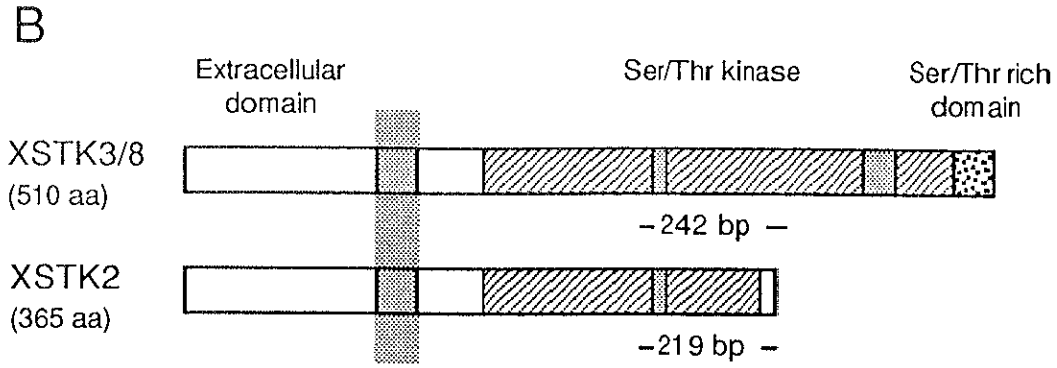

Figure 19. Structure of the truncated activin receptor cDNA, XSTK2, and the deduced precursor protein. (A) The nucleotide sequence of the full-length XSTK2 cDNA clone is shown along with the deduced amino acid sequence of the putative receptor protein. The signal sequence and the transmembrane domain are highlighted by thin underlining. The sequences used to synthesize oligonucleotide primers for RT-PCR are indicated by wavy underlines. The putative polyadenylation site at the 3' end of the mRNA is denoted by double underlines. The Ser/Thr kinase domain is shown by shading of the amino acid sequence. Roman numerals under the deduced amino acid sequence refer to the subdomains conserved among the various members of the protein kinase family. (B) Schematic representation of *Xenopus* activin receptor proteins. The extracellular domain, transmembrane region (shaded), Ser/Thr kinase domain and Ser/Thr rich domains are shown. Two characteristic inserts in the kinase domain are shaded [90]. The locations of the PCR primers are indicated by arrows and the predicted sizes of amplified products are also shown.

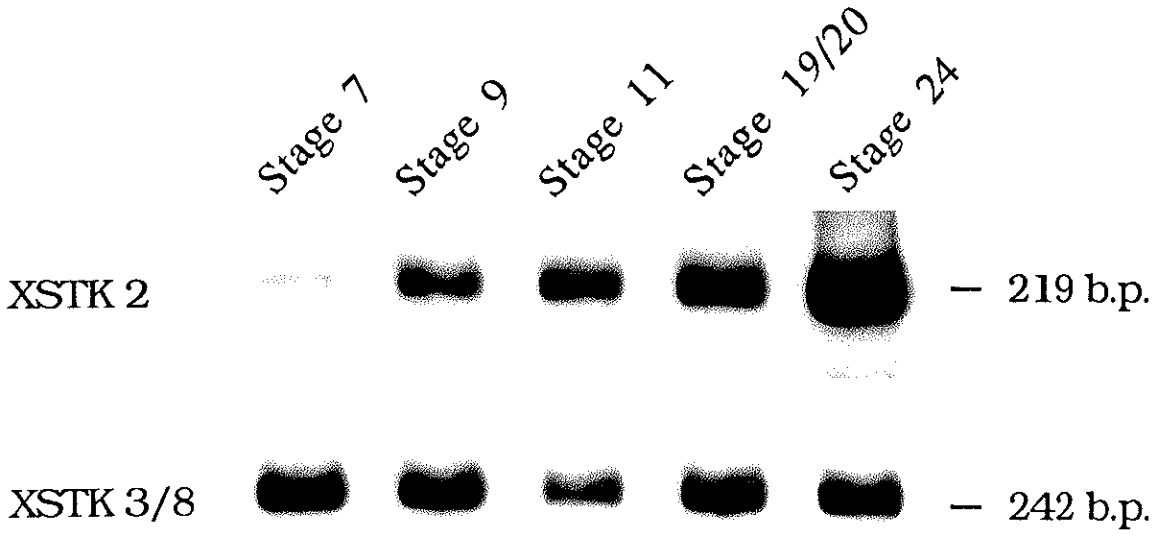

Figure 20. RT-PCR analysis of the truncated and intact activin receptor mRNA during early development. Total RNA (500 ng) from staged embryos was subjected to RT-PCR using specific 3' reverse primers for the truncated (XSTK2) and intact (XSTK3/8) receptor cDNAs. The upper strand primer lies in a region common among the three receptor types. The EF-1α transcript was amplified in a similar fashion to analyze both the quality and quantity of RNA (data not shown).

