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Original Article

Xtbx6r, a novel T-box gene expressed in the paraxial mesoderm, has anterior neural-inducing activity

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ABSTRACT T-box proteins are important transcriptional regulators in animal development. We searched the *Xenopus laevis* expressed sequence tag (EST) database using zebrafish *tbx24 (Ztbx24)* as a query and found a sequence. We then obtained corresponding clones from a neurula cDNA library. This novel gene has a T-box showing 53% homology with *Xenopus laevis tbx6 (Xtbx6)* and 51% with *Ztbx24* at the amino acid level and is relatively close to *Xtbx6* indicated by alignment analysis. *In situ* hybridization showed that it is expressed in the paraxial mesoderm in the caudal region in a very similar manner to *Xtbx6*, with a slight difference in that the former is emphasized more dorsally and has a more restricted distribution along the antero-posterior axis. From these results we named this gene *Xtbx6r* (*tbx6-related*). *Xtbx6r* or *Xtbx6r-En*^{R}, when overexpressed in animal caps, induced anterior neural markers but not mesodermal markers. In contrast, *Xtbx6r-VP16, Xtbx6* and *Ztbx24* induced various mesodermal markers. These results indicate that *Xtbx6r* is a transcriptional repressor and has activity different from that of *Xtbx6* or *Ztbx24*. *Xtbx6r* induced *Otx2, XAG* and *Pax6* in animal caps. This activity differed from that of *Xbra-En*^{R} or *Xtbx6-En*^{R}, suggesting that some differences in biological activity exist among the tested repressor-type T-box genes. Depletion of *Xtbx6r* by antisense morpholino oligo produced curved embryos, but did not affect expressions of *MyoD, Myf5, XWnt8* or *thylacine2*, nor inhibited muscle differentiation or segmentation. The results of knockdown and overexpression experiments suggest that *Xtbx6r* is involved in some morphogenesis in the paraxial mesoderm.

KEY WORDS: *Xenopus laevis, EST, wnt, neural induction, presomitic mesoderm*

Introduction

T-box genes are characterized by a conserved DNA-binding domain called the T-box. These genes constitute a family of transcription factors in animal genomes and are distributed widely in the animal kingdom (reviewed by Smith, 1999). It has been shown that T-box genes play important roles in various aspects of development, such as germ layer formation, specification and organogenesis. For instance, the founder member of this family, *brachyury*, is necessary for posterior mesoderm formation and axis development (Herrmann *et al*., 1990; Schulte-Merker *et al*., 1992; Halpern *et al*., 1993; Conlon *et al*., 1995; 1996). *VegT*, which is localized in vegetal regions in the frog egg, specifies endoderm (Zhang *et al*., 1996; 1998). When its expression level is diminished by an injection of antisense oligonucleotide, vegetal cells lose their ability to induce mesoderm, causing shifts in the geometry of the germ layers (Zhang *et al*., 1998).

The Tbx group of genes within the T-box family have been linked to human genetic disorders (Bollag *et al*., 1994; Agulnik *et al*., 1996; Gibson-Brown *et al*., 1996; reviewed by Packham and Brook, 2003). *Tbx1* is suggested to be responsible for DiGeorge syndrome, mutations in *Tbx3* cause ulnar-mammary syndrome and mutations in *Tbx5* lead to Holt-Oram syndrome (reviewed by Packham and Brook, 2003). Mouse *tbx6* is expressed first in the primitive streak and then spreads to the paraxial mesoderm and is retained in the presomitic mesoderm and tailbud mesenchyme (Chapman *et al*., 1996). *tbx6*-knockout mice lack somites but instead have two neural tube-like structures adjacent to the notochord and their tailbuds are enlarged with mesodermal cells (Chapman and Papaioannou, 1998). *As-T2*, a tunicate homolog of *tbx6*, induces muscle actin and myosin in non-muscle cells when overexpressed ectopically (Mitani *et al*., 1999). Injection of antisense morpholino oligos of *Ciona tbx6b* and *tbx6c* inhibits initiation of muscle-specific gene expression (Yagi *et al*., 2005). We previously cloned *Xenopus laevis tbx6* (*Xtbx6*) and showed

Abbreviations used in this paper: EST, expressed sequence tag; MO, morpholino oligo; *Xtbx6r, Xenopus* tbx6-related gene.

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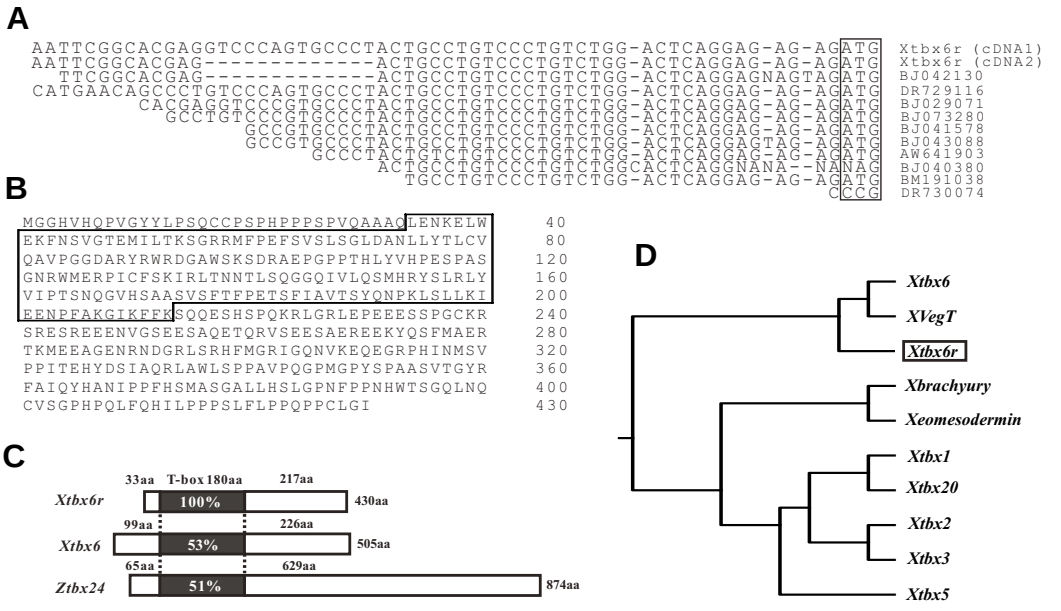


Fig. 1. Structure of *Xtbx6r*. (A) *Comparison of 5' end sequences among the two* Xtbx6r *cDNAs and 10 EST clones. A box displays the putative initiator ATG.* **(B)** *Deduced amino acid sequence of* Xtbx6r. *A box indicates the T-domain.* **(C)** *Protein alignment among* Xtbx6r, Xtbx6 *and* Ztbx24 *is shown.* **(D)** *A phylogenetic tree among the T-box genes. Alignment analysis was performed using only the T-domain. Amino acid sequences were aligned by clustalW method in the GenomeNet (Kyoto University Bioinformatics Center, http://www.geneme.ad.jp). X:* Xenopus laevis.

that it induces ventral mesodermal tissues in animal cap explants when overexpressed alone, but when co-expressed with the dorsalizing factor noggin, it induces muscle (Uchiyama *et al*., 2001). These results indicate that *tbx6* is implicated in somite and mesoderm differentiation. In a hypomorphic allele of mouse *tbx6*, the rv mutant, segmentation of the somites becomes aberrant, resulting in partial fusion of the ribs (Beckers *et al*., 2000; Watabe-Rudolph *et al*., 2002). Also the zebrafish *fused somite* (*fss*) mutant, in which segmentation is inhibited, is caused by *Ztbx24* (Nikaido *et al*., 2002), a gene that is closely related to the *tbx6* subfamily.

