In silico and *in vivo* identification of the intermediate filament vimentin that is downregulated downstream of Brachyury during *Xenopus* embryogenesis

Atsuko Yamada[a, 1], Kanako O. Koyanagi[a], and Hidemi Watanabe[a]*

[a]Graduate School of Information Science and Technology, Hokkaido University, Sapporo, Hokkaido 060-0814, Japan

*Correspondence to Hidemi Watanabe

Graduate School of Information Science and Technology, Hokkaido University, N14 W9, Kita-ku, Sapporo, Hokkaido 060-0814, Japan

Email: watanabe@ist.hokudai.ac.jp

Tel/Fax: 81-11-706-6146

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[1]Present address: Institute of Low Temperature Science, Hokkaido University, N19 W8, Kita-ku, Sapporo, Hokkaido 060-0819, Japan

Abstract

Brachyury, a member of the T-box transcription family, has been suggested to be essential for morphogenetic movements in various processes of animal development. However, little is known about its critical transcriptional targets. In order to identify targets of Brachyury and understand the molecular mechanisms underlying morphogenetic movements, we first searched the genome sequence of *Xenopus tropicalis*, the only amphibian genomic sequence available, for Brachyury-binding sequences known as T-half sites, and then screened for the ones conserved between vertebrate genomes. We found three genes that have evolutionarily conserved T-half sites in the promoter regions and examined these genes experimentally to determine whether their expressions were regulated by *Brachyury*, using the animal cap system of *Xenopus laevis* embryos. Eventually, we obtained evidence that *vimentin*, encoding an intermediate filament protein, was a potential target of Brachyury. This is the first report to demonstrate that Brachyury might affect the cytockeletal structure through regulating the expression of an intermediate filament protein, vimentin.

Keywords:

T/brachyury, T site, cell movement, clawed frog, *vimentin*

Abbreviations:

cdc42, cell division cycle 42; bp, base pair(s); PCR, polymerase chain reaction; eFGF, embryonic fibroblast growth factor; EMBOSS, The European Molecular Biology Open Software Suite; blast, basic local alignment search tool; RT-PCR, Reverse Transcription Polymerase Chain Reaction; ODC, ornithine decarboxylase;

1. Introduction

Brachyury or T protein is the founding member of the T-box family of transcription factors, characterized by the possession of a sequence-specific DNA-binding domain called the T-domain (Kispert and Herrmann, 1993; Kispert et al., 1995; Pflugfelder et al., 1992), and reported in multicellular animals from sponges to humans and certain unicellular opisthokonts (Mikhailov et al., 2009; Papaioannou, 2001). The *Brachyury* gene was first identified genetically in the mouse (Herrmann et al., 1990), and initially, its role in mesoderm formation and notochord differentiation was studied in various chordates. However, recent expression and functional analyses in various animals including invertebrates have demonstrated that the ancestral role of *Brachyury* is involved in regulating morphogenetic movements, i.e., cell movements, rather than specifying mesodermal fates, and is conserved in animals as diverse as invertebrates and vertebrates (Beddington et al., 1992; Gross and McClay, 2001; Tagawa et al., 1998; Technau, 2001; Yamada et al., 2010). For example, *Brachyury* plays a role in gastrulation in the sea urchin, *Xenopus*, and the mouse as well as in the invagination of the stomodeum in the sea urchin and ctenophore (Conlon and Smith,

1999; Gross and McClay, 2001; Kwan and Kirschner, 2003; Wilson and Beddington, 1997; Wilson, 1995; Yamada et al., 2010). In addition, *Brachyury* orthologs derived from various animals mimic the action of *Xenopus Brachyury* (*Xbra*) in experiments wherein their synthetic RNAs are introduced in the animal caps of *Xenopus laevis* (Marcellini et al., 2003; Yamada et al., 2010). These findings suggest the possibility that the *Brachyury*-downstream mechanisms are conserved among the metazoans.

