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Molecular analysis of muscle and neural cell determination and differentiation in *Xenopus* early development

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Molecular analysis of muscle and neural cell determination and differentiation

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Contents

General introduction

Formation of body in animal early development has been analyzing by many biologists. A supposed mechanism is two-step system, induction and differentiation (Fig. 1). The most popular inductive event is mesoderm and neural induction. Many studies of screening the inducer have made a trial in amphibian embryos. A decade has past since reports began appearing that peptide growth factors, such as family of TGF-β, FGF and WNT, and their binding protein, Noggin, Chordin, Follistatin, Frz-B and so on, play important roles in the mesoderm and the neural induction (Table 1). But the pathway of the inductive signals and direct inducing molecules of muscle and neuron *in vivo* are unclear. About muscle differentiation, after discovery of *MyoD* in mammalian, many analyses were carried out, and it was revealed that four myogenic factors play key roles (Reviewed by Weintraub, 1993; Olson and Klein, 1994; Rudunicki and Jaenisch, 1995). These genes encode bHLH-type protein that can bind to specific sequence of DNA and can activate muscle-specific gene expression. It was shown that the bHLH-type products function at heterodimmer with *E12* protein. *E12* also encoded bHLH-type protein. These four myogenic factors act different points of the steps, determination and differentiation (Fig. 2). The "determination" shows a state that cells are committed to a myogenic fate but the cells are proliferating. The cells are called blast cells. The "differentiation" indicates a state of cell obtained the function respectively and the cells withdraw from the cell cycle. On the other hand, key role molecules were

found in neural differentiation by genetics of *Drosophila* (Reveiwed by Campos-Ortega and Jan, 1991; Jan and Jan, 1993; Jarman *et al.*, 1993). It was revealed that one of the *Drosophila* proneural genes, *achaete-scute* complex (AS-C) and *atonal* encoding bHLH-type specific DNA binding protein, are required neural differentiation. The products of these genes act as partners for the *daughterless* protein, mammalian *E12* homolog, and regulate positively. These results from different tissue formation, led to a hypothesis, "muscle and neuron are using a same system for differentiation in both of vertebrate and insect". Then in *Drosophila*, *nautilus*, *myoD* homolog, was found and it was shown that fly uses a same system of mammalian in myogenesis. And in vertebrate, conceptual screening by PCR found *Mash1* that encodes homolog of *Drosophila* AS-C. These observation supported the hypothesis. In addition, it was expected from the hypothesis that some other genes act as key molecule in vertebrate neurogenesis like fly.

I tried to clarify, first, how inductive signals give the information to nuclei and induce the earliest myogenic factor. Therefore I analyzed the expression pattern of the myogenic factors and their regulator genes in *Xenopus* early development. And second, I tried cloning and functional analysis of the expected key molecule in vertebrate neurogenesis and clarify resemblance between the systems of neurogenesis and myogenesis.

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Fig. 1.

An outline of the mechanism of induction and differentiation

Many observations in developmental biology have shown that "induction" by growth factors to undifferentiated progenitor cell caused "differentiation".

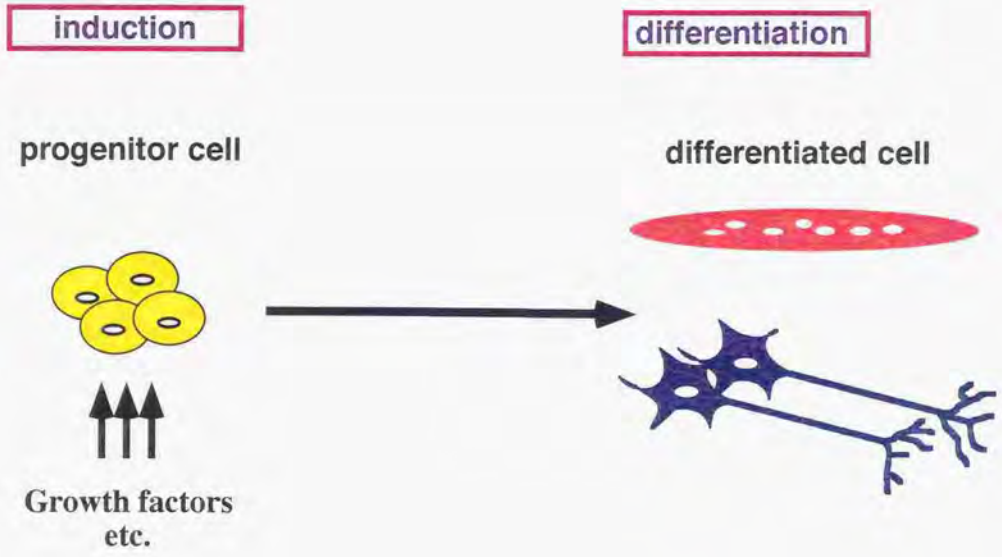
induction
differentiation
progenitor cell
differentiated cell
Growth factors
etc.

Fig. 2. Four myogenic factors act on different points of the steps, determination and differentiation

Myogenic factors are expressed at different developmental stages. The developmental stages can be distinguished, as determination and differentiation steps.

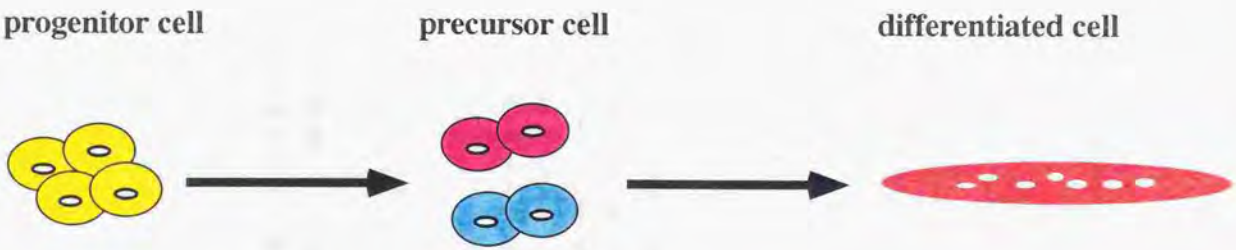
progenitor cell
precursor cell
differentiated cell
determination
differentiation
myf-5
myoD
myogenin
mrf4

Table 1. Peptide growth factors in the mesoderm and neural induction

Examples of PGF (With known receptors)	Most probable major natural role*	Sample reference
FGF		
bFGF	mesoderm induction	Amaya et al (1993)
	neural induction	Launay et al (1996)
	A/P neural patternmesoderm induction	Isaacs et al (1992)
TGF-b		
Activin A, B, D	dorsal mesoderm induction	Oda et al (1995)
Vg1	dorsal mesoderm induction	Thomsen and Melton (1993)
BMP 2, 4, 7	ventral mesoderm induction	Harland (1994)
Nodal Related 1, 2	organizer formation / mesoderm induction	Jones et al (1995)
Nodal Related 3	organizer formation / neural induction	Smith et al(1995)
		Hansen et al (1997)
Wnt		
Xwnt-11	contribute dorsal axis	Ku and Melton (1993)
Xwnt-8	ventral mesoderm formation	Christian et al (1991)
Xwnt-8b	possibly induce dorsal axis	Cui et al (1995)
Examples of PGF binding protein**		
noggin (BMP)	organizer formation / neural induction	Zimmerman et al (1996)
chordin (BMP)	organizer formation / neural induction	Smith and Harland (1992) Piccolo et al (1996)
follistatin (ACTIVIN, BMP)	organizer formation / neural induction	Sasai et al (1994) Nakamura et al (1990) Hemmati-Brivanlou et al (1994)
Xfrezzled (WNT)	organizer formation	Leyns et al (1997)
Frzb (WNT)	organizer formation	Wang et al (1997)
Sizzled (WNT)	most ventral region formation	Salic et al (1997)
Other PGF		
lunatic Fringe	mesoderm induction	Wu et al (1996)
cerberus	head induction	Bouwmeester et al (1993)

*based upon my interpretation of various analyses, whole embryo, animal cap overexpression studies and natural expression pattern.
**no known receptors and uncertain binding affinities for other PGFs.

Chapter I. Muscle development

A. Expression and regulation of *Xenopus* myogenic genes during early development

Abstract

In beginning of muscle development, determination is induced in the mesoderm, and then differentiation occurs with accumulation of muscle structural proteins. Mesoderm cells differentiate to many type cells, but the direct signaling activator for muscle determination is still unknown. In this paper I report some of the conditions required for determination of muscle. Muscle determination during *Xenopus* development was found to be marked by *Xmyf-5* and *XmyoD* expression, but not by expression of *Xmyogenin* or *Xmrf4*. *Xmyf-5* and *XmyoD* expression was first detected in the early gastrula stage. *Xmyf-5* expression was first detected on the dorsal side, whereas *XmyoD* was initially expressed on the ventral side. Subsequently, expression of both genes was strongly induced on the dorsal side. The expression of *Xmyf-5* and *XmyoD* did not continue to increase on the ventral side when it was separated from the dorsal side, although muscle originates from both sides. These findings suggest that a continuous increase in expression of both genes require the dorsalizing signal. The mesoderm inducers bFGF and Activin A induced both genes in animal caps, and the inductive activity of Activin A was stronger than that of bFGF. Overexpression of *Xbra*, a pan-mesoderm marker, alone induced both genes, but weakly. The inductive activity of *Xbra* was enhanced by co-injection with *noggin*. This suggests that

inhibition of *BMP4* by *noggin* in the mesoderm mediates dorsalizing signal, and may induce the direct dorsalizing activator genes of *Xmyf-5* and *XmyoD*.

Introduction

The discovery of *MyoD*, a mouse gene that can convert cultured fibroblasts into myoblasts (Davis *et al.*, 1987), has been followed by isolation of three more mammalian myogenic factors related to *MyoD*: *myogenin* (Edmonson and Olson, 1989; Write *et al.*, 1989), *myf-5* (Braun *et al.*, 1989), and *MRF4/myf-6/herculin* (Rhodes and Konieczny, 1989; Braun *et al.*, 1990; Miner and Wold, 1990). They are all members of the basic helix-loop-helix (bHLH) family of DNA-binding proteins (Murre *et al.*, 1989) and can bind to muscle-specific promoters (Lassar *et al.*, 1989; Brennan and Olson, 1990; Piette *et al.*, 1990). The pattern of expression of the all four myogenic factors has been reported in normal mouse development. In axial skeletal muscle, *myf-5* (day 8), *myogenin* (day 8.5), *MRF4* (day 9) and *MyoD* (day 10.5) are expressed sequentially, but a different sequence of expression of these genes is observed in the developing limb bud: *myf-5* was expressed transiently at day 10-12, *myogenin* and *MyoD* are expressed after day 10.5, and *MRF4* was detected after day 16 (Sassoon *et al.*, 1989; Bober *et al.*, 1991; Hinterberger *et al.*, 1991; Ott *et al.*, 1991). *In vitro* and gene-targeting studies suggest that *myf-5* and *MyoD* are involved in muscle cell determination and that *myogenin* and *MRF4* are involved in differentiation and maturation (reviewed by Weintraub, 1993; Olson and Klein, 1994; Rudunicki and Jaenisch, 1995). In *Xenopus*, the complete cDNAs of *XmyoD* (Hopwood *et al.*, 1989; Harvey, 1990; Scales *et al.*, 1990), *Xmyf-5* (Hopwood *et al.*, 1991), and *Xmrf4* (Jennings, 1992) have been cloned and

described, and a partial genomic *Xmyogenin* clone with *Xmrf4* was also described by Jennings (1992). Injection of both *Xmyf-5* and *XmyoD* mRNAs at the 2-cell stage results in fairly normal embryos (Hopwood *et al.*, 1991) with no large scale conversion of non-muscle cells into muscle (Gurdon *et al.*, 1992). Late blastula stage animal caps from embryos injected with 1-9 ng of *XmyoD* (Hopwood and Gurdon, 1990) or *Xmyf-5* mRNA (Hopwood *et al.*, 1991) were found to express muscle-specific cardiac actin, but the differentiated muscle-specific antigen 12/101 was not expressed in these explants. Injection of *XmyoD* mRNA together with RNA encoding its dimerization partner *Xenopus E12 (XE12)* appeared to lead to limited muscle differentiation (12/101 antigen expressed), although morphological muscle was still not observed (Rashbass *et al.*, 1992). A recent study, however, yielded different results, i.e., that injection of *XmyoD* or *Xmyf-5* mRNA at the 2- to 32- stage activates precocious and ectopic expression of muscle-specific antigens and induces the formation of ectopic muscle. Phenotypically, the embryos displayed enlarged myotomes with increased numbers of myocytes that were shown to be derived at least in part by recruitment of cells of nonsomitic lineage (Ludolph *et al.*, 1994). In either case, the results showed that *XmyoD* and *Xmyf-5* have inducing activity for some muscle-specific genes, and indicate that *XmyoD* and *Xmyf-5* play important roles in early muscle development in *Xenopus*.

The initial trigger of myogenesis in early development remains unknown. In amphibian development, mesoderm is formed in the equatorial region of the blastula by induction of the nearby animal pole by growth factors released by vegetal pole cells

(Nieuwkoop *et al.*, 1969; Nakamura *et al.*, 1971; Asashima, 1975). Recently, members of the TGFβ superfamily and basic fibroblast growth factor (bFGF) have been reported to induce mesoderm (reviewed in Asashima, 1994). Activin A has the strongest mesoderm-inducing activity of these factors (Asashima *et al.*, 1989,1990; Smith *et al.*, 1990; van den Eijnden-Van Raaij *et al.*, 1990). Activin A induces mesodermal gene-expression and tissues in a concentration-dependent manner (Green and Smith, 1990; Ariizumi *et al.*, 1991; Green *et al.*, 1992). *Xbra* has been reported as an early response gene (Smith *et al.*, 1991). Overexpression of *Xbra* induces ectopic muscle in the animal cap (Cunliffe and Smith, 1992), and acts synergistically with *noggin* (Cunliffe and Smith, 1994) and *pintallavis* (O'Reilly *et al.*, 1995). These observations may provide an important clue to the identity of the initial muscle determination gene. Very recently, a number of *Xenopus* genes encoding a T-box, a motif also found in *Xbra*, have been reported, including *Eomesodermin* (Ryan *et al.*, 1996), *Antipodean* (Stennard *et al.*, 1996), *Xombi* (Lustig *et al.*, 1996), *VegT* (Zhang and King, 1996), and *Brat* (Horb and Thomsen, 1997). These genes are expressed at an early stage of embryogenesis, suggesting that they play a role in mesoderm determination.

Materials and methods

Eggs and embryos

Xenopus laevis eggs were obtained by injecting of female animals with 600 IU of human chorionic gonadotropin (Gestron; Denka Seiyaku Co., Kanagawa Japan). Fertilized eggs were chemically dejellied by treatment containing 3% cystine hydrochloride in Steinberg's solution (pH 7.8) with kanamycin sulfate (100 mg/l; Banyu Pharmaceutical Co., Tokyo, Japan), then washed with sterile Steinberg's solution (pH 7.4). Embryos were transferred to Steinberg's solution and allowed to develop until stage 9 (Nieuwkoop and Faber, 1956).

RNA extraction, RT-PCR and Southern blotting

RT-PCR analysis of RNA samples was performed as described by Sambrook *et al.* (1989). Total RNAs were isolated by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method with several modifications (Chomczynski and Sacchi, 1987). Oligo(dT)-primed first strand cDNA was prepared from the total RNA of *Xenopus* whole embryos and explants, and PCR reactions were carried out in a Thermal Cycler (Perkin-Elmer Cetus). Internal negative controls to which no reverse transcriptase was added were prepared in parallel. After amplification, RT-PCR products were subcloned for Southern blotting, and the sequences were confirmed with an automatic DNA sequencing analyzer (ABI). ^{32}P-labeled probes were used to perform Southern blotting. The PCR products were transferred to a nylon membrane, and signals were

detected with X-ray film. The sequences of the primers used in this study were as follows: in the 5' to the 3' orientation, *Xmyf-5* at 27 cycles, upstream CAACTCCACTGAGCATCTTTCTAAG, downstream CGTCTTCATCCGATTCTTCAAGGTC; *XmyoD* at 27 cycles, upstream TGCCAAGAGTCCAGATTTCCTACAA, downstream TTATGGTGGGGTTCCTCTGGTTTCA; *Xmyogenin* at 27 cycles, upstream AGGTGTGCAAGAGGAAGACG, downstream GCCAATAGTGTCTGCAAGCG; *Xmrf4* at 27 cycles, upstream CACAGTTTGGATCAGCAGGACAAGC, downstream GGATAGTAGAGCAGTTGATCCTGTA; *alpha skeletal muscle actin (muscle specific actin; ms-actin)*, (Stutz and Spohr, 1986) at 27 cycles, upstream AACAGCAGCTTCTTCCTCAT, downstream TACACAGAGCGACTTGAACA; *ef1-α* (Kreig *et al.*, 1989) at 28 cycles, upstream TTGCCACACTGCTCACATTGCTTGC, downstream ATCCTGCTGCCTTCTTTTCCACTGC; *ornithine decarboxylase (odc)*, (Bassez *et al.*, 1990; Osborne *et al.*, 1991) at 27 cycles, upstream GTCAATGATGGAGTGTATGGATC, downstream TCCATTCCGCTCTCCTGAGCAC.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed according to the method described by Harland (1991). The subcloned RT-PCR products were used for synthesis of the digoxigenin-labeled RNA probe. Embryos obtained from albino females were used. Anti-digoxigenin antibodies were purchased from Boehringer Mannheim (GmbH. Mannheim, Germany).

mRNA synthesis and embryo manipulations

pSP64T vector cDNA was provided by Dr. D.A. Melton. Full-length *Xbra* of pXT1, provided by Dr. J.C. Smith, was ligated into pSP64T. The noggin template was Δ5'-noggin provided by Dr. W.C. Smith. Capped mRNA was synthesized *in vitro* as described previously (Krieg and Melton, 1984). The mRNAs dissolved in Gurdon's buffer (88 mM NaCl, 1 mM KCl, 15 mM Tris-HCl, pH 7.5) were injected into both blastomeres at the 2-cell stage in 5% Ficoll-Steinberg's solution. Animal caps were dissected from stage 9 embryos, then cultured in Steinberg's solution (pH 7.4) containing 0.1% BSA and 0.1 g/l kanamycin sulfate at 20°C, in the presence and absence of human recombinant Activin A or bFGF. Human recombinant Activin A was kindly provided by Dr. Yuzuru Eto of the Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan (Eto *et al.*, 1987; Murata *et al.*, 1988). Human recombinant bFGF was obtained from Mallinckropt Co. (Paris, France).

Preparation and screening of genomic DNA library

To construct genomic library, high-molecular-weight *Xenopus* genomic DNA was extracted from adult testis by standard methods (sambrook *et al.*, 1989) with some modifications. The high-molecular-weight *Xenopus* genomic DNA was partially digested with *Sau*3AI and cloned into λFIX II (Stratagene). About 2 x 10^6 recombinant clones were screened with random primed probes from PCR clones of *Xmyf5* and *Xmrf4*, to isolate the *Xenopus myf5* gene. After prehybridization at 65 °C for 16 hrs in 3 x SSC (1 x SSC is 0.15 M NaCl and 0.015 M sodium citrate), 1 x Denhardt's solution and 200 mM/ml denatured salmon sperm DNA, the filters were hybridized in hybridization buffer 0.5 M NaCl, 0.5 x

Denhard's solution, 40 mM Tris. HCl, 4 mM EDTA. Then the filters were washed for 20 min at room temperature in 2 x SSC, then washed for 20 min at 65 °C in 0.1 x SSC and 0.1 % SDS. Signals were detected by a Bioimage Analyzer, BAS2000 and X-lay films (Fuji Film). Positive clones were plaque-purified and the inserts subcloned into pBluescript II SK+ plasmid vector for restriction analysis and sequencing.

