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Evolutionary alterations of the minimal promoter for notochord-specific *Brachyury* expression in ascidian embryos

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SUMMARY

The *Brachyury* genes of two divergent ascidians, *As-T* of *Halocynthia roretzi* and *Ci-Bra* of *Ciona intestinalis*, are expressed exclusively in notochord precursor cells. A previous study showed that the notochord-specific expression of *Ci-Bra* is controlled by a minimal promoter that is composed of three distinct regions: a region responsible for repression of expression in non-notochord mesoderm cells, a region for activation of expression in notochord cells, and a region for activation of expression in non-notochord mesoderm cells, distal to proximal to the transcription initiation site, respectively. We examined various deletion constructs of the *As-T/lacZ* fusion gene and demonstrate that a module between −289 and −250 bp of the 5′-flanking region is responsible for notochord-specific expression of the reporter gene. Gel-shift assays suggested the binding of nuclear protein(s) to this module. The 5′-flanking region of *As-T* contains a potential T-binding motif (-ACCTAGGT-) around −160 bp. Deletion of this motif from the p(−289)As-T/lacZ diminished the reporter gene expression. In addition, coinjection of p(−289)As-T/lacZ and synthetic *As-T* mRNA resulted in ectopic expression of *lacZ* in non-notochord cells, suggesting that the T-binding motif is responsible for autoactivation of the gene. These findings revealed striking differences between the minimal promoters of *As-T* and *Ci-Bra* so far revealed, with respect to their notochord-specific expression. Furthermore, reciprocal injections of reporter gene constructs, namely *As-T/lacZ* into *Ciona* eggs and *Ci-Bra/lacZ* into *Halocynthia* eggs, suggest alterations in the *cis*-regulatory elements and trans-activation factors that have occurred during evolution of the two ascidian species.

Key words: Ascidians, *Brachyury*, Notochord-specific expression, Minimal promoter, T-binding motif, Divergence, Evolutionary alteration

INTRODUCTION

Brachyury encodes a sequence-specific transcriptional activator that contains a T-box DNA-binding domain (reviewed by Herrmann and Kispert, 1994; Smith, 1997; Papaioannou and Silver, 1998). The gene was originally cloned from mice, taking advantage of a *Brachyury* mutation (Herrmann et al., 1990). In vertebrates, *Brachyury* is initially expressed in the presumptive mesoderm, and during later stages the expression pattern is gradually restricted to the developing notochord and tailbud (Wilkinson et al., 1990; Smith et al., 1991; Schulte-Merker et al., 1992; Kispert et al., 1995). *Brachyury* expression is critical for notochord differentiation in all vertebrates that have been studied, including mice (Rashbass et al., 1991), frogs (Conlon et al., 1996) and zebrafish (Schulte-Merker et al., 1994).

Ascidians (urochordates) are one of the three chordate groups, and the ascidian tadpole is thought to represent the most simplified and primitive chordate body plan (reviewed by Satoh, 1994; Satoh and Jeffery, 1995; Di Gregorio and Levine, 1998). It contains a notochord composed of just 40 cells, of which the lineage has been completely described. Previous studies in two divergent ascidians, *Halocynthia roretzi* (Yasuo and Satoh, 1993) and *Ciona intestinalis* (Corbo et al., 1997a), revealed that *Brachyury* (*As-T* of the former species and *Ci-Bra* of the latter species) is expressed exclusively in the notochord precursor cells, and that the timing of the gene expression coincides with the clonal restriction of the notochord lineages. In *H. roretzi*, notochord formation is induced at the 32-cell stage by signals emanating from the adjacent endoderm (Nakatani and Nishida, 1994). Overexpression of *As-T* via its mRNA injection results in notochord formation without a requirement for the inductive event at the 32-cell stage (Yasuo and Satoh, 1998). Misexpression of both *As-T* (Yasuo and Satoh, 1998) and *Ci-Bra* (Takahashi et al., 1999) causes transformation of endoderm and neuronal lineages into notochord cells. These results indicate that the ascidian *Brachyury* gene is a critical determinant of the notochord.

The promoter for specific expression of *Brachyury* has been studied in mice (Clements et al., 1996) and *Xenopus* (Latinkic et al., 1997). In mice, the 5′-proximal region from −500 to +150 bp relative to the start of *Brachyury* transcription, is responsible for the primitive streak expression. However,

Clements et al. (1996) could not find any region within the 8.3 kb of 5′-flanking sequence that was associated with the activation of *Brachyury* in the node and notochord, suggesting that gene expression in the axial (node and notochord) and non-axial mesoderm (primitive-streak derived mesoderm) is differentially controlled. In *Xenopus*, the transcriptional regulation of *Xbra2*, a pseudoallele of *Xbra*, was examined (Latinkic et al., 1997), but identification of activin- and FGF-responsive elements in the *Xbra2* promoter has proved troublesome.

The minimal promoter for notochord-specific *Brachyury* expression has been best-characterized in the ascidian *C. intestinalis* (see Fig. 4C). Corbo et al. (1997a) demonstrated that the 434 bp of a minimal enhancer of *Ci-Bra* contains three distinctive regions. The distal one is a negative control region (from −434 to −299) that inhibits *Ci-Bra* expression in inappropriate embryonic lineages, including the trunk mesenchyme and the tail muscle. Within and around this region are *snail*-binding sites; *Ciona* snail protein acts as a repressor of *Ci-Bra* expression in the mesodermal region other than notochord, and therefore defines the boundary of the notochord (Fujiwara et al., 1998). The middle region (from −299 to −188 bp) is responsible for notochord enhancement, and the enhancer is activated by a regulatory element, which is closely related to the recognition sequence of *Suppressor of Hairless* transcription factor (Corbo et al., 1998). The proximal region is related to activation of expression in mesenchyme and muscle, and has E-box motifs.

We are interested in the changes in the control mechanisms of tissue-specific gene expression during animal evolution. Is the minimal promoter system for notochord-specific expression of *Ci-Bra* of *C. intestinalis* applicable to *As-T* of *H. roretzi*? Or has *As-T* adopted a different minimal promoter system to achieve its notochord-specific expression? The present study examined the *cis*-regulatory elements of the notochord-specific expression of *As-T.* The results showed that the notochord-specific expression of *As-T* is achieved by a minimal promoter that is different from the one that has been described for *Ci-Bra*. Our results also suggest evolutionary alterations in the minimal promoters between the two divergent ascidians.

MATERIALS AND METHODS

Animals and embryos

Halocynthia roretzi was purchased during the spawning season from fishermen in the vicinity of Otsuchi Marine Research Center, Ocean Research Institute, University of Tokyo, Iwate and Asamushi Marine Biological Station, Tohoku University, Aomori, Japan. *Ciona intestinalis* was collected in the vicinity of the Marine Bioresource Education Center of Tohoku University, Onagawa, Miyagi, Japan. Handling of eggs and embryos was described previously (Yasuo and Satoh, 1998; Corbo et al., 1997a).