Neural induction has long been a subject of study in embryology. Experiments using mainly *Xenopus* have shown that noggin and chordin, antagonists of bone morphogenetic protein (BMP), bind to BMP and impair its association with its receptor BMPR, thereby eliciting neural differentiation (Zimmerman *et al*., 1996; reviewed by De Robertis *et al*., 2000). *Fibroblast growth factor* (*FGF*) and *insulin-like growth factor* (*IGF*) also have neural-inducing activity (Hongo *et al*., 1999; Hardcastle *et al*., 2000; Pera *et al*., 2001; Richard-Parpaillon *et al*., 2002). FGF or IGF signaling causes phosphorylation of the linker region of smad1 via the MEK/ERK pathway and inhibits translocation of smad1 to the nucleus, resulting in neural induction whereas BMP antagonists inhibit phosphorylation of the C-terminus of smad1 via inhibition of BMPR activation (Kretzschmar *et al*., 1997; Pera *et al*., 2003).

In addition, there is growing evidence that T-box genes are also implicated in neural differentiation. *Xtbx2* and *Xtbx3/ET* are first expressed in the anterior neural plate and become localized later in the dorsal retina of the tadpole (Li *et al*., 1997; Hayata *et al*., 1999). Overexpression of these genes in the eye field causes defects in the ventral retina (Takabatake *et al*., 2002). *Eomesodermin* is first expressed in the dorsal mesoderm, but also expressed later in the telencephalon of the tadpole and is suggested to be involved in neural differentiation (Ryan *et al*., 1996; 1998). *Xbra3*, which is expressed in the notochord in a manner similar to its close relative *Xbra*, has neural-inducing activity independent of its mesoderm-inducing activity (Hayata *et al*., 1999; Strong *et al*., 2000). This neural induction is caused by some unknown secretory factor(s) that is distinct from BMP-antagonist or Wnt-antagonist (Hartman *et al*., 2002). *B304/B306* are forms of *Xbra* that has been artificially truncated at amino acid positions 304 and 306, respectively, have neural-inducing activity and induce neural tube and eyes in animal cap explants (Rao,

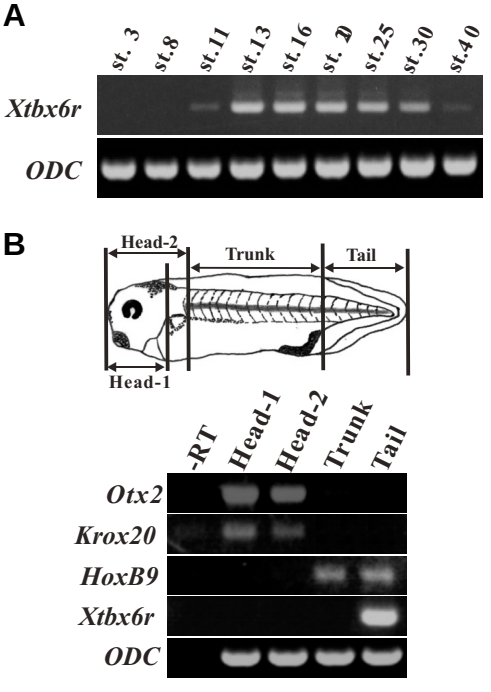


Fig. 2. Expression profile of *Xtbx6r*. (A) *Temporal expression of* Xtbx6r *as revealed by RT-PCR at indicated stages. Ornithine decarboxylase (ODC) was used as loading controls.* **(B)** *Spatial expression of* Xtbx6r *was examined by RT-PCR at st.33. Embryos were dissected into four fragments (head-1, head-2, trunk, tail) as shown and analyzed by RT-PCR for expression of the indicated genes.*

1994). Thus, T-box genes have been implicated in the development of all three germ layers. In the present study, we searched for a novel T-box gene in *Xenopus laevis* and found one (named *Xtbx6r*) that is expressed in the paraxial mesoderm. Knockdown of *Xtbx6r* function using an antisense morpholino oligo (MO) caused curved embryos, but did not affect muscle differentiation nor somitic segmentation. However it was demonstrated to have unique anterior neural-inducing activity when overexpressed in animal cap explants.

Results

Cloning of Xtbx6r

We performed a BLAST search using the T-box of *Ztbx24* as a query against the *Xenopus laevis* EST database (DNA data bank of Japan, http://www.ddbj.nig.ac.jp/ and Sanger institute, http://www.sanger.ac.uk/) and found a sequence encoding a novel T-box. We carried out 3' RACE based on this sequence and obtained a DNA fragment of approximately 1.30kb. Using this PCR product as a probe, we acquired four cDNA clones from a neurula cDNA library. The sequences of the four clones from the 5' end are shown in Fig.1A. All the sequences were completely identical, except for a stretch of 13 bases that were present in two clones and absent in the other two. We further searched the EST database using this 5' sequence as a query and found 10 entries (Fig.1A). The 5' termini of all the 10 sequences were found within this region and none had upstream stretches. Therefore, we think that the four cDNA clones are of a full length and that the first ATG sequence located at position 60 is the initiator methionine. The isolated clones have an open reading frame of 430 amino acids (Fig.1B). A BLAST search showed that this T-box protein is most closely–related to *Xenopus laevis tbx6* (*Xtbx6*), with 53% identity within the T-box at the amino acid level and is also related to *Ztbx24* with 51% identity (Fig.1C). We have not found homologues of this gene in other species. Since phylogenetic analysis showed that *Xtbx6r* is relatively close to *Xtbx6* (Fig.1D) and the expression pattern of the gene was similar to that of *Xtbx6*, as described later, we named it *tbx6-related* (*Xtbx6r*).

Temporal and spatial expression pattern of Xtbx6r

The temporal expression pattern of *Xtbx6r* was then examined by RT-PCR. Maternal expression of *Xtbx6r* was not detected and zygotic expression was first detected at st.11. The expression reached a maximal level at the neurula stage and decreased thereafter until st.40 (Fig.2A).

