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Expression Pattern and Transcriptional Control of *SoxB1* in Embryos of the Ascidian *Halocynthia roretzi*

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ABSTRACT—The Sox family is a large group of transcription factors that are characterized by the presence of a DNA-binding HMG domain. We isolated *HrSoxB1*, an ascidian homolog of the *Sox* gene that belongs to the B1 subclass of the Sox family, from *Halocynthia roretzi*. Expression was initiated as early as the 8-cell stage. During cleavage stages, *HrSoxB1* was expressed in three quarters of embryonic blastomeres but not in posterior-vegetal (B-line) blastomeres. Misexpression of mRNAs of *HrPEM* but not of *macho-1*, whose maternal mRNAs are localized to the posterior-vegetal cytoplasm of eggs and early embryos, repressed the anterior-vegetal expression of *HrSoxB1*. This result suggests that the zygotic expression of *HrSoxB1* is controlled by the localized maternal mRNA. When *HrSoxB1* was overexpressed in early embryos, ectopic expression of *HrBra*, a gene for a transcription factor expressed in notochord blastomeres, occurred in the most posterior blastomeres (B7.5), although these blastomeres did not eventually differentiate into notochord but developed into muscle, as they do in normal embryogenesis. In later embryogenesis, *HrSoxB1* was specifically expressed in neural plate cells. However, overexpression of *HrSoxB1* did not affect the expression of a neural plate marker gene, *HrETR-1*.

Key words: ascidian embryo, *SoxB1*, *HrPEM*, localized RNA, zygotic transcription

INTRODUCTION

The embryogenesis of ascidians represents basic characteristics of chordate development, but in a very simplified manner (Satoh, 1994; Nishida, 1997, 2002; Corbo *et al*., 2001). The fate specification of embryonic cells of ascidians greatly depends on localized maternal factors in egg cytoplasm. We are trying to identify genes whose zygotic expression is initiated at early cleavage stages, and investigating how their blastomere-specific expression is controlled by maternally localized ooplasmic factors. During our screening for such genes, we have found a clone showing sequence similarity to the Sox family of transcription factors (Miya and Nishida, 2002). As its expression pattern during cleavage is conspicuous, we isolated full-length cDNA to characterize the gene and to analyze maternal control of the zygotic transcription and its function during ascidian early development.

The Sox family is a large group of transcription factors that are characterized by the presence of a DNA-binding HMG (high mobility group) domain that is 70–80 amino acids long and are structurally related to the mammalian sex-determination factor Sry (Gubbay *et al*., 1990). Sox proteins bind to specific DNA sequences. The consensus binding motif for Sox proteins has been defined as the heptameric sequence 5'-(A/T)(A/T)CAAAG-3'. It has been proposed that Sox proteins function as architectural proteins by bending DNA and organizing local chromatin structure. Members of the family are found throughout the animal kingdom, and perform their function in a diverse range of developmental processes such as germ layer formation, organ development, and cell type specification (reviewed by Wegner, 1999). They are subdivided into eleven subgroups according to sequence similarity of the HMG domain, full-length protein structure, and gene organization (Bowles *et al*., 2000). Among those subgroups, subgroup B1 includes *Sox1*, *2*, and *3*, which are known to be involved in vertebrate neural development (reviewed by Sasai, 2001).

We report the detailed expression pattern of the *Halocynthia HrSoxB1* gene, evidence for the maternal control of its transcription, and results of overexpression of *HrSoxB1* mRNA.

MATERIALS AND METHODS

Animals and embryos

Halocynthia roretzi was purchased from fishermen near the Otsuchi Marine Research Center, Ocean Research Institute, Uni-

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versity of Tokyo, Iwate, Japan, and near the Asamushi Marine Biological Station, Tohoku University, Aomori, Japan. Naturally spawned eggs were fertilized with a suspension of non-self sperm. When fertilized eggs were cultured at about 12°C, they developed into gastrulae and early tailbud embryos at 12 and 24 hr, respectively after fertilization. Tadpole larvae hatched after 40 hr of development. In some experiments, cleavages of embryos were permanently arrested by treatment with 2 μg/ml Cytochalasin B at the 110-cell stage.

Cloning and sequence comparison of *HrSoxB1*

A cDNA library of 110-cell stage embryos was constructed by use of a uniZAP vector in a ZAP-cDNA synthesis kit (Stratagene, USA). A full-length cDNA for *HrSoxB1* was obtained by screening the library using an original partial cDNA as a probe (Miya and Nishida, 2002). The cDNA was cloned into the plasmid vector pBluescript (Stratagene), and was used for further analysis. Nucleotide sequences were determined for both strands with a SequiTherm Excel II kit (Epicentre Technologies, Madison, WI, USA) and an LIC-4000 DNA sequencer (Li-Cor Biosciences, Lincoln, NE, USA). Amino acid sequences of the Sox family gene products from various animals were aligned, and gaps were introduced to obtain alignment with maximal similarity. Molecular phylogenetic relationships of the Sox family gene products were estimated by means of neighbor-joining (Saitou and Nei, 1987) using the PHYLIP ver. 3.5c package (Felsenstein, 1993). A distance matrix was constructed according to the Dayhoff model (Dayhoff *et al*., 1978). Seventy-nine confidently aligned sites of the HMG box were analyzed.