DNA construction

pUC*Xmyf5-lacZ* was constructed by ligating genomic *Xmyf5* *Sau*3AI-*Bam*HI fragment into pUC19 with *lacZ* containing SV40-NLS into *Eco*RI site of *Xmyf*-5 first exon. Deletion constructs were made restriction enzymes and PCR technique.

Detection of *lacZ*

The embryos injected with *lacZ* constructs were visualized using X-gal (Sanes *et al.*, 1986) and detected CPRG (chlorophenol red-β-D-garactopyranoside) assay (Herbomel *et al.*, 1984). For X-gal assay, the injected embryos were fixed with 2 % paraformaldehyde, 2 % gluteraldehyde in 0.1 M phosphate buffer (pH 7.0) for 1 hour on ice. Then these were washed three times, detected X-gal solution (0.15 % X-gal, 10 mM $K_3Fe(CN)_6$, 10 mM $K_4Fe(CN)_6$, 0.1 % Triton X-100, in 0.1 M phosphate buffer pH 7.0). For CPRG assay, the embryos were homogenized with 0.25 M Tris-HCl and repeated "freeze-thaw" three times and centrifuged at 12000 rpm for 5 min. at 4°C. After measurement of protein volume, the supernatant (20 μg) was used for reaction in 250 μl of Z buffer (2x Z buffer 10ml; 4 ml of 0.5 M phosphate buffer pH

7.5, 0.2 ml of 1 M KCl, 0.2 ml of 0.1 M $MgSO_4$, 5.53 ml of sterile mQ and 70 µl of 14.4M 2-ME) with 50 µl of 15 mM CPRG. The mixture was reacted for 30 min.-2 hours at 37°C. Then reaction was stopped with 500 µl of 1M Na_2CO_3, and detected the absorbance at 574 nm.

Results

Xmyf-5 and *XmyoD* are expressed during determination of muscle

In *Xenopus*, the complete cDNAs of three myogenic factors, *Xmyf-5*, *XmyoD* and *Xmrf4*, and part of the sequence of genomic *Xmyogenin* DNA have been cloned. Analysis by Northern blotting detected expression of all three cDNAs during normal development, but did not detect *Xmyogenin* expression at any time. RT-PCR was used to examine the patterns of expression of these factors with greater sensitivity (Fig. 1). *Xmyf-5* and *XmyoD* were expressed at stage 10, with the level of transcripts increasing during gastrulation, as reported previously. Very weak *Xmyogenin* expression was detected at stage 15 but not during the early gastrula stage, similar to *Xmrf4* and muscle-specific actin. The highest expression of *Xmyogenin* was transient, at stage 35-40. Thus only *Xmyf-5* and *XmyoD* were expressed at the muscle-determination stage.

Comparison of *Xmyf-5* and *XmyoD* expression

Xmyf-5 transcripts increased up to the neurula stage and then decreased more rapidly than *XmyoD* (Fig. 1). This unique pattern of expression of *Xmyf-5* suggested that it may operate in a different activation pathway. I therefore closely compared the pattern of expression of *Xmyf-5* and *XmyoD* during muscle determination. Whole-mount *in situ* hybridization was used to compare the expression of *Xmyf-5* and *XmyoD* in early stage embryos (Fig. 2). No signals were detected in the late blastula (stage 9; Fig. 2a, f). In early gastrula (stage 10; Fig. 2b, g),

very weak expression of both *XmyoD* and *Xmyf-5* were detected by RT-PCR (Fig. 1), but the region of expression was ill-defined. In the mid-gastrula (stage 11; Fig.2c, h), both genes were strongly and specifically expressed in developing somitic mesoderm, but not in the presumptive notochord. *XmyoD* expression was detected in all somitic mesoderm, but *Xmyf-5* expression was restricted to the posterior region. In the late gastrula (stage 12; Fig. 2d, i) and neurula (stage 15; Fig. 2e, j), this difference became more distinct. *Xmyf-5* was transiently expressed, and expression then gradually decreased as the cells extended. *Xmyf-5* was expressed in a dorsal-to-ventral gradient (Fig. 2e, j). The region of highest *Xmyf-5* expression was immediately adjacent to the notochord, which did not express *Xmyf-5* at all.

Both *Xmyf-5* and *XmyoD* were expressed in the early gastrula stage (Fig. 1), but the specific sites of expression could not be clarified by whole-mount *in situ* hybridization. Therefore RT-PCR with divided embryos was used to resolve *Xmyf-5* and *XmyoD* expression (Fig. 3). Embryos were divided into dorsal and ventral explants at stage 10 and cultured until the stage at which they were sampled. *Xmyf-5* was expressed in stage 10 whole embryos, but expression began on the dorsal side and was not detected on the ventral side. In contrast, *XmyoD* was expressed on both sides, but more strongly on the ventral side than the dorsal side at stage 10. *Xmyf-5* expression increased greatly on the dorsal side as well as in the whole embryo, with some low-level expression becoming evident on the ventral side. *XmyoD* expression was also detected and increased on the dorsal side of

advanced stage explants, with increased transcript levels compared to the ventral side. The ratio of ventral/whole embryo *XmyoD* expression was higher than that of *Xmyf-5* expression. These results suggest that *Xmyf-5* induction was affected by dorsalizing and that this effect on *Xmyf-5* was larger than on *XmyoD*.

Xmyf-5 was induced by growth factors

bFGF and Activins are mesoderm-inducing factors, with bFGF generally inducing ventral mesoderm in animal caps, and Activins inducing both ventral and dorsal mesoderm, depending on the dose. Figure 4 shows *Xmyf-5* and *XmyoD* induction by these factors in animal caps. High-dose bFGF induced *Xmyf-5* in the animal caps, but a lower concentration of bFGF (1 ng/ml) did not induce *Xmyf-5*. The greatest induction of *Xmyf-5* by Activin A was at a dose of 10 ng/ml. *XmyoD* was induced at all concentrations of both growth factors, including 1 ng/ml bFGF. These results indicate that *Xmyf-5* and *XmyoD* can be induced by mesoderm-inducing factors, and that Activin A, which include a dorsalizing signal, is more effective than bFGF.

Xbra and *noggin* induce *Xmyf-5* expression

Other studies have shown that ectopic expression of *Xbra* in animal caps can induce muscle differentiation, and that *Xbra* is an activator of *XmyoD* and muscle-specific actin expression (Cunliffe and Smith, 1992; Horb and Thomsen, 1997). *Xbra* is also an early response gene for both bFGF and Activin A. I showed that bFGF and Activin A can induce *Xmyf-5* in animal caps,

similar to *XmyoD*. I therefore then examined whether *Xbra* could also induce *Xmyf-5* expression (Fig. 5a). When a lower dose of *Xbra* (0.5 - 2 ng/embryo) was injected into both blastomeres at the 2-cell stage, *Xmyf-5* and *XmyoD* were not induced in the animal caps, but high-dose *Xbra* (4 ng/embryo) induced expression of both genes. Only very weak *Xmyf-5* expression was induced, however, and required a long exposure time for detection (compare with whole embryos; WE, at the right of Fig. 5a and b). I therefore co-injected *noggin* and *Xbra*, since this has been described as leading to high induction of muscle-specific actin at a low dose of *Xbra* (Cunliffe and Smith, 1994). Neither *noggin* (200 pg/embryo) nor *Xbra* (1 ng/embryo) alone induced *Xmyf-5* or *XmyoD* (Fig. 5b), but when *noggin* (200 pg/embryo) and *Xbra* (1 ng/embryo) were injected together at the same doses they induced both *Xmyf-5* and *XmyoD*.

Isolation of genomic DNA encoding *Xmyf5*

I tried different approach against the regulator of the early myogenic factor, *Xmyf-5*. To isolate the *Xmyf-5* gene, I made a *Xenopus* genomic library and screened the with the probe that obtained from RT-PCR products using primers, *Xmyf-5* (B) and *Xmrf4*. From screening with *Xmyf-5* fragment, one of the positive clones containing a 19 Kb genomic fragment was chosen for further characterization. Southern blot analysis showed that this clone contained a 6 Kb *Sau*3AI-*Xho*I fragment which hybridized strongly to *Xmyf-5* probe and this clone was mapped using restriction enzymes. Tandem location of *Xmrf4* and *Xmyf-5* gene has described for mouse, human and Chick (Miner and Wold,

1990; Braun *et al.*, 1990; Saitoh *et al.*, 1993). However the 19 Kb clone was not hybridized to *Xmrf4* (data not shown). I tried to screen with *Xmrf4* fragment probe to isolate a clone containing *Xmrf4* and *Xmyf-5* genes located tandemly. But isolated *Xmrf4* clones were not contained *Xmyf-5* gene (data not shown) and could not be clarified the tandem location in *Xenopus*.

Characterization of the *Xmyf5* gene

Using site-deletion clones, I determined the nucleotide sequence of the about 6Kb *Sau3AI-Xho*I fragment of *Xmyf-5* genomic DNA. Based on the consensus sequence of the donor and acceptor splice junction and comparison to *Xmyf-5* cDNA sequence, intron sequences were identified (Fig. 6). The *Xmyf-5* gene is organized by three exon and two introns, and contains some bHLH protein binding element (E-box (CANNTG)), *MEF2* binding element (CTA(T/A)$_4$TA). An element like consensus sequence of *Xbra* (TGACACCTAGGTGTGAAATT) exists in 5' flanking region of *Xmyf-5*.

Promoter analysis of *Xmyf-5*

To determine element at 5'-untraslated region of *Xmyf-5* gene, which is required to promote *Xmyf-5*, I made *lacZ* fusion constructs (Fig. 7A). The *lacZ* fusion constructs were injected into *Xenopus* 8-cell embryos. X-gal staining showed expession patterns of lacZ by the longest version promoter were almost similar to a pattern of whole mount *in situ* hybridization (data not shown). The injected embryos were cultured to gastrula stage and were used to measure lacZ (β-galactosidase) activity (Fig. 7). Unlikely to pENL; mouse *EF1-α* promoter (positive control),

Xmyf-5 promoter showed site specificity. Vegetal cells showed the stronger activity than animal cells. Dorsal- and ventral-difference was appeared only in the animal cells (Fig. 7B). Difference of the activity from length of 5'-untranslated region indicated existance of two regulatory elements at upstream -728~-392 and -391~-196 from ATG (Fig. 7A). The element at -728~-392 included a similar sequence of consensus sequence of *Xbra* (Fig. 6, 7A).

Discussion

Four myogenic factors that contain a bHLH domain are expressed sequentially and play an important role in determination and differentiation of muscle. I have identified the initial stage of *Xmyogenin* expression in *Xenopus* for the first time. Jennings (1992) isolated a genomic DNA fragment of *Xmyogenin*, but was unable to detect any transcripts and could not isolate any cDNAs. Highly sensitive RT-PCR Southern blotting analysis detected very low levels of transcripts at the same stage as *Xmrf4* transcripts were detected before. A transient peak of expression was observed at stage 35-40. At this point in the development of the muscle cell lineage, myofibers accumulate before the start of multinucleation (Boujelida and Muntz, 1987). Transient expression of *Xmyogenin* transcripts in forming myotubes has been reported during regeneration of adult muscle following cardiotoxin injury (Nicolas *et al.*, 1996). These observations indicate that *Xmyogenin* may play a role in muscle determination and may not function in muscle determination. I therefore concluded that only two of the four factors, *Xmyf-5* and *XmyoD*, function in the muscle determination step.

Xmyf-5 and *XmyoD* expression was previously reported to begin in the early gastrula (stage 10) (Hopwood *et al.*, 1989,1991; Harvey, 1990). Our present study showed that *Xmyf-5* expression begins at the early stage in the dorsal hemisphere, and that in contrast to *XmyoD*, it is expressed in the ventrolateral mesoderm (Frank and Harland, 1991). *Xmyf-5* was subsequently detected in the divided ventral side, but was only

weakly and unstably expressed (Fig. 3). *XmyoD* did not show continuous expression on the ventral side too. Moreover, the ventral explants expressed very little muscle-specific actin at stage 28 (data not shown). It is well known that the presumptive fate of muscle in *Xenopus* blastula lies in the mesoderm region of both the dorsal and ventral hemispheres (Keller, 1975, 1976; Dale and Slack, 1987; Moody, 1987a,b; Moody and Kline, 1990). Previous reports and our current whole-mount *in situ* hybridization study have shown that *Xmyf-5* and *XmyoD* are also expressed at the neurula stage in the posterior region derived from the ventral hemisphere of early gastrula embryos. However, *Xmyf-5* transcripts did not accumulate in ventral explants from which the dorsal side had been cut off. Therefore, these phenomena suggest that the dorsalizing effect that was released from the organizer and led to muscle formation actually persists during gastrulation, and that this continuing dorsalization occurs under convergent-extending movement (Vogt, 1925; Gerhart and Keller, 1986). The cells may continuously express the muscle determination genes *Xmyf-5* and *XmyoD* in response to a dorsalizing signal from the extended presumptive notochord when the presumptive muscle cells derived from the ventral hemisphere of early gastrula embryos move to the dorsal side.

Activins and bFGF are potent mesoderm-inducing factors (reviewed by Slack, 1994; Asashima, 1994), and *XmyoD* is induced by bFGF and XTC-MIF (Harvey, 1990). I examined the ability of bFGF and Activin A to induce *Xmyf-5*. Both mesoderm-inducing factors induced *Xmyf-5*, similar to *XmyoD*, but Activin A was the stronger inducer of both genes. Thus, both factors may have

basal inducing activity, and Activin A may also be a dorsalization signal. Activin A induces gene expression and differentiation of dorsal mesoderm depending on the dose (Green *et al.*, 1990; Ariizumi *et al.*, 1991). Activin A induced the strongest expression of both myogenic factors at 10 ng/ml, the concentration at which explant elongation and muscle tissue induction occur. Treatment of Activin A at 100 ng/ml showed weaker inductive activity than at 10 ng/ml. The reason for this is suspected of being that high-dose Activin A mainly induces the notochord. If the dorsalization signal is excessive, it may cause deactivation of organizer genes and notochord formation, and the myogenic factors *Xmyf-5* and *XmyoD* may be suppressed. Thus, myogenesis may be both up- and down-regulated by dorsalization.

Both bFGF and Activin A have been reported to induce the T-box gene *Xbra*, a pan-mesoderm marker, and ectopic expression of *Xbra* induces mesoderm, including muscle (Cunliffe and Smith, 1992). Our experiments suggest that induction of muscle by *Xbra* is mediated by *Xmyf-5* and *XmyoD* expression. Injection of high doses of *Xbra* was required to induce of these genes, especially *Xmyf-5*, and lower doses acted synergistically action at lower doses with *noggin*, a dorsalization molecule. Therefore, muscle determination may be activated by two different signals, basal mesoderm induction and dorsalization, and *Xbra* and *noggin* may be the mediating molecules *in vivo*. *Xbra* encodes a DNA-binding nuclear protein containing a T-box and may direct activation of *Xmyf-5* and *XmyoD*. Other recently cloned T-box genes may have similar functions. The secreted proteins *noggin* and *chordin* have

been reported to act as *BMP4* suppressors by direct binding (Piccolo *et al.*, 1996; Zimmerman *et al.*, 1996). This suggests that inhibition of *BMP4* by *noggin* and *chordin* in the mesoderm mediates dorsalization signal and may induce the direct dorsalizing activator genes of *Xmyf-5* and *XmyoD*. A candidate direct dorsalizing activator is *pintallavis*, which encodes a nuclear protein, and has been reported to act synergistically with *Xbra* (O'Reilly *et al.*, 1995).

In order to research a real regulator, I have injected chicken *myf-5* upstream region ligated *lacZ*. But the constructs were not expressed specifically on time in early *Xenopus* embryos. Therefore I isolated *Xenopus* genomic clones containing *Xmyf-5* gene and determined the sequence. A tandem location of *mrf4* and *myf-5* genes has been described for mouse, human and Chick (Miner and Wold, 1990; Braun *et al.*, 1990; Saitoh *et al.*, 1993). In *Xenopus* the clones isolated in this study were not containing each other. The sequence isolated in this study contain some regulatory elements (Fig. 6). One recognizable binding site motif is E-box (CANNTG), a consensus b-HLH protein homo- and hetrodimers binding site. It is possible that the E-boxes in *Xmyf-5* regulatory region are enhancer elements auto-regulating by *Xmyf-5* and the other b-HLH proteins and negative regulator elements by them. However the earlier bHLH gene than *Xmyf-5* is not known. Another motif is *MEF2* binding site. *MEF2* was initially identified as a binding activity (Gossett *et al.*, 1989) to an AT-rich sequence ($CTA(T/A)_4TA$) shown to be important for the activation of many muscle genes in differentiating myotubes. *MEF2* levels are very low at the time when *Xmyf-5* and

XmyoD are first activated. Injection of functional *MEF2* into animal caps did not lead to activation of the *XmyoD* (Chambers, 1992). The E-box and *MEF-2* consensus sequence exist in *Xmyf-5* upstream region and introns, but for that reason, it is hard to think that these can regulate *Xmyf-5* in first expression. To determine element at 5'-untraslated region of *Xmyf-5* gene, I made *lacZ* fusion constructs (Fig. 7A). The *lacZ* fusion constructs were injected into *Xenopus* 8-cell embryos. Unlikely to pENL, mouse *EF1-α* promoter (positive control), *Xmyf-5* promoter showed the site-specific expression. Vegetal cells showed the stronger activity than animal cells. In Vegetal cell, Dorsal- and ventral- difference was did not observe, it appeared only in the animal cell (Fig. 7B). Therefore another enhancer element is indicated. The different activity in length of 5'-untranslated region indicated existance of two regulatory elements at upstream -728~-392 and -391~-196 from ATG. The element at -728~-392 included similar sequence of consensus sequence of *Xbra* (Fig. 7A). These results support a possibility that *Xbra* or/and other T-box gene may be a direct inducer of *Xmyf-5*. Mutation into the similar sequence of consensus *Xbra* did not show clear difference wild type sequence (data not shown). Because *Xbra can* induce *Xmyf-5*, but very weakly, it may be not detected by this assay. The sequence of -390~-195 did not include major known element.

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Fig. 1. Temporal expression of four myogenic factors.

Total RNA, isolated from embryos indicated developing stage (f.egg, st. 6 - 50) and adult leg muscle (AM) and adult heart (AH), was analysed by RT-PCR for levels of myogenic factors and *odc* RT+, which serves as a loading control. *odc* RT- is internal negative control. Only *Xmyf-5* and *XmyoD* were expressed at muscle-determination stage.