Xtbx6r spatial expression pattern was analyzed by *in situ* hybridization. At the late gastrula stage, *Xtbx6r* expression was first detected in the dorsolateral region around the blastopore (Fig.3A). At the neurula and tailbud stages, *Xtbx6r* expression persisted in the posterior paraxial region (Fig.3B), localizing near to, but not at the very tip of the tail (Fig.3C, D) in a characteristic U-shaped pattern when viewed from the dorsal side. Transverse sections showed that *Xtbx6r* was expressed in the paraxial mesoderm, but not in the axial mesoderm, lateral plate or ventral mesoderm (Fig.3E). Parasagittal sections revealed that the expression was localized to the presomitic mesoderm (Fig.3F). We also detected signals in the head region (Fig.3C, D, F). However, our methodology also detected similar signals with a sense probe. Therefore embryos were dissected into four fragments (head-1, head-2, trunk, tail) as shown in the diagram and analyzed by RT-PCR (Fig.2B). We concluded that the signal in the head region was non-specific. We compared the expression of *Xtbx6r* with *Xtbx6* by double *in situ* hybridization. At all stages examined, the anterior border of *Xtbx6* expression was slightly more anteriorly spreading than that of *Xtbx6r* expression (Fig.4B, C) and the former extended more ventrolaterally (Fig.4A-D). At the neurula stage, expression of *Xtbx6* extended more posteriorly (Fig.4A).

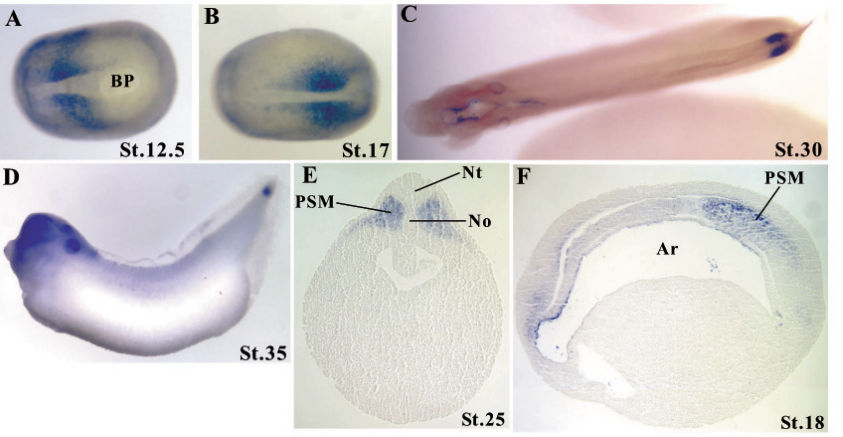


Fig. 3. Spatial expression pattern of *Xtbx6r* revealed by *in situ* hybridization. (A-C) *Dorsal view.* **(D)** *Lateral view. (A-D) Anterior is to the left.* **(E)** *Transverse sections, dorsal is to the top.* **(F)** *Parasagittal sections, anterior is to the left. Ar, archenteron; Bc, blastocoel; Bp, blastopore; No, notochord; Nt, neural tube; PSM, presomitic mesoderm.*

Overexpression of Xtbx6r *inhibits gastrulation movement and elongation of animal caps caused by activin treatment*

The biological activity of Xtbx6r was then examined by overexpression experiments. Dorsal overexpression of *Xtbx6r* inhibited

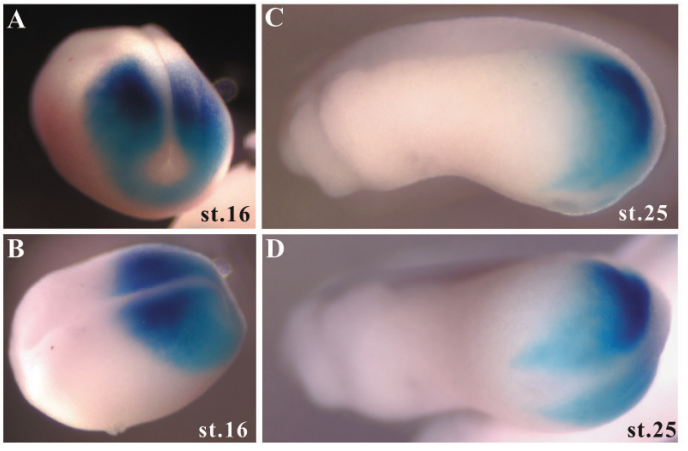


Fig. 4. Comparison of expression of *Xtbx6r* and *Xtbx6* by double in situ *hybridization*. (A-D) Xtbx6r *expression was detected with MagentaPhos, while* Xtbx6 *expression (turquoise) was detected with BCIP. Since the area of* Xtbx6r *expression was completely included in the* Xtbx6 *area, there is no magenta domain and the overlap is stained in dark blue.* **(A)** *Posterior view.* **(B)** *Dorsolateral view.* **(C)** *Lateral view.* **(D)** *Ventrolateral view.*

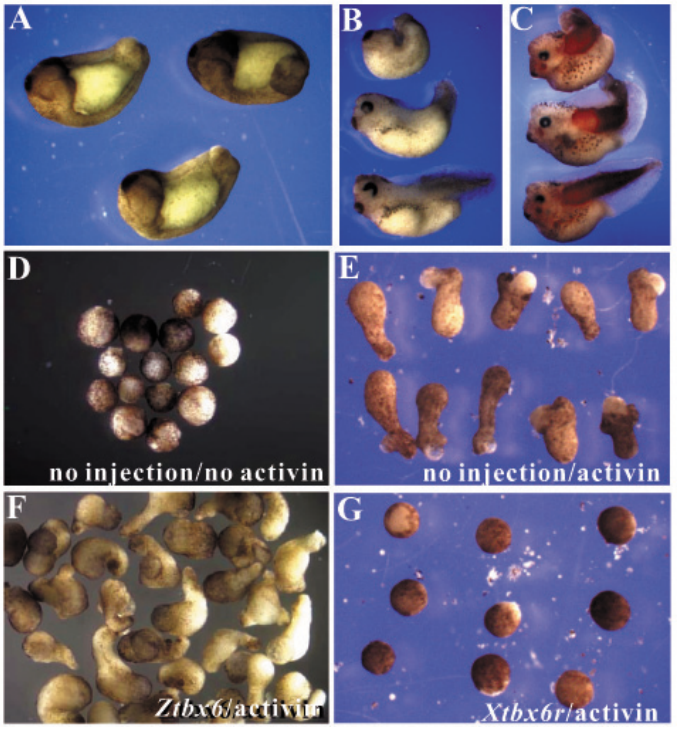


Fig. 5 (Left). Overexpression of *Xtbx6r* inhibits convergent extension movements. (A-C) Xtbx6r *mRNA (200 pg/embryo) was injected into two dorsal blastomeres of 4-cell stage embryos.* **(A)** *st.24,* **(B)** *st.35,* **(C)** *st.38, stained with 12/101 monoclonal antibody.* **(D-G)** *Effect of Xtbx6r on convergent extension elicited by activin. mRNA doses were* Xtbx6r*: 150 pg,* Ztbx6*: 400 pg and the activin concentration was 2.0 ng/ml. Explants were photographed at st.19.*