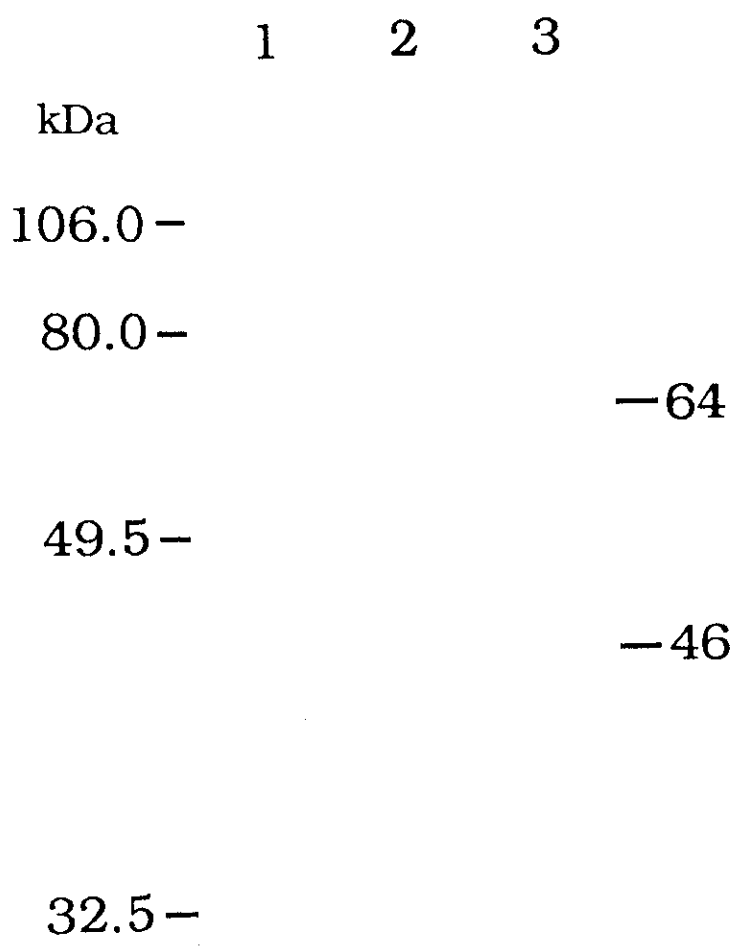

Figure 21. ***In vitro*** **synthesis of** ***Xenopus*** **activin receptor protein.** The products produced by a nuclease-treated rabbit reticulocyte lysate were loaded onto 10% SDS-PAGE. Lysate containing XSTK8 mRNA (lane 1), XSTK2 mRNA (lane 2) and lysate with no added RNA (lane 3). Molecular mass standards are indicated in kDa on the left.

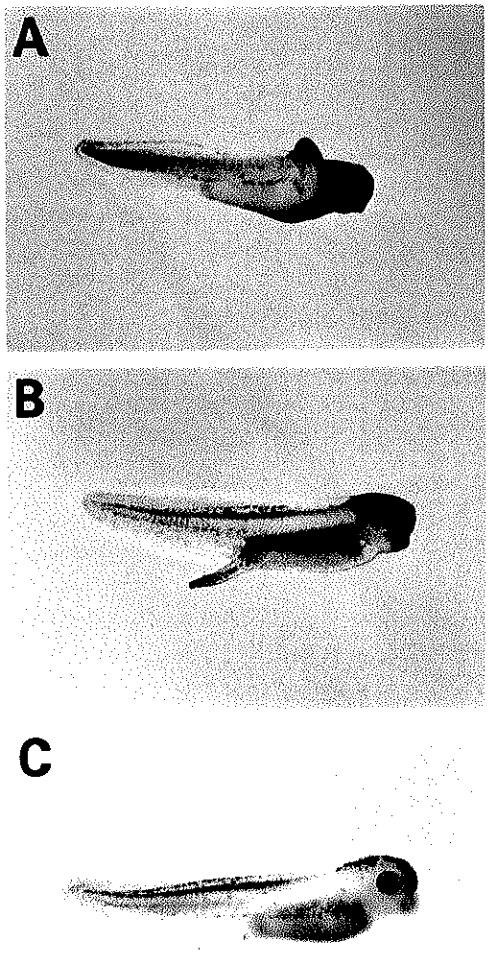

Figure 22. Developmental defects in embryos injected with activin receptor XSTK2 and XSTK8 mRNAs. Embryos were injected at the 4-cell stage with mRNA synthesized *in vitro* (900 pg mRNA/embryo). (A) represents an embryo injected with XSTK8 mRNA and (B) represents an embryo injected with XSTK2 mRNA. A control embryo injected with buffer alone is shown in (C).