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed as described by Miya *et al*. (1997). After hybridization, the specimens were washed in 50% formamide, 2×SSC, 1% SDS (15 min, 50°C). The specimens were digested with 10 μg/mL RNase A in 2×SSC, 0.1% Tween 20 (20 min, 37°C), then washed in 2×SSC, 0.1% Tween 20 (2×20 min, 50°C), and 0.2×SSC, 0.1% Tween 20 (2×20 min, 37°C). Finally, the specimens were washed twice in PBS containing 0.1% Tween 20 at room temperature and visualized by the alkaline phos-

Fig. 1. Comparison of amino acid sequences of HrSoxB1 with those of mouse Sox1 (shown as mSox1; GenBank accession no. CAA63846), Sox2 (mSox2; AAC31791), Sox3 (mSox3; CAA63845), *Xenopus* Sox2 (xeSox2; AAC14215), Sox3 (xeSox3; CAA68828), and sea urchin SpSoxB1 (suSoxB1; AAD40688). Amino acids identical to HrSoxB1 are highlighted. Conserved amino acids are indicated by asterisks. HMG box is indicated.

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phatase reaction.

Injection of synthetic mRNA

The entire open reading frame of *HrPEM*, *macho-1* (Nishida and Sawada 2001), *HrSoxB1* or *lacZ* as a control was cloned into the pBluescriptHTB transcription vector, which contains both 5' and 3' UTR regions of HrTBB2, a *Halocynthia* beta-tubulin gene (Akanuma and Nishida, unpublished data). The recombinant plasmid was linearized with *Xho*I and transcribed with T3 polymerase in the presence of $m^7G(5')ppp(5')G$ by using an mMessage mMachine kit (Ambion, USA). Synthetic mRNAs of *HrPEM*, *macho-1*, and *HrSoxB1* were injected into fertilized eggs. Eggs injected with mRNA were allowed to develop to appropriate stages, then embryos were fixed for *in situ* hybridization or for immunohistochemistry with Mu-2 and Not-1 monoclonal antibodies as described in Nishikata *et al*. (1987) and Nishikata and Satoh (1990). A portion of the embryos were allowed to develop into larvae to examine the larval phenotypes.