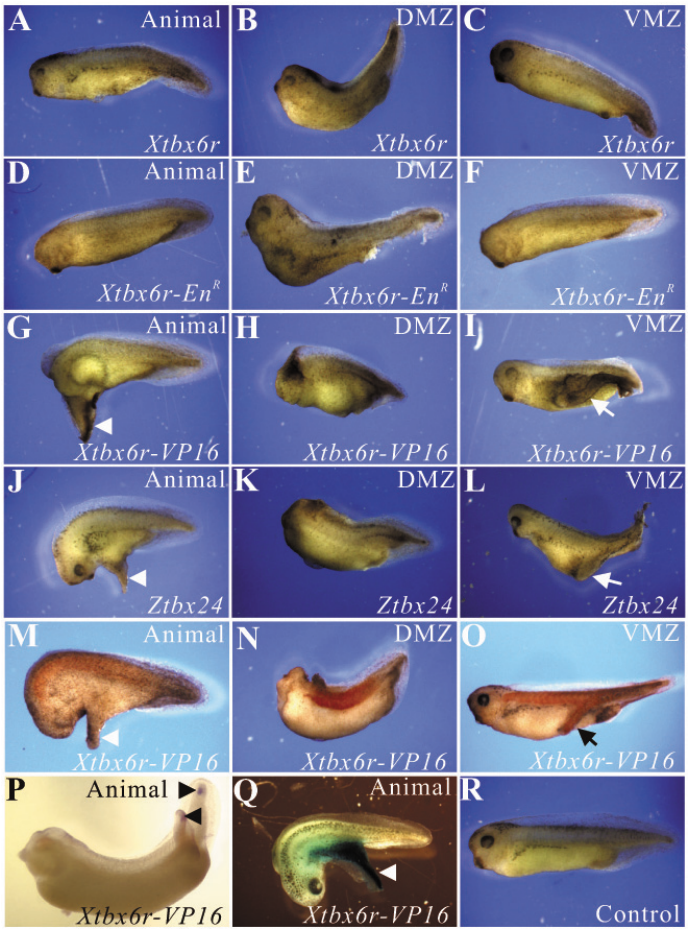


Fig. 6 (Right). The effect of overexpression of *Xtbx6r* and its derivatives or *Ztbx24* on development. (A-Q) *mRNA was injected into the animal (Animal), dorsal (DMZ), or ventral (VMZ) region.* **(M-O)** *Immunohistochemistry with 12/101 monoclonal antibody.* **(P)** Xtbx6 *expression was detected by whole-mount* in situ *hybridization (black arrowheads).* **(Q)** *Embryos were injected with* Xtbx6r-VP16 *mRNA and* nlacZ *mRNA and stained with X-Gal. White arrowhead indicates ectopic tail-like structures. Black arrow displays the secondary axis.*

convergent extension morphogenesis and caused gastrulation defects. When 200 pg of *Xtbx6r* mRNA was injected dorsally, 92% (n=73) of embryos showed gastrulation defects (Fig.5A). Similarly 100 pg or 25 pg mRNA produced defective embryos in 70% (n=75) or 21% (n=55) of cases, respectively, showing that the gastrulation defects are dose-dependent. The resulting embryos were dorsally curved and shortened (Fig.5B). Muscle differentiation in these embryos was not inhibited, as revealed by immunohistochemistry using 12/101 monoclonal antibody (Fig.5C). We examined whether overexpression of *Xtbx6r* inhibited the elongation of animal caps induced by activin (Fig.5D, E) and found that it did (Fig.5G). Although *Xtbx6* has been reported to inhibit this morphogenetic property (Hamaguchi *et al.*, 2004), *Ztbx6* did not (Fig.5F). These results indicate that inhibition of elongation in activin-treated animal caps by *Xtbx6r* is not a common property of T-box genes.

Xtbx6r *behaves as a transcriptional repressor*

To determine whether *Xtbx6r* acts as a transcriptional activator or a repressor, we fused *Xtbx6r* to the *VP16* activation domain (Friedman *et al.*, 1988) or the repressor domain of *Drosophila Engrailed* (*En*^{R}) (Han and Manley, 1993). As already described, dorsal *Xtbx6r* overexpression caused gastrulation defects (Fig.6B), while animal or ventral *Xtbx6r* overexpression had little effect (Fig.6A, C). *Xtbx6r* overexpression gave similar results to *Xtbx6r-En*^{R} overexpression (Fig.6D-F). In contrast, dorsal overexpression of *Xtbx6r-VP16* produced acephalic embryos lacking both eyes and cement glands (Fig.6H). *Xtbx6r-VP16* induced the secondary axis containing skeletal muscle when overexpressed ventrally (Fig.6I, O). Animal *Xtbx6r-VP16* overexpression resulted in ectopic tail-like structures (Fig.6G). These structures were devoid of skeletal muscle (Fig.6M) but expressed *Xtbx6* at their tips (Fig.6P), indicating that the ectopic tail-like structures have tail identity. X-Gal-stained cells were detected in these ectopic tail-like structures using *nLacZ* mRNA as a lineage tracer (Fig.6Q), showing that the structures were formed by the *Xtbx6r-VP16* injected cells. The morphological changes caused by *Xtbx6r-VP16* overexpression were similar to those embryos with *Ztbx24* overexpression.

Xtbx6r *overexpression induces anterior neuroectodermal markers in animal caps*

The biological activity of *Xtbx6r* was next examined in animal caps. *Xtbx6r*-injected caps formed cement glands (45% of cases,