To identify the genes expressed downstream of *Brachyury*, several groups have conducted subtractive screens in the frog (*X. laevis*), ascidian (*Ciona intestinalis*), and sea urchin (*Strongylocentrotus purpuratus*) using *Brachyury*-misexpressing embryos and embryos in which *Brachyury* was either normally expressed or repressed (Hotta et al., 2008; Rast et al., 2002; Saka et al., 2000; Tada et al., 1998; Takahashi et al., 1999). Several genes identified in these screens encoded proteins similar to those associated with cell shape modification and cell movements although these genes were not orthologs conserved among all 3 species. Only the *cdc42* gene, a member of the Rho GTPase family mediating changes in the actin cytoskeleton, has been detected in the ascidian and sea urchin (Hotta et al., 2008; Rast et al., 2002), but its roles

downstream of *Brachyury* have not been demonstrated. Moreover, studies in *X. laevis* and *C. intestinalis* have suggested roles for *Xwnt11* and *prickle*, respectively, downstream of *Brachyury* (Hotta et al., 2007; Tada and Smith, 2000), both of which are members of the noncanonical Wnt/planar cell polarity signal transduction pathway that has been implicated in directed cell migration and mediolateral intercalation of embryonic cells (Barrow, 2006; Keller, 2002; Roszko et al., 2009). *Xwnt11* particularly acts on *Xenopus* gastrulation as a direct Xbra target but is not capable of rescuing gastrulation-like movements induced by human recombinant activin in animal caps derived from embryos wherein Xbra functions were suppressed (Saka et al., 2000; Tada and Smith, 2000). This suggests that additional Xbra targets are involved in the gastrulation movements in *X. laevis*. In addition, a putative tumor suppressor gene homolog, *Xbtg1*, is involved in gastrulation movements downstream of *Xbra* during embryogenesis in *X. laevis*, but it has not been shown to be bound by Xbra (Saka et al., 2000). Taken together, these results suggest that the *Brachyury*-downstream mechanisms underlying cell movements such as gastrulation and stomodeal invagination are not well understood.

Studies by Kispert and Herrmann have reported a type of Brachyury-binding site, the T site, which includes a 20-bp palindromic sequence identified by a PCR-based selection procedure using mouse Brachyury and random oligonucleotide DNA (Kispert and Herrmann, 1993; Kispert et al., 1995). Brachyury activates the expression of reporter genes through binding to the T site (Kispert et al., 1995); in addition, not only mouse Brachyury but also its orthologous proteins in *X. laevis* and zebrafish bind to the T site proposed in the mouse in a gel shift assay (Kispert and Herrmann, 1993). Indeed, sequences similar to the T site have been found in the regulatory region of *Ciona tropomyosin-like* gene, *Brachyury* itself in an ascidian, *Halocynthia roretzi*, and a mouse, *nanog* (Di Gregorio and Levine, 1999; Suzuki et al., 2006; Takahashi et al., 1999). In *eFGF* and *bix* of *X. laevis*, both of which are Xbra target genes, a sequence similar to the palindromic half unit of the T site, identified in their upstream regions, is sufficient for the binding of Xbra (Casey et al., 1998; Tada et al., 1998). These studies suggest that the palindromic half unit of the T site (the T-half site) has been conserved in the upstream regions of Xbra target genes, at least in *X. laevis*, and could function to activate gene transcription.

In order to understand the *Brachyury*-downstream mechanisms, which are thought to be conserved among animals, we sought to isolate further targets of Xbra from *X. laevis* by identifying the T-half site. Since the complete genome of *X. laevis* has not been reported yet, we used the genome of *X. tropicalis*, a closely related species of *X. laevis*. Similarly to *X. laevis*, it has been demonstrated that *eFGF* and *bix* orthologs of *X. tropicalis* possess sequences similar to the T-half site in their upstream regions with which the Brachyury ortholog of *X. tropicalis* interacts (Messenger et al., 2005). The *xom* gene is activated by injection of *Xbra* RNA in the animal caps of *X. laevis*; its ortholog in *X. tropicalis* also includes an upstream T-half site-like sequence and is bound by Brachyury of *X. tropicalis* (Messenger et al., 2005). These results support the contention that the T-half site has been conserved between *X. laevis* and *X. tropicalis*. Therefore, we searched putative regulatory regions of *X. tropicalis* genes for the T-half site sequences to obtain potential Xbra target genes. In addition, we performed comparative genomic searches using other vertebrate genomes for the T-half site sequences since there is a possibility that evolutionarily conserved targets of Brachyury possess the T-half sites. We then determined whether the *Xenopus* genes