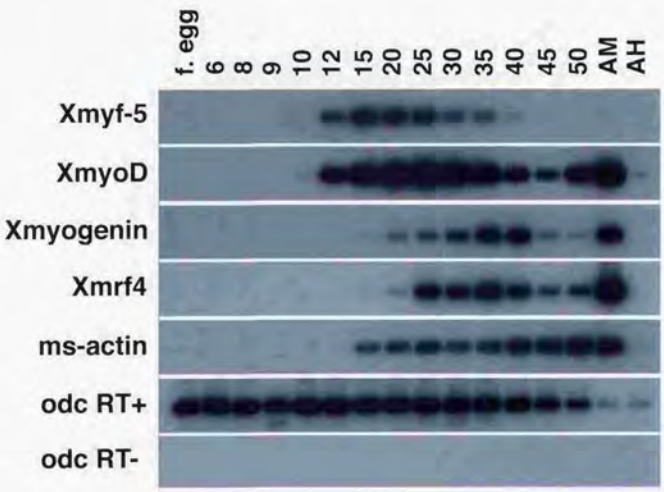
f. egg
6
8
9
10
12
15
20
25
30
35
40
45
50
AM
AH
Xmyf-5
XmyoD
Xmyogenin
Xmrf4
ms-actin
odc RT+
odc RT-

Fig. 2. Spatial distribution of *Xmyf-5* and *XmyoD*.

Whole-mount *in situ* hybridization showed different expression of these genes at determination stage (a,f: st.9, b,g: st.10, c,h: st.11, d,i: st.12, e,j: st.15).

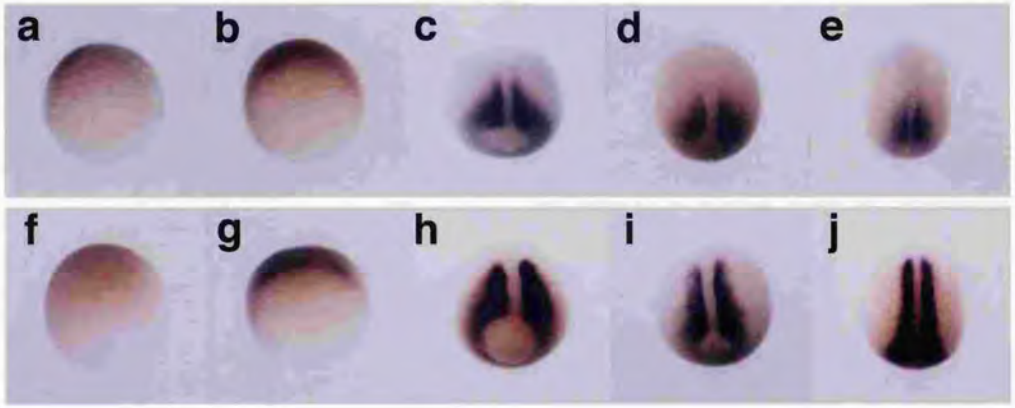
a
b
c
d
e
f
g
h
i
j

Fig. 3. Activation of *Xmyf-5* and *XmyoD* by dorsalizing signal.

The embryos were divided in two, dorsal and ventral, at st.10 and were cultured until the sampling stage that sibling embryos developed (WE: Whole embryos), and analyzed by RT-PCR. *ef1-α* RT+ is loading control and *ef1-α* RT- is internal negative control.

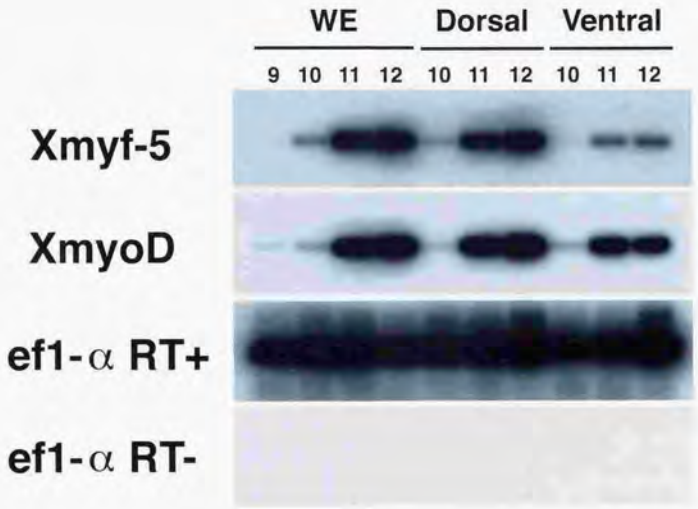
WE
Dorsal
Ventral
9 10 11 12 10 11 12 10 11 12
Xmyf-5
XmyoD
ef1-α RT+
ef1-α RT-

Fig. 4. bFGF and Activin A induced *Xmyf-5* and *XmyoD*.

Animal caps were dissected at stage 9 and cultured in presence or absence of growth factors for 6 hours, and analyzed by RT-PCR. *ef1-α* RT+ is loading control and *ef1-α* RT- is internal negative control.

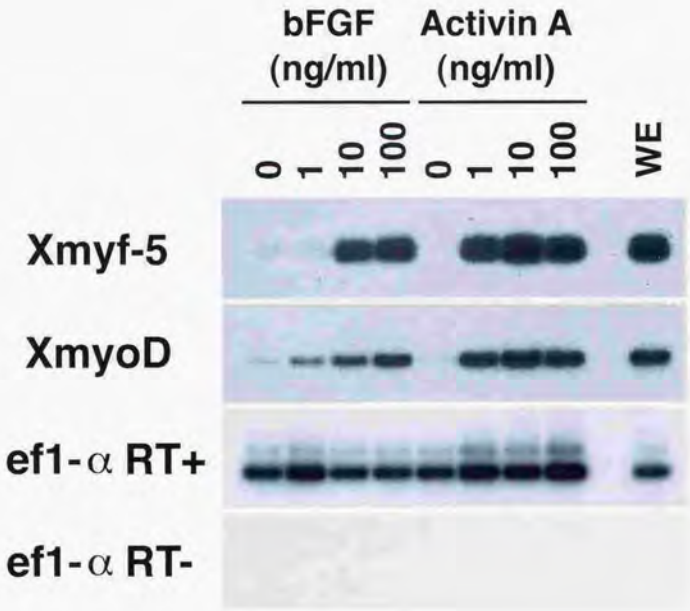
bFGF (ng/ml)
Activin A (ng/ml)
0
1
10
100
0
1
10
100
WE
Xmyf-5
XmyoD
ef1-α RT+
ef1-α RT-

Fig. 5. *Xbra* induced *Xmyf-5* and *XmyoD*, and synergized with *noggin*.

Synthetic mRNA was injected at 2-cell stage, then animal caps isolated at stage 10. and analyzed by RT-PCR. *ef1-α* RT+ serves loading control and *ef1-α* RT- is internal negative control.(a) High dose of *Xbra* induced *Xmyf-5* and *XmyoD*, alone, at low level. (b) Co-injection of *Xbra* and *noggin* induced these genes at low dose, that did not induce alone, of *Xbra*.

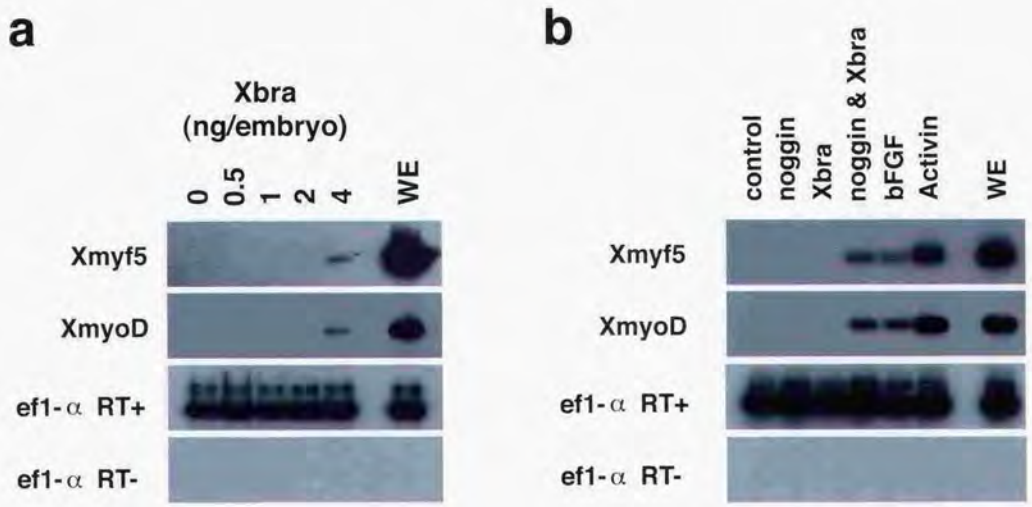
a
Xbra
(ng/embryo)
0
0.5
1
2
4
WE
Xmyf5
XmyoD
ef1-α RT+
ef1-α RT-
b
control
noggin
Xbra
noggin & Xbra
bFGF
Activin
WE
Xmyf5
XmyoD
ef1-α RT+
ef1-α RT-

Fig. 6. *Xmyf-5* genomic DNA sequence and the deduced amino acid sequence.

Sequences matching a consensus E-box (CANNTG) are underlined, *MEF2* binding motif is shown by a green box, *Xbra* binding motif is shown by red box, TATA-box is shown with blue and ATG overlined with an arrow. bHLH domain in amino acid sequence is indicated with pink. The poly-A adding signal is shown by yellow box.

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-1750      ATCAAA CTATTAATTG TTGATATTTT TTATAAAGCC CCAGGATTTA
-1700 TAAAAGGACC AGTTTCTTGG TCTGCACTGA GCCGAGTGTG TAATGAACAT
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-1500 TTAATTACCC TTAACATTTT CAGTGTTGTG CTGATGATTT GGGAAGAACA
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 -600 TTTCCCTAAA CAACAGTTGG TATTGTAATT ACAATGACTT TGCCCTTGAA
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 -200 AAAGTATGCT AATGTTATTA GCCTCTGTGG ACCAGCCTCC TTTGGTTGAA
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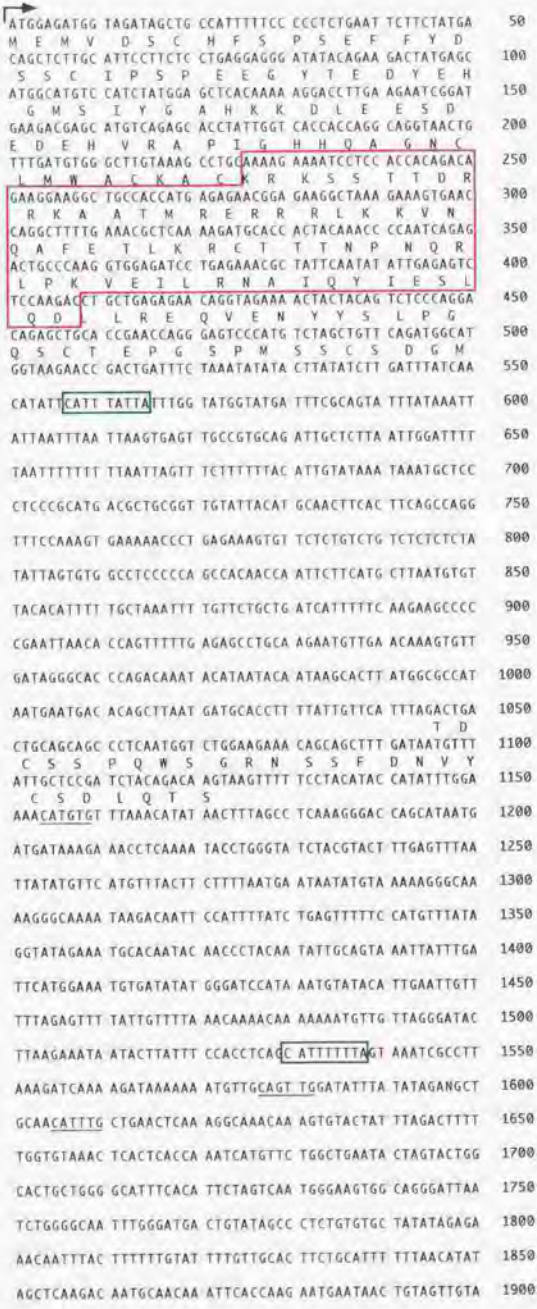

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CCTAGAATTA GCTCCAGGAC ACAATACTAA TGTCATTTAG TCATGATCCC  2100

CTGTGATGTC ATTTCTCAAA ACTGCCCATT GTAAACAAAT TACCAGACTT  2150

TTGTTGTTGA GGTCTAACTG CAATGCCAAT GTACCCCTCT GGTCATCACT  2200

GCACTTGCAG GTATTTGTTT GCCAGCAACT GATCACAATG GTTTTCAGGC  2250

AAGTTTCTTT AAGAACCAGT GTGTCAATCA CAGGTATTAT GTAAACTGAG  2300

CAGCAAGGAA GTGAAGAAGT GCTGGTTCTG GCAAGGGGTC CCTTAGTGAT  2350

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CAAGTATGCT TATACAACTG AATATGCTAA GTAAGTTTTT ATTTTTCTTT  2500

TATAGGTTTC TCTTCAACCA AACTGACATT GTCCAGCCTT GACTGCTTAT  2550
      F   S   S   T   K     L   T   L     S   S   L     D   C   L   S
CTAGTATTGT GGATCGGATC TCCTCTCCTC AGCAATGCTC CCTCCCTATT  2600
  S   I   V     D   R   I     S   S   P   Q     Q   C   S     L   P   I
CCAGACTCTA TCACCCCGTC TCCTACCAGT AGCACTGACT CCCTTCCGCG  2650
P   D   S   I     T   P   S     P   T   S     S   T   D   S     L   P   R
GTCACCTGAT GCGCATGATT GCCGACCAAT CTACCATGTG TTATAAGCCT  2700
  S   P   D     A   H   D   C     R   P   I     Y   H   V     L
CTCTTAATTC CATTAACATG TACATACTAG ATATTAATCC AAGACATTTC  2750

CTTACACCCA ACAAACCAGC GGATAAGGAC TGATGTTATT ATTTGTATAA  2800

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TTCTACTTGG CTGCATTTTG TATTTAACAT AAATAACCAA ACCTACAGCA  2900

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GGTAAAACTC CCTTTATTGT GATAAACAAT ATCACTTTCT CCATTGTTTT  3000

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GCGCAGTACC CTGCCGAGCA TAACGTCGGT CATTTGTATA AAGCACCGGA  3300

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ATGAGTGTAG ATCAATCCCA GACAATGGAA TATTCAATTG CATCATGAGG  3650

TGCAGCACAG GTACAGGATG CTTGGGAAGG GTGGGGGGTG TCCGGAGCTT  3700

ACACTGTAAC AAAAGCTCTA GCTGATGGAG CTGAGATTTC TCTACATGGC  3750

TGGGGGAATG TCCAGTGGAG CTTGTACTGA ACTATTACTG TATCCCCCCC  3800
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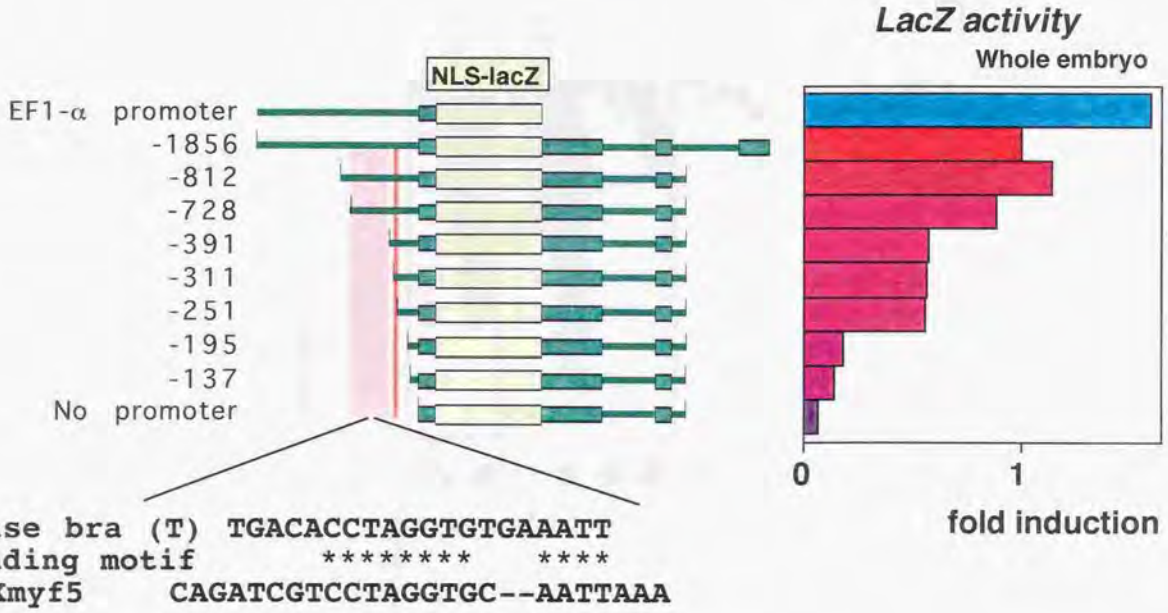
Fig. 7. Promoting activity of *Xmyf-5* promoter.

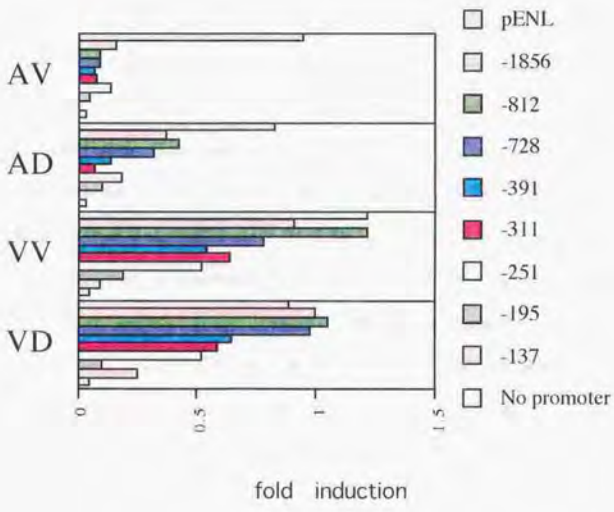
Promoter activity of *Xmyf-5* in *Xenopus* embryos is indicated by fold induction under a circumstance, longest promoter (-1856) activity = 1.0. Tested constructs are shown in the left of A. A pink and an orange line show two effective elements. Mouse bra binding motif and the similar sequence in *Xmyf-5* are indicated in the pink line. Right graph indicates activity by fold induction in case of injection into all cells at 8-cell stage . B shows activity by fold induction a case of site specific injection at 8-cell stage. The injected sites are four animal ventral; AV, Animal dorsal; AD, Vegetal ventral; VV, Vegetal dorsal; DA. Color bars in the right of graph indicate the tested constructs.

A



Mouse bra (T) TGACACCTAGGTGTGAAATT
binding motif ******** ****
Xmyf5 CAGATCGTCCTAGGTGC--AATTAAA

B


AV
AD
VV
VD
0
0.5
1
1.5
fold induction
pENL
-1856
-812
-728
-391
-311
-251
-195
-137
No promoter

B. PCR-cloning and expression pattern of *Xenopus prx-1* during early development

Abstract

Homeobox genes are expressed both temporally and spatially in vertebrate development, and regulate the tissue-specific expression of other genes. I cloned a *Xenopus paired-related homeobox-1* (*Xprx-1*) cDNA. *Xprx-1* had a *paired*-related homeodomain, but did not contain a *paired*-box. The sequence of *Xprx-1* had a high level of homology with *K-2* (mouse) and *Prx-1* (chicken), *Xprx-1* is assumed to be a *Xenopus* homolog of these genes. In *Xenopus* embryos, *Xprx-1* transcript were maternally restricted, and then decreased in the late blastula stage, with a subsequent increase in zygotic transcripts after gastrulation. The transcripts were localized to the animal hemisphere of the late blastula and were concentrated in the branchial arches of the tailbud stage embryo. In animal cap experiments, Activin A dose-dependently induced *Xprx-1* gene expression. These results suggest that *Xprx-1* plays a role in the *Xenopus* early development, similarly to other species.