Screening of genomic library and nucleotide sequencing

An *H. roretzi* genomic library was constructed in λFIX II (Stratagene; Kusakabe et al., 1992). Screening of the libraries was performed using standard procedures (Sambrook et al., 1989). Nucleotide sequences were determined for both strands with dye primer cycle sequencing FS ready reaction kits and an ABI PRISM 377 DNA sequencer (Perkin Elmer).

Fusion gene constructs

The *As-T* constructs were made in the following manner. The 4.0 kb and 1.7 kb *As-T* genomic fragments were subcloned into the multicloning site of plasmid p46.21, a version of pPD1.27 that lacks the *C. elegans* sup-7 gene (Fire et al., 1990). p46.21 encodes a bacterial gene for β-galactosidase (*lacZ*) with a nuclear localization signal in the multicloning site, and was kindly provided by Dr A. Fire (Carnegie Institution of Washington). Constructs p(−897)As-T/lacZ, p(−490)As-T/lacZ, p(−334)As-T/lacZ, p(−289)As-T/lacZ, p(−164)As-T/lacZ and p(−58)As-T/lacZ were gel-purified after digestion of p(−1,759)As-T/lacZ DNA with *Spe*I, *Pvu*I, *Eco*RV, *Nsp*I, *Eco*T14I and *Pst*I, respectively.

To construct p(−270)As-T/lacZ, primers −270 and pPD-R were used for PCR amplification. The amplified fragments were digested with *Hin*dIII and *Pst*I, and ligated with *Hin*dIII/*Pst*I-digested p(−289)As-T/lacZ. p(−250)As-T/lacZ was also constructed in the same manner using the primers −250 and pPD-R. These primer sequences were: primer −270*Hin*dIII, 5′-AACCCAAGCTTGTATCCATTTCGCAT-3′; primer −250*Hin*dIII, 5′-AACCCAAGCTTTGCCAATGAACTCGT-3′; pPD-R, 5′-TAGCGCAACATTTTGCTG-3′.

Mutations were introduced using synthetic oligonucleotides. Essentially, in each case, the region to be mutated was replaced by synthetic double-stranded oligonucleotides in which the sequence had been transversionally changed. QuikChange site-directed mutagenesis kit oligonucleotides (Stratagene) used in the procedure were as follows:

μA: CGAAGTGAAaccGACATCAGTATCCATTTCGC+GCGAA-ATGGATACTGATGTCggtTTCACTTCG;
μB: CGAAGTGAACAAtcaATCAGTATCCATTTCGC+GCGAA-ATGGATACTGATtgaTTGTTCACTTCG;
μC: CGAAGTGAACAAGACcgaAGTATCCATTTCGC+GCGAA-ATGGATACTtcgGTCTTGTTCACTTCG;
μD: GAACAAGACATCctgATCCATTTCGCATTTAGC+GCTAA-ATGCGAAATGGATcagGATGTCTTGTTC;
μE: GAACAAGACATCAGTcgaCATTTCGCATTTAGC+GCTAA-ATGCGAAATGtcgACTGATGTCTTGTTC;
μF: CAAGACATCAGTATCacgTTCGCATTTAGCTGCG+CAGC-TAAATGCGAAcgtGATACTGATGTCTTG;
μG: CAAGACATCAGTATCCATggaGCATTTAGCTGC+GCAGC-TAAATGCtccATGGATACTGATGTCTTG;
μH: CAAGACATCAGTATCCATTTCtacTTTAGCTGCCAATGA-A+TTCATTGGCAGCTAAAgtaGAAATGGATACTGATGTC-TTG;
μI: CAAGACATCAGTATCCATTTCGCAgggAGCTGCCAATG-AA+TTCATTGGCAGCTcccTGCGAAATGGATACTGATGT-CTTG;
μJ: CAAGACATCAGTATCCATTTCGCATTTctaTGCCAATGA-A+TTCATTGGCAtagAAATGCGAAATGGATACTGATGTC-TTG.

The p(−289del(−170-150))As-T/lacZ construct was made by site-directed mutagenesis of the construct p(−289)As-T/lacZ using the following oligonucleotides: 5′-GCATTCATTTCTAACGTTGAG-GGCATGG-3′; 5′-CCATGCCCTCAACGTTAGAAATGAATGC-3′ (underlines indicate the nucleotides between which the deletion was made).

Microinjection of fusion gene constructs and histochemical detection of β-galactosidase activity

Microinjection of fusion constructs and histochemical detection of β-galactosidase (β-gal) activity were performed as described previously (Hikosaka et al., 1994; Kusakabe et al., 1995). Ascidian eggs are enclosed by a vitelline coat, or chorion. Fertilized eggs were treated with a solution (1% sodium thioglycolate, 0.05% actinase E (Kaken Pharmaceutical Co., Ltd., Tokyo), adjusted to pH 10 with NaOH) for 2 minutes with pipetting to remove follicle cells. After this treatment, the eggs were easily fixed on cover glasses, which made

microinjection of recombinant DNAs into the eggs easy. The microinjection was performed through the vitelline coat.

Plasmid DNAs were linearized by digestion with *Pst*I and dissolved in 1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0. Microinjection was carried out using injection pipettes held by micromanipulators (model MN-151; Narishige Scientific Instruments Lab., Tokyo) on a cover glass under a stereomicroscope. Micropipettes were made on a horizontal puller (model PG-1; Narishige) from 1.2-mm fiber-filled glass capillary tubing (Microcaps; Drummond Sci. Co., Broomall, PA) and were sterilized. The DNA solution (2 μg) was injected into the cytoplasm of fertilized eggs under pressure. Injected eggs were incubated on plastic dishes coated with 1.5% agar in Millipore-filtered sea water containing 50 mg/l streptomycin sulfate (Meiji Seika Co. Ltd., Tokyo) until late tailbud stage.

Injected embryos were fixed for 30 minutes at room temperature in 0.5 M NaCl, 27 mM KCl, 2 mM EDTA (pH 8.0) containing 1% glutaraldehyde. Fixed embryos were rinsed in phosphate-buffered saline (PBS) and incubated in PBS that contained 250 μM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 0.1% Triton X-100, 1 mM $MgCl_2$, 3 mM $K_4[Fe(CN)_6]$, and 3 mM $K_3[Fe(CN)_6]$ at 37°C for 30 minutes. The stained embryos were washed in PBS to stop the staining reaction and observed under an Olympus stereomicroscope.

Electroporation of fusion gene constructs

Electroporation was carried out as described (Corbo et al., 1997a).

