Regulation of the muscle-specific expression and function of an ascidian T-box gene, As-T2

Yasuo Mitani¹,*,§, Hiroki Takahashi¹,2,4,§ and Nori Satoh¹,¶

¹Department of Zoology, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
²PRESTO, Japan Science and Technology Corporation, Japan
*Present address: National Institute of Advanced Industrial Science and Technology, 2-17-2-1 Tsukisamu-Higashi, Toyohira-ku, Sapporo 062-8517, Japan
§These authors contributed equally to this work
¶Author for correspondence (e-mail: satoh@ascidian.zool.kyoto-u.ac.jp)

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SUMMARY

The Tbx6 T-box genes are expressed in somite precursor cells of vertebrate embryos and are essential for the differentiation of paraxial mesoderm. However, it is unclear how spatial regulation of the gene expression is controlled and how the genes function to promote muscle differentiation. The Tbx6-related gene As-T2 of the ascidian Halocynthia roretzi is first expressed very transiently in endodermal cells around the 32-~44-cell stage, is then expressed distinctly and continuously in muscle precursor cells, and later in epidermal cells situated in the distal tip region of the elongating tail. We now show that inhibition of As-T2-mediated transcriptional activation by microinjection of As-T2/EnR into one-cell embryos resulted in suppression of the expression of the muscle-specific actin gene (HrMA4) and myosin heavy chain gene (HrMHC), but the injection did not affect the differentiation of endodermal cells or tail tip cells, suggesting that the primary function of As-T2 is associated with muscle cell differentiation. The 5′ flanking region of As-T2 contains two promoter modules that regulate its specific expression: a distal module that responsible for its specific expression in the tail, and a proximal module required for its muscle-specific expression. Around the proximal module, there are two putative T protein-binding motifs (TTCACACTT). Co-injection of an As-T2/lacZ construct with or without the T-binding motifs together with As-T2 mRNA revealed that these motifs are essential for autoregulatory activation of the gene itself. In addition, we found that the minimal promoter regions of HrMA4 and HrMHC contain T-binding motifs. Co-injection of HrMA4/lacZ or HrMHC/lacZ containing the T-binding motifs along with As-T2 mRNA revealed that As-T2 protein binds to these motifs to upregulate the gene activity. Taking into account the recent finding of maternal molecules for muscle differentiation, we propose a model for a genetic cascade that includes As-T2 as a regulator of muscle cell differentiation in the ascidian embryo.

Key words: Ascidian T-box gene, As-T2, Function, Minimal promoter, T protein-binding motif, Autoregulation, Muscle-specific gene expression

INTRODUCTION

The T-box genes encode a family of transcription factors that share an evolutionarily conserved T DNA-binding domain first defined in the product of the Brachyury (T) gene (Herrmann et al., 1990; reviewed by Herrmann and Kispert, 1994; Smith, 1997; Smith, 1999; Papaioannou and Silver, 1998). The Tbx6 subfamily includes mouse Tbx6 (Chapman et al., 1996), chick Tbx6L (Knezevic et al., 1997), Xenopus VegT (Zhang and King, 1996) and zebrafish ttx6 (Hug et al., 1997). The embryonic expression of all of these genes is similar during mesoderm formation at gastrulation, although VegT is also expressed maternally (Zhang and King, 1996). Mouse Tbx6 expression is first seen in the primitive streak, extending laterally into the newly formed paraxial mesoderm, and is subsequently restricted to the unsegmented, presomitic, paraxial mesoderm and the tail bud, and disappears when new mesoderm production ceases (Chapman et al., 1996). In mouse Tbx6 knockouts, irregular somites form in the neck region of mutant embryos, while the more posterior paraxial tissue does not form somites but instead differentiates along the neural pathway (Chapman and Papaioannou, 1998). These results strongly suggest an essential role of Tbx6 genes in the specification of somites, including muscle cells. Therefore, further questions that need to be answered are (1) how is the spatially regulated expression of Tbx6 controlled, (2) how does Tbx6 function in the specification of somites (for example, how does Tbx6 identify its target genes), and (3) how does Tbx6 regulate the activity of the target genes?