RESULTS

Cloning and Structure of *HrSoxB1*

During our screening for genes whose expression are

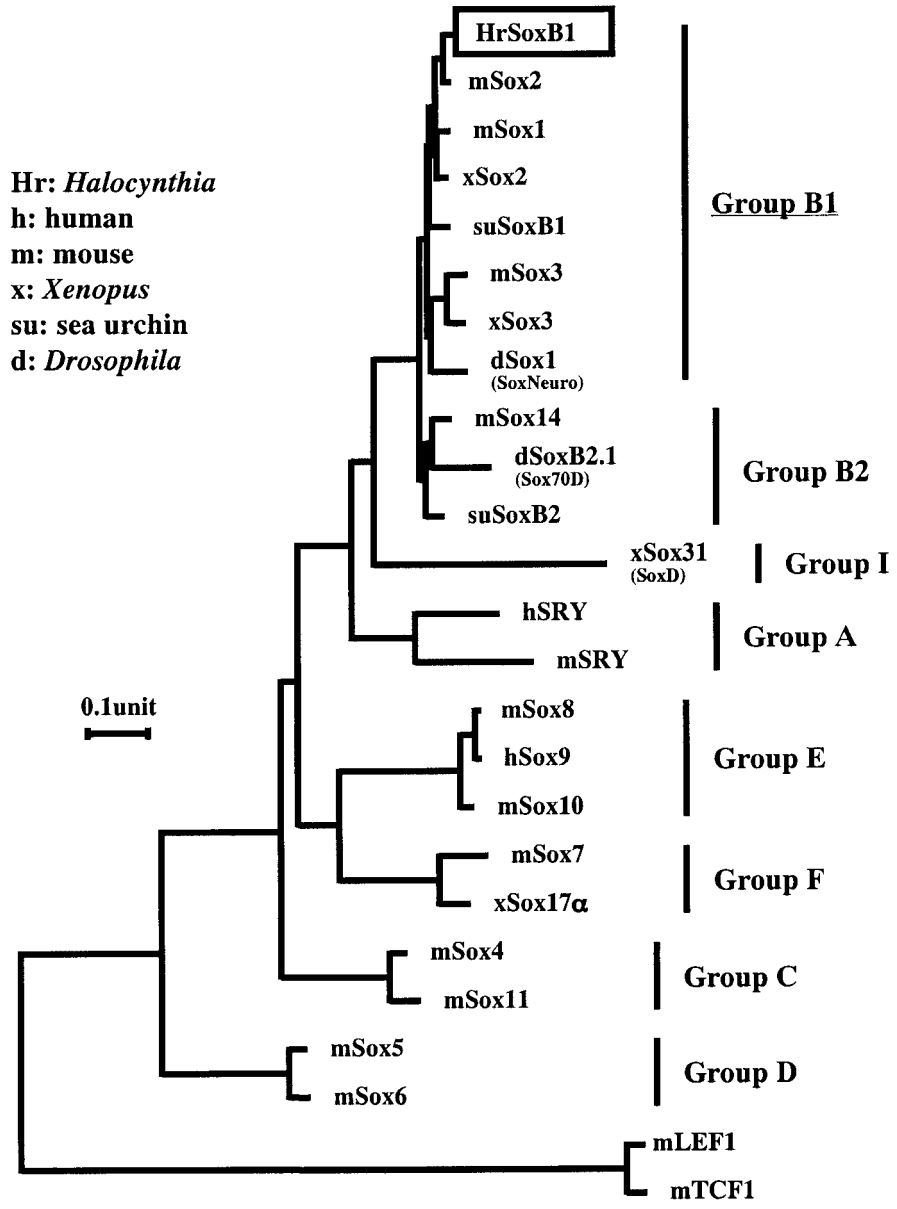

Fig. 2. Molecular phylogenetic relationship among the Sox family estimated by the neighbor-joining method. The name of each gene and group follows Bowles *et al*. (2000). In addition to those listed in Fig. 1, we included for analysis mouse Sox4 (shown as mSOX4; GenBank accession no. CAA49779), Sox5 (mSOX5; CAA09269), Sox6 (mSOX6; BAA09618), Sox7 (mSOX7; BAA78765), Sox8 (mSOX8; AAF35837), Sox10 (mSOX10; AAC24564), Sox14 (mSOX14; AAF62397), Sry (mSRY; AAC53433), human Sox9 (hSOX9; CAA86598), Sry (hSRY; CAA65281), *Xenopus* Sox17α (xSOX17α; CAA04957), Sox31 (xSOX31; BAA32249), sea urchin SoxB1 (suSOXB1; AF157389), SoxB2 (suSOXB2; AAD40687), *Drosophila* SoxB1 (dSOXB1; CAB64386), and SoxB2.1 (dSOXB2.1; CAA65279). Mouse Lef1 (mLEF1; P27782) and Tcf1 (mTCF1; Q00417) were used for the outgroup rooting. The results suggested that HrSoxB1 is a member of group B1.

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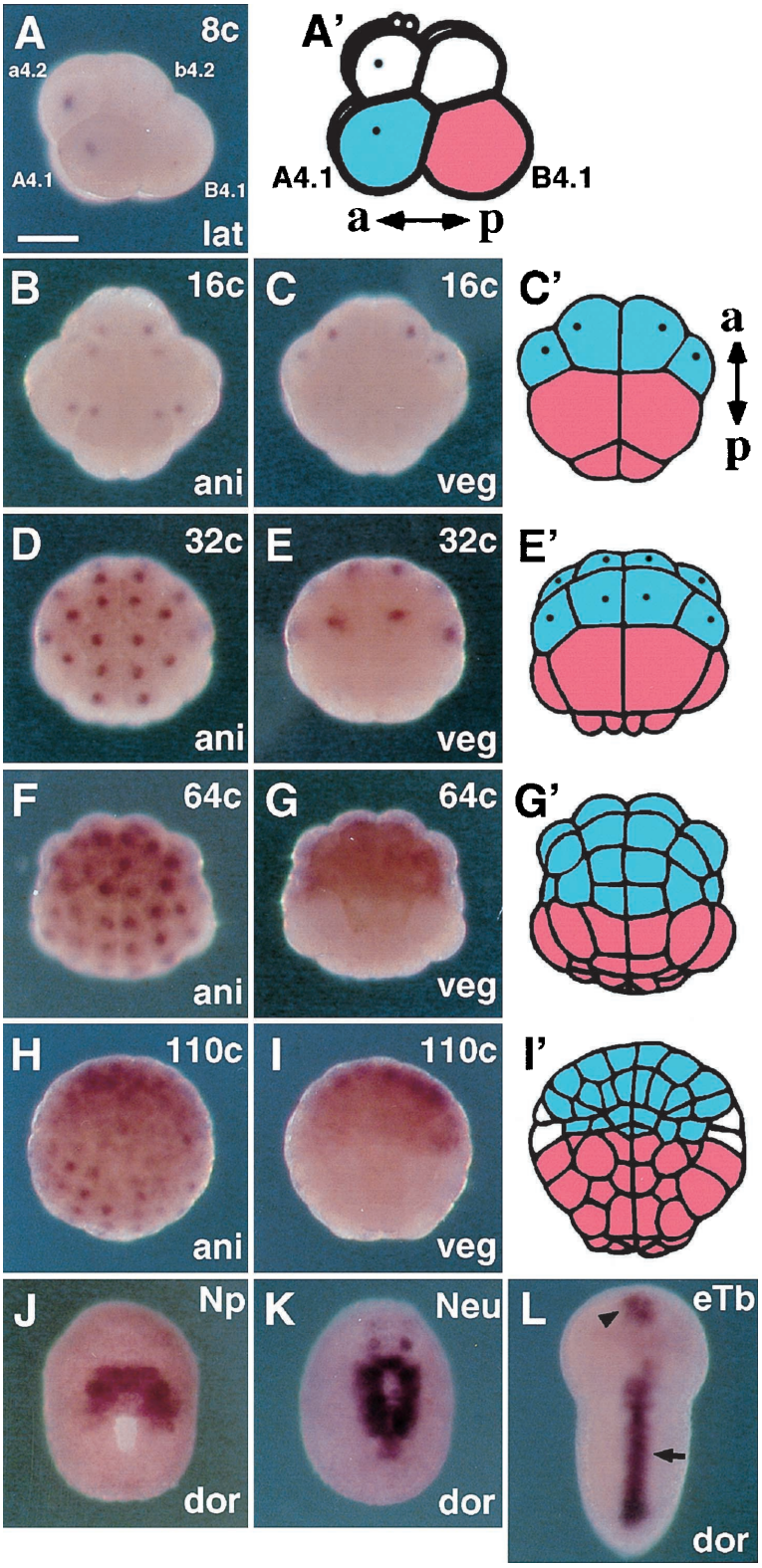