Preparation of nuclear extracts

All buffers contained 0.2 mM PMSF, 0.5 mM DTT, 2 mM Na_3VO_4, 50 mM NaF, 10 mg/ml aprotinin and 0.5 mM spermidine. Dechorionated frozen *H. roretzi* gastrula stage embryos (5 g) were thawed and washed twice with 100 ml of extraction buffer (10 mM Hepes, pH 8.0, 1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 0.36 M sucrose) by stirring at 4°C. After centrifugation at 1,100 ***g*** for 10 minutes, the resulting precipitates were suspended in extraction buffer containing 0.05% Nonidet P(NP)40, and homogenized using 15 up-and-down strokes using a glass Dounce homogenizer (type B pestle). The homogenate was centrifuged at 1,100 ***g*** for 10 minutes and the resulting precipitates (nuclear preparation) were washed once with the same buffer and centrifuged. The nuclei were resuspended in 1 ml of extraction buffer containing 0.05% NP40, and supplemented by dropwise addition with continuous stirring of 1 ml of high-salt buffer (20 mM Hepes, pH 8.0, 0.2 mM EDTA, 1.6 M KCl, 1.5 mM $MgCl_2$, 25% glycerol). The nuclei were extracted for 30 minutes by stirring at 4°C, and then the extracted nuclei were pelleted by centrifugation at 20,000 ***g*** for 15 minutes. The samples were dialyzed twice against 500 ml of dialysis buffer (20 mM Hepes, pH 8.0, 0.2 mM EDTA, 100 mM KCl, 20% glycerol). After centrifugation of the extract at 20,000 ***g*** for 20 minutes, the supernatant was frozen at −80°C.

Gel-shift assays

First, PCR was performed to amplify a part of the 5′-flanking region of p(−289)As-T/lacZ as a template. The amplified fragments were cut with *Hin*dIII/*Bam*HI and subcloned into the *Hin*dIII/*Bam*HI site of pBSII SK(+). Insert DNA fragments were gel-purified and labeled with [α-^{32}P]dCTP using Klenow fragment. The following oligonucleotides were used to prepare these DNA fragments:

a(290-250): GCGCAAGCTTCGAAGTGAACAAGACATG+CGCGGGATCCGCTAAATGCGAAATGGAT;
b(290-260): GCGCAAGCTTCGAAGTGAACAAGACATG+CGCGGGATCCAAATGGATACTGATGTCTTG;
c(280-250): GCGCAAGCTTCAAGACATCAGTATCCA+CGCGGGATCCGCTAAATGCGAAATGGAT;
d(290-270): GCGCAAGCTTCGAAGTGAACAAGACATG+CGCGGGATCCTGATGTCTTGTTCACTT;
e(280-260): GCGCAAGCTTCAAGACATCAGTATCCA+CGCGGGATCCAAATGGATACTGATGTCTTG;
f(270-250): AACCCAAGCTTGTATCCATTTCGCAT+CGCGGGATCCGCTAAATGCGAAATGGAT.

Binding assays were done with *H. roretzi* gastrula stage nuclear extracts. The nuclear extract was incubated with 10 ng/ml of cold competitor DNAs in a solution containing 10 mM Hepes, pH 7.9, 50

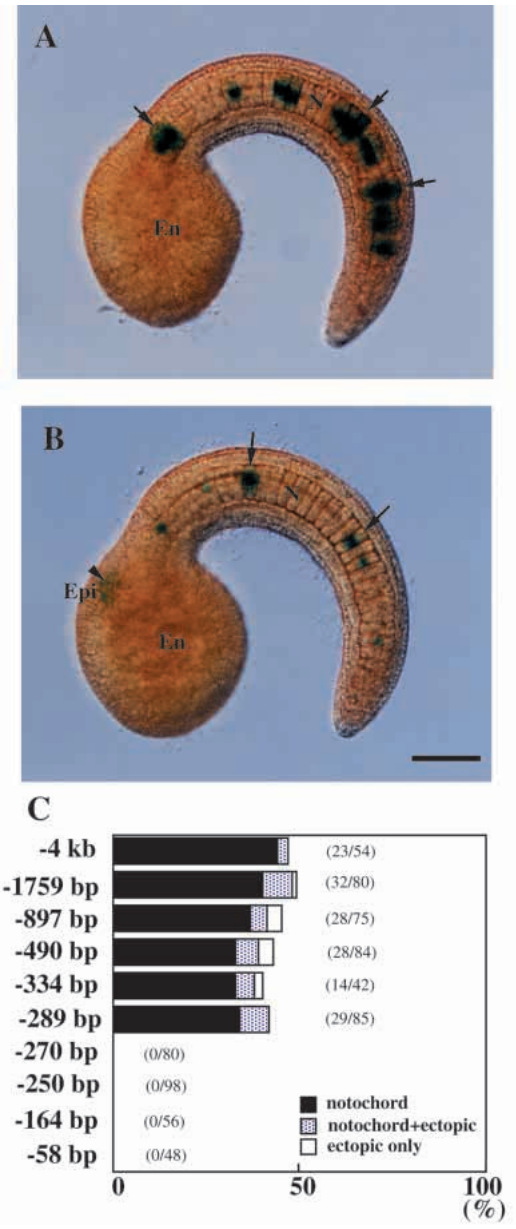

Fig. 1. Expression of *lacZ* in *Halocynthia* tailbud-stage embryos that developed from eggs injected with various deletion constructs of pAs-T/lacZ. (A) Injection of p(−289)As-T/lacZ resulted in notochord-specific expression of the reporter gene (arrows). En, endoderm; N, notochord. (B) Injection of p(−289)As-T/lacZ sometimes resulted in ectopic expression of *lacZ* in epidermal cells (Epi, arrowhead). Scale bar, 50 μm. (C) Frequency of embryos with the reporter gene expression and sites of expression. Numbers in parentheses indicate the number of positive embryos relative to total embryos examined. The deletion constructs are indicated on the left.

mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 10 mg/ml aprotinin, 5 mM Na_3VO_4, 2 mM PMSF and 0.1 mg/ml poly(dI-dC) on ice for 5 minutes. Samples containing 0.1 ng/ml of labeled DNA were then added and incubated at room temperature for 15 minutes. Afterwards, the reaction mixtures were fractionated on a 9% polyacrylamide gel and subsequently prepared for autoradiography (Ip et al., 1991).

RESULTS

The 289 bp 5′-flanking region of *As-T* contains a minimal promoter for the notochord-specific reporter gene expression

In order to determine the minimal promoter required for the notochord-specific expression of *As-T*, we first made a fusion gene construct, p(–4 kb)As-T/lacZ, in which 4 kb of the 5′-flanking region of *As-T* (from –4,000 to +110, including the first 19 bp of the coding region; see also Fig. 4A) was linked with the reporter gene *lacZ*. From this construct, we made various deletion constructs, including p(–1,759)As-T/lacZ, p(–897)As-T/lacZ, p(–490)As-T/lacZ, p(–334)As-T/lacZ, p(–289)As-T/lacZ, p(–270)As-T/lacZ, p(–250)As-T/lacZ, p(–164)As-T/lacZ, and p(–58)As-T/lacZ (cf. Fig. 4A). We injected linearized pAs-T/lacZ into *H. roretzi* eggs about 30-90 minutes after insemination at concentrations of 16×, 8× and 4×10^4 copies per egg, respectively. We found that injection of 8×10^4 copies of pAs-T/lacZ yielded the best results, while injection of 16×10^4 copies sometimes resulted in an ectopic reporter gene expression in addition to the notochord-specific expression observed in the case of injection of 8×10^4 copies. Injection of 4×10^4 copies resulted in few embryos with the reporter gene expression (data not shown). On average, about half of the eggs injected with fusion constructs cleaved normally and developed to tailbud embryos with normal morphology. We scored the reporter gene expression only in manipulated embryos that exhibited normal morphology. No endogenous β-gal activity was detected in control non-injected embryos (data not shown).