including the evolutionarily conserved T-half sites behaved as *Xbra* downstream genes by *in vivo* experiments using the animal cap system of *X. laevis*.

2. Materials and methods

2.1. Genomic datasets

All sequences of the putative regulatory regions and the amino acid sequences of the *X. tropicalis* genes as well as those of *Mus musculus* (mouse), *Gallus gallus* (chicken), and *Danio rerio* (fish) were obtained from the Ensembl database release 56 (http://www.ensembl.org). Upstream intergenic regions were defined as the putative regulatory regions of each gene. The length of these regions was limited to at most 6 kb upstream of the transcription start site defined in the Ensembl database.

2.2. Computational detection of the T-half site

We scanned the transcription regulatory regions of *X. tropicalis* and the other three vertebrate genomes for potential Brachyury (T)-binding sites using "fuzznuc," an EMBOSS program used for nucleic pattern searching (http://emboss.sourceforge.net/). As Brachyury-binding site sequences, we utilized the T-half site sequence corresponding to the right half unit of the palindromic T site, which is a consensus sequence of the Brachyury-binding sites identified in a study using mouse Brachyury

and random oligonucleotide DNA (Kispert and Herrmann, 1993). The reason why the right half of the T site was used is that the right side is longer and more conserved than the left (Kispert and Herrmann, 1993), and sequences similar to the right side sequence have been detected in Xbra target genes (Casey et al., 1998; Messenger et al., 2005; Tada et al., 1998). We searched for the right half-site sequence and its complementary sequence, i.e., 5′-AGGTGTGAAATT-3′ and 5′-AATTTCACACCT-3′, respectively (one mismatch allowed). Genes having such sites in the regulatory region are hereafter referred to as T-half site-containing genes or THS genes.

2.3. Identification of gene orthologs

Orthologous relationships between the *X. tropicalis* THS genes and the vertebrate THS genes were determined by BLASTP (blast-2.2.18) and synteny analyses. Homologs were defined as proteins with a BLASTP score of >54 bits. Orthologous gene pairs between the *X. tropicalis* THS genes and their homologous THS genes in the three vertebrates were then determined by a reciprocal best hit search using BLASTP, except for *bix*. *Xenopus bix* appears to have resulted from *Xenopus* lineage-specific

duplication (Supplementary Figure 1A). Thus, we considered duplicate *bix* genes as an orthologous group to the mouse *mixl1* gene (ENSMUSG00000026497; Supplementary Table 3). Syntenic regions were also determined by examining whether genes around each *X. tropicalis* THS gene were orthologous to those around its orthologous THS genes in the vertebrates using BLASTP.

X. laevis orthologs of *X. tropicalis* THS genes were isolated from the GenBank database, including 33,998 protein sequences of *X. laevis* by a reciprocal best hit search using BLASTP. In cases where two or more genes of *X. laevis* were more closely related to each other than to other possible paralogs of *X. tropicalis* and *X. laevis*, possible orthologs were determined by phylogenetic analyses: the protein sequences of these genes in *X. laevis* and *X. tropicalis* were aligned with those of related genes in other vertebrates using MAFFT (v6.713b) with default parameters. After removing gaps, the alignments were used to construct phylogenetic trees. Trees were calculated by the neighbor-joining method using the Phylip-3.69 software under the Jones–Taylor–Thornton model of protein evolution (Supplementary Figure 1).