Fig. 3. Spatial expression of *HrSoxB1*. (A–L) Embryos hybridized *in situ* with *HrSoxB1* antisense probe. (A', C', E', G', I') Diagrams corresponding to lateral view (A) and the vegetal views (C, E, G, I) of embryos. The anterior-vegetal blastomeres derived from A4.1 blastomeres of the 8-cell embryo in which mRNA was detected are colored light blue. The posterior-vegetal blastomeres derived from B4.1 blastomeres of the 8-cell embryo in which mRNA is absent are shown in red. Black dots represent signals detected in blastomere nuclei. Anterior is to the left in panel A and up in B–L. Arrow in (L) indicates nerve cord expression; arrowhead indicates expression in the anterior neural tissue. Scale bar, 100 μm. 32c, 32-cell stage; 64c, 64-cell stage; 110c, 110-cell stage; Np, neural plate stage; Neu, neurula stage; eTb, early tailbud stage; lat, lateral view; ani, animal view; veg, vegetal view; dor, dorsal view.

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initiated during the early cleavage stage of the ascidian *Halocynthia roretzi*, we isolated a clone showing sequence similarity to the Sox family of transcription factors (Miya and Nishida, 2002). Since the clone appeared to lack an amino terminus, we screened the cDNA library of the 110-cell stage by using the partial clone as a probe. The longest clone we obtained was 2160 bp long, and had 18 adenyl residues at the 3' end and a putative open reading frame of 360 amino acids (DDBJ/EMBL/GenBank accession number: AB087830). A BLAST search showed that it is most similar to the B1 subclass of Sox family transcription factors, and we named it *HrSoxB1*. Fig. 1 shows the alignment of the predicted amino acid sequences of HrSoxB1 and other B1 subclass *Sox* gene products.

To verify that HrSoxB1 belongs to the B1 subclass of the Sox family, we constructed a molecular phylogenetic tree using the sequences of the well conserved HMG box. The tree shown in Fig. 2 was constructed by the neighbor-joining method (Saitou and Nei, 1987), and mouse Lef1 and Tcf1 were used as the outgroup for rooting. The tree supported the view that HrSoxB1 is a member of the B1 subclass, although we could not tell the relationship within the subclass from the tree.

Expression pattern of *HrSoxB1*

Zygotic expression of *HrSoxB1* was first detected as early as the 8-cell stage (Fig. 3A) by whole-mount *in situ* hybridization. Signals were apparent in a4.2 and A4.1 blastomeres, which lie in the anterior half of embryos. Sometimes a weak signal was also observed in b4.2 blastomeres, which are posterior-animal blastomeres. At the 16-cell stage, expression was detected in all eight blastomeres of the animal hemisphere, which are derived from a4.2 and b4.2 blastomeres of the 8-cell embryo, as well as in the four anterior-vegetal blastomeres derived from A4.1 blastomeres (Figs. 3B, C). In contrast, no signal was detected in the four posterior-vegetal blastomeres derived from B4.1 blastomeres. At the 32- to 110-cell stages, *HrSoxB1* expression was maintained in the entire animal hemisphere and in the anterior-vegetal blastomeres, but no signal was detected in the posterior-vegetal blastomeres (Figs. 3D–I). Therefore, the posterior-vegetal blastomeres that were derived from B4.1 blastomeres never expressed *HrSoxB1* during cleavage stage, but all other blastomeres did. In later stages, *HrSoxB1* was expressed in the neural plate (Figs. 3J, K), and after neural tube formation in the nerve cord (posterior neural tube) and anterior neural tissues (Fig. 3L).