The results of expression analyses of the deletion constructs summarized in Fig. 1 were clearcut. Fusion constructs with 289 bp or more upstream relative to the transcription start site of *As-T* were able to direct notochord-specific reporter gene expression in nearly half of injected embryos (Fig. 1A-C). In contrast, fusion genes with 270 bp or less of *As-T* 5′-flanking sequences were not able to direct the reporter gene expression (Fig. 1C). Expression of *lacZ* in notochord cells was usually mosaic (Fig. 1A,B), presumably due to random incorporation of the constructs into early blastomeres. In a few cases, the reporter gene was expressed ectopically in embryonic cells other than notochord (Fig. 1B,C). However, the ectopic expression was usually detected only in epidermal cells (Fig. 1B). This pattern of ectopic expression is different from the case of the ectopic *lacZ* expression in mesenchyme and muscle cells when pCi-Bra/lacZ deletion constructs were electroporated into *Ciona* eggs (Corbo et al., 1997a). The results suggest that at least the module of sequences between –289 and –270 is associated with the notochord-specific *As-T* expression.

Nuclear protein(s) is bound to modules around –270 bp of *As-T*, and mutations in the modules diminish the notochord-specific reporter gene expression

The analyses of the deletion constructs described above demonstrated that the sequence of about 20 bp between –289 and –270 bp is required for the notochord-specific expression of the reporter gene. Gel-shift assays were carried out to determine whether factor(s) in nuclear extracts from gastrula stage embryos is bound to element(s) in and around this region. As shown in Fig. 2A, gel-shift assays of six types of oligonucleotides corresponding to regions ‘a’ (–290 to approx. –250 bp), ‘b’ (–290 to approx. –260 bp), ‘c’ (–280 to approx. –250 bp), ‘d’ (–290 to approx. –270 bp), ‘e’ (–280 to approx. –260 bp) and ‘f’ (–270 to approx. –250 bp), showed that nuclear protein(s) is bound to the ‘a’ region (Fig. 2B). This binding seemed specific because the binding was downregulated by ‘a’ competitor.

Three overlapping oligonucleotides, ‘d’, ‘e’ and ‘f’, were designed to cover the entire ‘a’ sequence (Fig. 2A). However, none of them could completely compete with ‘a’, although the intensity of binding was weaker than that without any competitor (Fig. 2B). This suggests that the module contains

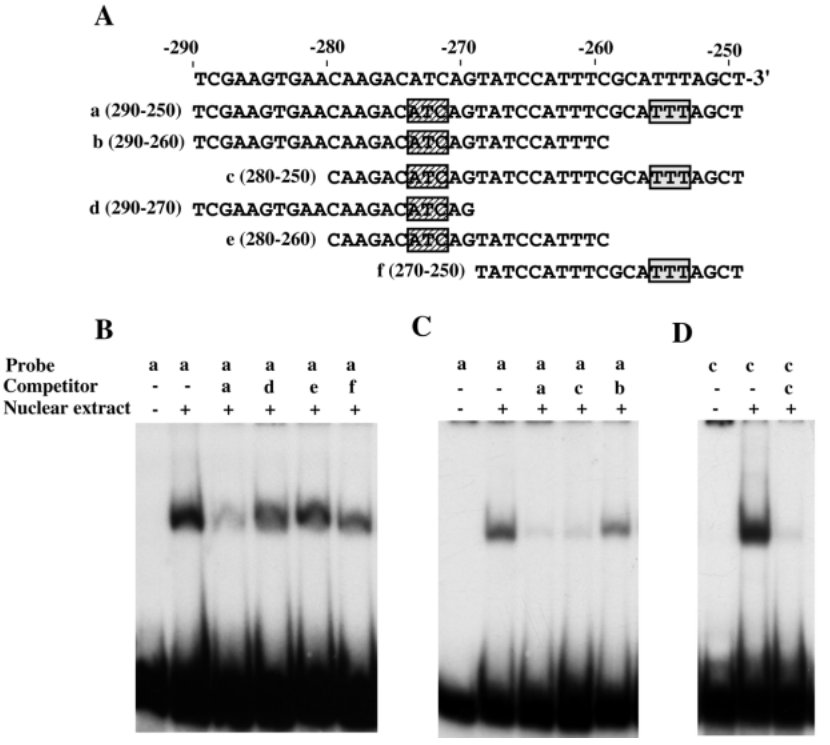

Fig. 2. Gel-shift assay of binding of nuclear extracts from gastrula stage embryos to oligonucleotides corresponding to various regions between –290 and –250 bp of *As-T*. (A) Six types of oligonucleotide (a-f) were examined. Two 3-bp sequences for potential binding sites are shown in shaded boxes. (B-D) Gel-shift assays of binding of nuclear extracts to the oligonucleotides shown above. All competitor probes were added in 50-fold molar excess.

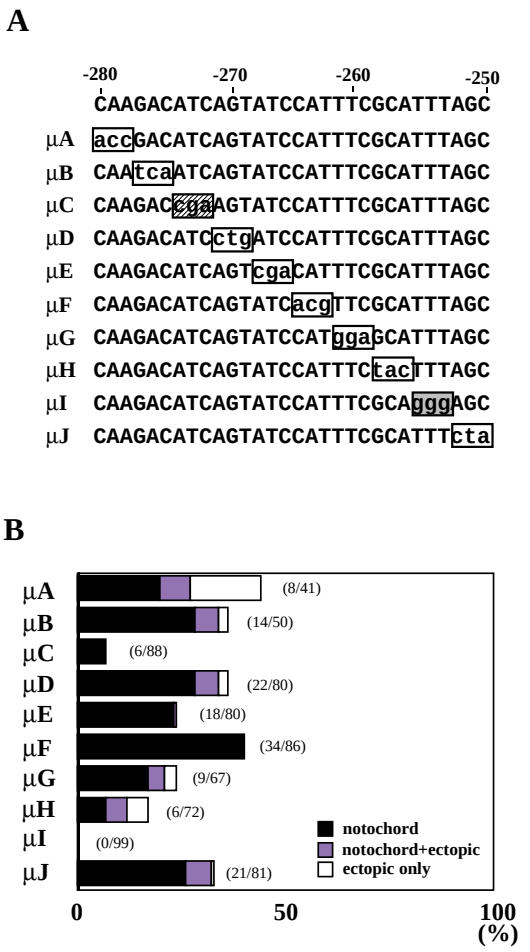

Fig. 3. Insertion of mutations affects the notochord-specific expression of the reporter gene. (A) Mutations inserted into the 5′-flanking region between –280 and –250 bp of *As-T*. (B) Frequency of embryos with *lacZ* expression that developed from eggs injected with various mutation constructs (indicated on left) and sites of expression. Numbers in parentheses indicate the number of positive embryos relative to total embryos examined.