2.4. Injection into embryos from *X. laevis*

For animal cap assays and disruption of *Brachyury* function, African clawed frogs (*X. laevis*) were used. Eggs were obtained from female *X. laevis*, and artificially fertilized eggs were maintained in Steinberg's solution (SBS; 58.2 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, 4.6 mM Tris-HCl, pH 7.4–7.6). Capped RNAs were synthesized from pCS2+ plasmids containing the protein coding regions of wild-type Xbra, VegT, and Eomesodermin of *X. laevis* using the mMessage mMachine kit (Ambion, USA). A dominant-interfering construct of Xbra (*Xbra-EnR*) were made by cloning the region encoding amino acids 1-228 of Xbra into pCS2-EnR vectors (Addgene plasmid 11028) and its capped RNA were synthesized in the same way as above. For animal cap assays, 100pg of capped RNAs were injected into the animal poles of each cell of the 4-cell stage embryos that had been dejellied. Animal caps were dissected at stage 8 and cultured in SBS until these were harvested for RT-PCR analyses at stage 11. Total RNA was prepared from 5 animal caps for each assay using ISOGEN (Wako, Japan), and cDNA was synthesized using Superscript II $RNaseH^-$ (Invitrogen, USA). The primer sequences used for RT-PCR and the numbers of PCR

cycles were as follows: *bix*, 5′-AGCACCTACTTCTCCTCCAGTG-3′, 5′-TGGTGGATGTCTGGGTACATATT-3′ and 30 cycles; *col8a1*, 5′-GAGGGTCCTGATGGACCAAT-3′, 5′-GCATCCCAGGTTCTCCTTTG-3′ and 33 cycles; the *ornithine decarboxylase* (*ODC*) gene, 5′-GTC AAT GAT GGA GTG TAT GGA TC-3′, 5′-TCC ATT CCG CTC TCC TGA GCA C-3′ and 30 cycles; *vimentin*, 5′-GCAACCTGCAGTCATTCAGA-3′, 5′-GATTAGCAGCTTCCGACAGG-3′ and 33 cycles. *ODC* was used as an internal reference based on previous studies of *X. laevis* mRNA expression (Isaacs et al., 1992; Šindelka et al., 2006). For disruption of Xbra function, 50 pg of *Xbra-EnR* RNA was injected into the marginal zone of the two dorsal cells of a 4-cell embryo. Under some experiments, RNA was co-injected with 400 pg of *lacZ* RNA as a lineage tracer. Injected embryos were cultured in 5% Ficoll 400/1x SBS overnight and then in 0.1x SBS until the appropriate stage for each experiment.

3. Results and Discussion

3.1. *In silico* identification of conserved THS genes

To identify potential Xbra target genes, a comprehensive search for T-half site sequences in *X. tropicalis* genome was performed. The putative transcription regulatory regions of *X. tropicalis* were scanned for T-half sites using "fuzznuc", an EMBOSS program used for nucleic pattern searching. The length of these regions was limited to at most 6 kb upstream of the transcription start site defined in the Ensembl database because among the known T sites, the T site of the mouse *nanog* is the most distantly located, about 5 kb from its transcription start site (Suzuki et al., 2006). The search allowing a maximum of one mismatch in the 12-bp T-half site sequences found 965 THS genes in *X. tropicalis* genome (Supplementary Table 1 and 2). We did not think that all of the T-half site sequences of the 965 genes function as Xbra-binding motifs *in vivo*. We assumed that the T-half sites properly actually act as Xbra-target sites in the following cases: (1) these sites were found in the regulatory regions of a *X. tropicalis* gene and its orthologs in other vertebrates, and (2) the *X. tropicalis* gene and the vertebrate orthologs were also within a syntenic region on the *X. tropicalis* and the vertebrate genomes, and that such T-half sites would be involved in conserved and crucial developmental functions. Thus, we scanned the putative transcription