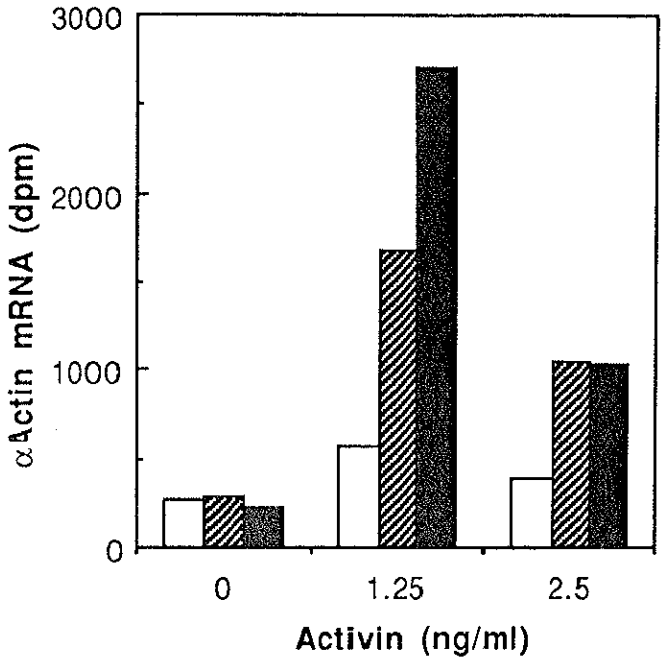

Figure 23. Induction of the α-actin gene by activin in animal caps isolated from embryos. Embryos were injected at the 4-cell stage with receptor XSTK2 and XSTK8 mRNAs synthesized *in vitro* (400 pg mRNA/embryo). Embryos were then allowed to develop to the blastula stage and animal caps obtained from five blastulae were incubated in the presence of various concentrations of activin for 1 day and pooled for RNA isolation. The relative expression of α-actin mRNA in animal caps injected with XSTK8 and XSTK2 mRNA is represented by the hatched and the shaded boxes respectively. Control embryos injected with buffer alone are shown by the open box.

	RNA injected	Total	Embryos with phenotype (%)				
	(pg/embryo)	(n)	Normal	Short	Anterior	Posterior	Spina bifida
XSTK8	500	66	68	5	0	17	11
	900	47	72	0	4	13	6
XSTK2	500	63	78	2	0	14	6
	900	42	90	0	0	7	2
Oocyte mRNA	900	12	92	0	0	0	8
Mock		26	88	0	0	0	11

Table 1. Defects produced in embryos injected with *Xenopus* activin receptor RNAs.
Embryos with extra-dorsal structures laterally or ventrally placed were classified into several groups based on appearance, namely normal, no defect; short, short embryo length; anterior, duplicated head structures; posterior, extra tail- and trunk-like protrusions; spina bifida, bifurcation at posterior end. Controls with and without injected oocyte mRNA are presented for comparison.

CONCLUSION

Recent studies on peptide growth factors have revealed that growth factors, including fibroblast growth factors (FGFs) [18, 19] and platelet-derived growth factor (PDGF) [102], are maternally encoded in the *Xenopus laevis* oocyte. These growth factors are implicated in the regulation of early amphibian development including embryonic inductions and establishment of the body axis [103, 104]. Asashima *et al.* have revealed that human recombinant activin can induce mesoderm in amphibian embryonic explants [25]. My initial experiments shown in this thesis, were designed to prove the working hypothesis that activin is present in early embryos as a natural mesoderm-inducer.

Activin and its receptor in early embryos

I demonstrated that there are several activin-related genes in the frog *Xenopus*, which were cloned by hybridization at low stringency, using mammalian activin βA cDNA as a probe. I determined by Northern blotting whether these activin-related genes are transcribed in the fertilized egg and early embryos, using the respective DNAs cloned from *Xenopus laevis* as a probe. I failed to detect activin mRNA in embryos before the tailbud stage. Thomsen *et al.* have also shown by means of the RNase protection assay, which is more sensitive than Northern blotting, that activin mRNA is transcribed from late blastula embryos (Stage 9) [79]. These results suggest that activin is not present in embryos until that time. Most of the embryonic mesoderm is already induced by stage 9, although activin may be important for further patterning and induction of mesoderm at a

later stage. However, recent studies by Asashima *et al.* [81] have provided evidence for an activin-like activity in the *Xenopus* eggs and early embryos. It is possible therefore, that maternally derived activin proteins are present in early *Xenopus* embryos in the absence of their corresponding mRNAs.