Expression of *HrSoxB1* is suppressed by overexpression of *HrPEM* but not by *macho-1*

From the peculiar but simple expression pattern, one may assume that transcription of *HrSoxB1* is repressed in the B4.1 blastomere and its descendants by maternal mRNAs localized to the posterior-vegetal egg cytoplasm. Of several mRNAs reported to be localized to the posterior cytoplasm of fertilized eggs and partitioned into the B4.1 blastomeres, we tested *HrPEM* and *macho-1* that were cloned by Nishida and Sawada (2001). We injected synthetic mRNA of *HrPEM* and *macho-1* into fertilized eggs and examined the expression of *HrSoxB1* and two other marker genes, *HrBra* and *HrMA4*. *HrBra* is an ascidian homolog of the *brachyury* gene and is expressed in notochord precursor cells, many of which lie in the anterior-vegetal hemisphere (Yasuo and Satoh, 1994). *HrMA4* encodes muscle actin and

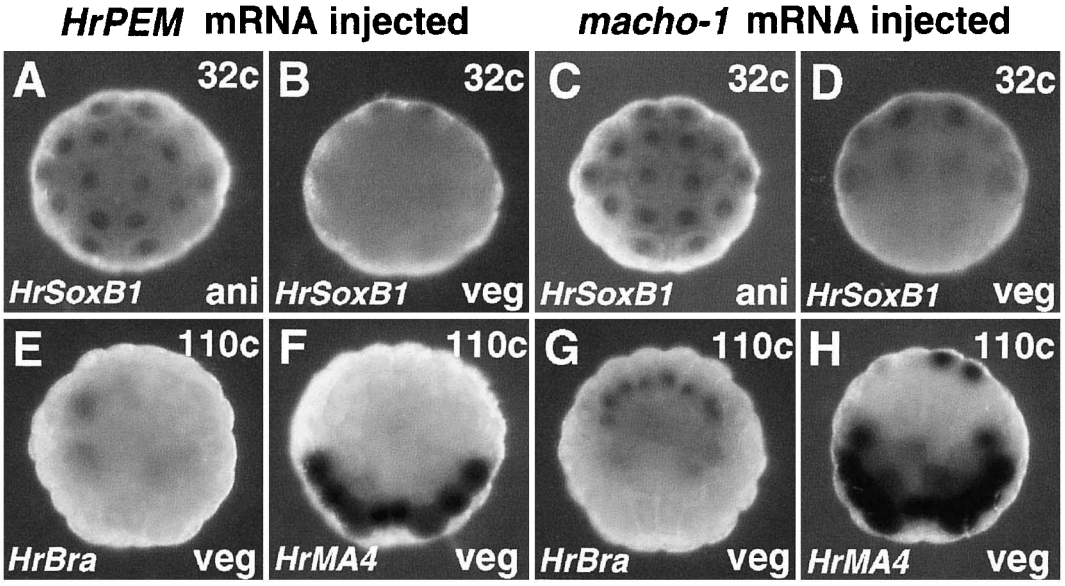

Fig. 4. Effects of *HrPEM* and *macho-1* overexpression on early expression pattern of *HrSoxB1*, *HrBra*, and *HrMA4*. Fertilized eggs were injected with 10 pg of *HrPEM* mRNA (A, B, E, F) or 20 pg of *macho-1* mRNA (C, D, G, H). (A–D) Expression of *HrSoxB1* in the 32-cell embryo detected by *in situ* hybridization. Expression of *HrSoxB1* in the vegetal hemispheres was suppressed in *HrPEM*-injected embryo. (E, G) Expression of *HrBra*, an ascidian ortholog of *brachyury*, in the 110-cell embryo. (F, H) Expression of *HrMA4*, an embryonic muscle actin gene, in the 110-cell embryo. ani, animal view; veg, vegetal view; 32c, 32-cell stage; 110c, 110-cell stage.

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is expressed in muscle precursor cells in the posterior-vegetal hemisphere (Satou *et al*., 1995).

The injection of 10 pg of *HrPEM* mRNA into fertilized eggs suppressed *HrSoxB1* expression in the anterior-vegetal blastomeres but not in the animal hemisphere of the 32-cell embryos (Figs. 4A, B). The repression of *HrSoxB1* in the