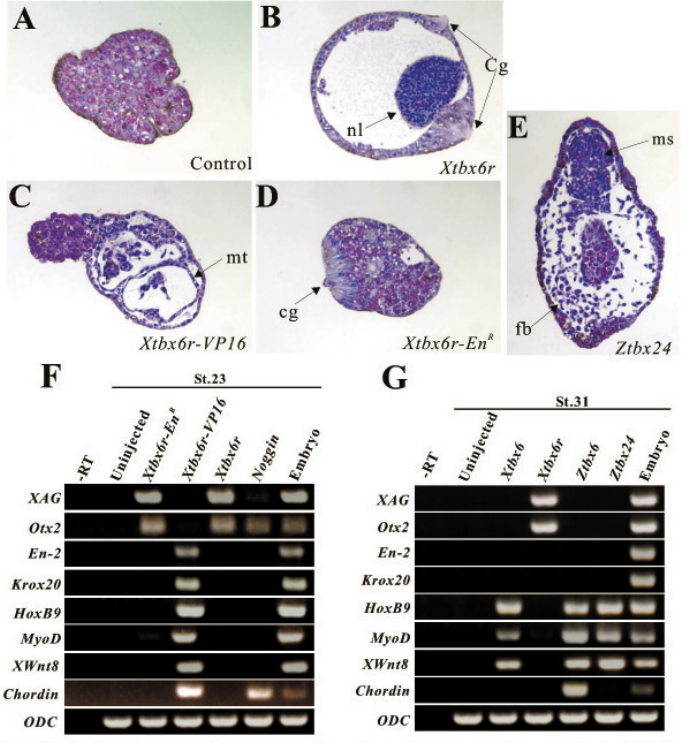
n=60) and – on rare occasions (2%, n=60) – neural-like tissues characterized by high cell density and nuclei that were well stained with hematoxylin (Fig.7B). *Xtbx6r-En*^{R}-injected caps also formed cement glands (Fig.7D). On the other hand, *Xtbx6r-VP16*-injected caps differentiated into mesodermal tissues such as mesothelium (Fig.7C). *Ztbx24*-injected caps differentiated into fibroblasts or muscle tissue (Fig.7E). Examination of animal caps by RT-PCR showed that *Xtbx6r* and *Xtbx6r-En*^{R} induced the cement gland marker *XAG* and forebrain-midbrain marker *Otx2*, but did not induce the more posterior neural markers *En-2*, *Krox20* and *HoxB9*, or mesodermal markers (Fig.7F). In contrast, *Xtbx6r-VP16* induced various mesodermal markers such as *MyoD* and *XWnt8*, but did not induce *XAG* or *Otx2*. *Xtbx6*, *Ztbx6* and *Ztbx24* induced various mesodermal markers but not anterior neural markers (Fig.7G).

The activity of *Xtbx6r* in the animal cap was compared with those of other repressor T-box genes, such as *Xbra-En*^{R} and *Xtbx6-En*^{R}, since we think that *Xtbx6r* acts as a transcriptional repressor (Fig.8). Also compared were *Xbra3* and *B306*, whose neural-inducing activities have been reported previously (Rao 1994; Strong *et al*., 2000; Hartman *et al*., 2002). *Xtbx6r* and *Xtbx6r-En*^{R} induced *XAG*, *Otx2* and *Pax6*. *Xbra-En*^{R} and *Xtbx6-En*^{R} also induced *XAG* and *Otx2*, but not *Pax6*. *Xbra3* induced *HoxB9* and *Pax6* and also mesodermal markers. *B306* had the strongest neural-inducing activity among the genes tested. It induced anterior neural markers, the more posterior neural markers *En2* and *Krox20* and also a pan-neural marker *Sox2*. These results show that the neural-inducing activity of these genes was variable.

Anterior neural markers are mainly induced in animal caps when BMP or zygotic Wnt signaling is inhibited. It was shown previously that elongation of animal caps is induced by co-expression of *Xtbx6* and a BMP antagonist (Uchiyama *et al*., 2001). Therefore we performed co-expression of *Xtbx6* and *Xtbx6r* in animal caps. Co-expression of *Xtbx6* and *noggin* caused strong elongation of the animal cap (Fig.9F), whereas no such elongation was obtained by co-expression of *Xtbx6* and *Xtbx6r* (Fig.9E, G). We then investigated whether *Xtbx6r* overexpression inhibited Wnt signaling and found that when *Xtbx6r* was dorsally overexpressed, only 38% (n=77) of embryos showed normal gastrulation (Fig.9B). In contrast, when *Xtbx6r* and *XWnt8*/pCSKA were injected, the gastrulation defects were cancelled and gastrulation proceeded normally in 93% (n=68) of the embryos (Fig.9C). 54% (n=56) of *Xtbx6* and *Xtbx6r*-coinjected embryos displayed normal gastrulation (data not shown).

Curved embryos occurred by knockdown of Xtbx6r *function*

To study the function of *Xtbx6r in vivo*, loss of function experiments was performed using an antisense MO (Heasman. 2002). An antisense MO targeting exon1-intron1 junction (exint-MO) was designed (Draper *et al*., 2001) based on *Xtbx6r* genomic sequence obtained by screening of a genomic library (Fig.10A). To examine whether exint-MO alter the splicing of *Xtbx6r* mRNA precursor, RT-PCR analysis was carried out (Fig.10B). Two splicing variant mRNAs (long and short) were detected in exint-MO-injected embryos. Long splicing variant mRNA (1228bp) contained complete intron1 sequence, while the short variant (706bp) had a cryptic splice donor site at 189bp downstream of



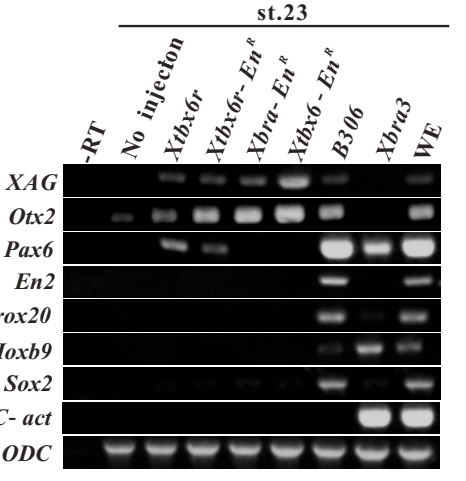


Fig. 7 (Left). *Xtbx6r* induces anterior neuroectodermal markers in animal cap explants. (A-E) *Histological sections of animal cap explants at st.31. Injected mRNA and its doses were:* **(A)** *uninjected control,* **(B)** Xtbx6r *(200 pg),* **(C)** Xtbx6r-VP16 *(200 pg),* **(D)** Xtbx6r-En^R *(200 pg),* **(E)** Ztbx24 *(200 pg). Cg, cement gland; fg, fibroblast; nl, neural-like tissue; ms, muscle; mt, mesothelium.* **(F-G)** *Animal cap explants were analyzed at st.23* **(F)** *and at stage 31* **(G)** *by RT-PCR. Injected mRNA and its doses were: (F)* Xtbx6r *(200 pg),* Xtbx6r-En^R, Xtbx6r-VP16 *and noggin (100 pg, respectively). (G)* Xtbx6, Xtbx6r, Ztbx6 *and* Ztbx24 *(200 pg, respectively).*

Fig. 8 (Right). Comparison of neural inducing activities among T-box genes. *Injected mRNA and its doses are,* Xtbx6r *(200pg),* Xtbx6r-En^R, Xbra-En^R, Xtbx6-En^R *(100 pg, respectively),* B306 *(1.8ng),* Xbra3 *(200 pg).*