To understand the activin signal transduction pathway, I also studied the activin receptor in early *Xenopus* development. Four independent genes for *Xenopus* activin receptors have been cloned from a oocyte cDNA library. Each of these contain extracellular, single transmembrane and cytoplasmic Ser/Thr kinase domains and XSTK2 lacks the carboxyl-terminal portion of the Ser/Thr kinase located distal to domain VIII. Northern blots showed that all receptor genes are maternally expressed and differentially regulated during early embryogenesis. In this thesis, I demonstrated that the truncated activin receptor can mediate activin signals and function in a manner similar to the intact receptor by investigating the effects of receptor overexpression on morphogenesis and α-actin gene activation. It has been shown that there are three types of activin receptor in mammalian cells, based upon their molecular mass [24]. These have been designated types I (42kDa), II (51kDa) and III (151kDa). Nither proteins nor genes for type I and III receptors have yet been isolated. Since the molecular mass of the truncated receptor is similar to that of the type I activin receptor, it is possible that it represents the type I receptor. Precise investigations focusing upon structural-functional relationships of the activin receptor are necessary in order to determine the active core of the Ser/Thr kinase domain and to correlate autophosphorylation of the receptor with various biological phenomena.

A recent study by Hemmati-Brivanlou and Melton [105] has shown

that injection of a mutant activin receptor mRNA which predicts a receptor that lacks the entire intracellular kinase region, inhibited the gastrulation of the embryo by blocking activin signalling in the embryo. This suggests that activin is required for the induction of mesoderm and the patterning of the body plan *in vivo*. Taken together, activin is most likely to be the natural mesoderm-inducer in *Xenopus*.

Bone morphogenetic proteins

In this study, I also cloned genes for three different types of bone morphogenetic proteins (BMPs) from *Xenopus* oocyte cDNA library. Transcpripts of BMPs are differentialy regulated. Western blots using a specific antibody also showed that the embryonic BMP-4 was a 27 kDa monomer peptide in developing embryos.

As described in Chapter III, *Xenopus* BMPs have significant amino acid homology to the TGF-β family of proteins, especially the DPP subfamily, that includes the products of the *Drosophila decapentaplegic* gene (*dpp*) [43] and *Xenopus* Vg1 of which the mRNA is localized to the vegetal hemisphere of the oocytes [13]. The Vg1 has been cloned from an oocyte cDNA library based upon the hypothesis that mesoderm-inducing factor is localized to vegetal cells [12]. However, the Vg1 protein appears not to be able to induce mesoderm, and its function during embryogenesis remains unclear. All BMPs do not have mesoderm-inducing activity, according to animal cap assay (data not shown). Regardless, the fact is that at least 4 different BMP family members including Vg1 are already present in the oocyte [106, 107]. It has been reported recently that *Xenopus* BMP-4 (xBMP-4) functions as a ventralizing factor in the early development of *Xenopus laevis*[108, 109]. Overexpression of xBMP-4 mRNA causes

embryos to replace part of the dorsal mesoderm with ventral mesodermal tissues such as blood cells, whereas the closely related activin induces a secondary dorsal axial structure. However, the physiological role of the other two BMPs, except BMP-4 in *Xenopus* development, remains unknown.

Most proteins of the BMP family in oocytes, are monomer peptides which do not have biological activity. By analogy to TGF-β, of which crystal structure has been recently characterized [110, 111], the active mature BMP protein should dimerize through one of the 7 cysteines either with itself or with another BMP protein. Shoda *et al.* have demonstrated that there are minute amounts of dimeric forms of BMP-2 in *Xenopus* embryo extracts[112]. These data suggest that the formation of either homodimers or heterodimers that yield active BMP protein may be regulated. It is possible that post-translational controls that generate active BMP protein play an important role in not only early development but also later morphogenesis such as bone formation. Finally, the isolation and characterization of the BMP family of receptors will also allow us to determine the precise role in the early embryo.

I hope that this molecular analysis of *Xenopus* bone morphogenetic proteins, activin and its receptor will provide useful tools with which to elucidate the mechanism of embryonic induction, which was first described by Spemann and Mangold [1] and which has attracted the attention of many developmental biologists for almost 70 years.

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