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Regulation of the *Xmyf-5* and *XmyoD* Expression Pattern during Early *Xenopus* Development

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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 we 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 the 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

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(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, 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 guanidinium 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* (Krieg *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 Drs. W. C. Smith and R. M. Harland (Smith and Harland, 1992). 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).

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 re-

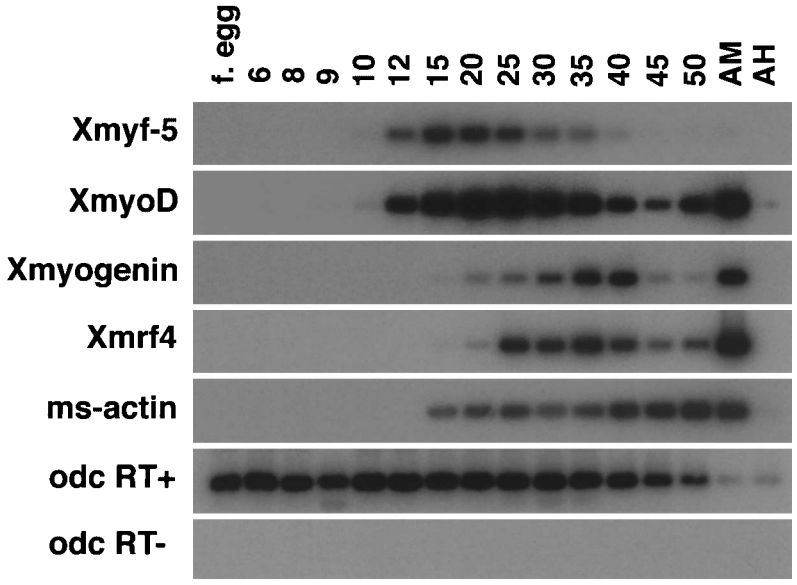

Fig. 1. Temporal expression of four myogenic factors. Total RNA isolated from embryos at the indicated stage of development (f. egg, st. 6 - 50) and from adult leg muscle (AM) and adult heart (AH) was analyzed by RT-PCR for levels of expression of myogenic factors and *odc* RT+, which served as a loading control. *odc* RT- is an internal negative control. Only *Xmyf-5* and *XmyoD* were expressed at the muscle-determination stage.

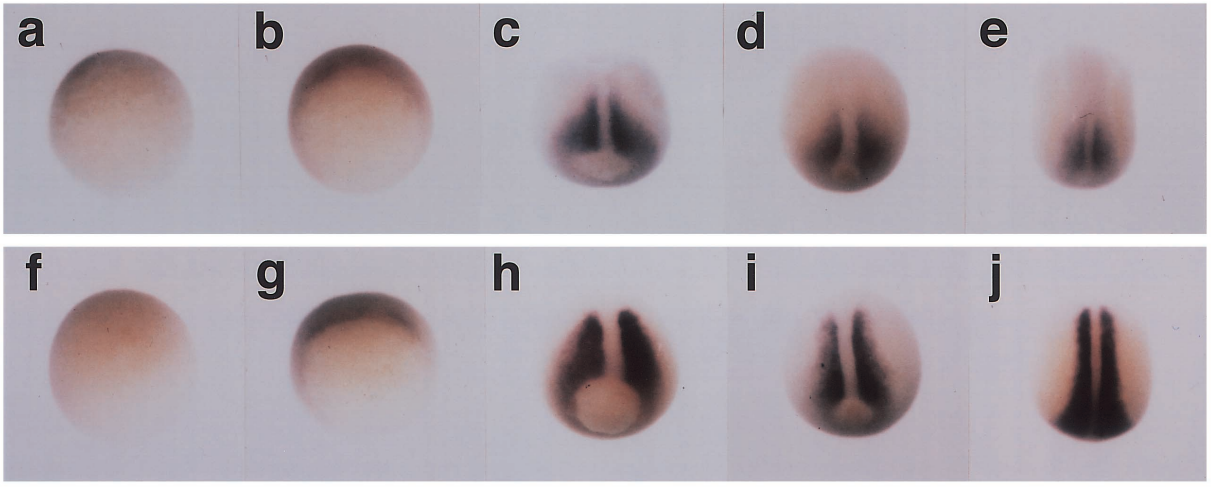

Fig. 2. Spatial distribution of *Xmyf-5* and *XmyoD*. Whole-mount *in situ* hybridization showed different expression of *Xmyf-5* (**a**–**e**) and *XmyoD* (**f**–**j**) at the determination stage [(**a**,**f**) st.9, (**b**,**g**) st.10, (**c**,**h**) st.11, (**d**,**i**) st.12, (**e**,**j**) st.15].

ported 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. We 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. We showed that bFGF and Activin A can induce *Xmyf-5* in animal caps, similar to *XmyoD*. We 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). We 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*.