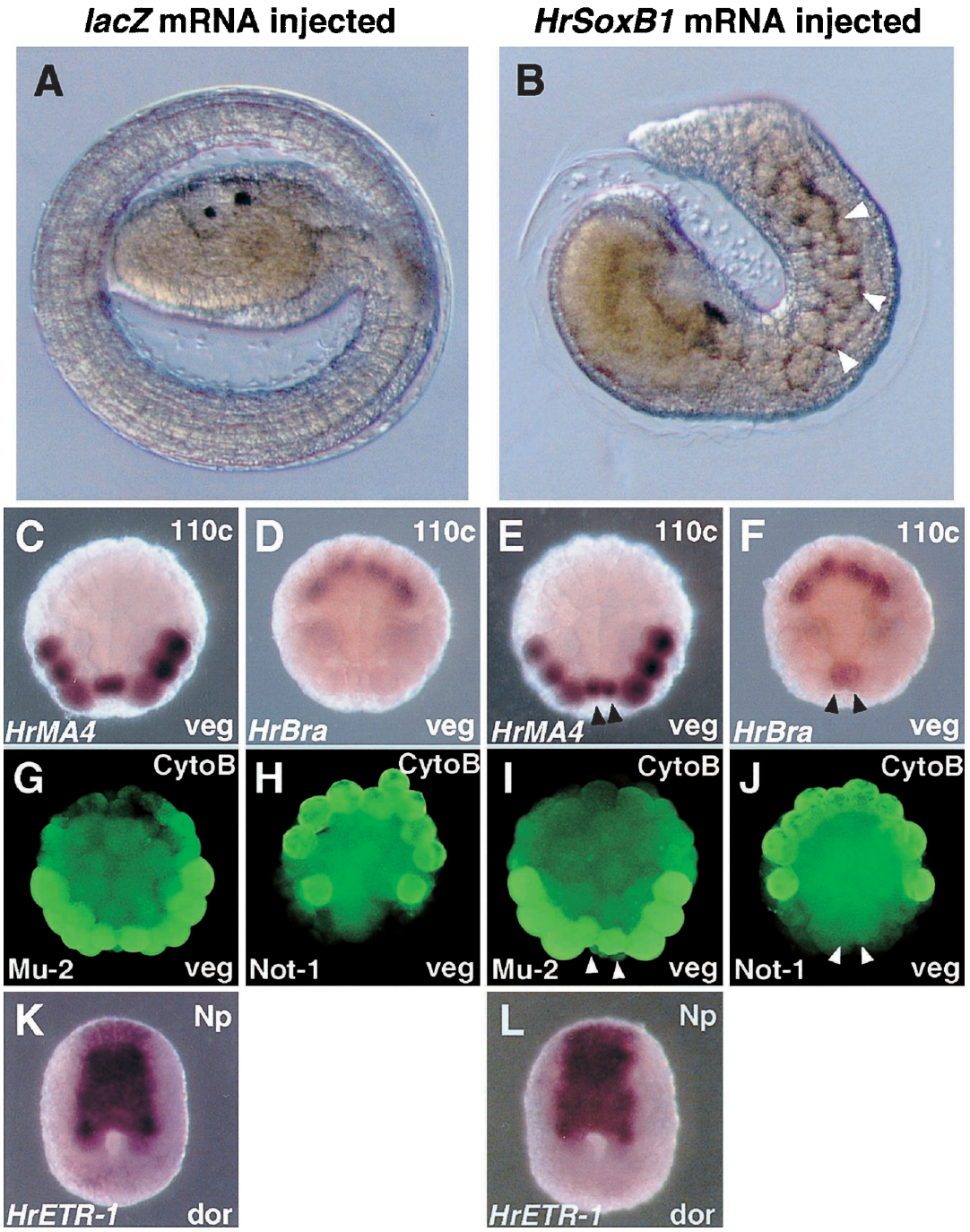

Fig. 5. Effects of *HrSoxB1* overexpression in ascidian embryos. Fertilized eggs were injected with 50 pg of either *lacZ* mRNA (A, C, D, G, H, K) as a control or *HrSoxB1* mRNA (B, E, F, I, J, L). (A, B) Morphology of larvae. Notochord cells (arrowheads) failed to align and the tail did not elongate. (C, E) Expression of *HrMA4* in the 110-cell embryo. (D, F) Expression of *HrBra* in the 110-cell embryo. In *HrSoxB1*-injected embryo, ectopic expression of *HrBra* (black arrowheads) was observed. (G–J) After injection, embryos were cleavage-arrested at the 110-cell stage and raised until control embryos reached the tailbud stage. (G, I) Embryos were immunostained with Mu-2 monoclonal antibody, which recognizes myosin heavy chain in muscle cells. (H, J) Embryos were immunostained with Not-1 monoclonal antibody, which stains differentiated notochord cells. In *HrSoxB1*-injected embryos and control embryos, B7.5 blastomeres (white arrowheads) differentiated into muscle but not into notochord. (K, L) Expression of neural-plate marker gene, *HrETR-1*, at neural-plate stage. No significant difference was observed between *HrSoxB1*-injected embryo (L) and control *lacZ*-injected embryo (K). veg, vegetal view; dor, dorsal view; 110c, 110-cell stage; CytoB, cleavage arrest with Cytochalasin B.

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vegetal hemisphere was confirmed in all cases examined (n=12). This amount of *HrPEM* RNA suppressed *HrBra* expression in the notochord precursors (12 out of 13 cases; Fig. 4E), but did not affect muscle actin expression (all 14 cases; Fig. 4F) at the 110-cell stage.

The injection of 20 pg of *macho-1* mRNA into fertilized eggs was sufficient to promote ectopic muscle formation as reported in Nishida and Sawada (2001). This amount of *macho-1* mRNA induced ectopic expression of *HrMA4* in the posterior-vegetal region (11 out of 12 cases), and in some embryos (3 out of 12 cases) ectopic expression was also observed in anterior nerve cord precursors (Fig. 4H). However, in all 13 cases we examined, this amount of *macho-1* did not affect the expression of *HrBra* in the notochord precursors (Fig. 4G). In embryos injected with *macho-1*, *HrSoxB1* expression was not altered (Figs. 4C, D). Normal expression of *HrSoxB1* was confirmed in all 15 embryos. Thus, the repression of *HrSoxB1* expression was specific to *HrPEM* mRNA.

Overexpression of *HrSoxB1* promoted ectopic expression of *HrBra* but not ectopic notochord formation

To investigate the function of *HrSoxB1*, we injected synthetic *HrSoxB1* mRNA into eggs. When 50 pg of *HrSoxB1* mRNA was injected, the result was short-tailed larvae (Fig. 5B). In 60% (40 out of 67) of the larvae, notochord cells failed to correctly intercalate with each other, and tail elongation was interfered. The rest showed milder abnormalities. In control larvae injected with *lacZ* mRNA, development was normal (Fig. 5A).