Introduction

Recent years have brought important advances in my understanding of the early events that guide formation of vertebrate body pattern. A mesoderm induction assay using *Xenopus* animal caps have implicated factors such as the Activins, Vg1, Wnts and BMPs as being important in directing the formation of dorso-ventral axis patterning (reviewed in Harland, 1994). In response to the early signals, the expression of multiple zygotic genes commences in the mid-blastula and multiple position- and stage-specific genes are sequentially expressed. A number of *Xenopus* genes have been cloned and their expressions and functions during embryogenesis have been analyzed.

Homeodomain proteins are known to act as regulatory factors in both invertebrate and vertebrate body patterning. *Pax* genes, a group of the *paired*-type genes, encode sequence-specific DNA-binding proteins which contain a homeobox and a 128-amino acid DNA binding domain called the *paired*-box. They are highly conserved across vertebrate species and are thought to be related to neural patterning (reviewed in Gruss and Walther, 1992). Another group belonging to the small subfamily of *paired*-type homeobox genes, *paired*-related homeobox (*prx*) genes, are also expressed during vertebrate embryogenesis. *prx* genes encode a homeodomain sequence related to *Drosophila paired* and *gooseberry* homeodomains like *pax* genes, but lack the *paired*-box. The related mouse *prx* genes were previously termed *Mhox* (Cserjesi *et al.*, 1992), *K-2* (Kern *et al.*, 1992) and *S8* (Opstelten *et al.*, 1991). *prx* genes in other species were reported previously include human *Phox* (Grueneberg *et al.*, 1992) and chicken *Prx-1* and *Prx-2* (Nohno

et al., 1993; Leussink *et al.*, 1995). The proteins of the *prx* family have A/T rich sequence-specific binding activity, and regulate gene transcription (Cserjesi *et al.*, 1992; de Jong *et al.*, 1993; Cserjesi *et al.*, 1994; Takeda *et al.*, 1995). During mouse and chicken development, *prx* genes are expressed primarily in mesenchymal or mesodermal regions, but are not found in ectodermal derivatives (Nohno *et al.*, 1993; Kuratani *et al.*, 1994; Leussink *et al.*, 1995; Martin *et al.*, 1995). *Mhox* loss-of-function mutant mice have a range of skeletal defects, involving loss or shortening of structures in the skull, face and limbs. These analyses have revealed that the function of this gene involves regulation of the formation of preskeletal condensations from undifferentiated mesenchyme (Martin *et al.*, 1995). In *Xenopus*, several genes with the *paired*-type homeodomain such as *goosecoid* (*gsc*) (Cho *et al.*, 1991), *Mix1* (Rosa, 1989) and *siamois* (Lemaire *et al.*, 1995) reportedly play important roles in the formation of *Xenopus* axis patterning. The present study focused on the isolation of *Xenopus prx-1* gene and analysis of the expression during development.

Materials and methods

Eggs and embryos

Xenopus laevis eggs were obtained by injection of male and female animals with human chorionic gonadotropin (Gestron; Denka Seiyaku Co., Kanagawa Japan) at a dose of 600 IU. Fertilized eggs were dejellied by treatment with 3 % cysteine hydrochloride in Steinberg's solution (pH 7.8) with kanamycin sulfate (100 mg/l; Banyu Pharmaceutical Co., Tokyo, Japan), then washed thoroughly with sterile Steinberg's solution (pH 7.4). Embryos were transferred to culture dishes containing Steinberg's solution and allowed to develop until stage 9 (Nieuwkoop and Faber, 1967).

Cloning of *Xprx-1*

Total RNA was isolated from *Xenopus* embryos. First-strand cDNA was synthesized using MMLV reverse transcriptase (BRL) with oligo dT primer, and cDNA was amplified using degenerate primers encoding the amino acid sequences EAGDMV and VWFQNR (antisense), with flanking BamHI and KpnI restriction sites at the 5'-ends, respectively. Temperature cycling was as follows: initial 1.5 min. incubation at 94°C followed by 35 cycles of 40-sec incubation at 92°C, 40sec incubation at 44°C, and 1.5 min. incubation at 72°C. The PCR products were electrophoresed, blotted onto nylon membranes and hybridized with chicken *Prx-1* cDNA (Nohno *et al.*, 1992) according to an established method (Sambrook *et al.*, 1989). A positive band at 320 bp was isolated using QIAEX (Qiagen), cloned into pGEM7Zf(+) after BamHI and KpnI digestion, and identified by nucleotide sequencing. Based on the

nucleotide sequence obtained from the PCR products, the following primers were synthesized: 5'-TGACCAGTGGAAGTGACACT-3 (XPX3), 5'-CGGGATCCAAGAAAATGAACAGATGAC-3 (XPX4), 5'-TGTTCTGCAGTCATCTGTTC-3' (XPX7), 5'-AGGAGTGTCACTTCCACTGG-3 (XPX8). Additional primers to obtain the 5'- and 3'-coding sequences were also synthesized as follows: 5-GCTCTAGAT24-3' (T24), 5-CCGGTAC14-3' (D12-1). To obtain the 5'-coding sequence, G-tailed cDNA from stage 40-41 was prepared according to Hirzmann *et al.* (1993), and used as a template for PCR. First round PCR was carried out at annealing temperatures of 63°C to 53°C, decreasing 0.5°C per cycle for 20 cycles, followed by an additional 15 cycles at an annealing temperature of 53 °C with XPX7 and D12-1. The PCR product was diluted to 2,000-fold, and used as a template for second round PCR with XPX8 and D12-1. To obtain the 3'-coding sequence, first round PCR was carried out with XPX3 and T24 using stage 40-41 cDNA as a template, and then with XPX4 and T24 with the same temperature cycle as that used to obtain 5'-coding sequence. Final PCR products longer than 300 bp for the 5'-coding region and 450 bp for the 3'-coding region were purified using QIAEX, and ligated with pCR-Script SK(+) (Clontech) at the SrfI site. To obtain the full-length coding sequence, the following primers were synthesized: 5'-CAATAGTATAGTATTTCTGC-3' (XPX-10), 5'-CGGGATCCGATTGGAAGCAGCATGAGCT-3' (XPX-11), 5'-CGGAATTCTAGCTGGTGGCATTTTTCC-3' (XPX-12). First round PCR was carried out with XPX-11 and XPX-12 using stage 40-41 cDNA as a template, and then with XPX-11 and XPX-10. A 790 bp DNA fragment (*Xprx-1*) was amplified and purified using QIAEX, and ligated with EcoRV-BamHI-digested pBluescript SK(+) after BamHI digestion. The

nucleotide sequence was determined with an automatic DNA sequencing Analyzer (Applied Biosystems Model 373S). Both strands were sequenced with significant overlapping. Multiple sequence analysis was carried out using GeneWorks software (Intelligenetics). The *Xprx-1* was finally ligated into expression vector pSP64TL (Ishikawa *et al.*, 1995). mRNAs were synthesized using SP6 polymerase after linearization of the plasmids with restriction digestion.

RNA extraction, quantitative reverse transcription-polymerase chain reaction (RT-PCR) and Southern blotting

RT-PCR analysis of RNA samples was performed as described by Sambrook *et al.* (1989) except that total RNAs were isolated by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method with several modifications (Chomczynski and Sacchi, 1987; Hatada *et al.*, 1995). Oligo(dT)-primed first strand cDNA was prepared from the total RNA of *Xenopus* whole embryos and explants and PCR reactions were carried out with a Thermal Cycler (Perkin-Elmer Cetus). Negative controls to which reverse transcriptase was not added (RT-) were prepared in parallel against all samples. *Xprx-1* primers were used at 27 and 30 cycles, *EF1-α* (Kreig *et al.*, 1989) primers at 28 cycles, and *ornithine decarboxylase (ODC)* (Bassez *et al.*, 1990; Osborne *et al.*, 1991) primers at 28 cycles. After amplification, RT-PCR products were subcloned for Southern blotting and the sequences were determined with an automatic DNA sequencing Analyzer (ABI). Using the ^{32}P-labeled probes, Southern blotting was carried out and the signals were detected with X-ray film. The sequences of the primers used in this study were as

follows, in the 5' to the 3' orientation: *Xprx-1*; upstream XPX-11, downstream XPX-12. *EF1-α*; upstream TTGCCACACTGCTCACATTGCTTGC, downstream ATCCTGCTGCCTTCTTTTCCACTGC. *ODC*; upstream GTCAATGATGGAGTGTATGGATC, downstream TCCATTCCGCTCTCCTGAGCAC.

Animal cap assays

Animal caps were dissected from stage 9 embryos, then cultured in Steinberg's solution (pH 7.4) containing 0.1% BSA and 0.1 g/l kanamycin sulfate at 20°C, in the presence or absence of Human recombinant Activin A or bFGF. Human recombinant Activin A was kindly provided by Dr. Yuzuru Eto of the Central Research Laboratory, Ajinomoto Co., Kawasaki, Japan (Eto *et al.*, 1987; Murata *et al.*, 1988). Human recombinant bFGF was obtained from Mallinckropt Co.(Paris, France).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed following the method described in Harland (1991). Embryos obtained from albino females were used. Anti-digoxigenin antibodies were purchased from Boehringer Mannheim (GmbH. Mannheim, Germany). To prepare sections, embryos stained by *in situ* hybridization were dehydrated in ethanol, transferred to xylene and embedded in paraffin. Sections were observed by differential interference contrast microscopy.

RESULTS

PCR-cloning of *Xprx-1*

Sequence analysis of isolated cDNA clones revealed that the open reading frame encoded 245 amino acids. Sequence of the amino acid had a *paired*-related homeodomain but did not contain a *paired*-box as had been recognized in other vertebrate *prx* genes homologs, and was therefore named *Xprx-1* (Fig. 1a). Comparison of the amino acid sequence of *Xprx-1* with other related proteins revealed phylogenic relationships (Fig. 1b). Human, mouse and chicken homologs are also known by different names, as indicated in Fig. 1b. The amino acid sequence of *Xprx-1* was closely related to those of mouse *K-2* (Kern, *et al.*, 1992) and chicken *Prx-1* (Nohno, *et al.*, 1993) with overall identities of 85% and 84%, respectively. *Xprx-1* was distantly related to mouse *S8* (Opstelten, *et al.*, 1991) and chicken *Prx-2* (Leussink *et al.*, 1995). In comparisons of the amino acid sequences with *Xenopus paired*-type homeodomain genes, such as *gsc*, *Mix1* and *siamois*, *Xprx-1* was found to be distantly related to these genes. Within a portion of the homeodomain, *Xprx-1* showed amino acid identities of 59% (*gsc*), 50% (*Mix1*) and 38% (*siamois*).

Temporal expressions of *Xprx-1* during early development

The Northern blotting pattern of the stage 40 embryo is shown in Fig. 2a. Blots were probed with the entire *Xprx-1* coding region. A single band of approximately 3.7 Kb was visualized. Expression of *Xprx-1* in early stage embryos was not detected by Northern blotting until stage 30 (data not shown). As shown in

Fig. 2b, the developmental expression pattern of *Xprx-1* was examined by RT-PCR and Southern blotting. In low cycle (27 cycles) reactions, the expression of *Xprx-1* was observed in stage 30 embryos (Fig. 2b top). An additional 3 cycles in this reaction detected transcripts exist at low levels in earlier stages (Fig. 2b middle). Maternal *Xprx-1* transcripts were present but these transcripts did not increase during cleavage stages. The amounts of the maternal transcripts gradually decreased after stage 8, leaving faint signals at stage 10. After gastrulation, the level of expression of *Xprx-1* significantly increased. Fig.3 shows the localization of the transcripts in the stage 9 blastula. The *Xprx-1* transcripts were localized in the animal cap region with very little expression observed in the other regions.

Xprx-1 is expressed in prospective head mesenchyme including branchial arches at tailbud stage

Xprx-1 expression pattern was examined by whole-mount *in situ* hybridization after tailbud stage embryos (Fig.4). *Xprx-1* expression was not detected at any time prior to gastrulation by this method. At stage 25, the early tailbud embryo (a), *Xprx-1* was weakly but specifically expressed in the head region. At stage 30 (b, c, d), the transcripts were found in head mesenchyme and branchial arches. At stage 35 (e), the transcripts were also seen in the ventral region of the tailbud, in the mesenchyme under the posterior somite. Transverse sections of the stage 30 embryo head region (f) revealed that *Xprx-1* was expressed in presumptive head mesenchyme but not in either brain or epidermis.

Xprx-1 induction in animal caps treated with peptide growth factors

Activins and FGFs are potent mesoderm inducing factors and a number of position-specific genes can be activated by these factors (reviewed in Smith *et al.*, 1993; Asashima, 1994). Therefore, I examined the induction activity of these growth factors by RT-PCR and Southern blotting. Fig. 5 compares the induction of *Xprx-1* by Activin A and bFGF in animal caps. The animal caps were treated with various concentrations of growth factors and cultured for 6 hours. Little expression of *Xprx-1* was detected in animal caps treated with bFGF and non-treated caps. In contrast, Activin A induced dose-dependent expression of *Xprx-1*.

Alteration of *Xprx-1* expression in animal cap treated with Activin A

To test the temporal pattern of *Xprx-1* expression, animal caps were treated with a high concentration of Activin A (100 ng/ml) for 30 min, then cultured in Steinberg's solution for different periods of time (0-24 hour). It have been reported that the treatment of Activin A at high doses for 30 min. had an enough effect similar to a longer treatment (Ariizumi *et al.*, 1991). Expression was examined by RT-PCR and Southern blotting (Fig. 6). As already shown in Fig. 2B, *Xprx-1* transcripts were found in stage 9 animal caps. In non-treated explants, *Xprx-1* transcripts disappeared at 6 hours, which corresponds to the beginning of neuralization, and no further zygotic expression as detected. In the animal caps treated with Activin A, in contrast,

though the transcripts showed similar decrease at 3 hours , a significant increase was seen after 6 hours, and this increase in zygotic transcripts was still apparent at 24 hours.

Discussion

Multiple homeobox genes play a major role in the regulation of animal body patterning (for review see De Robertis, 1994). In the present study, I identified a *paired*-related *Xenopus* homeobox gene using a reverse transcription PCR cloning strategy. This gene, called *Xprx-1*, contained a *paired*-type homeodomain, but no *paired* box, and the amino acid sequence showed high homology with chicken *Prx-1*, mouse *K-2*, mouse *Mhox* and human *Phox1*. Therefore, the *Xprx-1* appears to belong to the *paired*-related homeobox (prx) family, and is the *Xenopus* homolog of *K-2* (mouse) / *Prx-1* (chicken).

Expression of *Xprx-1* during early development

Expression of *Xprx-1* by Northern blotting in early stage embryos from stage 30 to 40 was detected. However, RT-PCR analysis showed that the transcripts were maternally derived, stored in the animal cap region, and then decreased during the blastula stage. It is possible that the maternal *Xprx-1* transcripts act in the early stage, like the other *paired*-type homeobox genes. Zygotic *Xprx-1* transcripts were localized in head mesenchyme including the branchial arches of tailbud and tadpole stage embryos. The region will form head structure, for example, bones and gills. *Xprx-1* expression was also detected in mesenchyme under the posterior somites. This position includes the region responsible for posterior limb bud formation. It is possible that the posterior expression found in the stage 35 embryo corresponds to that in the limb bud formation reported in

other species. *Xprx-1* was not expressed in axial mesoderm and epidermis in this stage. These patterns of expression are in general agreement with those reported in mouse and chicken embryos. During mouse development, *prx* genes are expressed primarily in cranial mesenchyme, limb buds, axial mesoderm, branchial arches and their derivatives. These genes are not found in the ectoderm region (Kuratani *et al.*, 1994; Leussink *et al.*, 1995; Martin *et al.*, 1995). In the chicken embryo, the *Prx-1* gene is shown to be expressed predominantly in the limb bud and visceral arches (Nohno *et al.*, 1993). These observations indicate that *Xprx-1* may have a conserved function in the differentiation of head mesenchyme and branchial arches.

Activin A induces *Xprx-1* expression.

Activins and FGFs are potent mesoderm-inducing factors and a number of position specific genes can be activated by these factors (reviewed in Smith *et al.*, 1993; Asashima, 1994). It was reported that Activin A induced gene expression and differentiation of dorsal mesoderm depending on dose, and anterior structures can be formed at the highest concentrations (Ariizumi *et al.*, 1991; Ariizumi and Asashima 1995; Green *et al.*, 1990, 1994; Henry *et al.*, 1996; Wilson and Melton, 1994). These factors are thought to be capable of affecting dorso-ventral axis patterning. The *paired*-type homeodomain genes, *gsc* and *Mix.1*, are early response genes induced by Activin, which do not require any translation events before they are transcribed (Cho *et al.*, 1991; Rosa, 1989). I examined the activity of Activin A and bFGF on *Xprx-1* induction. In contrast to bFGF, Activin A induced

expression of *Xprx-1* in a dose-dependent manner in animal caps. High doses (100 ng/ml) of Activin A induced maximal expression of *Xprx-1*. These results resemble *gsc* and *Mix1*. But it is a different point that the zygotic expression of *Xprx-1* induced by Activin A was required for 3-6 hours after treatment. The similar patterns were seen in normal *Xenopus* development, maternal mRNA decreased once and then zygotic mRNA synthesis started after gastrulation. Because the temporal patterns of expression after Activin A treatment were different from those of the early response genes that play an important role in axis formation and dorso-ventral patterning, *Xprx-1* expression induced by Activin A may correspond later stage embryo development and such as the differentiation of head structure.

Function of *Xprx-1* gene

The early (maternal) *Xprx-1* was uniquely localized in the animal cap region of the blastula. Other *Xenopus paired*-type homeodomain genes show various patterns of expression. *gsc* is expressed in the blastula organizer region (Cho *et al.*, 1991), *siamois* in the dorsal vegetal region (Lemaire *et al.*, 1995), and *Mix.1* throughout the vegetal region (Rosa, 1989). Although *gsc* and *siamois* transcripts can induce dorsal structures when injected into the ventral region of embryo, *Mix.1* can not. It was very recently reported that injection of *Mix.1* mRNA results in the transformation of dorsal mesoderm to a ventral fate (Mead *et al.*, 1996). Furthermore, *Mix.1* can form heterodimers with the dorsalizing gene *siamois* and block the secondary axis formation induced by ectopic *siamois* expression. The authors suggest a

model by which dimerization of homeodomain proteins regulates dorsal-ventral patterning. Therefore, the function of *Xprx-1* was examined in the early stage as well. Although *Xprx-1* mRNA (100 pg/embryo) was injected separately into the two ventral vegetal blastomeres of an 8-cell stage embryo, *Xprx-1* mRNA did not induce obvious secondary axis formation. Furthermore, the mRNA did not affected normal development including head structure formation (data not shown). The maternal *Xprx-1* is present during cleavage stages and may act in *Xenopus* early development, but their functions remain unclear.