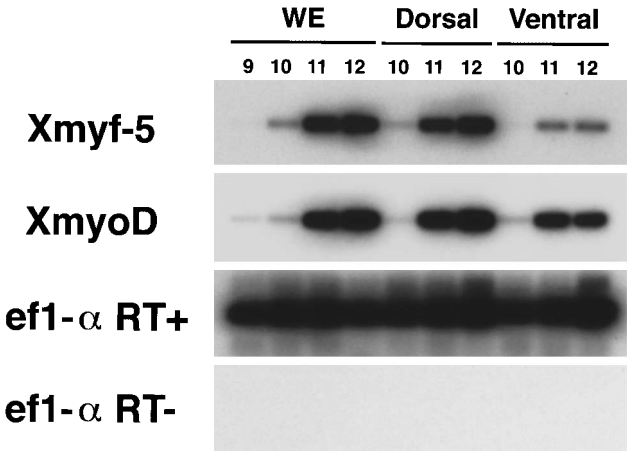

Fig. 3. Activation of *Xmyf-5* and *XmyoD* by the dorsalizing signal. The embryos were divided in two, a dorsal half and a ventral half, at st.10 and cultured until the sampling stage at which sibling embryos developed (WE: whole embryos), and then were analyzed by RT-PCR. *ef1-α* RT+ is a loading control, and *ef1-α* RT- is an internal negative control.

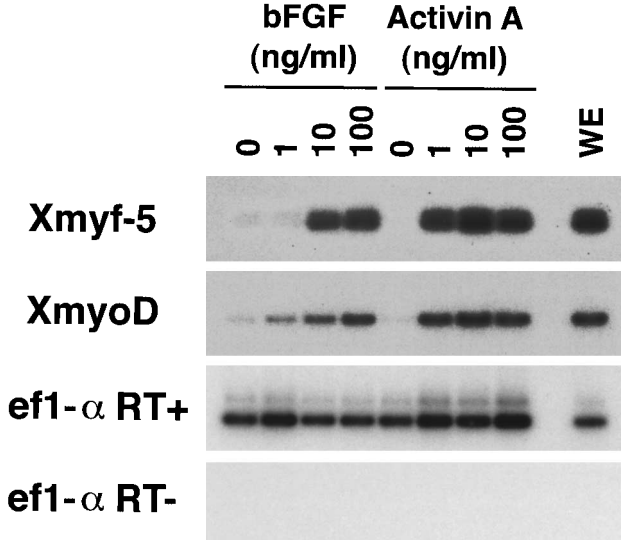

Fig. 4. bFGF and Activin A induced *Xmyf-5* and *XmyoD*. Animal caps were dissected at stage 9 and cultured in the presence and absence of growth factors for 6 hr, and then were analyzed by RT-PCR. *ef1-α* RT+ is a loading control, and *ef1-α* RT- is an internal negative control.

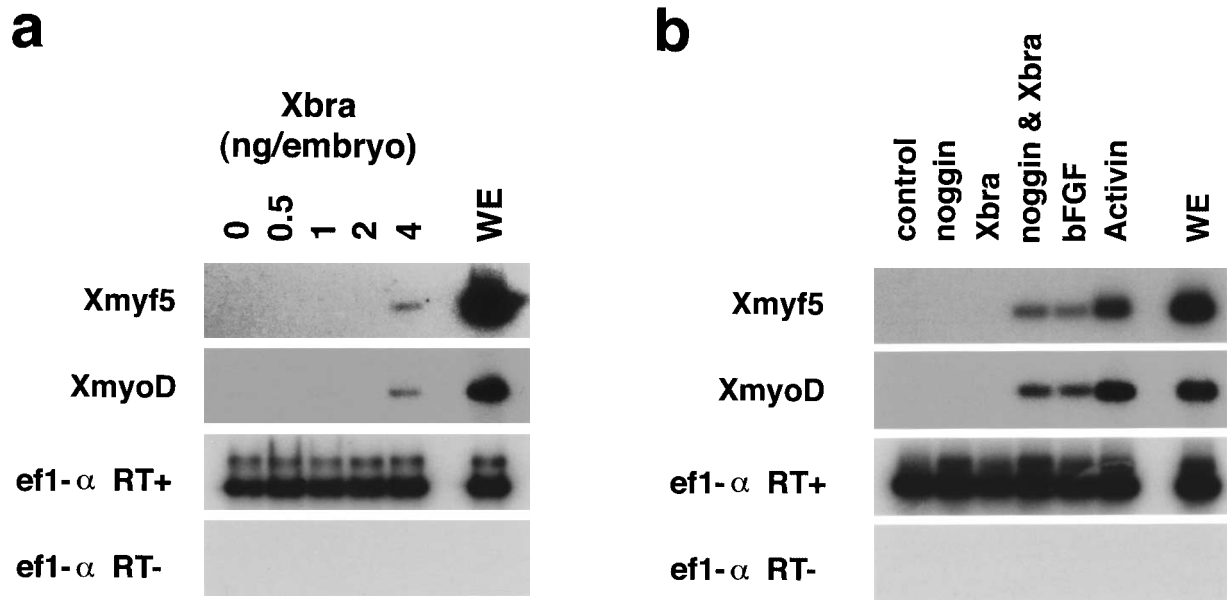

Fig. 5. *Xbra* induced *Xmyf-5* and *XmyoD*, and acts in synergy with *noggin*. Synthetic mRNA was injected at the 2-cell stage, and animal caps were isolated at stage 10 and analyzed by RT-PCR. *ef1-α* RT+ served as a loading control, and *ef1-α* RT- is an internal negative control. (**a**) High-dose *Xbra* alone weakly induced both *Xmyf-5* and *XmyoD*. (**b**) Co-injection of low doses of *Xbra* and *noggin* induced both genes.

DISCUSSION

Four myogenic factors that contain a bHLH domain are expressed sequentially and play an important role in determination and differentiation of muscle. We 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 differentiation and may not function in muscle determination. We 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, 1929; 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). We 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 the 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).

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