We examined marker gene expression in *HrSoxB1*-injected embryos. We first examined the expression of a muscle actin gene, *HrMA4*, at the 110-cell stage. In all 19 cases, expression was normal (Figs. 5C, E). In contrast, 77% (17 of 22) showed ectopic expression of *HrBra* in B7.5 blastomeres, which lie posteriorly in the vegetal hemisphere (Fig. 5F, arrowhead). B7.5 blastomeres give rise to larval muscle cells and trunk ventral cells (TVCs), which produce adult body-wall muscle and heart after metamorphosis (Hirano and Nishida, 1997). The B7.5 blastomeres never expressed the *brachyury* gene in normal or control embryos (Fig. 5D). Therefore, in B7.5 blastomeres of *HrSoxB1*-injected embryos, *brachyury* and *HrMA4* were expressed together. This never happens in normal development.

With these puzzling results in *HrSoxB1*-injected embryos, we further investigated which tissue the B7.5 blastomeres developed into in later embryogenesis. We carried out experiments with cleavage-arrested embryos. Embryos were treated with 2 μg/ml Cytochalasin B at the 110-cell stage to inhibit further cell divisions, and cultured until the control embryos reached tailbud-stage. Even when cleavages of ascidian embryos were permanently arrested at 110-cell stage, cleavage-arrested blastomeres continues some differentiation processes and eventually express muscle and notochord differentiation features (Whittaker, 1973; Nishikata *et al*., 1987; Nishikata and Satoh, 1990). When we stained the embryos with the muscle-specific Mu-2 antibody, an antibody for myosin heavy chain, cleavage-arrested muscle blastomeres, including the B7.5 blastomeres, expressed muscle myosin. In embryos injected with *HrSoxB1* mRNA, the pattern of myosin expression was not altered in all cases (n=15) (Figs. 5G, I). The Not-1 antibody stain differentiated notochord cells. In control, this antibody stained cleavage-arrested notochord blastomeres that expressed the *brachyury* gene at the 110-cell stage (Fig. 5H). In all embryos injected with *HrSoxB1* mRNA (n=15), the pattern of antibody staining was normal and signals were never detected in B7.5 blastomeres (Fig. 5J, arrowhead). These results clearly indicate that in *HrSoxB1*-injected embryos, B7.5 blastomeres differentiate into muscle cells, although they expressed ectopic *HrBra* at the 110-cell stage. When we increased the amount of injected mRNA, so that it might overcome the muscle differentiation, the cleavage pattern of embryos was disturbed and we could not analyze the results.

The vertebrate *Sox2* gene, as well as *Sox1* and *Sox3*, is known to be involved in neural development (for review, Sasai, 2000), and *HrSoxB1* is expressed in the neural plate and neural tube in the ascidian (Figs. 3J–L). Therefore, we examined the expression of a neural marker gene, *HrETR-1*, in *HrSoxB1*-overexpressed embryos at the neural-plate stage. However, in all 14 cases, the expression pattern of *HrETR-1* was normal (Figs. 5K, L).

DISCUSSION

We isolated *HrSoxB1*, an ascidian homolog of the *Sox* gene, which belongs to the B1 subclass of the Sox family. During cleavage, *HrSoxB1* was expressed in many blastomeres, but not in the posterior-vegetal B-line blastomeres. Overexpression of *HrPEM* but not *macho-1* repressed the anterior-vegetal expression of *HrSoxB1*. When *HrSoxB1* was overexpressed in early embryos, ectopic expression of *HrBra* in the most posterior blastomeres (B7.5) was observed. However, those blastomeres did not eventually differentiate into notochord but developed into muscle cells, as they do in normal embryogenesis.

***HrSoxB1* is an ascidian homolog of B1 subclass *Sox* genes**

The Sox family is a large group of transcription factors that have a DNA-binding HMG domain. On the basis of the sequence of the HMG domain, full-length protein structure, and gene organization, the Sox family is subdivided into eleven subgroups (Bowles *et al*., 2000). Subgroup B1 includes vertebrate Sox1, 2, and 3. Sequence similarity suggested that the ascidian HrSoxB1 is a B1 member. This conclusion is supported by the molecular phylogenetic analysis of the HMG domain shown in Fig. 2.

Regulation of *HrSoxB1* expression

The zygotic expression of *HrSoxB1* begins as early as

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the 8-cell stage. Some ascidian genes whose zygotic expression starts at the 8-cell stage have been reported (Shimauchi *et al*., 1997; Chiba *et al*., 1998; Nishikata *et al*., 2001; Miya and Nishida, 2002). However, no gene is so far known to be zygotically expressed at the 4-cell stage. Therefore, *HrSoxB1* is one of the earliest genes to be zygotically expressed in ascidian embryos. This early initiation suggests that the expression of *HrSoxB1* is regulated by maternal factors. Accordingly, the expression pattern of *HrSoxB1* was simple: it began and continued in three-quarters of each embryo except for the posterior-vegetal B-line blastomeres. There are two possibilities for the absence of expression from the B-line blastomeres. One is that *HrSoxB1* is transcribed by maternal factors localized in a4.2, b4.2, and A4.1 blastomeres but not present in B4.1 blastomeres. The other is that there are transcriptional repressors that are present only in B4.1 blastomeres, and *HrSoxB1* is transcribed by general transcription factors that exist throughout the entire embryo.