In knock-out experiments, *Mhox* (-/-) mouse mutants have a range of skeletal defects, involving loss or shortening of structures in the skull, face and limbs, indicating that this gene functions in the regulation of preskeletal condensations formed from undifferentiated mesenchyme (Martin, *et al.*, 1995). The similar amino acid sequence and expression pattern of *Xprx-1* and *Mhox* suggests that the zygotic expression of *Xprx-1* functions in head mesenchyme and posterior mesenchyme at a later stage. A similar observation has been reported where Activin A (-/-) mice showed craniofacial defects (Matzuk, *et al.*, 1995). These reports suggest that Activin A may induce *Xprx-1* genes in normal vertebrate head structure development in the same cascade *in vivo* as in animal cap experiments.

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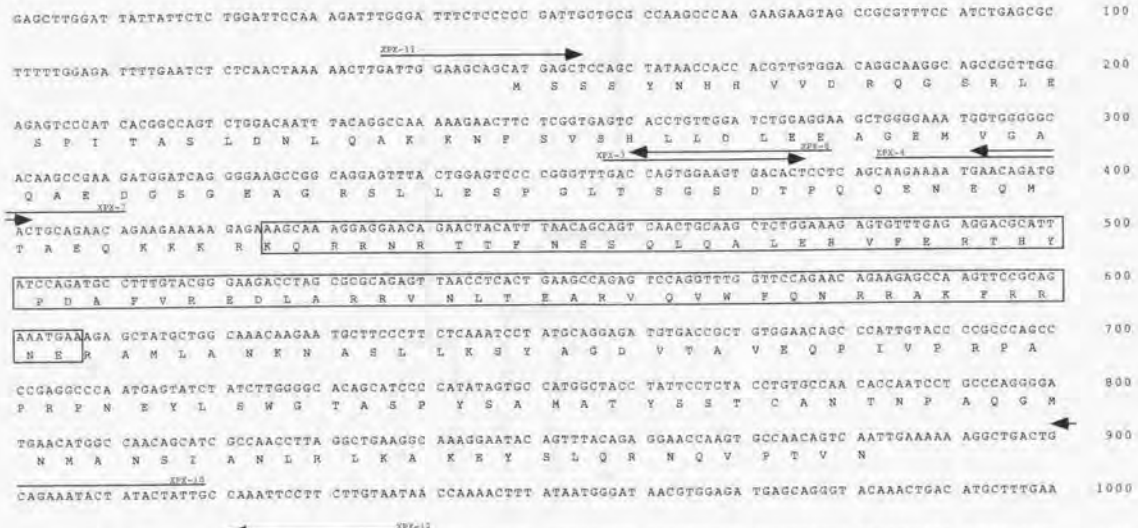
Fig. 1. Sequence of *Xprx-1* and comparison with related homeodomain proteins.

(a) Sequence of *Xprx-1* cDNA and the amino acid. The homeodomain was boxed. Used primers for cloning and detection of the expression were pointed by arrows. (b) A phylogenetic tree of *Xprx-1* and related homeodomain proteins.

a



b

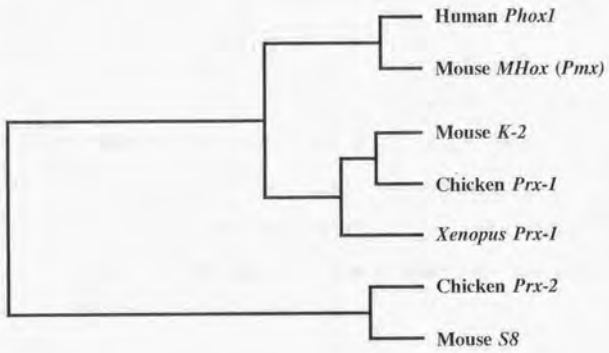

Human *Phox1*
Mouse *MHox* (*Pmx*)
Mouse *K-2*
Chicken *Prx-1*
Xenopus Prx-1
Chicken *Prx-2*
Mouse *S8*

Fig. 2. Embryonic expressions of *Xprx-1*.

(a) Northern blot analysis was performed with total RNA (20 µg) from embryos at stage 40. The full length *Xprx-1* coding region was used as a probe. (b) The RT-PCR and Southern blot analysis showing the temporal expression of *Xprx-1*. Total RNA for RT-PCR was isolated from five embryos at various stages. (egg) fertilized egg; (8) stage 8, mid-blastula; (9) stage 9, late blastula; (10) stage 10, early gastrula; (12) stage 12, late gastrula; (15) stage 15, mid-neurula; (20)stage 20, late neurula; (30) stage 30, tailbud. Subcloned PCR products were sequenced and used as probes. The RT-PCR was performed under two conditions, 27 cycles (top) and 30 cycles (middle). *ODC* is a loading control which is uniformly expressed throughout early developmental stages (bottom) (Bassez *et al.*, 1990; Osborne *et al.*, 1991). RT- (reverse transcriptase was not added to the total WE RNA) was a negative control, showed genomic DNA contamination was not detected.

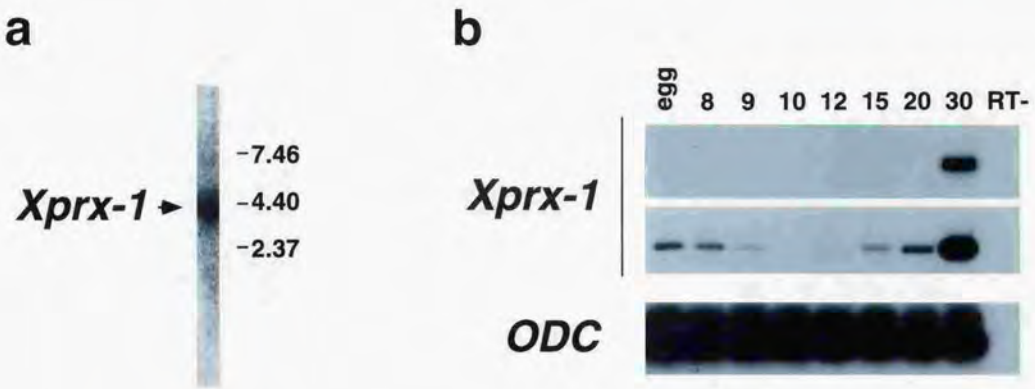
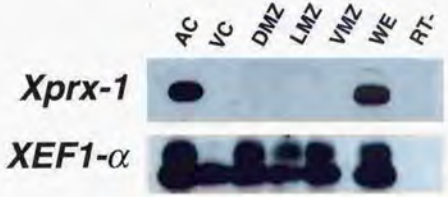

a
Xprx-1
-7.46
-4.40
-2.37
b
egg
8
9
10
12
15
20
30
RT-
Xprx-1
ODC

Fig. 3. Spatial expression of *Xprx-1* at stage 9.

The RT-PCR and Southern blot analysis showing localized expressions of *Xprx-1*. Fifty embryos were dissected into an animal cap (AC), vegetal cap (VC), the dorsal marginal zone (DMZ), lateral marginal zone (LMZ) and ventral marginal zone (VMZ), as shown on the right. Total RNA was isolated and used for RT-PCR and Southern blot analysis. *EF1-α* is an internal loading control that is expressed ubiquitously in embryos (Krieg *et al.*, 1989). WE (whole embryo, stage 9) was a positive control and RT- (reverse transcriptase was not added to the total WE RNA) was a negative control.


AC
VC
DMZ
LMZ
VMZ
WE
RT-
Xprx-1
XEF1-α

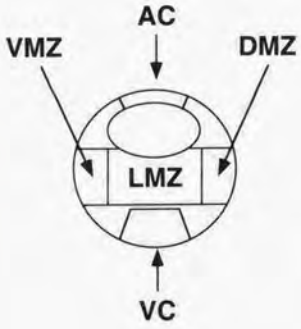

AC
VMZ
DMZ
LMZ
VC

Fig. 4. Spatial expressions of *Xprx-1* during taibud stages.

Xprx-1 expression was detected by whole-mount *in situ* hybridization using albino embryos. In (a)-(e), anterior is to the left. (a) Lateral view of stage 25 embryo. *Xprx-1* was weakly expressed in the head region (arrowhead). (b) Lateral view of stage 30 embryo. *Xprx-1* was expressed in head mesenchymal areas (arrowhead) and branchial arches (arrows, 1,2,3,4, are showing the arch's number). (c) Enlarged view of (b). (d) Dorsal view of stage 30 embryo. (e) Lateral view of stage 35 embryo. *Xprx-1* was expressed in the same region (left-arrowhead) and the ventral region of the tailbud (right-arrowhead). (f) Transverse section of the head region at stage 30 embryo, pointed by black bar in (b). *Xprx-1* was expressed in the head mesenchyme (arrowheads).

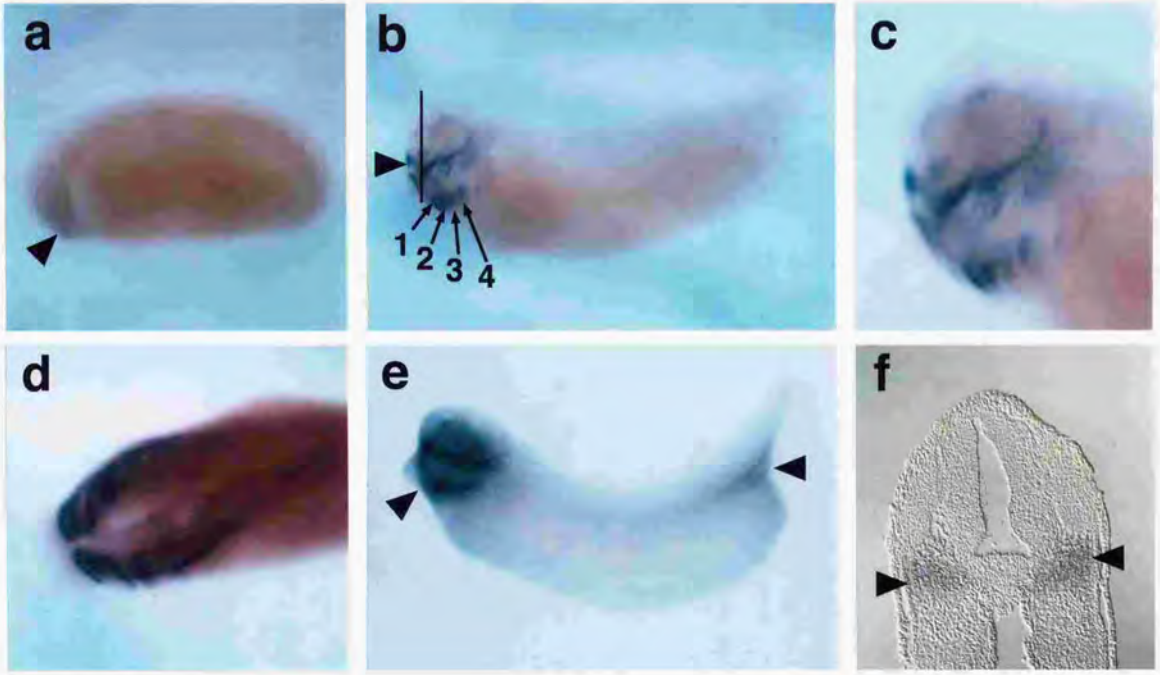

a
b
1
2
3
4
c
d
e
f

Fig. 5. Expression of *Xprx-1* in animal caps treated with peptide growth factors.

Total RNA was isolated from animal caps treated with different concentrations of Activin A or bFGF for 6 hours. RT-PCR and Southern blot analysis showed that Activin A dose-dependently induced expression of *Xprx-1* genes, whereas bFGF did not. *EF1-α* served as an internal loading control. WE (whole embryo, stage 20) was a positive control and RT- (reverse transcriptase was not added to the total WE RNA) was a negative control.

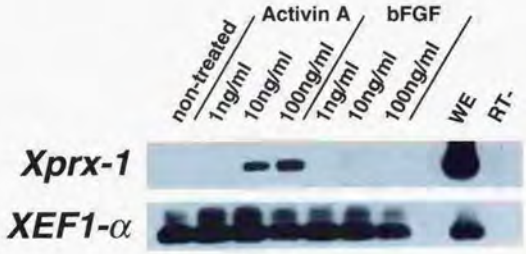
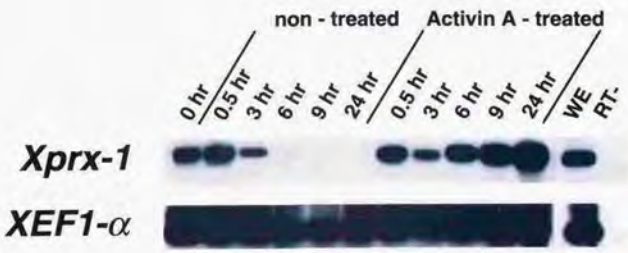

Activin A
bFGF
non-treated
1ng/ml
10ng/ml
100ng/ml
1ng/ml
10ng/ml
100ng/ml
WE
RT-
Xprx-1
XEF1-α

Fig. 6. Temporal expression of *Xprx-1* in animal caps treated with Activin A.

Animal caps were treated in Steinberg's solution with or without 100 ng/ml Activin A for 30 min. After treatment, the animal caps were maintained in normal Steinberg's solution for various durations (0-24 hours). RT-PCR and Southern blot analysis showed the induction of zygotic *Xprx-1* expression after 6 hours in Activin A-treated animal caps, in contrast to non-treated animal caps. *EF1-α* served as an internal loading control. WE (whole embryo, stage 20) was a positive control and RT- (reverse transcriptase was not added to the total WE RNA) was a negative control.


non - treated
Activin A - treated
0 hr
0.5 hr
3 hr
6 hr
9 hr
24 hr
0.5 hr
3 hr
6 hr
9 hr
24 hr
WE
RT-
Xprx-1
XEF1-α

Chapter II. Neural development

A. Expression and function of *Xenopus ATH-3*.

Abstract

I have isolated a novel basic helix-loop-helix (bHLH) gene homologous to the *Drosophila* proneural gene *atonal*, termed *ATH-3*, from *Xenopus*. *ATH-3* is expressed in the developing nervous system, with high levels of expression in the brain, retina and cranial ganglions. Injection of *ATH-3* RNA into *Xenopus* embryos dramatically expands the neural tube and induces ectopic neural tissues in the epidermis but inhibits non-neural development. This *ATH-3*-induced neural hyperplasia does not require cell division, indicating that surrounding cells which are normally non-neural types adopt a neural fate. In *Xenopus* animal cap assay, *ATH-3* is able to convert ectodermal cells into neurons expressing anterior markers without inducing mesoderm. Interestingly, a single amino acid change from Ser to Asp in the basic region, which mimics phosphrylation of Ser, severely impairs the anterior marker-inducing ability without affecting general neurogenic activities. These results provide evidence that ATH-3 can directly convert non-neural or undetermined cells into a neural fate, and suggest that the Ser residue in the basic region may be critical for the regulation of ATH-3 activity by phosphorylation.

Introduction

In *Drosophila*, neural development is controlled positively or negatively by multiple basic helix-loop-helix (bHLH) genes (Campos-Ortega and Jan, 1991; Jan and Jan, 1993; Jarman *et al.*, 1993; 1994). For example, *achaete-scute* complex (AS-C) and *atonal* are proneural genes, and the former is required for external sensory organ development while the latter is required for photoreceptor and chordotonal organ development. In contrast, *hairy* and *Enhancer of split* [*E(spl)*] inhibit neural development by antagonizing the proneural genes.

As in *Drosophila*, vertebrate neurogenesis is also controlled positively or negatively by multiple bHLH genes (Guillemot *et al.*, 1993; Ishibashi *et al.*, 1994; Ferreiro *et al.*, 1994; Turner and Weintraub, 1994; Ishibashi *et al.*, 1995; Lee *et al.*, 1995; Kageyama *et al.*, 1995; Tomita *et al.*, 1996). *XASH-3*, a *Xenopus* bHLH gene homologous to the *Drosophila* proneural gene complex AS-C (Zimmerman *et al.*, 1993), converts ectodermal cells to a neural fate and therefore acts as a proneural gene (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). *Mash-1*, a mammalian bHLH gene homologous to AS-C, regulates early stages of neuronal differentiation, and *NeuroD*, another vertebrate bHLH gene, functions in terminal differentiation of neurons (Johnson *et al.*, 1990; Guillemot *et al.*, 1993; Lee *et al.*, 1995). Thus, multiple bHLH genes positively regulate vertebrate neurogenesis at different stages. In contrast, *HES-1*, a mammalian bHLH gene homologous to *Drosophila hairy* and *E(spl)*, is expressed in neural precursor

cells and acts as a negative regulator of neurogenesis (Sasai *et al.*, 1992). Forced expression of *HES-1* blocks neuronal differentiation in the brain (Ishibashi *et al.*, 1994) and retina (Tomita *et al.*, 1996). Conversely, a *HES-1*-null mutation accelerates neuronal differentiation and results in severe anomalies of the brain (Ishibashi *et al.*, 1995) and eye (Tomita *et al.*, 1996). Thus, *HES-1* prevents premature neurogenesis and regulates brain and eye morphogenesis.

The exact mechanism of how *HES-1* prevents premature neurogenesis is unclear, but it is likely that *HES-1* antagonizes bHLH genes that positively regulate neurogenesis, as in the case of *Drosophila*. Interestingly, expression of *Mash-1* is up-regulated in *HES-1*-null mice (Ishibashi *et al.*, 1995), suggesting that *Mash-1* up-regulation may contribute to premature neurogenesis. However, null mutation of *Mash-1* does not cause any apparent abnormalities in the central nervous system (CNS) (Guillemot *et al.*, 1993) and, based upon the expression patterns, *XASH-3* or its mammalian equivalent and *NeuroD* are unlikely to compensate for *Mash-1*. Therefore, additional bHLH genes may be required for the CNS development.

Materials and Methods

Isolation of the mouse *ATH-3* gene and cDNA

Oligo(dT)-primed cDNA was prepared from E9.5 mouse CNS and subjected to PCR with fully degenerate oligonucleotide primers corresponding to the amino acid sequences NARER and TLQMA of Atonal, as previously described (Akazawa *et al.*, 1995; Shimizu *et al.*, 1995). The amplified fragments were subcloned and sequenced and *ATH-3* sequence was obtained. The PCR clone of *ATH-3* was labeled with [a-^{32}P]dCTP by a random-primer method and used as a probe for screening a mouse genomic library (Stratagene) (Takebayashi *et al.*, 1994). Sequence analysis indicated that an approximately 7.5 kb BglII fragment of the *ATH-3* gene contained the open reading frame (Isaka *et al.*, 1996). Subsequently, a 685 bp HincII-PstI fragment from the mouse *ATH-3* gene which contained most of the coding region was used as a probe for screening the P0-P10 mouse retina cDNA library. Approximately one million plaques were screened and 200 positive clones were obtained. Thirty clones were sequenced, and all contained the *ATH-3* sequence. This step was carried out by collaborator, Dr. Takebayashi (Kyoto University).

Isolation of *Xenopus ATH-3* cDNA

The 685 bp HincII-PstI fragment of the mouse *ATH-3* gene was used as a probe for screening the *Xenopus* stage 30 embryo cDNA library (Stratagene). Four positive clones were isolated and sequenced, and all contained *Xenopus ATH-3* cDNA.