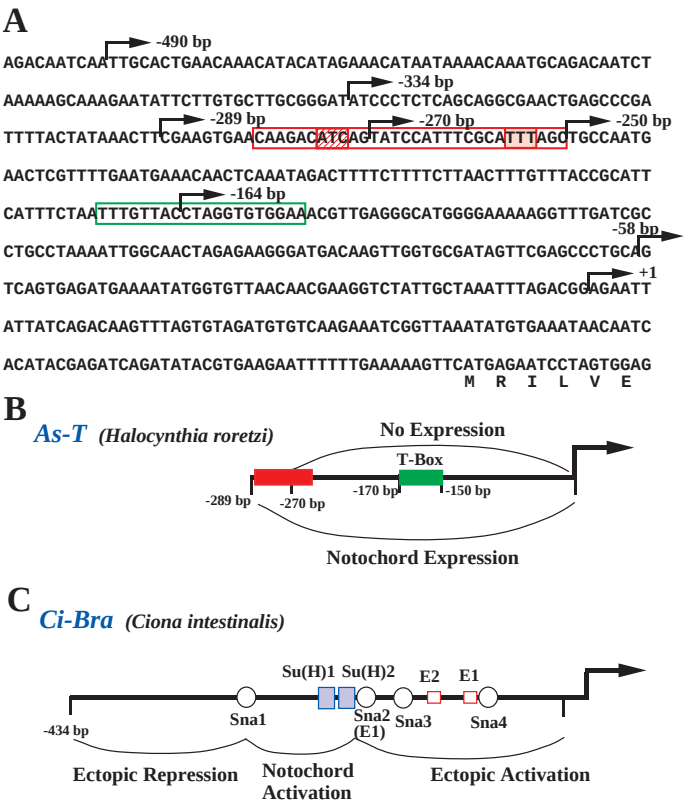

Fig. 4. Nucleotide sequence of the minimal promoter for notochord-specific *As-T* expression (A) and suggested mechanism of action of *As-T* of *Halocynthia roretzi* (B) and *Ci-Bra* of *Ciona intestinalis* (C) (from Corbo et al., 1997a; Fujiwara et al., 1998, with permission).

two or more binding sites that cannot be covered by any of the three competitors. Two other overlapping oligonucleotides, ‘b’ and ‘c’, were designed to examine this possibility (Fig. 2A). As shown in Fig. 2C, ‘c’ but not ‘b’ competed with the binding of ‘a’. In addition, nuclear protein(s) is bound to the ‘c’ region, and the binding was inhibited by ‘c’ competitor (Fig. 2D). These results suggest that nuclear protein(s) recognize two *cis*-elements in this module of *As-T*.

To substantiate the specific binding of nuclear protein(s) to the two potential *cis*-elements, we inserted mutations for every three bases between –280 and –250 of pAs-T/lacZ, and examined the effects of three-base mutations on expression of the reporter gene by injecting recombinant constructs into *Halocynthia* eggs. The results, shown in Fig. 3, indicate that mutations in two elements, μC (-273 to approx. –271) and μI (–257 to approx. –255), markedly reduced the frequency of embryos with notochord-specific reporter gene expression. Together with the results of the gel-shift assay, this result suggests that there are at least two elements between –280 and –250 that are associated with the notochord-specific *As-T* expression.

The minimal promoter region contains a potential T protein-binding motif

Kispert and Herrmann (1993) examined specific DNA binding of the mouse Brachyury (T) protein to DNA fragments, which were selected from a mixture of random oligomers. They identified a 20 bp palindrome, TG(C)ACACCTAGGTG-TGAAATT, as a possible consensus sequence that binds to the Brachyury DNA-binding domain. However, no full-length copy of this proposed sequence has been reported to be present in any genome yet, although half of the palindrome has been reported in the promoter region of *Xenopus eFGF*, which is a direct target of *Xbra* (Casey et al., 1998). The nucleotide sequence of about 500 bp of the 5′-flanking region of *As-T* is shown in Fig. 4A. We noticed that the promoter of *As-T* contains a 21 bp palindrome-like sequence, TTTGTTACCTAGGTGTGGAAA, between –171 and –151 from the transcription start site (+1). We therefore examined whether or not this sequence is essential for *As-T* expression. We made a fusion gene of p(–289 del(–170 to approx. –150))As-T/lacZ, in which the palindrome-like sequence was deleted from p(–289)As-T/lacZ (Fig. 5A). This deletion completely diminished the *lacZ* expression when the construct was injected into *Halocynthia* fertilized eggs (Fig. 5A),

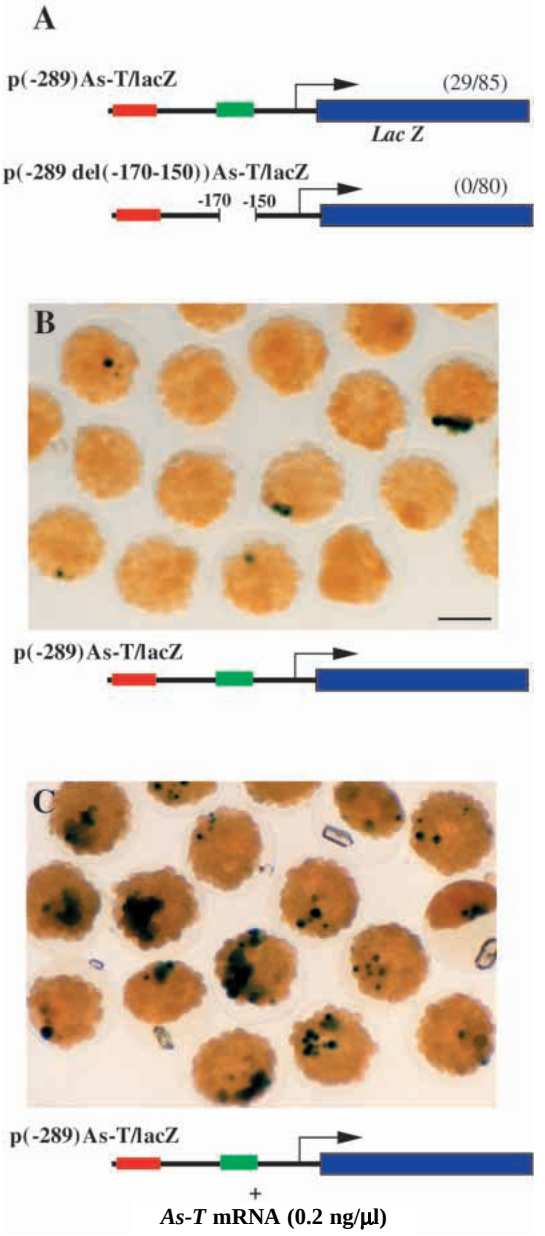

Fig. 5. Requirement of a potential T-binding domain for notochord-specific *lacZ* expression. (A) Control p(−289)As-T/lacZ, and p(−289)As-T/lacZ without the potential T-binding domain (−170 to approx. −150 bp). The latter failed to direct notochord-specific *lacZ* expression. Numbers in parentheses indicate the number of positive embryos relative to total embryos examined. (B,C) Misexpression of As-T protein induces ectopic *lacZ* expression in blastomeres of non-notochord lineages. Eggs were injected with p(−289)As-T/lacZ either (B) without or (C) with *As-T* mRNA. Injected eggs were allowed to develop to the 110-cell stage, and then cleavage was arrested for about 12 hours before detection of the reporter gene expression. Scale bar, 250 μm.