Our results support the latter possibility. When we injected *HrPEM* mRNA into fertilized eggs, anterior-vegetal expression of *HrSoxB1* was repressed, suggesting the presence of maternal control of *HrSoxB1* expression. *Pem* was first reported in the ascidian *Ciona savignyi* as a cDNA clone whose mRNA is abundant and is localized to the posterior egg cytoplasm, and is then segregated into B4.1 blastomeres (Yoshida *et al*., 1996). Although its function in early embryogenesis is still unclear, Pem of both *Halocynthia* and *Ciona* has a WRPW tetrapetide in its C-terminus. Since the WRPW motif is characteristic of a group of transcriptional repressors including Hairy/Enhancer of Split family, Pem might also act as a transcriptional repressor. In our experiment, 10 pg of *HrPEM* RNA did not repress the expression of *HrSoxB1* in the animal hemisphere. The reason is still unclear at the moment. Injected mRNA could be preferentially partitioned into the vegetal blastomeres during cleavages. But this is not the case because when we injected lacZ mRNA, the enzyme activity was evenly detected in descendant cells of both animal and vegetal hemispheres. Expression in the animal hemisphere might be controlled by some positive factors. A larger amount of *HrPEM* RNA might repress *HrSoxB1* expression throughout the entire embryo. But when we increased the amount of injected RNA, the cleavage pattern was so disturbed that we could not analyze the gene expression.

In sea urchin embryos, zygotic expression of *SpSoxB1* is preferentially activated in the animal hemisphere (Kenny *et al*., 1999). This factor is the earliest known spatially restricted regulator of transcription along the animal-vegetal axis of the sea urchin embryo. The SpSoxB1 protein interacts with a *cis* element that is essential for transcription of *SpAN*, a gene that is activated in the animal hemisphere at early blastula stage. Therefore, the expression of ascidian *HrSoxB1* in the animal hemisphere may also have a roles in establishing the fate of animal blastomeres.

Role of *HrSoxB1* in early embryogenesis

When *HrSoxB1* mRNA was injected into eggs, they developed into short-tailed larvae with a malformed notochord. Therefore, we examined the expression of *HrBra*, a transcription factor that is essential for notochord formation (Yasuo and Satoh, 1998). We showed that *HrBra* is expressed normally in the notochord precursor cells. However, in addition to the expression in notochord precursors, ectopic expression was observed in B7.5 muscle/trunk ventral cell precursors. It is hard to simply explain at the moment why ectopic expression is restricted to the B7.5 blastomeres. The ectopic expression of *HrBra* in *HrSoxB1*-injected embryos and the absence of expression of *HrBra* and *HrSoxB1* in *HrPEM*-injected embryos (Figs. 4B, E) may suggest that *HrSoxB1* is involved in proper expression of *HrBra* in notochord precursors, although the possibility should be carefully examined in further experiments.

B7.5 blastomeres in *HrSoxB1*-injected embryos did not eventually differentiate into notochord but developed into muscle cells, as they do in normal embryogenesis, although they expressed ectopic *HrBra* at the 110-cell stage. This result coincides with the observation that overexpression of *HrBra* mRNA does not transform all the embryonic blastomeres into notochord (Yasuo and Satoh, 1998). Shimauchi et al. (2001) have revealed that both *HNF-3* and *HrBra* are required for notochord differentiation of ascidian embryos, and that *HNF-3* is not expressed in B7.5 blastomeres. This may be the reason why B7.5 blastomeres in *HrSoxB1*-injected embryos did not eventually differentiate into notochord.

HrSoxB1 and neural development

Vertebrate group B1 Sox genes, Sox2, Sox1, and Sox3, are known to be involved in neural development (reviewed by Sasai, 2000). *HrSoxB1* is also specifically expressed in neural tissues during embryogenesis. This suggests a possibility that *HrSoxB1* is involved in ascidian neural development. However, the expression of ascidian neural plate marker *HrETR-1* precedes the neural expression of *HrSoxB1* (Yagi and Makabe, 2001). In addition, overexpression of *HrSoxB1* did not affect the expression pattern of *HrETR-1* (Fig. 5). In *Xenopus, Sox2* alone cannot induce neural development (Mizuseki *et al*., 1998). Therefore, in ascidian, sole misexpression of *HrSoxB1* also might not be sufficient to promote ectopic neural development. Because a *Sox2* construct lacking the HMG box is able to act as a dominant negative form and inhibit neural differentiation in *Xenopus* (Kishi *et al*., 2000), we made a similar construct with *HrSoxB1* lacking the HMG box and injected mutated mRNA into *Halocynthia* eggs. However, the eggs still developed into normal swimming tadpoles (data not shown).

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