Whole mount *in situ* hybridization analysis of *ATH-3*

Whole mount *in situ* hybridization experiments were performed, essentially as described previously (Harland, 1991; Takebayashi *et al.*, 1995; Hatada *et al.*, 1995). Digoxigenin (DIG)-labeled antisense RNAs corresponding to the 634 bp XbaI-EcoRI *Xenopus ATH-3* cDNA fragment were synthesized *in vitro*. These probes were hybridized to whole *Xenopus* albino embryos.

Analysis of RNA-injected *Xenopus* embryos

The full-length coding region of the *Xenopus ATH-3* cDNA was subcloned into pSP64T vector, and capped *ATH-3* RNA was produced *in vitro* as described before (Krieg and Melton, 1984). The *ATH-3* and *β-galactosidase* RNAs were injected into one cell of 2-cell or 4-cell stage *Xenopus* embryos. At various stages, the injected embryos were subjected to histological study with hematoxylin-eosin (HE) staining, immunological study with NEU-1 monoclonal antibody (Itoh and Kubota, 1989) and anti-myosin monoclonal antibody MF 20 (Bader *et al.*, 1982), or *in situ* hybridization of *N-CAM*, *twist* and *keratin*. The probe regions for *N-CAM*, *twist* and *keratin* are nucleotide residues 1466-2020 (Accession number M76710), 155-652 (M27730) and 615-1171 (M11940), respectively. To identify the injected side, X-gal staining was performed.

For HUA treatment, injected embryos were transferred to 20 mM hydroxyurea-0.15 mM aphidicoline at stage 10.5, as previously described (Harris and Hartenstein, 1991).

Xenopus animal cap assay

Mutation was introduced into *ATH-3* cDNA by hybridizing oligonucleotides 5'-AAGTCCATGCATTCTATCACGCTCCCTGGCATTGGC-3' and 5'-AAGTCCATGCATTCTGTTACGCTCCCTGGCATTGGC-3' with heat-denatured *ATH-3* cDNA for S89D and S89N, respectively. Each mutant was cloned into pSP64T and sequenced.

In vitro synthesized *ATH-3*, S89D, or S89N RNA was injected into both cells of two-cell stage *Xenopus* embryos (1 ng/embryo). Animal caps that had been either injected or uninjected with RNA were explanted at the late blastula stage (stage 9) and cultured in Steinberg's solution for 3 hr, one day, or three days. Total RNA was extracted from animal caps by using the acidic guanidine thiocyanate method. Reverse transcription-PCR (RT-PCR) analysis for *XANF-1* (Zaraisky *et al.*, 1992), *EF-1α* (Krieg *et al.*, 1989), *N-CAM* (Kintner and Melton, 1987), *XlHbox6* (Wright *et al.*, 1990), *NF-M* (Sharpe *et al.*, 1988), *F-spondin* (Ruiz i Altaba *et al.*, 1993), *XIF3* (Sharpe *et al.*, 1989), and *S-actin ; alpha skeletal muscle actin* (=*muscle specific actin; ms-actin*) (Stutz and Spohr, 1986) was done, as previously described (Hemmati-Brivanlou and Melton, 1994; Sasai *et al.*, 1995; Hatada *et al.*, 1995). In addition, I performed RT-PCR by using the following primers; *opsin* (Saha and Grainger, 1993), 5'-TTCGGATGGTCCAGATACATCC-3' and 5'-GGTGGTAAGAGATTCCTGTTGC-3' (36 cycles); type-II *b-tubulin* (Good *et al.*, 1989), 5'-ATTAACAAGTCGTGGCAGCC-3' and 5'-TCTGGACATTGCATCTACC-3' (36 cycles).

Results

Structural analysis of *Xenopus* and mouse *ATH-3*

Polymerase chain reaction (PCR) with degenerate oligonucleotide primers was carried out to search for a novel bHLH gene expressed in the developing nervous system. A PCR fragment from a bHLH gene was termed *ATH-3* because of structural similarity to the *Drosophila* proneural gene *atonal*. This PCR fragment was used to screen mouse genomic library to obtain mouse *ATH-3* gene (Isaka *et al.*, 1996). By using this *ATH-3* genomic clone as a probe, *Xenopus* and mouse cDNA libraries were screened to determine the full-length coding sequences.

Xenopus and mouse ATH-3 consisted of 315 (Fig. 1A) and 330 amino acid residues (Fig. 1B), respectively, and shared 93% identity in the bHLH domain (Fig. 1 *underlined* and Fig. 2B). The structural similarity extended to the upstream and downstream regions of the bHLH domain, with an overall identity of 67% (Fig. 2A). In addition, ATH-3 showed significant sequence homology to MATH-1 (Akazawa *et al.*, 1995), MATH-2/NEX-1 (Bartholomä and Nave, 1994; Shimizu *et al.*, 1995), NeuroD/BETA2 (Lee *et al.*, 1995; Naya *et al.*, 1995), NDRF/KW8 (Kume *et al.*, 1996; Yasunami *et al.*, 1996), and Atonal (Jarman *et al.*, 1993) in the bHLH domain (Fig. 2B), suggesting that these factors share a recent common ancestral gene. However, unlike the latter factors, ATH-3 contained serine (amino acid residue 89 in *Xenopus*) or threonine (98 in mouse) in the basic region, which forms a potential phosphorylation site. It has been shown that myogenic bHLH factors contain a threonine residue in the basic

region and that its phosphorylation inactivates the myogenic activity (Li *et al.*, 1992). Thus, the activity of ATH-3 could be also regulated by phosphorylation and dephosphorylation of the basic region. Another structural feature common to *Xenopus* and mouse ATH-3 is the acidic region (Glu-Asp stretch; amino acid residues 38-56 of *Xenopus* and 49-65 of mouse) and the basic region (Lys-Arg stretch; amino acid residues 57-72 of *Xenopus* and 66-81 of mouse), which are located upstream of the bHLH domain. These acidic and basic regions are also present in MATH-2, NeuroD, and NDRF, and could be involved in transcriptional activity.

Spatial and temporal distribution of *ATH-3*

The spatiotemporal expression patterns of *ATH-3* were determined by *in situ* hybridization. In *Xenopus*, *ATH-3* expression was first detected weakly at stage 12 in two stripes within the presumptive neural plate (Fig. 3A *arrowheads*). At stage 14, *ATH-3* was expressed in three stripes on both sides of the midline of the neural plate (Fig. 3B *arrowheads*). Assessed by their positions, cells in these stripes may correspond to primary neuronal precursors, which differentiate into motor and sensory neurons and interneurons. At stages 18 and 21, strong *ATH-3* expression appeared in the cranial ganglions (Fig. 3C,D *arrowheads*) while weaker expression remained in the spinal cord. Later, *ATH-3* was expressed strongly in the eye, forebrain, and cranial ganglions, where active neuronal differentiation occurs (Fig. 3E,F). Thus, *ATH-3* may be initially expressed in neural

progenitor cells and later in differentiating postmitotic neurons of the anterior nervous system in *Xenopus* embryos.

Neural hyperplasia by injection of *ATH-3* RNA

To assess the function of *ATH-3* in neural development, *in vitro* generated *Xenopus ATH-3* RNA was injected into *Xenopus* embryos. When 50 or 100 pg of *ATH-3* RNA was injected into one cell of two-cell stage *Xenopus* embryos, the most notable and frequent phenotype was expansion of the CNS on the injected side (Table 1). Co-injection of *β-galactosidase* RNA allowed us to confirm the injected side by X-gal staining (data not shown).

Immunological analyses with the pan-neural marker NEU-1 (Itoh and Kubota, 1989) showed that the neural tube was significantly enlarged laterally at stages 25 and 30 (Fig. 4A,B *arrowheads*). The optic cup was deformed and displaced on the injected side (Fig. 4B *arrow*). This *ATH-3* phenotype of neural tube enlargement is different from that of *NeuroD*, which does not cause neural tube hyperplasia (Lee *et al.*, 1995), but seems quite similar to that of *XASH-3*, which expands the neural tube (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). In addition to the expanded neural tube, ectopic NEU-1 or *N-CAM* staining was detected in the epidermis on the injected side (Fig. 4B,G,H). Ectopic neurogenesis was also induced in the epidermis when *ATH-3* RNA was injected into a ventral cell of four-cell stage embryos (Fig. 4E). Ectopic NEU-1-positive cells in the epidermis showed morphology of neurons with multiple processes (Fig. 4E). This *ATH-3* phenotype is quite similar to that of *NeuroD*, which ectopically converts neural crest and epidermal cells into

neurons (Lee *et al.*, 1995). These results suggest that *ATH-3* can not only expand the neural tube like *XASH-3* but also induce ectopic neurogenesis in the epidermis like *NeuroD*.

At the tadpole stage, the brain was significantly enlarged (Fig. 4I *arrowhead*), and the eye was deformed and displaced ventrally on the injected side. Histological analyses revealed that the forebrain dramatically expanded laterally and anteriorly on the injected side (Fig. 4J *arrowheads*). As a result, the development of the surrounding regions was disturbed. The hindbrain was also expanded by overexpression of *ATH-3* (Fig. 4K *arrowheads*). Because the injected *ATH-3* RNA was likely to have disappeared by this time, the above results indicate that neural hyperplasia remained permanently by transient expression of *ATH-3*.

The observed neural hyperplasia could be due to either cell proliferation or conversion of surrounding non-neural cells to a neural fate. To distinguish these possibilities, cell division of *ATH-3* RNA-injected embryos was blocked by hydroxyurea/aphidicoline (HUA) treatment at mid-gastrulation. It has been shown that embryos treated with HUA at mid-gastrulation stop cell division but develop almost normally until tailbud stages (Harris and Hartenstein, 1991; Turner and Weintraub, 1994). As shown in Fig. 4C, *ATH-3* overexpression expanded the CNS and induced ectopic neurogenesis in the epidermis on the injected side even in the presence of HUA. Thus, cell proliferation is not necessary for neural hyperplasia induced by *ATH-3*, suggesting that overexpression of *ATH-3* converts adjacent non-neural cells to a neural fate.

Suppression of non-neural tissue development by *ATH-3*

If the surrounding non-neural cells are converted into a neural fate, development of non-neural tissues should be reduced in *ATH-3* RNA-injected embryos. To test this possibility, I next determined expression of non-neural markers in the injected embryos. On the *ATH-3* RNA-injected side, expression of *twist*, a marker for non-neural types of neural crest cells (Hopwood *et al.*, 1989; Turner and Weintraub, 1994), was severely reduced (Fig. 5B,C), suggesting that ectopic *ATH-3* expression decreased the population of neural crest cells with the potential to differentiate into non-neural cells. These cells may instead become ectopic neurons, which were observed in the epidermis of *ATH-3* RNA-injected embryos (see Fig. 4E,G,H).

In the injected embryos, expression of *keratin*, an epidermal marker (Jonas *et al.*, 1985), was also significantly reduced (Fig. 5E,F). In addition, somite formation was also severely blocked, as revealed by impaired myosin expression (Fig. 5H *arrows*). These results support the hypothesis that *ATH-3* expands the neural tissues at the expense of adjacent non-neural cells.

Activation of neural gene expression in animal caps by *ATH-3*

To assess more clearly the *ATH-3* function in neurogenesis, additional markers were examined in the injected embryos. The *ATH-3* RNA was injected into both cells of two-cell stage *Xenopus* embryos, and subsequently the animal caps were isolated from the injected embryos at stage 9. These explants were cultured for an

additional 3hr (equivalent to stage 11), one day (stages 25-30), or three days (stages 35-40). Uninjected animal caps are known to become atypical epidermis, and therefore neural and mesodermal markers were not expressed (Fig. 6 lane 2). In contrast, injection of *ATH-3* RNA induced significant expression of the pan-neural marker *N-CAM* (Kintner and Melton, 1987) and the neuronal markers type-II *β-tubulin* (Good *et al.*, 1989) and *neurofilament-M* (*NF-M*) (Sharpe, 1988) in stage 25-30 animal caps (Fig. 6 lane 3), indicating that *ATH-3* can induce neuronal differentiation. In addition, expression of the anterior neural markers *XANF-1* (Zaraisky *et al.*, 1992) and *XIF-3* (Sharpe *et al.*, 1989) and the retinal marker *opsin* (Saha and Grainger, 1993) was also significantly induced in *ATH-3* RNA-injected animal caps (Fig. 6 lane 3). However, other markers such as the posterior neural marker *XlHbox6* (Wright *et al.*, 1990) and the floor-plate marker *F-spondin* (Ruiz i Altaba *et al.*, 1993) were not induced by *ATH-3* (Fig. 6 lane 3). Thus, *ATH-3* can promote development of neurons with anterior features, suggesting that *ATH-3* may have a role in specification of anterior neuronal types. This neural induction by *ATH-3* was also observed when the culture was continued for three days (equivalent to stages 35-40) (data not shown), indicating that the activation of neural gene expressions by *ATH-3* is stable. This is in sharp contrast to the action of *XASH-3*, which only transiently induces neural gene expression in animal caps (Ferreiro *et al.*, 1994).

In *ATH-3* RNA-injected animal caps, expression of mesodermal markers was not detected; *Xbra* (Smith *et al.*, 1991) and *goosecoid* (Blumberg *et al.*, 1991) were not expressed in the

animal cap explants that had been cultured for 3hr (data not shown). In addition, *alpha skeletal muscle actin; S-actin (=muscle specific actin; ms-actin)* (Stutz and Spohr, 1986) was not expressed in the injected animal caps that had been cultured for one day (equivalent to stages 25-30) (Fig. 6 lane 3). Thus, *ATH-3* promoted neuronal differentiation without inducing mesoderm, suggesting that *ATH-3* may directly convert ectodermal cells into neurons.

Modification of ATH-3 activities by a single amino acid change in the basic region

Among the vertebrate neural bHLH factors that have been characterized, ATH-3 has a unique structural feature; a serine or threonine residue in the basic region (Fig. 2B), which forms a potential phosphorylation site. As an initial step to relate the possible phosphorylation with the neurogenic activity of ATH-3, a Ser (89)-to-Asp mutation was introduced into *Xenopus* ATH-3 (S89D), which mimics the phosphorylation of Ser. Another mutation was also introduced to change Ser (89) to Asn (S89N), which could represent a non-phosphorylated form. On the animal cap analysis, S89D was able to induce *N-CAM*, typeII *β-tubulin*, and *NF-M* expression (Fig. 6 lane 4), indicating that S89D keeps the general neurogenic activities. However, it failed to induce the anterior neural markers *XANF-1* and *opsin* (Fig. 6 lane 4). In contrast, S89N induced anterior neural gene expression as well as general neural markers, like wild type *ATH-3* (Fig. 6 lane 5). These results indicate that Ser (89) is critical for the regulation of ATH-3 activity by phosphorylation and that

modification of a single amino acid residue in the basic region can regulate some of the neurogenic activities of a bHLH factor.

Discussion

ATH-3 may function in both determination and differentiation steps of *Xenopus* neural development

In this study, I showed that overexpression of *ATH-3* can induce ectopic neurons and hyperplasia of the CNS but suppress development of non-neural tissues in *Xenopus* embryos. Interestingly, *ATH-3*-induced neural hyperplasia does not require cell division, indicating that the adjacent non-neural lineage cells adopt a neural fate. In addition, *ATH-3* can induce neurons without inducing mesoderm in the animal cap assay. These results provide evidence that *ATH-3* can directly convert non-neural or undetermined cells into neurons.

It has been proposed that there are at least two separate developmental choices for generation of neurons in vertebrates; first, whether or not to adopt a neural lineage, and second, whether or not to differentiate as a neuron (Ferreiro *et al.*, 1994; Turner and weintraub, 1994; Lee *et al.*, 1995). The first step involves the initial decision between neural and epidermal fates in the ectoderm by a proneural gene, while the second step is the subsequent process of neuronal differentiation. It has been shown that *Xenopus* bHLH gene *XASH-3* can expand the neural tube at the expense of adjacent non-neural ectoderm and therefore acts as a proneural gene (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). I showed that *ATH-3* can induce similar effects in *Xenopus* embryos, i.e. expansion of the neural tube and disturbance of development of the surrounding non-neural tissues. These results suggest that, like *XASH-3*, *ATH-3*

can function as a proneural gene in *Xenopus* embryos. The early onset of *ATH-3* expression before the neural plate appears is also consistent with the notion that *ATH-3* is a proneural gene. Interestingly, whereas in animal cap assays *XASH-3* can only transiently induce neural gene expressions and requires the neural inducer noggin for stable induction (Ferreiro *et al.*, 1994), *ATH-3* can stably induce neural gene expression without noggin. Thus, ATH-3 seems to have a stronger neurogenic activity than XASH-3, or ATH-3 may be less susceptible to inhibitory signals such as Notch and HES-1.

Another bHLH gene, *NeuroD*, regulates the terminal differentiation step of neural development. It has been shown that *NeuroD* can induce ectopic neural tissues in the epidermis of *Xenopus* embryos. *ATH-3* also induces ectopic neural tissues in the epidermis, like *NeuroD*, suggesting that *ATH-3* and *NeuroD* have similar neurogenic functions. Furthermore, *ATH-3* is expressed at a high level in the nervous system during neuronal differentiation stages, suggesting that *ATH-3* can also act as a *NeuroD*-like differentiation gene. Thus, *ATH-3* may function in both proneural and neuronal differentiation stages in *Xenopus* embryos.

ATH-3 activity may be regulated by phosphorylation of Ser (or Thr) in the basic region

ATH-3 has a serine or threonine residue in the basic region, which could serve as a phosphorylation site. Other neural bHLH factors contain asparagine or arginine in the corresponding position, thus indicating that the amino acid

residue in this position is not conserved among the neural bHLH factors (Fig. 2B). Interestingly, the myogenic bHLH factors contain a threonine residue in the basic region, and it has been shown that its phosphorylation inactivates the myogenic activities (Li *et al.*, 1992). Change from Thr to Asp, which mimics the phosphorylation in the basic region of myogenin, causes loss of DNA-binding and myogenic activities (Brennan *et al.*, 1991). In the case of *Xenopus* ATH-3, mutation of Ser (89) to Asp (S89D) maintains a general neurogenic activity but severely impairs anterior marker-inducing abilities in the *Xenopus* animal cap assay. In contrast, S89N, which could represent a non-phosphorylated form, can induce both general and anterior neural markers. These results thus point to the importance of possible phosphorylation of Ser/Thr in the basic region of ATH-3, which can modify the anterior-specific neurogenic activities, although it remains to be determined whether or not the basic region of ATH-3 is phosphorylated in vivo.

The mechanism of how S89D, which loses anterior neurogenic activity, can induce general neural markers is quite interesting, and I can offer two possibilities. One is that S89D could lose the DNA-binding activity and titrate negative regulators by forming a non DNA-binding heterodimer complex, thereby activating gene expression. The other possibility is that the DNA-binding specificity of S89D could be changed by addition of a negative charge in the basic region; it could bind to the promoter of general neural genes but not of anterior

genes. Determination of ATH-3-binding sequences will be necessary to answer these problems.