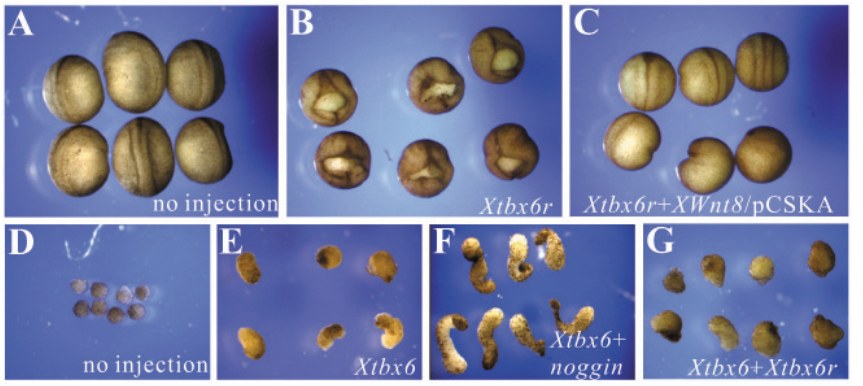


Fig. 9. Relationship between *Xtbx6r* and BMP or Wnt signaling. (A-C) *Indicated genes were overexpressed dorsally.* **(D-G)** *Animal cap explants. Injected mRNA and its doses were:* **(A)** *no injection,* **(B)** Xtbx6r *(50 pg),* **(C)** Xtbx6r *(50 pg) and* XWnt8*/pCSKA plasmid (50 pg),* **(D)** *no injection,* **(E)** Xtbx6 *(200 pg),* **(F)** *co-expression of* Xtbx6 *(200 pg) and* noggin *(100 pg),* **(G)** *co-expression of* Xtbx6 *(200 pg) and* Xtbx6r *(50 pg).*

the original splice donor site as revealed by sequencing analysis. These two splicing variants had several in-frame stop codons at random. Knockdown of *Xtbx6r* function *in vivo* by injecting exint-MO (50ng) produced ventrally curved and slightly shortened embryos (Fig.10D). 12/101 immunohistochemistry showed that muscle differentiation in these embryos was not perturbed (Fig.10E). Expression of several genes in exint-MO-injected embryos were examined by *in situ* hybridization, only to find normal *MyoD*, *Myf5* and *XWnt8* expressions at the neurula and tail bud stages (data not shown). When *nlacZ* mRNA was coinjected with exint-MO, regular nuclear staining of X-gal was observed in the resulting somite at st.35 (Fig.10E), indicating that somitogenesis in these embryos were normal and it was also demonstrated by *in situ* hybridization using antisense *MyoD* or *thylacine2* probe (data not shown). Next we tried to rescue the ventral curved phenotype of exint-MO-injected embryos. Since dorsal *Xtbx6r* overexpression caused gastrulation defects as described earlier, we injected 100 pg of a synthetic mRNA which correspons to the short splicing variant of 706bp from exon1 to 4 of Fig.10B, together with 25ng exint-MO. The result of this coinjection showed that the curved phenotype elicited by exint-MO was rescued and the body length at st.32 restored from 0.3392 ± 0.035mm (mean ± standard deviation, n=35 in exint-MO-injected embryos) to 0.3993 ± 0.0288 mm (n=37 in MO-mRNA-coinjected embryos) (Fig.10F, G).

Discussion

We cloned a novel T-box gene, *tbx6r*, from *Xenopus laevis*. We found no homologous genes in other organisms, but it is possible that a homologue may exist in animals such as *Rana*, axolotl or newt, for which large-scale databases have not yet been created. On the basis of a comparison of 5' upstream sequences among four *Xtbx6r* cDNA clones and ten EST clones, we conclude that they constitute a full length. This is supported by the *Xtbx6r* genomic sequence. Phylogenetic analysis revealed that *Xtbx6r* is relatively close to *Xtbx6* and the anterior border of its expression almost coincides with that of *Xtbx6* and the overall expression patterns of these genes are quite similar (Uchiyama *et al.*, 2001), indicating that the two genes may have diverged from a common ancestor and that their mode of expression regulation may be similar.

Xtbx6r inhibited gastrulation movement when overexpressed in the dorsal side of the embryo, coinciding well with its inhibitory activity on elongation morphogenesis in animal caps that had been treated with activin. Wnt inhibitors also interfere with gastrulation and inhibit convergent extension morphogenesis of animal caps (Xu *et al.*, 1998; Itoh and Sokol, 1999), raising the question of whether *Xtbx6r* has anti-Wnt activity, as will be discussed later. *Xtbx6r* induced *Otx2* in animal caps and *Otx2* can inhibit gastrulation (Andreazzoli *et al.*, 1997), suggesting a further possibility that *Xtbx6r* inhibits gastrulation via *Otx2* expression. However, injection of *Otx2* into the dorsal region causes multiple cement gland formation along with defective gastrulation (Andreazzoli *et*

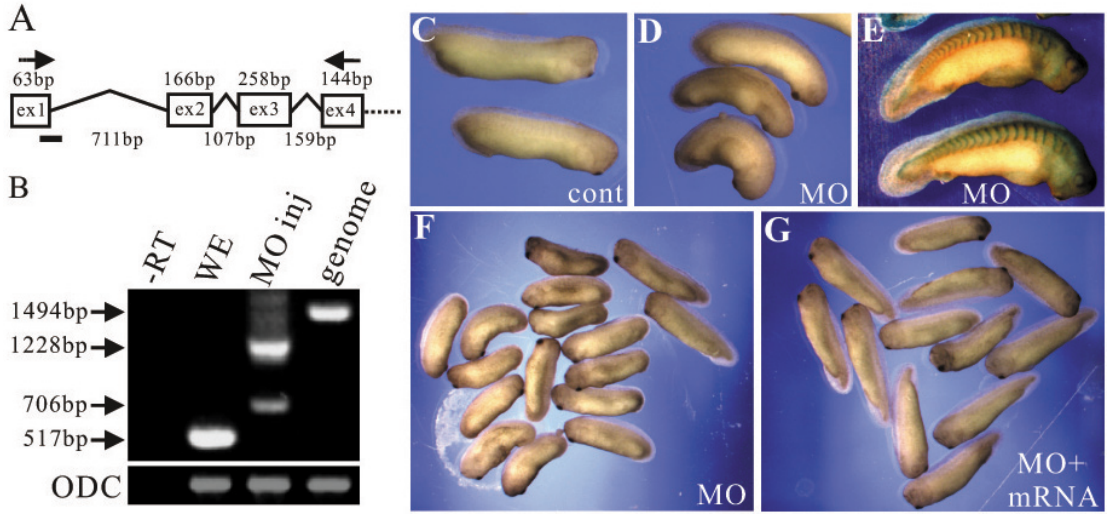


Fig.10. Knockdown experiments using exint-MO. (A) *Partial genomic structure of the* Xtbx6r *gene. Boxes show exons and folded lines display introns. Arrows indicate primers used in (B). Black bar display exint-MO.* **(B)** *RT-PCR analysis of MO-injected embryos. MO was injected into 4 blastomeres at the equatorial region of 4 cell stage embryos (50ng/embryo). WE, whole embryo; MO inj, MO-injected embryos; genome,* Xenopus laevis *genomic DNA.* **(C)** *Control embryos.* **(D)** *MO-injected embryos.* **(E)** *MO and* nlacZ *mRNA-coinjected embryos were stained with X-Gal and carried out immunohistochemistory with 12/101 antibody.* **(F)** *MO (25 ng) injected embryos.* **(G)** *MO (25 ng) and short splicing variant mRNA (100 pg)-coinjected embryos.*