Ascidians are excellent experimental animals with which to explore genetic cascades of cell specification and differentiation (reviewed by Satoh, 1994; Satoh, 1999; Di Gregorio and Levine, 1998; Satou and Satoh, 1999). The fertilized egg of Halocynthia...
roretzi, for example, develops relatively quickly into a tadpole-type larva, in which exactly 42 unicellular, striated muscle cells are formed in the tail region within approx. 24 hours of fertilization. The embryonic lineage of muscle cells has been completely described (Nishida, 1987), and the spatial and temporal expression of muscle-related structural genes has been precisely described at the single cell level (Satou et al., 1995; Satoh et al., 1996). The solitary ascidian Ciona intestinalis has a small, compact genome of about 1.6x10^8 bp/haploid (Simmen et al., 1998), whereas the compound ascidian Botryllus schlosseri has a comparatively large genome of about 7.3x10^8 bp/haploid (De Tomaso et al., 1998). Owing to the small genome sizes, it is relatively easy to isolate specific genes and associated 5′ regulatory regions with the minimal promoter required for correct spatial expression, which is usually located within about 300 bp upstream of the transcription start site of the gene (e.g. Satou and Satoh, 1996; Corbo et al., 1997; Takahashi et al., 1999).

Muscle cell differentiation in ascidian embryos takes place autonomously and depends on maternal cytoplasmic information in the egg (reviewed by Satoh, 1994; Satoh, 1999; Nishida, 1997; Jeffery, 2001). In H. roretzi, the expression of muscle-specific structural genes, such as the muscle actin gene (HrMA4) and the myosin heavy chain gene (HrMHC), begins at the 32-cell stage (Satou et al., 1995). By contrast, the expression of an ascidian MyoD homolog (HrMD1, formerly AMD1) begins at the 64-cell stage (Araki and Satoh, 1996; Satoh et al., 1996). The T-box gene AMD1 begins at the 64-cell stage (Araki and Satoh, 1996; Jeffery, 2001). Molecular phylogenetic analysis suggests that AMD1 and/or overexpression of T2 regulates the activity of the As-T2 lineage cells (Mitani et al., 1999). These results suggest the involvement of As-T2 in the differentiation of muscle cells.

In this study, we have further extended our investigation of the function and regulation of specific expression of As-T2. We have determined how the spatially regulated expression of As-T2 is controlled and how As-T2 regulates the activity of the target genes.

**MATERIALS AND METHODS**

**Animals and embryos**

Halocynthia roretzi was purchased during the spawning season from fishermen. H. roretzi is a self-sterile hermaphroditic. Naturally spawned eggs were fertilized with a suspension of non-self sperm. When fertilized eggs were raised at about 12°C, they developed into embryos that were at the 110-cell stage and at the early tailbud stage about 10 and 24 hours after fertilization, respectively.

**Screening of As-T2 genomic clones**

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 of As-T2 genomic clones were determined for both strands with dye primer cycle sequencing FS ready reaction kits and an ABI PRISM 377 DNA sequencer (Perkin Elmer).

**Preparation of fusion gene constructs**

As-T2/En<sup>R</sup> construct

The As-T2/En<sup>R</sup> construct was generated by fusing the As-T2 DNA-binding domain (amino acids 1-232) to a fragment encoding amino acids 2-298 of the Drosophila Engrailed protein. This region of engrailed was derived from plasmid MENt (Badiani et al., 1994). All constructs were cloned in-frame in the pBluescript RN3 vector (Lemaire et al., 1995).

As-T2/lacZ constructs

The As-T2/lacZ constructs were made in the following manner. The 2164-bp As-T2 genomic fragment was subcloned into the multicloning site of plasmid pPD46.21, a version of pPD1.27 which lacks the C. elegans sup-7 gene (Fire et al., 1990). pPD46.21 harbors a gene for bacterial β-galactosidase (lacZ) and a nuclear localization signal in the multicloning site and was kindly provided by Dr A. Fire (Carnegie Institution of Washington).

The p(−2164 ~ −556)As-T2/lacZ construct was made by digesting pl(−2164)As-T2/lacZ with HindIII/HinII, and subcloned into the HindIII/SmaI site of pPD46.21. To construct p(−1754 ~ −556)As-T2/lacZ, primers Hld1754F and Hbl556R were used for PCR amplification. The amplified fragments were digested with HindIII and BamHI, and ligated with HindIII/BamHI-digested pPD46.21. p(−1454 ~ −556)As-T2/lacZ, p(1154 ~ −556)As-T2/lacZ, and p(−854 ~ −556)As-T2/lacZ were also constructed in the same manner using the primers Hld1454F, Hld1154F, Hld556F and Hbl556R. These primer sequences were: Hld1754F 5′-cccaagcttgggAATA-TTTTCAAATCGGGAG-3′; Hld1454F 5′-cccaagcttgggGTAGTTAGGAAAGATAAATA-3′; Hld1154F 5′-cccaagcttgggAGTGGTAAAGAGGAAATA-3′; Hld556F 5′-cccaagcttgggTAATGTCTTATCGGGTTT-3′; and Hbl556R 5′-gggtatccggGACAACGTGTGGTAAAG-3′.