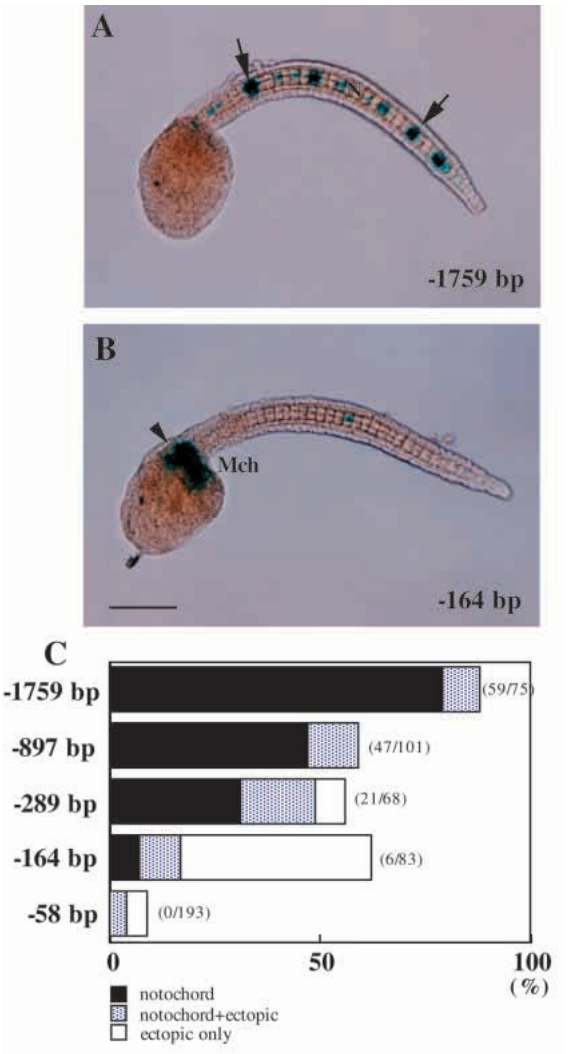

Fig. 6. Expression of *lacZ* in *Ciona* embryos that developed from eggs injected with various deletion constructs of pAs-T/lacZ. (A) Injection of p(−1759)As-T/lacZ resulted in reporter gene expression in notochord cells (N, arrows). (B) Injection of p(−164)As-T/lacZ resulted in *lacZ* expression very rarely in notochord cells, but frequently in mesenchyme cells (Mch, arrowhead). Scale bar, 50 μm. (C) Frequency of embryos with the reporter gene expression that developed from eggs injected with deletion constructs (left) of pAs-T/lacZ and sites of expression. Numbers in parentheses indicate the number of positive embryos relative to total embryos examined.

suggesting that the palindrome-like sequence is essential for *As-T* expression.

The presence of a potential T-binding sequence and the requirement of this domain for *As-T* expression suggest that this domain is associated with the autoregulative activation of *As-T*. In order to examine this possibility, we coinjected p(−289)As-T/lacZ and synthetic *As-T* mRNA. If injection of synthetic *As-T* mRNA causes ectopic expression of As-T protein in blastomeres of non-notochord lineages, and this in turn activates expression of the fusion construct, the reporter gene should be expressed ectopically. In this experiment, we took advantage of cleavage-arrested 110-cell-stage embryos, because ectopic expression is more easily detected in them. The 110-cell-stage embryo contains 10 notochord precursor cells. These cells divide twice to form 40 notochord cells in the tailbud-stage embryo. Cleavage-arrested control embryos showed a few blastomeres with *lacZ* expression (Fig. 5B; average 0.7 cells/embryo, maximum 6 cells/embryo, 44 embryos examined). However, a 110-cell stage embryo with

two positive blastomeres, for example, corresponds to a tailbud stage embryo with eight positive cells. This seems compatible with the number of cells with *lacZ* expression in the tailbud-stage embryo (Fig. 1A,B). As shown in Fig. 5C, coinjection of p(−289)As-T/lacZ and synthetic *As-T* mRNA resulted in an ectopic expression of the reporter gene (average 5.9 cells/embryo, maximum 32 cells/embryo, 76 embryos examined). These results suggest that the T-binding sequence of *As-T* is important for enhancement of the gene expression. In other words, this domain seems to be responsible for autoregulative activation of *As-T*.

Comparison of the notochord-specific minimal promoters of *As-T* and *Ci-Bra*

The nucleotide sequences of the 5′-flanking regions of *As-T* are shown in Fig. 4A, and the minimal promoters of *As-T* of *H. roretzi* and *Ci-Bra* of *C. intestinalis* genes are compared in Fig. 4B,C. Corbo et al. (1997a) demonstrated that the 434 bp minimal enhancer of *Ci-Bra* contains three distinctive regions (Fig. 4C), including a negative control region (from –434 to –299), which inhibits *Ci-Bra* expression in inappropriate embryonic lineages, including the trunk mesenchyme and the tail muscle. Within and around this region are *snail*-binding sites; *Ciona* snail protein acts as repressor of *Ci-Bra* so that it is not expressed in the mesodermal regions other than the notochord, and therefore defines the boundary of the notochord (Fujiwara et al., 1998). The second region (from –299 to –188) is responsible for notochord enhancement, and the enhancer is activated by a regulatory element, which is closely related to the recognition sequence of the *Suppressor of Hairless* transcription factor (Corbo et al., 1998). The proximal region (from –188 to –1) is associated with enhancement of *Ci-Bra* expression in mesenchyme and muscle, and has E-box motifs (Fig. 4C).

The structure of the *As-T* minimal promoter that we infer from the experiments described above is quite different from that of *Ci-Bra*. We examined seven different types of deletion construct between –490 and –59, and the results showed that elements between –290 and –250 are associated with the notochord-specific reporter gene expression (Fig. 4B). This suggests that the two divergent ascidian species have evolved different types of the minimal promoter organization responsible for the notochord-specific expression of the genes. We therefore examined the reporter gene expression when pAs-T/lacZ was injected into *Ciona* eggs and when pCi-Bra/lacZ was injected into *Halocynthia* eggs.

Pattern of reporter gene expression in *Ciona* eggs electroporated with pAs-T/lacZ

The results of this series of reciprocal-injection experiment are summarized in Fig. 6. As in the case of *As-T/lacZ* in *Halocynthia* embryos, fusion constructs with –289 or more upstream sequences of *As-T* directed notochord-specific reporter gene expression in more than half of injected *Ciona* embryos (Fig. 6A,C), although the frequency of embryos with ectopic expression was slightly higher than that in *Halocynthia* embryos.