al., 1997), unlike the effect of *Xtbx6r* which does not result in extra cement gland formation. *Xbra* and *XWnt11* are implicated in gastrulation movement (Conlon *et al*., 1996; Conlon and Smith, 1999; Tada and Smith, 2000). However, we found that *Xtbx6r* does not inhibit the expression of *Xbra* or *XWnt11* in activin-treated animal caps (data not shown).

Xtbx6r-VP16 and *Ztbx24* inhibited head differentiation when overexpressed in the dorsal side of the embryo. This may be attributed to *XWnt8* which have posteriorizing activity (Christian and Moon, 1993), since it was shown to be induced by *Xtbx6r-VP16* or *Ztbx24* in the animal cap assay (current study). *Xtbx6r-VP16* and *Ztbx24* induce tail-like structures when overexpressed in the animal region. This phenomenon has also been observed in injection experiments involving *Xbra* (Tada–*et al*., 1997), *Xtbx6* (Yabe, data not shown), or *Xtbx2/3-VP16* (Takabatake *et al*., 2002), suggesting that this activity is common to transcriptional activator-type T-box genes. These results also indicate that *Xtbx6r* and *Ztbx24* have quite opposite effects, suggesting that *Xtbx6r* is not an ortholog of *Ztbx24*.

Anterior neural markers are expressed in the animal cap assay when BMP signaling or zygotic Wnt signaling is inhibited (Glinka *et al*., 1997; 1998; reviewed by Stern, 2005). If BMP signaling is inhibited on the ventral side of the embryo, a secondary axis arises (reviewed by De Robertis, 2000). *Xtbx6r* did not induce a secondary axis when overexpressed in the ventral side, nor did it induce elongation morphogenesis when co-expressed with *Xtbx6* in the animal cap assay, in contrast to *noggin*, which clearly induced elongation when expressed with *Xtbx6*, suggesting that *Xtbx6r* does not inhibit BMP signaling. Overexpression of Wnt-inhibitory factors *in vivo* induces enlargement of head structures and ectopic cement gland formation (Itoh *et al*., 1995; Glinka *et al*., 1997). *Xtbx6r* did not have these activities. However, the inhibition of gastrulation by dorsal overexpression of *Xtbx6r in vivo* was abrogated by co-injection of the plasmid *XWnt8*/pCSKA, suggesting that *Xtbx6r* may weakly antagonize Wnt signaling. Although the mechanism of neural induction by *B304/B306* in animal caps has not been fully elucidated, it is proposed that *B304/B306* inhibits a low level of *Xbra* that is present maternally in the animal cap, thereby inducing nervous tissue (Smith *et al*., 1991; Rao, 1994). In this study we compared the activity of *Xtbx6r*, *Xtbx6r-En*^{R}, *Xbra-En*^{R}, *B306* and *Xbra3* in the animal cap assay. *Otx2* expression was sometimes weakly detectable or undetectable in control animal caps, depending on experimental conditions. However, *Xtbx6r* and *Xtbx6r-En*^{R} increased *Otx2* expression consistently. *Xbra-En*^{R} and *Xtbx6-En*^{R} induced *XAG* and *Otx2*, but not *Pax6*, suggesting that the activity of *Xtbx6r* is not common to all repressor-type T-box genes. Even between *Xbra-En*^{R} and *Xtbx6-En*^{R}, the latter induced *XAG* more potently than the former, indicating a difference in activity. Among the genes tested, only *B306* induced posterior neural markers such as *En2* and *Krox20 and* a pan-neural gene *Sox2*, in addition to the anterior markers. *Xbra-En*^{R} did not induce *Pax6*, *En2*, or *Krox20* and induced *Sox2* only weakly in comparison with *B306*, indicating that the activity of *B306* differs greatly from that of *Xbra-En*^{R}. In our experiment, *Xbra3* had only weak neural-inducing activity, possibly because we used a low dose of 200 pg mRNA per embryo as compared with the previous study (Strong *et al*., 2000). *Xtbx6r-VP16* did not induce anterior neural markers, but rather posterior neural markers such as *En2*, *Krox20*, *HoxB9*. This may well be attributable to the induction of chordin, a neural-inducing factor and also the induction of *XWnt8*, which posteriorizes nervous tissue (Sasai *et al*., 1994; Kiecker and Niehrs, 2001). *HoxB9* is also induced by *Xtbx6* or *Ztbx24*, but this could be attributable to an induced mesodermal component, because *HoxB9* is not a strict neural marker and is also expressed in the mesoderm (Wright *et al*., 1990; Taira *et al*., 1997).

Xtbx6 is expressed widely in tailbud from presomitic to ventral mesoderm, while *Xtbx6r* is expressed only in presomitic mesoderm, suggesting that *Xtbx6r* inhibits expression of some genes activated by *Xtbx6*, thus contributing to the establishment of somite. However, the loss-of-function experiments suggest that *Xtbx6r* is not concerned with somitic differentiation or segmentation. On the basis of *in situ* hybridization, *Xtbx6r* expression was weak especially at early and mid gastrula stages. Therefore, curved phenotypes produced by injection of exint-MO may be due to changes in convergent extension movement of paraxial mesoderm after gastrulation. The phenotype caused by exint-MO was rescued by an artificial splice variant mRNA. We can expect some part of exint-MO to bind to the coinjected mRNA, but if the molar ratio of these two molecules are considered, the rationale for this rescue is not clear. We intend to investigate the *in vivo* function of this gene further, as this aspect is still unclear.

Materials and Methods

Microinjection and animal cap assay

Preparation of *Xenopus laevis* eggs and *in vitro* transcription of mRNA were carried out according to Uchiyama *et al*. (2001). The staging of embryos was according to Nieuwkoop and Faber (1956). For *in vivo* overexpression experiments, mRNA or DNA was injected into both animal blastomeres of 2-cell-stage embryos, or injected into two dorsal or two ventral blastomeres of 4-cell-stage embryos. The injected embryos were cultured in 1X Steinberg's solution containing 2% Ficoll until the desired stages. The animal cap assay was performed as described by Hamaguchi *et al.* (2004). The RNA was extracted from animal caps by using Isogen (Nippon Gene, Tokyo, Japan) and dissolved in DEPC-treated water. Following overnight LiCl precipitation, total RNA was digested with DNase I (TaKaRa, Otsu, Japan), purified with phenol/chloroform, chloroform and precipitated with ethanol. cDNA was synthesized with an oligo-dT primer and Reverscript II (Nippon Gene). Histological analysis was carried out as described by Uchiyama *et al*. (2001).