regulatory regions of the mouse genome for potential T-half sites in the same manner as those of *X. tropicalis* (Supplementary Table 1 and 2). Then, orthologs of *X. tropicalis* THS genes were searched against the THS genes of the mouse using BLASTP. The *X. tropicalis* THS genes were first screened only if they had homologous THS genes in mouse. Homologs were defined as proteins with a BLASTP score of >54 bits. Of the 965 THS genes in *X. tropicalis*, 307 showed homology to the THS genes in *X. tropicalis* and mouse. To indentify further evolutionarily-conserved THS genes, we screened for the 307 genes on the condition that they had homologous THS genes in chicken and zebrafish in the same way with "fuznuzz" searches and homology analyses. And then, orthologous relationships between the *X. tropicalis* THS genes and their homologous THS genes in the other three vertebrates were determined by a reciprocal best hit search using BLASTP. Syntenic regions were determined by examining whether genes around each *X. tropicalis* THS gene were orthologous to those around its orthologous THS genes in each vertebrate using a reciprocal best-hit BLASTP search. Nonannotated genes and genomic rearrangements were not considered in this study.

As a result, we found three THS genes conserved between *X. tropicalis* and the mouse (Table 1 and Supplementary Table 3).

As we expected, *bix*, which is a direct Xbra target (Tada et al., 1998), was included (Table 1and Supplementary Table 3). The remaining two genes have been suggested to be associated with cell migration and the cytoskeleton (Ivaska et al., 2007; Shuttleworth, 1997), implying that they are related to cell movements.

3.2. *In vivo* analysis of the conserved THS genes in *Xenopus* animal caps

To investigate if Xbra regulates the non-*bix* genes in Table 1 as well as *bix*, we used the animal cap system of *X. laevis*, based on the assumption that the T-half sites of Brachyury target genes would be conserved in *X. tropicalis* and *X. laevis* (see 1. Introduction). The animal cap tissues, which normally develop into undifferentiated epidermis, can be differentiated into mesoderm through the activation of *Xbra* downstream genes when synthetic *Xbra* RNA is injected into the tissues (Cunliffe and Smith, 1994). In addition, the nature of a *Brachyury*-inducible *bix* has been revealed using this system of *X. laevis* (Tada et al., 1998).

Putative *X. laevis* orthologs of the three *X. tropicalis* genes were identified and are shown in Table 1. We considered five and two *X. laevis* genes as putative orthologs of *bix* and *vimentin*, respectively using phylogenetic analyses (Supplementary Figure 1). One of the reasons why these genes seem to be duplicated in *X. laevis* is tetraploidization in the *X. laevis* lineage (Graf and Kobel, 1991; Hellsten et al., 2007). Like other studies, we did not distinguish these *X. laevis*-specific duplicated genes individually (Herrmann et al., 1989; Mead et al., 1998; Tada et al., 1998); we designed RT-PCR primers for the animal cap assay so that they would anneal to all the duplicated *X. laevis* orthologs of a *X. tropicalis* gene. This system would have been less sensitive than those using gene-specific primers, but it is still probable that it would detect expression changes/differences in 1 of the duplicated orthologs if it was regulated by *Xbra*. Using these two primers and another primer pair for the duplicated and nonduplicated *X. laevis* orthologs, each expression of the three gene sets was analyzed in the animal caps injected with synthetic *Xbra* RNA when the sibling embryos reached the mid-gastrula stage (Figure 1). It was confirmed that the expression of *bix* was strongly induced in *Xbra*-injected animal caps. In contrast, injection of *Xbra* RNA