In the case of *As-T/lacZ* in *Halocynthia* eggs, p(–164)As-T/lacZ did not direct reporter expression (Fig. 1C). In contrast, p(–164)As-T/lacZ in *Ciona* eggs showed distinct reporter expression in mesenchyme cells (Fig. 6B,C). Interestingly, this pattern was similar to the case of p(–190)Ci-Bra/lacZ in *Ciona* eggs. p(–58)As-T/lacZ does not promote reporter gene expression in *Halocynthia* eggs (Fig. 1C), and this construct showed no reporter gene expression in *Ciona* eggs (Fig. 6C).

Pattern of reporter gene expression in *Halocynthia* eggs injected with Ci-Bra/lacZ

In this series of experiments, we injected p(–3.5 kb)Ci-Bra/lacZ, p(–434)Ci-Bra/lacZ and p(–250)Ci-Bra/lacZ into *Halocynthia* eggs. We injected pCi-Bra/lacZ at concentration of 8×10^4 copies per egg, the same as pAs-T/lacZ into *H. roretzi* eggs. The first two constructs show notochord-predominant reporter gene expression when electroporated into *Ciona* eggs, whereas the third construct shows reporter gene expression not only in notochord but also in two other mesodermal regions, mesenchyme and muscle (Corbo et al., 1997a).

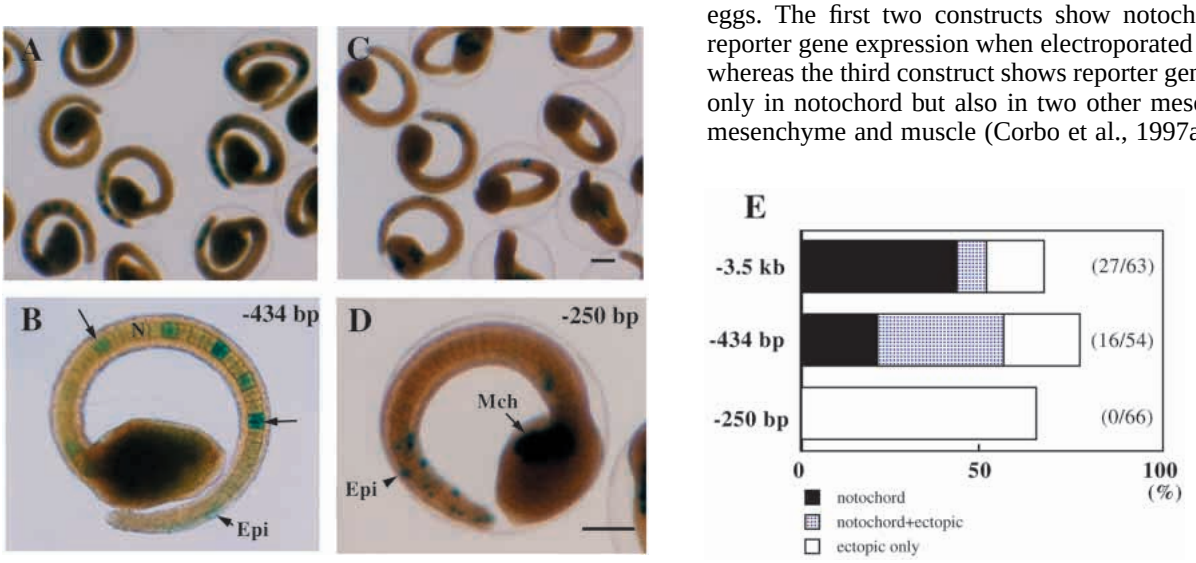

Fig. 7. Expression of *lacZ* in *Halocynthia* embryos that developed from eggs injected with various deletion constructs of pCi-Bra/lacZ. (A,B) Injection of p(–434)Ci-Bra/lacZ resulted in reporter gene expression in notochord cells (N, arrows), and sometimes ectopically in epidermal cells (Epi, arrowhead). (C,D) Injection of p(–250)Ci-Bra/lacZ resulted in *lacZ* expression not in notochord cells (N), but ectopically in mesenchyme cells (Mch, arrows) and epidermal cells (Epi, arrowhead). Scale bar, 50 μm. (E) Frequency of embryos with the reporter gene expression, which developed from eggs injected with deletion constructs (left) of pCi-Bra/lacZ and sites of expression. Numerals in parentheses indicate number of positive embryos relative to total embryos examined.

When p(−3.5 kb)Ci-Bra/lacZ and p(−434)Ci-Bra/lacZ were injected into *Halocynthia* eggs, the reporter gene was mainly expressed in notochord cells and a few ectopic cells (Fig. 7A,B,E). The ectopic expression was usually found in epidermal cells (Fig. 7B). Although the rate of embryos with ectopic reporter gene expression was higher than that when these constructs were injected into *Ciona* eggs, this pattern of reporter gene expression is similar to that seen in the case of electroporation of these constructs into *Ciona* eggs.

However, when p(−250)Ci-Bra/lacZ was injected into *Halocynthia* eggs, the reporter gene was not expressed in the notochord, but instead only in ectopic regions (Fig. 7C,D,E). This was different from the pattern seen when p(−250)Ci-Bra/lacZ was electroporated into *Ciona* eggs, because this construct shows the reporter gene expression in notochord and ectopic regions (mesenchyme and muscle) of *Ciona* embryos (Corbo et al., 1997a).

DISCUSSION

Both *As-T* and *Ci-Bra* are exclusively expressed in notochord precursor cells of *H. roretzi* and *C. intestinalis* embryos. The present study demonstrated that (1) the 5′-flanking region of *As-T* contains the minimal promoter system for notochord-specific expression of the gene, to which nuclear protein(s) is bound; (2) the minimal promoter of *As-T* contains a potential Brachyury protein-binding site; and (3) complicated alterations might have occurred in the minimal promoters during evolution of these two ascidian species, which was suggested by reciprocal injection of pAs-T/lacZ into *Ciona* eggs and pCi-Bra/lacZ into *Halocynthia* eggs.

The minimal promoter for notochord-specific expression of *As-T*

The promoter for specific expression of *Brachyury* has been studied in mice (Clements et al., 1996) and *Xenopus* (Latinkic et al., 1997). In mice, the 5′-proximal region between −500 and +150 bp relative to the start of *Brachyury* transcription is responsible for the primitive streak expression. However, Clements et al. (1996) could not find the region associated with the activation of *Brachyury* in the node and notochord within the 8.3 kb of 5′ sequence, suggesting that the gene expression in the axial (node and notochord) and non-axial mesoderm (primitive streak-derived mesoderm) is differentially controlled. In *Xbra2*, identification of activin- and FGF-responsive elements in the *Xbra2* promoter has proved troublesome.