X-Gal staining

Embryos were fixed in 2% formaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM $Na_2HPO_4.12H_2O$, 2.68 mM KCl, 1.47 mM KH_2PO_4) for 20 min at room temperature (RT) and washed twice for 10 min in PBS. The embryos were then stained at 37ºC in PBS containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 1 mg/ml X-Gal. After staining, the embryos were refixed in 2% formaldehyde for 20 min at RT and stored in 99.5% ethanol.

Immunohistochemistry

Immunohistochemistry was carried out as described by Sive *et al*. (2000).

In situ *hybridization*

A template for *Xtbx6r* was prepared from the *Xtbx6r* 3' RACE product/pBluescript II linearized with *Bam* H I. cRNA probes were transcribed with T3 RNA polymerase (Stratagene, La Jolla, USA) according to Uchiyama *et al*. (2001). *In situ* hybridization and the *Xtbx6* cRNA probe were performed as described by Uchiyama *et al*. (2001). For double *in situ* hybridization, *Xtbx6r* cRNA was labeled with digoxigenin-UTP (Roche,

Basel, Switzerland) and *Xtbx6* cRNA with fluorescein-UTP (Roche). Hybridization with the DIG-labeled and fluorescein-labeled probes was done simultaneously and the embryos were incubated with anti-fluorescein-AP-Fab (Roche) and stained using MagentaPhos (BIOSYNTH, Naperville, USA). After staining, the phosphatase activity was diminished with 10 mM EDTA in MAB (Sive *et al*., 2000) for 30 min at 60ºC. Subsequently, anti-DIG-AP-Fab (Roche) was applied and the chromogenic reaction was performed using BCIP (Roche).

3' RACE and cDNA library screening

For the first PCR, st.25 whole embryonic cDNA was amplified for 20 cycles with a forward primer (5'-TCTTCTCTACACTCTGTGTGTCCAGG-3') and a reverse primer (5'-GCCACGCGTCGACTAGTAGC-3'). The product was amplified again using 1.5 μl of the first PCR reaction mixture included in 50 μl of a reaction and a forward primer (5'-AGAGGGAGATGCCCGTTACC-3') and a reverse primer (5'-GCCACGCGTCGACTAGTAGC-3') for 35 cycles and the product was ligated into a T-vector. The *Eco* RI-*Xho* I fragment from this plasmid was labeled with [^{32}P-dCTP] using a Multiprime DNA-labeling system (Amersham Biosciences, Piscataway, USA). Approximately one million plaques of a Lambda ZAP II (Stratagene) cDNA library derived from stage 22-25 embryos were blotted onto a Biodyne B membrane (PALL, Pensacola, USA), hybridized with the probe in the hybridization solution (17.6% PEG, 1.55X SSPE, 7.25% SDS) and washed in stringent conditions.

Genomic library screening

To generate a template for genomic library screening, PCR products were amplified form *Xtbx6r*/pBluescript I with forward primer (5'-ATGGGTGGGCATGTTCACC-3') and reverse primer (5'-GTGGGAATGACGTACAAGCG-3') and ligated into the T-vector. *Eco* RI-*Xho* I fragment from this plasmid was labeled as described above. Approximately a million plaques of *Xenopus laevis* muscle genomic library (Clontech, CA, USA) were blotted onto Biodyne B membrane, hybridized and washed as described above. DNA fragment from the obtained EMBL3 SP6/T7 clone (# 7-11) was cut with *Sal* I and subcloned into pBluescript II and its DNA sequence was determined by shotgun sequencing (Hitachi Instruments Service, Tokyo, Japan). The clone contained all the exons and introns.

Plasmid constructs

The *Xtbx6r-Bgl* II fragment was PCR-amplified from *Xtbx6r*/pBluescript II with a forward primer (5'-ATGGGTGGGCATGTTCAC-3') and a reverse primer (5'- AGATCTTGATGCCAAGGCAGGGGG-3'). The *Xtbx6r-Eco* RI fragment was PCR-amplified from *Xtbx6r*/pBluescript II with a forward primer (5'-ATGGGTGGGCATGTTCAC-3') and a reverse primer (5'-GAATTCCGATGCCAAGGCAGGGG-3'). To produce *Xbra-En*^{R}, the *Xbra-Eco* RI fragment was PCR-amplified from *Xbra*/pSP73 with a forward primer (5'-TAATGAGTGCGACCGAGAGC-3') and a reverse primer (5'-GAATTCTAAGGGTAGACCAGTTATCATGG-3'). These products were ligated into the T-vector. Subsequently, *Xtbx6r-VP16*/pCS2+, *Xtbx6r-En*^{R}/pCS2+ and–*Xbra-En*^{R}/pCS2+ were constructed according to Uchiyama *et al*. (2001). To obtain *B306*/pCS2+, the PCR product was amplified from *Xbra*/pSP73 with a forward primer (5'-TAATGAGTGCGACCGAGAGC-3') and a reverse primer (5'-AGGCTTAAGAATTATCTGCTAAATTG-3') and subcloned into the T-vector. It was then cut with *Xho* I and *Xba* I and ligated into pCS2+. To construct *noggin*/pCS2+, the *Eco* RI-*Eco* RV fragment of *nogginD5*/pGEM5Zf(-) was subcloned into the *Eco* RI -*Stu* I site of pCS2+. To generate *Ztbx6*/pCS2+, *Ztbx6*/pBluescript II was digested with *Xho* I and *Xba* I and ligated into pCS2+. St.15 whole embryonic cDNA was amplified with a forward primer (5'-CTGTTTCTATGGAGCTGTTGCC-3') and a reverse primer (5'-GCCTATAAGACGTGATAGATGGTGC-3') and ligated into a T-vector to generate MyoD/pBluescript II. All PCR products were sequenced and ascertained to have no mutation.

Xtbx6r/pCS2+, *Xtbx6r*-VP16/pCS2+, *noggin*/pCS2+, *Xtbx6*/pCS2+ (Uchiyama *et al.,* 2001), *Ztbx6*/pCS2+, *Ztbx24*/pCS2+ (Nikaido *et al*., 2002), *B306*/pCS2+ and *Xbra3*/pCS2+ were digested with *Not* I to linearize the templates. *Xtbx6r-En*^{R}/pCS2+, *Xbra-En*^{R}/pCS2+ and *Xtbx6-En*^{R}/pCS2+ (Uchiyama *et al*., 2001) were cut with *Apa* I and blunted with T4 DNA polymerase (New England Biolabs, Beverly, USA).

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