ATH-3 in retinal development

It is striking that *ATH-3*, expressed at a high level in the retina, can induce *opsin* expression in the animal cap assay, raising the possibility that *ATH-3* may be involved in retinal specification. However, induction of anterior neural markers by *ATH-3* could reflect a general property of *Xenopus* ectoderm since neural inducers like *noggin*, *chordin*, and *follistatin* also induce a similar spectrum of anterior markers (Lamb *et al.*, 1993; Hemmati-Brivanlou and Melton, 1994; Sasai *et al.*, 1995). Nevertheless, I speculate that the *opsin*-inducing activity may be an intrinsic property of ATH-3, because S89D loses the *opsin*-inducing ability but maintains general neurogenic activities in the animal cap assay.

Eye morphogenesis is known to be regulated by the eye master control gene *Pax-6* (Halder *et al.*, 1995), and it is therefore possible that *ATH-3* expression is regulated by *Pax-6*. However, *ATH-3* was similarly expressed in the optic vesicles of wild type and *Small eye* mutant mice, which have a *Pax-6* mutation (Hill *et al.*, 1991; Walther and Gruss, 1991), indicating that *ATH-3* expression does not depend upon *Pax-6*. Thus, *ATH-3* and *Pax-6* may constitute different genetic pathways for retinal development. Because only a part of *Pax-6*-expression domains becomes retina, it has been suggested that another factor not regulated by *Pax-6* may be required for retinal specification (Macdonald and Wilson, 1996). Thus, the results that *ATH-3* can

induce *opsin* expression suggest that *ATH-3* may have an independent function in the process of retinal specification within *Pax-6*-expressing regions.

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Fig. 1. Primary structure of *Xenopus* and mouse *ATH-3*.

(A) Nucleotide and deduced amino acid sequences of the *Xenopus ATH-3* cDNA. The putative bHLH domain and the in-frame stop codons in the 5'-noncoding region are underlined. (B) Nucleotide and deduced amino acid sequences of the mouse *ATH-3* cDNA. The putative bHLH domain and the in-frame stop codon in the 5'-noncoding region are underlined.

A

```
                                                                    TAAGGATACAGTTTGAAGAACACATAAAATCCTGCTAAT    -1

MetSerGluMetValAsnValHisGlyTrpMetGluGluAlaLeuSerSerGlnAspGlu      20
ATGTCAGAGATGGTCAATGTGCATGGGTGGATGGAGGAAGCCCTTAGTTCCCAGGATGAG      60

MetLysGluArgAsnGlnSerAlaTyrAspIleIleSerGlyLeuCysHisGluGluArg      40
ATGAAGGAGAGGAATCAGTCTGCCTATGATATCATTTCAGGTCTATGCCATGAGGAAAGG     120

GlySerIleAspGlyGluGluAspGluGluGluGluAspGlyGluLysProLysLys        60
GGCAGCATTGATGGAGAAGAGGATGATGAAGAAGAAGGATGGAGAGAAACCAAAAAAG     180

ArgGlyProLysLysLysLysMetThrLysAlaArgValGluArgPheArgValArgArg      80
AGGGGACCCAAAAAAAAGAAGATGACCAAGGCTAGAGTGGAGAGGTTCCGTGTCCGTAGA     240

ValLysAlaAsnAlaArgGluArgSerArgMetHisGlyLeuAsnAspAlaLeuGluAsn     100
GTAAAAGCCAATGCCAGGGAGCGTTCAAGAATGCATGGACTTAATGATGCCCTGGAAAAT     300

LeuArgArgValMetProCysTyrSerLysThrGlnLysLeuSerLysIleGluThrLeu     120
TTGAGAAGGGTTATGCCTTGCTATTCCAAAACACAAAAGTTGTCTAAAATTGAGACTCTT     360

ArgLeuAlaArgAsnTyrIleTrpAlaLeuSerAspIleLeuGluGlnGlyGlnAsnAla     140
AGACTGGCCAGAAACTATATATGGGCATTATCTGATATTCTAGAACAAGGTCAAAATGCA     420

GluGlyLysGlyPheLeuGluIleLeuCysLysGlyLeuSerGlnProThrSerAsnLeu     160
GAGGGAAAGGGCTTTCTGGAAATACTCTGCAAAGGTCTTTCTCAGCCAACAAGCAACTTA     480

ValAlaGlyCysLeuGlnLeuGlyProGlnAlaMetPheLeuAspLysHisGluGluLys     180
GTAGCTGGCTGCTTGCAACTTGGACCTCAGGCCATGTTCTTGGATAAACACGAAGAAAAG     540

SerHisIleCysAspSerSerLeuThrGlyHisThrTyrAsnTyrGlnSerProGlyLeu     200
TCTCATATATGTGATTCCTCTCTTACTGGTCATACTTATAATTACCAGTCCCCAGGACTA     600

ProSerProProTyrGlyAsnIleAspValHisHisLeuHisLeuLysProSerSerPhe     220
CCCAGTCCTCCTTATGGTAACATTGATGTTCACCACTTGCACTTGAAACCCTCTTCTTTC     660

LysProValMetAspProSerValValThrHisThrLeuAsnCysThrThrProProTyr     240
AAACCAGTAATGGATCCTTCTGTGGTAACCCATACACTTAACTGTACCACTCCACCATAT     720

GluGlyAlaLeuThrProProLeuSerIleGlyGlyAsnPheSerLeuLysGlnAspSer     260
GAAGGAGCTCTAACACCTCCACTCAGCATCGGTGGTAATTTTTCTTTGAAGCAAGATAGT     780

SerProAspMetAspLysSerTyrAlaPheArgSerProTyrProAlaLeuGlyLeuGly     280
TCACCCGATATGGATAAATCATATGCATTCAGGTCCCCCTATCCAGCTCTTGGGCTTGGT     840

GlySerHisGlyHisAlaSerHisPheHisThrSerValProArgTyrGluLeuProIle     300
GGATCTCATGGACATGCGTCACACTTTCATACCAGTGTTCCAAGGTATGAACTACCCATA     900

AspMetAlaTyrGluProTyrProHisHisAlaIlePheThrGlu***                315
GACATGGCTTACGAGCCTTACCCACACCATGCTATATTCACTGAATAAACACTCTACACA     960
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B

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                                        TAGAAGGTAGCTACCAGTTTAACATGGACGACTGAA   -121
AGGGTCTTCTGTTTCCCACGATCTGCCTGGTCAGGTCAGGGTAGAACTGACTGCTCTGAT    -61
AGTTCTTCAGGACACAGATTAGAGTTTAATCTTGGAACTGGACTTCCAGAGAATCTATAC     -1

MetAlaLysMetTyrMetLysSerLysAspMetValGluLeuValAsnThrGlnSerTrp     20
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MetAspLysGlyLeuSerSerGlnAsnGluMetLysGluGlnGluArgArgProGlySer     40
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TyrGlyMetLeuGlyThrLeuThrGluGluHisAspSerIleGluGluAspGluGluGlu     60
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GluGluAspGlyAspLysProLysArgArgGlyProLysLysLysLysMetThrLysAla     80
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ArgLeuGluArgPheArgAlaArgArgValLysAlaAsnAlaArgGluArgThrArgMet    100
CGCCTTGAAAGATTCAGGGCTCGAAGAGTCAAGGCCAATGCTAGAGAACGGACCCGGATG    300

HisGlyLeuAsnAspAlaLeuAspAsnLeuArgArgValMetProCysTyrSerLysThr    120
CATGGCCTGAATGATGCCTTGGATAATCTTAGGAGAGTCATGCCATGTTACTCTAAAACT    360

GlnLysLeuSerLysIleGluThrLeuArgLeuAlaArgAsnTyrIleTrpAlaLeuSer    140
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GluValLeuGluThrGlyGlnThrLeuGluGlyLysGlyPheValGluMetLeuCysLys    160
GAAGTCCTGGAGACTGGTCAGACACTTGAAGGGAAGGGATTTGTAGAGATGCTATGTAAA    480

GlyLeuSerGlnProThrSerAsnLeuValAlaGlyCysLeuGlnLeuGlyProGlnSer    180
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ThrLeuLeuGluLysHisGluGluLysSerSerIleCysAspSerThrIleSerValHis    200
ACCCTCCTGGAGAAGCATGAGGAAAAATCTTCAATTTGTGACTCTACTATCTCTGTCCAC    600

SerPheAsnTyrGlnSerProGlyLeuProSerProProTyrGlyHisMetGluThrHis    220
AGCTTCAACTATCAGTCTCCAGGGCTCCCCAGCCCTCCTTATGGCCATATGGAAACACAT    660

SerLeuHisLeuLysProGlnProPheLysSerLeuGlyAspSerPheGlySerHisPro    240
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ThrProArgTyrAspValProValAspLeuSerTyrAspSerTyrSerHisHisSerIle    320
ACTCCCCGCTATGATGTTCCTGTAGACCTGAGCTATGATTCCTACTCCCACCATAGCATT    960

GlyThrGlnLeuAsnThrIlePheSerAsp***                               330
GGAACTCAGCTCAATACGATCTTCTCTGATTAGAGCAATAAGATAAGCACCAATATTTCA   1020
```
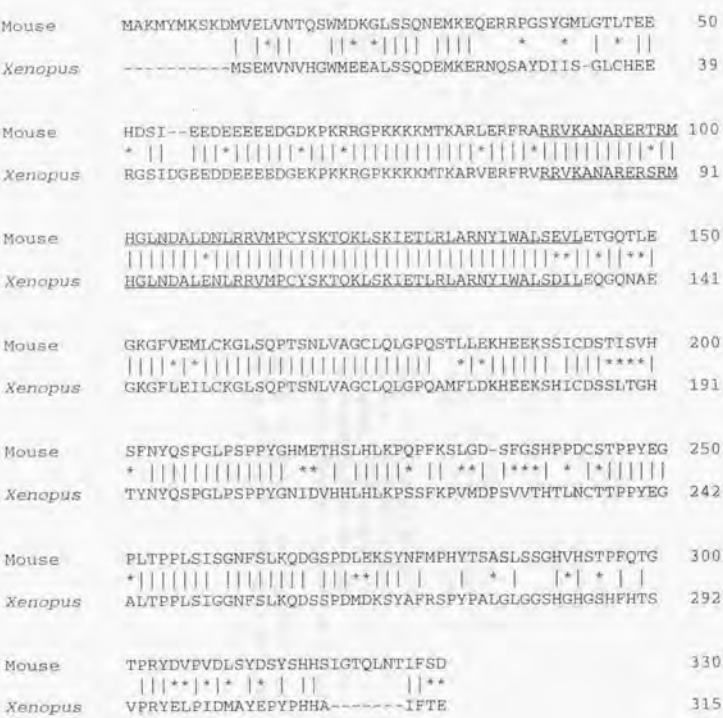

Fig. 2. Structural comparison of *Xenopus* and mouse ATH-3 and other HLH factors.

(A) Amino acid sequence comparison of mouse and *Xenopus* ATH-3. Identical residues between the two are indicated by a bar (|), and conservative changes are shown by an asterisk. The putative bHLH domain is underlined. (B) Sequence comparison of mouse ATH-3 (MATH-3), *Xenopus* ATH-3 (XATH-3), and other HLH factors. The positions of the basic region, helices 1 and 2, and loop are indicated above. The conserved residues in comparison to ATH-3 are shown in bold. The identity (%) to MATH-3 is indicated on the right. Sources for sequences are as follows: mouse MATH-1 (Akazawa *et al.*, 1995); mouse MATH-2 (Bartholomä and Nave, 1994; Shimizu *et al.*, 1995); mouse NeuroD (Lee *et al.*, 1995); mouse NDRF (Yasunami *et al.*, 1996); *Drosophila* Atonal (Jarman *et al.*, 1993); rat Mash-1 (Johnson *et al.*, 1990); rat Mash-2 (Johnson *et al.*, 1990); *Xenopus* Xash-3 (Zimmerman *et al.*, 1993); rat HES-1 (Sasai *et al.*, 1992); human E47 (Murre *et al.*, 1989); mouse MyoD (Davis *et al.*, 1987); mouse Id-1 (Benezra *et al.*, 1990); and human N-myc (Kohl *et al.*, 1986).

A


Mouse MAKMYMKSKDMVELVNTQSWMDKGLSSQNEMKEQERRPGSYGMLGTLTEE 50
Xenopus ----------MSEMVNVHGWMEEALSSQDEMKERNQSAYDIIS-GLCHEE 39
Mouse HDSI--EEDEEEEEDGDKPKRRGPKKKKMTKARLERFRARRVKANARERTRM 100
Xenopus RGSIDGEEDDEEEEDGEKPKKRGPKKKKMTKARVERFRVRRVKANARERSRM 91
Mouse HGLNDALDNLRRVMPCYSKTQKLSKIETLRLARNYIWALSEVLETGQTLE 150
Xenopus HGLNDALENLRRVMPCYSKTQKLSKIETLRLARNYIWALSDILEQGQNAE 141
Mouse GKGFVEMLCKGLSQPTSNLVAGCLQLGPQSTLLEKHEEKSSICDSTISVH 200
Xenopus GKGFLEILCKGLSQPTSNLVAGCLQLGPQAMFLDKHEEKSHICDSSLTGH 191
Mouse SFNYQSPGLPSPPYGHMETHSLHLKPQPFKSLGD-SFGSHPPDCSTPPYEG 250
Xenopus TYNYQSPGLPSPPYGNIDVHHLHLKPSSFKPVMDPSVVTHTLNCTTPPYEG 242
Mouse PLTPPLSISGGNFSLKQDGSPDLEKSYNFMPHYTSASLSSGHVHSTPFQTG 300
Xenopus ALTPPLSIGGGNFSLKQDSSPDMDKSYAFRSPYPALGLGGSHGHGSHFHTS 292
Mouse TPRYDVPVDLSYDSYSHHSIGTQLNTIFSD 330
Xenopus VPRYELPIDMAYEPYPHHA-------IFTE 315

B

	< BASIC >< HELIX-1 >< LOOP >< HELIX-2 >	Identity
MATH-3	RRVKANARERTRMHGLNDALDNLRRVMPCY--SK---TQKLSKIETLRLARNYIWALSEVL	100%
XATH-3	RRVKANARERSRMHGLNDALENLRRVMPCY--SK---TQKLSKIETLRLARNYIWALSDIL	93%
MATH-1	RRLAANARERRRMHGLNHAFDQLRNVIPSF--NN---DKKLSKYETLQMAQIYINALSELL	63%
MATH-2	RRQEANARERNRMHGLNDALDNLRKVVPCY--SK---TQKLSKIETLRLAKNYIWALSEIL	88%
NeuroD	RRMKANARERNRMHGLNAALDNLRKVVPCY--SK---TQKLSKIETLRLAKNYIWALSEIL	88%
NDRF	RRQKANARERNRMHDLNAALDNLRKVVPCY--SK---TQKLSKIETLRLAKNYIWALSEIL	86%
Atonal	RRLAANARERRRMQNLNQAFDRLRQYLPCL--GN---DRQLSKHETLQMAQTYISALGDLL	54%
Mash-1	RR---NERERNRVKLVNLGFATLREHVPNG-AAN----KKMSKVETLRSAVEYIRALQQLL	44%
Mash-2	RR---NERERNRVKLVNLGFQALRQHVPHG-GAN----KKLSKVETLRSAVEYIRALQRLL	45%
Xash-3	RR---NERERNRVKLVNMGFAKLRQHVPQAQGPN----KKMSKVETLRSAVEYIRALQSLL	44%
HES-1	RKSSKPIMEKRRRARINESLSQLKTLILDALKKDSSRHSKLEKADILEMTVKHLRNLQRAQ	19%
E47	RRMANNARERVRVRDINEAFRELGRMCQMHL--K--SDKAQTKLLILQQAVQVILGLEQQV	32%
MyoD	RRKAATMRERRRLSKVNEAFETLKRCTSSNP--N----QRLPKVEILRNAIRYIEGLQALL	39%
Id-1	PALLDEQQVNVLLYDMNGCYSRLKELVPTLP-----QNRKVSKVEILQHVIDYIRDLQLEL	21%
N-myc	RRRNHNILERQRRNDLRSSFLTLRDHVPELV--K---NEKAAKVVILKKATEYVHSLQAEE	30%

Fig. 3. *In situ* hybridization of *Xenopus ATH-3*.

(A) Posterio-dorsal view of a stage 12 embryo. *ATH-3* is weakly expressed in the presumptive neural plate (*arrowheads*). The blastopore is shown by an *arrow*. (B) Posterior view of a stage 14 embryo. *ATH-3* is expressed in three stripes on either side of the midline of the neural plate (*arrowheads*). (C) Anterior view of a stage 18 embryo. Strong *ATH-3* expression occurs in the cranial ganglions (*arrowheads*). Weaker *ATH-3* expression remains in the neural plate. (D) Lateral view of a stage 21 embryo. Anterior is toward the left. *ATH-3* is expressed in the cranial ganglions (*arrowheads*). (E) Lateral view of a stage 33 tadpole. Anterior is toward the left. *ATH-3* is expressed in the forebrain (*arrow*), eye (*arrowhead*), and cranial ganglions. (F) Anterior view of a stage 33 tadpole. A high level of the *ATH-3* transcript is present in the forebrain (*arrow*). Dorsal is toward the top in all figures.

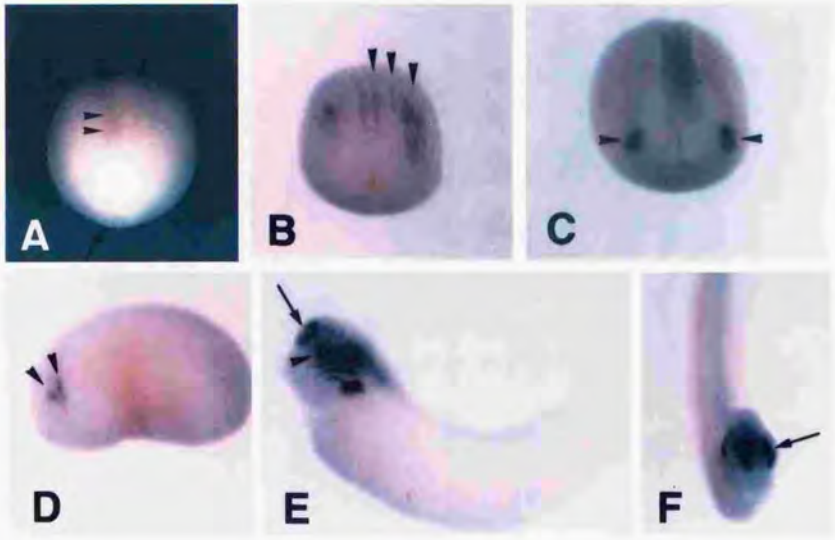

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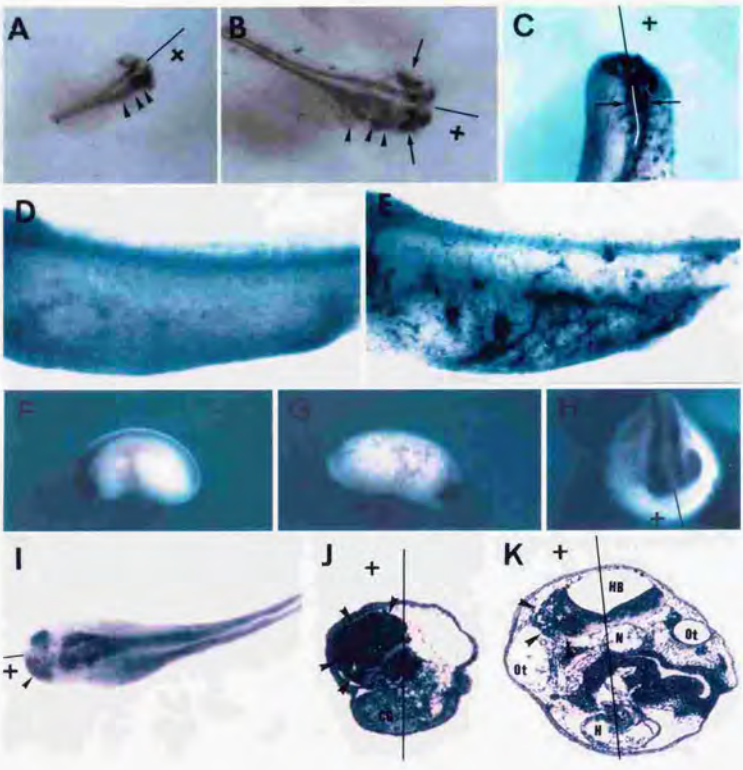
Fig. 4. Hyperplasia of the neural tube and ectopic neural tissues by injection of *ATH-3* RNA.