reduced the expression of *vimentin* in the caps. This reduction was also observed in *Xbra*-injected caps of neural plate stage embryos (data not shown). *Xenopus* orthologs of the other t-box genes, *VegT* and *Eomesodermin*, both of which are thought to be transcriptional activators, have been suggested to bind the sequences similar to the T-half site *in vitro* (Conlon et al., 2001; Hyde and Old, 2000; Tada et al., 1998). Therefore, the effects of these genes on *vimentin* expression were examined in animal caps. Injection of synthetic *VegT* or *Eomesodermin* RNA induced *Xbra* expression, nevertheless either RNA did not suppress the expression of *vimentin* (Supplementary Figure 2A, B). The expression levels of *Xbra* in *VegT*- or *Eomesodermin*-injected caps were lower than those in *Xbra*-injected ones (Supplementary Figure 2C), implying the possibility that the reduction of *vimentin* expression in *VegT*- or *Eomesodermin*-injected caps could have been below the sensitivity of RT-PCR. Taken together, these results suggested that *vimentin* might be suppressed by Xbra directly or indirectly, but not VegT or Eomesodermin. This apparent inhibitory activity of Xbra was unexpected since no report has indicated that Xbra influences the expression of *vimentin* and acts as a repressor. We also examined whether *vimentin* was upregulated

in embryos where Xbra function was disrupted through the use of dominant interfering *Xbra* constructs (*Xbra-EnR*). Whole mount *in situ* hybridization to detect *vimentin* transcripts revealed that injection of *Xbra-EnR* did not induce the ectopic expression of *vimentin* (data not shown), although this result did not necessarily mean that Xbra could not regulate the *vimentin* expression We detected no expression of *col8a1*, even in the whole embryos used in this study (data not shown), implying that this gene was not affected by injected *Xbra* at the mid-gastrula stage.

Vimentin is the major intermediate filament protein and participates in a number of developmental events such as adhesion, migration, and cell signaling (Dent et al., 1989; Ivaska et al., 2007), implying that the roles of Brachyury in cell movements may be executed through these functions of vimentin. In *Xenopus*, zygotic transcripts of *vimentin* are first detected at the mid-gastrula stage (Herrmann et al., 1989; Sharpe, 1988) approximately 2 hours after those of *Xbra* are detected. Recently, a whole-mount *in situ* hybridization analysis revealed that *vimentin* is expressed on lateral surfaces of the neural tube at the neural stage and later in the central nervous system broadly (Kiyota et al., 2008). Unlike *vimentin*, *Xbra* is expressed transiently during

gastrulation around the blastopore, in involuting mesoderm, and subsequently becomes restricted to the notochord (Smith et al., 1991), indicating that the spatial expression patterns of *vimentin* transcripts do not overlap those of *Xbra*. Thus, we have no data contradicting the hypothesis that Xbra could regulate the expression of *vimentin* in cell-autonomous manner. In this study, *vimentin* has not been demonstrated to be a direct Xbra target. A further investigation of the binding ability of Xbra to the regulatory regions of the *vimentin* genes in *X. laevis* and *X. tropicalis* will clarify whether the inhibitory activity of Xbra against *vimentin* expression originates from Xbra's direct transcriptional regulation.

It is possible that another conserved THS gene, *col81a*, is regulated by Xbra at other stages or in specific cell types that were not investigated in this study. Indeed, *col8a1* has been reported to be expressed in the notochord of zebrafish embryos (Gansner and Gitlin, 2008) where convergent extension movements occur (Glickman et al., 2003) and a *Brachyury* ortholog of zebrafish is also expressed (Martin and Kimelman, 2008). In addition, optimization of the number of mismatches allowed in the searched sequences might facilitate the identification of Brachyury target genes.

4. Conclusions

Cell movement is one of the important developmental processes of animals. Recently, the T-box transcription factor Brachyury has been suggested to be involved in regulating morphogenetic movements in various animals, but its downstream mechanism is poorly understood. In this study using *in silico* and *in vivo* analyses, we identified a gene, *vimentin*, as a candidate for *Brachyury* downstream gene. *vimentin* encodes an intermediate filament protein and has been known to be involved in cell adhesion and migration. Therefore, our study demonstrated that Brachyury affects the cytoskeleton to regulate cell movement. Further studies on the binding ability of Brachyury to the regulatory regions of *vimentin* remains to be examined.