As shown in this study, the 5′-flanking region of *As-T* contains a module of sequences between −290 and −250 bp that is associated with the notochord-specific expression of the gene. Various pAs-T/lacZ constructs sometimes showed ectopic reporter gene expression. However, the ectopic expression was detected primarily in epidermal cells and very rarely found in mesenchyme and muscle cells. In this regard, the *As-T* minimal promoter is different from the *Ci-Bra* minimal promoter in which the suppression system on the ectopic (mesenchyme and muscle) gene expression is incorporated. In addition, nuclear protein is bound to the *As-T* minimal promoter, and mutations in this module markedly reduced the rate of the reporter gene expression. Therefore, together with the minimal promoter of *Ci-Bra*, the ascidian *Brachyury* genes provide experimental systems with which to explore the regulatory machinery for the notochord-specific expression of the genes.

Potential Brachyury protein-binding site in the *As-T* promoter and autoregulative activity of the gene

In the 5′-upstream region of *As-T* at around −160 bp, there is a 10 bp core sequence (ACCTAGGT) of a palindrome (TTTC-ACACCTAGGTGTGAAA), which is a potential T-protein-binding site (Kispert and Herrmann, 1993; Kispert et al., 1995). Deletion of this element from p(−289)As-T/lacZ diminished the potential of this construct to direct notochord-specific reporter gene expression. In addition, coinjection of p(−289)As-T/lacZ and *As-T* mRNA induced additional and ectopic reporter gene expression. These results strongly suggest that this palindrome-like element is associated with enhancement of the gene activity. If *As-T* is once activated to produce As-T protein, the produced As-T protein may bind to the autoregulative elements to accelerate the activity of *As-T* transcription. Casey et al. (1998) examined the genes downstream of *Xenopus Brachyury*, *Xbra*. One candidate is *eFGF*, the promoter sequence of which contains half of the T-binding sequence. This finding, together with the present results, suggests that the potential Brachyury protein-binding sequence functions in vivo.

Nakatani and Nishida (1994) reported that differentiation of notochord cells of *H. roretzi* requires an induction in the early phase of the 32-cell stage, and that induction occurs not only in a heterogeneous combination of presumptive notochord cells and presumptive endoderm cells but also in a homogeneous combination of two presumptive notochord cells. It is likely that the above-mentioned autoregulative system is involved in this induction process. The palindromic sequence (ACCTAGGT), however, is not found in the minimal promoter of *Ci-Bra*. If the absence of the sequence means no autoregulative activation of *Ci-Bra*, homogeneous combination of presumptive notochord cells of *Ciona* 32-cell-stage embryos may not be involved in notochord differentiation. This should be examined in future studies.

Evolutionary aspects of minimal promoters for specific gene expression

The minimal promoter for notochord-specific expression of *Ci-Bra* was well characterized (Corbo et al., 1997a, 1998; Fujiwara et al., 1998) (Fig. 4C). The 434 bp minimal enhancer of *Ci-Bra* contains three distinctive regions: the first region (from −434 to −299 bp) prevents *Ci-Bra* expression in inappropriate embryonic lineages; the second region (from −299 to −188 bp) is responsible for notochord enhancement; and the third region (from −187 bp to the transcription start site) is responsible for activation of transcription in mesenchyme and muscle cells.

In contrast, *As-T* seems to have a simple module for the notochord-specific expression. Therefore, the *Ci-Bra* minimal promoter system for notochord-specific expression does not always correspond to the *As-T* minimal promoter. The second region of *Ci-Bra* contains the Suppressor of Hairless [Su(H)] binding site, which may be associated with notochord enhancement (Corbo et al., 1997b, 1998). However there is no recognition sequence (-GTGGGAA-) (Tun et al., 1994) for the

Suppressor of Hairless transcription factor in the minimal promoter of *As-T* (Fig. 4A). In *Ciona* embryos, the *snail* gene (*Ci-sna*) is expressed in the mesenchyme and muscle, to prevent *Ci-Bra* expression in the inappropriate embryonic lineages (Fujiwara et al., 1998). However, the expression pattern of the *snail* gene (*HrSna*) of *H. roretzi* (Wada and Saiga, 1999) is different from that of *Ci-sna* (Corbo et al., 1997b; Fujiwara et al., 1998). In addition to its expression in muscle and mesenchyme lineages, *HrSna* is also first expressed in an overlapping pattern with *As-T* at the 64-cell, 110-cell and early gastrula stages. Therefore, it is unlikely that *HrSna* expression defines the boundary between the notochord and non-notochord mesoderm in *Halocynthia* embryos.

There are several possible explanations for these evolutionary changes. Ascidians are a large group of the subphylum Urochordata, which consists of two major orders, Enterogona and Pleurogona. *C. intestinalis* belongs to the former group, which has one gonad, and *H. roretzi* to the latter group, with one pair of gonads. The two orders diverged rather deep in the history of ascidian evolution. During evolution of the two divergent ascidian groups, each group independently evolved a minimal promoter system for notochord-specific expression of *Brachyury*, and therefore the systems of *Ci-Bra* and *As-T* are rather different. However, this possibility seems unlikely in light of fact that p(−434)Ci-Bra/lacZ showed notochord-specific expression of the reporter gene in *Halocynthia* embryos, and p(−298)As-T/lacZ showed notochord-specific expression of the reporter gene in *Ciona* embryos. If the minimal promoters for the notochord-specific gene expression had evolved completely independently, it is very difficult to explain this phenomenon.

One possible explanation is that the minimal promoter of ascidian *Brachyury* was modified drastically during evolution of the two divergent species. Therefore, the promoter systems appear quite different superficially, but both retain to some extent notochord-specific expression in the case of reciprocal injection. In the present study, we have only focused on the minimal promoter in the 5′-flanking region. It remains a possibility that there are additional regulatory motifs in more upstream region than the 4 kb-flanking region of *As-T* or in the 3′-flanking region of *As-T*, which may correspond to the *Ci-Bra* promoter system. Mesenchyme-specific reporter gene expression of p(−164)As-T/lacZ in *Ciona* embryos seems unexpected because all kinds of pAs-T/lacZ construct we tested here did not show mesenchyme-specific reporter gene expression (Fig. 1C). However, it is possible that some other motifs within −164 of *As-T* may act as suppressor of the gene expression in mesenchyme cells of *Halocynthia* embryos, or certain motifs within this region of *As-T* may respond to the *Ciona* enhancement factors for the mesenchyme expression in *Ciona* embryos.

Another possible explanation would be that the expression of *Brachyury* genes in ascidians is controlled in a biphasic mode, initiation and maintenance. The elements found in *Ciona* could be responsible for the initiation of the gene expression. The presence of binding sites for snail and a putative Su(H) factor supports this notion. On the other hand, the *Halocynthia* enhancer could be involved in the maintenance part. Expression of p(−298)As-T/lacZ in *Ciona* embryos may be interpreted in terms of maintenance and/or autoregulation. That is, Ci-Bra protein is produced as usual in the injected notochord cells, and this Ci-Bra protein might bind to the T-binding sequence of *As-T*, and thereby promote *lacZ* expression specific to the notochord cells. However, it remains to be elucidated why the promoter-proximal region of *As-T* did not show the same pattern of the reporter gene expression as the promoter-proximal region of *Ci-Bra*.

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