The *in vitro* synthesized *Xenopus ATH-3* RNA was injected into one cell of two cell-stage embryos (A-C, F-K) or a ventral animal cell of four cell-stage embryos (D,E). Injected embryos were examined at stage 25 (A,C,F-H), stage 30 (B), and the tadpole stage (D,E,I-K). (A-E) Whole-mount immunological staining with NEU-1 monoclonal antibody. Some embryos were cleared to enhance the signals (A,B). (A) Dorsal view. NEU-1-positive CNS (brown) is expanded laterally in the anterior region (*arrowheads*) on the injected side (+). (B) Dorsal view. The anterior CNS is significantly expanded (brown, *arrowheads*) on the injected side (+). The optic cup (*arrow*) is deformed and displaced on the injected side (+). (C) Dorsal view. Expanded CNS and ectopic neurogenesis in the epidermis (blue staining) are observed on the injected side of an embryo treated with HUA. (D) Lateral view of the uninjected side. No apparent NEU-1 staining is detected. (E) Lateral view of the injected side. Ectopic NEU-1 staining (blue) exhibits morphology of neurons with processes. (F-H) Whole-mount in situ hybridization of *N-CAM*. (F) Lateral view of the uninjected side. (G) Lateral view of the injected side. Ectopic *N-CAM* staining is observed in the epidermis. (H) Anterior view. The forebrain is expanded and ectopic neurogenesis occurs in the epidermis on the injected side (+). (I) Dorsal view. The forebrain (*arrowhead*) is expanded on the injected side (+). (J) Frontal section anterior to the eye. The forebrain dramatically expands anteriorly (*arrowheads*) on the injected side (+). (K) Frontal section at the level of

hindbrain. The hindbrain is expanded laterally (*arrowheads*) on the injected side (+). CG, cement gland; H, heart; HB, hindbrain; N, notochord; Ot, otic vesicle.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HB
Ot
Ot
N
N
CB

Fig. 5. Suppression of non-neural tissue development by injection of *ATH-3* RNA.

The *in vitro* synthesized *Xenopus ATH-3* RNA was injected into one cell of two cell-stage embryos and the phenotypes were examined at stages 19 (D-F) and 25 (A-C) and the tadpole stage (G,H). (A-C) *In situ* hybridization of *twist*. (A) Lateral view of the uninjected side. Anterior is toward the left. (B) Lateral view of the injected side. *twist* expression is severely reduced. Anterior is toward the right. (C) Anterior view. On the injected side (+), *twist* expression is reduced or almost absent. (D-F) *In situ* hybridization of *keratin*. (D) Anterior view of an uninjected embryo. *keratin* is expressed outside the neural tube. (E,F) Anterior (E) and dorsal (F) views. *keratin* expression is reduced and the unstained forebrain is expanded on the injected side. In (F), anterior is toward the top. (G,H) Whole-mount immunological staining with anti-myosin monoclonal antibody. (G) Lateral view of the uninjected side. Somites are visible. (H) Lateral view of the injected side. Formation of some somites is blocked (*arrows*). The injected side is confirmed by blue staining with X-gal solution (*arrowheads*).

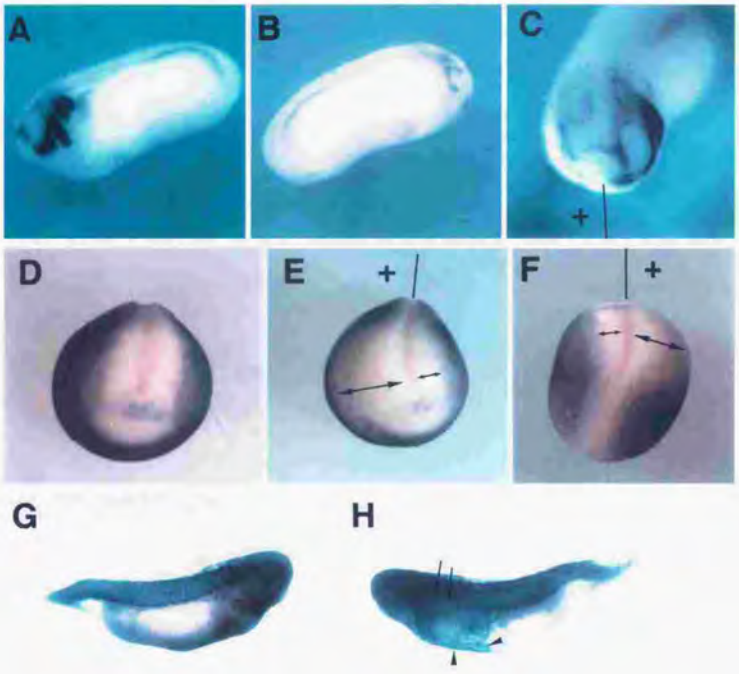
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Fig. 6. *Xenopus* animal cap assay.

The *in vitro* synthesized wild type *Xenopus ATH-3*, S89D or S89N RNA was injected into both cells of two-cell stage *Xenopus* embryos, and subsequently the animal caps were isolated from the injected or uninjected embryos at stage 9. These explants were cultured for an additional day (equivalent to stages 25-30), and then RNA was extracted and subjected to RT-PCR analysis, as indicated on the left. Lane 1, Control RT-PCR experiment using RNA from whole embryos. Lane 2, Uninjected animal caps. Lane 3, *ATH-3* RNA-injected animal caps. Lane 4, S89D RNA-injected animal caps. Lane 5, S89N RNA-injected animal caps. I performed at least two independent experiments and obtained the same results.

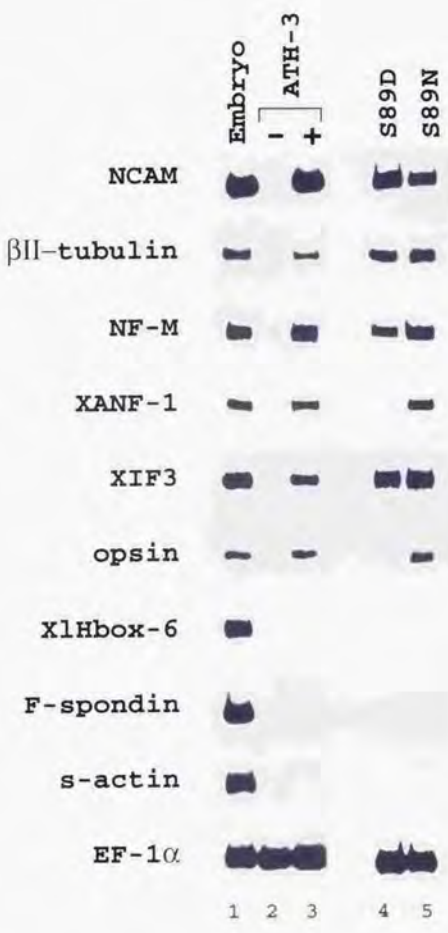
Embryo
ATH-3
-
+
S89D
S89N
NCAM
βII-tubulin
NF-M
XANF-1
XIF3
opsin
XlHbox-6
F-spondin
s-actin
EF-1α
1 2 3 4 5

Table 1. Phenotypes of *ATH-3* injected *Xenopus* embryos*.

Injected amount per embryo	Control injection**	*Xenopus ATH-3* 50 pg	100 pg
Tested embryos	21	36	30
No effect	21 (100%)	1 (3%)	0 (0%)
Expansion of forebrain***	0 (0%)	34 (94%)	25 (83%)
Anterior anomalies****	0 (0%)	1 (3%)	5 (17%)

**ATH-3* RNA was injected into one cell of two-cell stage *Xenopus* embryos, and the phenotypes on the injected side were examined. The numbers and ratios (%) of tested embryos are indicated.
**Globin RNA (100 pg) (Krieg and Melton, 1984) was injected.
***The forebrain was expanded laterally towards the eye region, and the eye became deformed and displaced. No apparent abnormalities were detected in other regions.
****The anterior neural tube was expanded and the adjacent tissues in addition to eyes were severely deformed.

General discussion

Many open questions remain in the system of cell differentiation. I tried to clarify the problems in two systems, muscle and neuron development.

First, I investigated how inductive signals give the information to nuclei and induce the earliest myogenic factor. Therefore I analyzed the expression pattern of myogenic factors and their regulators in *Xenopus* early development. I found that the earliest myogenic factor that is involved in determination step is only both *Xmyf-5* and *XmyoD*, but *Xmyogenin* and *Xmrf4* in *Xenopus*. The expression pattern resembles but is slightly different. My results suggest that "the upstream regulator" of the genes may be different. Both *Xmyf-5* and *XmyoD* are activated by mesoderm inducers, Activin A and bFGF. But Activin A showed stronger activity than bFGF. *Xbra*, which are able to be induced by mesoderm inducers, both Activin A and bFGF, single mRNA injection induced *Xmyf-5* and *XmyoD* expression, therefore it was suggested that *Xbra* is one of candidates for the regulator of *Xmyf-5* and *XmyoD*. However the activity was very weak especially in *Xmyf-5* expression, the other T-box family genes may play a role in the reguration. *Xbra* co-injection with *noggin* that is induced by Activin A but bFGF, caused to express strongly *Xmyf-5* and *XmyoD*. It was reported that Noggin is binding protein against BMP directly, and can suppress the signaling (Zimmerman *et al.*, 1996). The suppression of BMP signaling alone in animal caps induces neuralization. Therefore *Xmyf-5* and *XmyoD* expression may require both suppression of BMP and expression of

Xbra. *noggin* and *chordin*, which was also reported as BMP binding protein (Piccolo *et al.*, 1996) are induced by WNT/β-catenin signaling too (Carnac *et al.*, 1996). Accordingly the real down-regulator of BMP signaling *in vivo* may be maternal Activin/Vg1 and, or maternal WNT/β-catenin signaling. "BMP signal-less signal" target is not unclear. A candidate of direct dorsalizing and myogenic activator is *pintallavis* reported synergizing ability with *Xbra* (O'Reilly *et al.*, 1995). I also tried to isolate genes that directly induce *Xmyf-5* by different approach. Isolated gene, *Xprx-1* was not expressed in axial mesoderm including muscle. And *Xprx-1* mRNA injection caused no effect and did not induce ectopic muscle. Therefore *Xprx-1* is not a candidate for *Xmyf-5* inducer and may not be involved in developing early muscle. These observations are summarized in Fig. 1.

Second, I tried cloning and functional analysis of the expected key molecule in vertebrate neurogenesis and clarify resemblance between the systems of neurogenesis and myogenesis. I isolated a novel basic helix-loop-helix (bHLH) gene homologous to the *Drosophila* proneural gene *atonal*, termed *ATH-3*, from *Xenopus*. *ATH-3* is expressed in the developing nervous system, with high levels of expression in the brain, retina and cranial ganglions. Experiments of injection of *ATH-3* RNA into Xenopus embryos provide evidence that ATH-3 can directly convert non-neural or undetermined cells into a neural fate. It has been proposed that there are at least two separate developmental choices for generation of neurons in vertebrates; first, whether or not to adopt a neural lineage, and second, whether or not to

differentiate as a neuron (Ferreiro *et al.*, 1994; Turner and weintraub, 1994; Lee *et al.*, 1995). The first step involves the initial decision between neural and epidermal fates in the ectoderm by a proneural gene, while the second step is the subsequent process of neuronal differentiation. This model is similar to myogenesis model. The evidence of model is given by discovering the molecules, act on the steps. It has been shown that *Xenopus* bHLH gene *XASH-3* can expand the neural tube at the expense of adjacent non-neural ectoderm and therefore acts as a proneural gene (Ferreiro *et al.*, 1994; Turner and Weintraub, 1994). I showed that *ATH-3* can induce similar effects in *Xenopus* embryos, i.e. expansion of the neural tube and disturbance of development of the surrounding non-neural tissues. These results suggest that, like *XASH-3*, *ATH-3* can function as a proneural gene in *Xenopus* embryos. The early onset of *ATH-3* expression before the neural plate appears is also consistent with the notion that *ATH-3* is a proneural gene. Interestingly, whereas in animal cap assays *XASH-3* can only transiently induce neural gene expressions and requires the neural inducer noggin for stable induction (Ferreiro *et al.*, 1994), *ATH-3* can stably induce neural gene expression without *noggin*. Thus, ATH-3 seems to have a stronger neurogenic activity than XASH-3, or ATH-3 may be less susceptible to inhibitory signals such as Notch and HES-1. Another bHLH gene, *NeuroD*, regulates the terminal differentiation step of neural development. It has been shown that *NeuroD* can induce ectopic neural tissues in the epidermis of *Xenopus* embryos. *ATH-3* also induces ectopic neural tissues in the epidermis, like *NeuroD*, suggesting that ATH-3 and NeuroD

have similar neurogenic functions. Furthermore, *ATH-3* is expressed at a high level in the nervous system during neuronal differentiation stages, suggesting that *ATH-3* can also act as a *NeuroD*-like differentiation gene. Thus, ATH-3 may function in both proneural determination and neuronal differentiation stages in *Xenopus* embryos. Therefore I think that both insect and vertebrate are similar system in the both muscle and neural cell determination and differentiation. These observations are summarized in Fig. 1 too.

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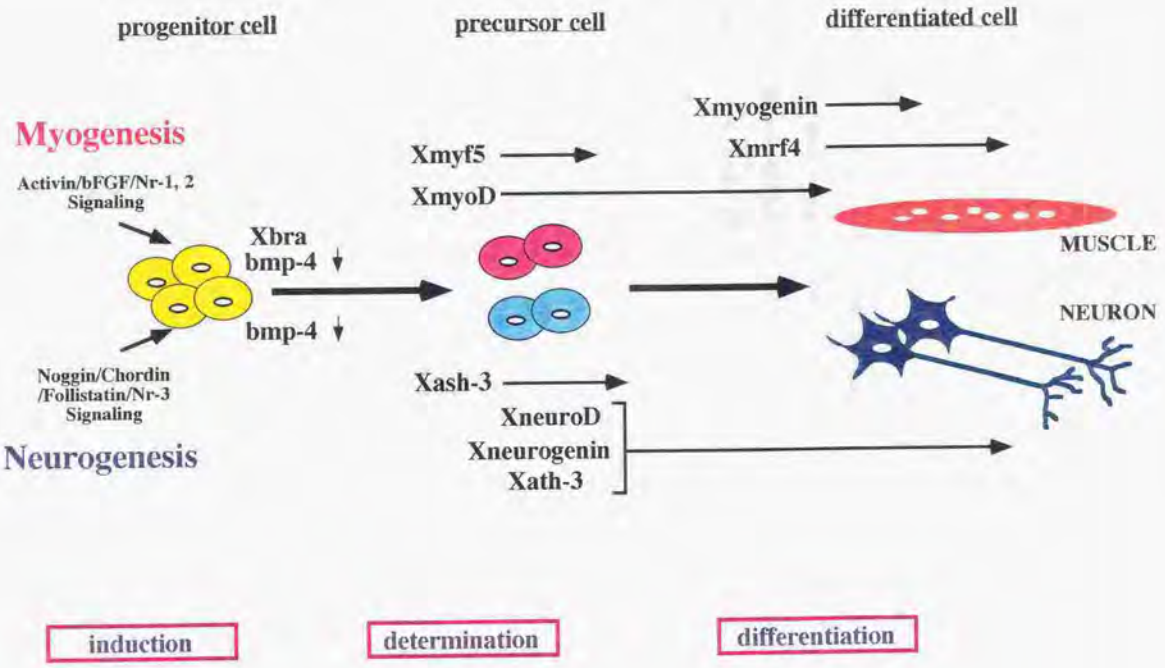
Fig. 1. A model of myogenesis and neurogenesis in *Xenopus* development

The model was constructed by previous data and my results. Slender line with arrow following genes shows the expressing and functional term of the gene.


progenitor cell
precursor cell
differentiated cell
Myogenesis
Activin/bFGF/Nr-1, 2
Signaling
Xbra
bmp-4
bmp-4
Noggin/Chordin
/Follistatin/Nr-3
Signaling
Neurogenesis
Xmyf5
XmyoD
Xash-3
XneuroD
Xneurogenin
Xath-3
Xmyogenin
Xmrf4
MUSCLE
NEURON
induction
determination
differentiation

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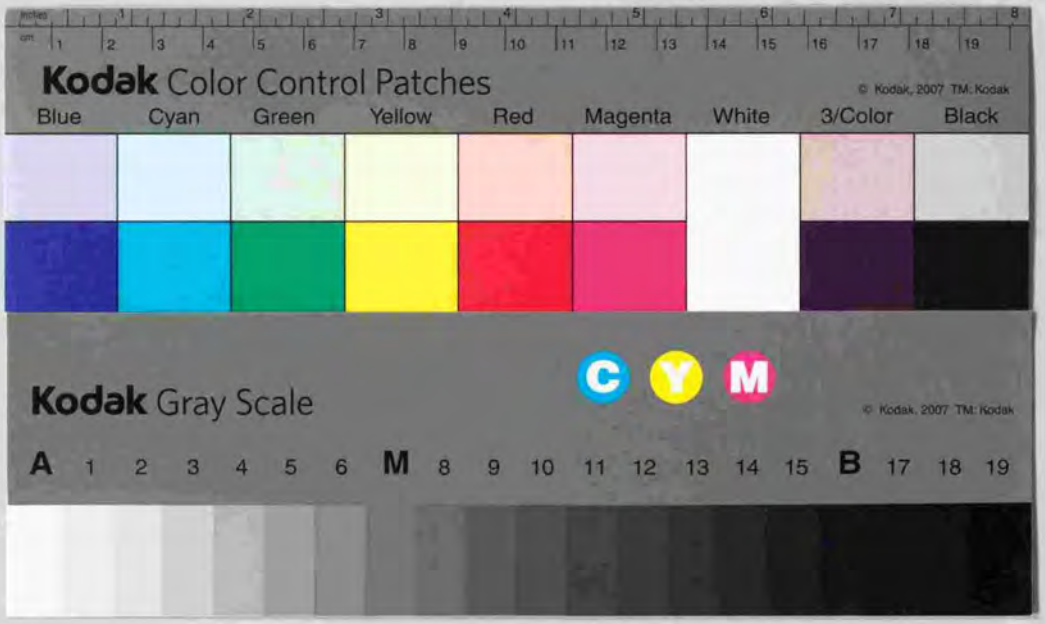
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A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19