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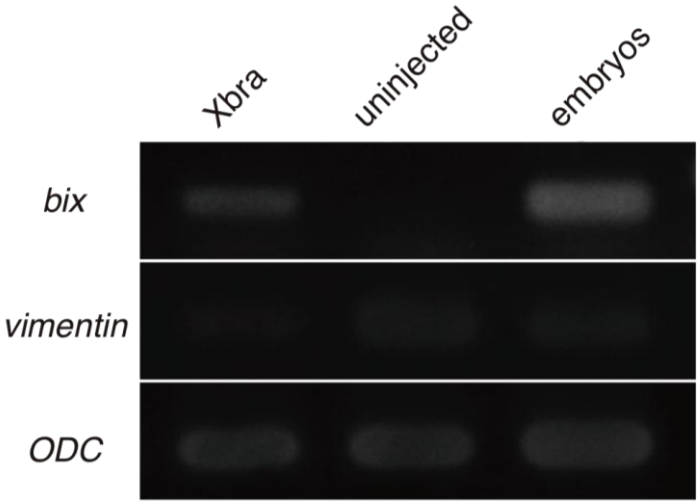
Figure legends

Figure 1. RT-PCR analysis of animal caps injected with 100 pg of *Xbra* RNA. cDNAs derived from *Xbra*-injected animal caps, uninjected animal caps, and intact whole embryos were used as templates for RT-PCR. The numbers of PCR cycles were 30 for *bix*, 33 for *vimentin*, and 30 for ornithine decarboxylase gene (*ODC*). Expression of the *ODC* was used as an internal control. In 2 independent experiments, *Xbra* induced the expression of *bix* in animal caps but suppressed the expression of *vimentin*. Without reverse transcriptase, no PCR products were amplified (data not shown).


Xbra
uninjected
embryos
bix
vimentin
ODC

Supplementary figure legends

Supplementary Figure 1. Phylogenetic trees of the bix (**A**) and vimentin (**B**) proteins constructed by the neighbor-joining method. Bootstrap values obtained with 1000 replicates are given around branches in positions important for the classification. The *X. tropicalis* THS proteins and their possible *X. laevis* orthologs, which are shown with the accession numbers for their nucleotide sequences in Table 1, are indicated with a *box* and in *bold*, respectively. Species abbreviations: Dr, *Danio rerio*; Gg, *Gallus gallus*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Xt, *Xenopus tropicalis*.

Supplementary Figure 2. RT-PCR analysis of animal caps injected with 100 pg of *VegT* RNA and *Eomesodermin* RNA.

cDNAs derived from *VegT*- or *Eomesodermin*-injected animal caps and uninjected animal caps were used as templates for RT-PCR. The numbers of PCR cycles were 33 for *vimentin*, 30 for *Xbra*, and 30 for *ODC*. Expression of the *ODC* was used as an internal control. In *VegT*- or *Eomesodermin*-injected caps, the expression of *Xbra* was induced while the expression of *vimentin* was not affected (**A**, **B**). The expression

levels of *Xbra* induced by the injection of *VegT* or *Eomesodermin* were lower than those by *Xbra* injection. Two independent experiments each were performed.

Table 1. *X. tropicalis* genes including the T-site half sequences within 6 kb upstream of their transcription start sites and their orthologs in *X. laevis*

Gene name	Description	*X. tropicalis*[a]	*X. laevis*[b]
bix	*Brachyury*-inducible homeobox protein	ENSXETG00000027670	NM_001088363.1 NM_001085602.1 NM_001085603.1 NM_001088365.1 AF081351.1
collagen alpha-1(VIII) chain precursor (*col8a1*)	nonfibrillar short-chain collagen	ENSXETG00000005782	BC082620.1
vimentin	intermediate filament	ENSXETG00000007462	NM_001087438.1 NM_001087439.1

[a]Ensembl gene ID of *X. tropicalis* genes identified in this analysis.

[b]GenBank accession number of *X. laevis* orthologs.