The p(−2164 (−575 ~ −301))As-T2/lacZ construct was made by self-ligation digesting p(−1754)As-T2/lacZ with EcoRI. The p(−301)As-T2/lacZ construct was made by digestion of p(−2164)As-T2/lacZ with HindIII/EcoRI, followed by gel purification. To construct p(−230)As-T2/lacZ, primers Hld230F and Hbl40R were used for PCR amplification. The amplified fragments were digested with HindIII and BamHI, and ligated with HindIII/BamHI-digested pPD46.21. p(−140)As-T2/lacZ, p(−104)As-T2/lacZ and p(−50)As-T2/lacZ were also constructed in the same manner using the primers Hld140F, Hld104F, Hld50F and Hbl40R. These primer sequences were: Hld140F 5′-cccaagcttgggACTACGCGTTAACTTTTTGCTGCTTTA-3′; Hld104F 5′-cccaagcttgggACGCAAGGTATTAAGATTAA-3′; Hld50F 5′-cccaagcttgggGTGGTTTCCGAAGTGATA-3′; and Hbl40R 5′-gggtatccggATGAGACTTCACCAAG-3′.

As-T2/lacZ constructs without T-binding domains

To construct p(−351)As-T2/lacZ, primers Hld854F, HdΔ1D, HdΔDf and Hbl40R were used for PCR amplification. The PCR products amplified with primers Hld854F and HdΔD, and with primers HdΔD and Hbl40R, were used as templates for the second PCR amplification. The second PCR was performed using primers Hld854F and Hbl40R. The amplified fragment was digested with HindIII and BamHI, and ligated with HindIII/BamHI-digested pPD46.21. p(−140)As-T2/lacZ, p(−104)As-T2/lacZ and p(−50)As-T2/lacZ were also constructed in the same manner using the primers Hld140F, Hld104F, Hld50F and Hbl40R. These primer sequences were: Hld140F 5′-cccaagcttgggCTACGCGTTAACTTTTTGCTGCTTTA-3′; Hld104F 5′-cccaagcttgggATGAGACTTCACCAAG-3′; Hld50F 5′-cccaagcttgggGTGGTTTCCGAAGTGATA-3′; and Hbl40R 5′-gggtatccggATGAGACTTCACCAAG-3′.

**GFP constructs**

To construct As-T2 tagged with GFP, primers T3 and KpTbx6R were used for PCR amplification. The amplified fragment was digested with SmaI and KpnI, and ligated in EcoRI(blunted)/KpnI-digested pRN3:GFP, which was made in the following manner, pEGFP-
N1 (Clontech) was digested with ApII, blunted with T4 DNA polymerase, and then digested with BglII. This fragment containing EGFP and the SV40 polyadenylation signal was ligated in NotI/blunted/BglII-digested pBSRN3. To construct HrBra tagged with GFP, primers T7 and KpBrAR were used for PCR amplification. The amplified fragment was digested with HindIII and KpnI, and ligated in EcoR1/blunted/KpnI-digested pRN3.GFP. These primers were: T3′ -5′-AATATACCTCACTAAAAGGG-3′; KpBr6R 5′-ggggtacctcc-CATTGTCGTAATGTTAGTCTG-3′; T7′ -5′-GTAATACGACTCACTAGGGC-3′; and KpBrAR 5′-ggggtacctccAAAGTCTCAATTCTGTAATG-3′.

HrMA4/IacZ and HrMHC/IacZ constructs

In the present study, we also used gene constructs containing the muscle-specific actin gene HrMA4 or myosin heavy chain gene HrMHC. HrMA4/IacZ and HrMHC/IacZ were originally constructed by Satou et al. (Satou et al., 1995) and Araki and Satoh (Araki and Satoh, 1996). To construct primer(-216)(Δ)HrMA4/IacZ, primers MAF, dMAR, dMAF and BhMAR were used for PCR amplification. The amplified fragment was digested with BamHI and PstI, and ligated in BamHI/PstI-digested p46.21. To construct primer(-132)(Δ)HrMHC/IacZ, primers pPDF, dMHR, dMHP and pPDR were used for PCR amplification. The amplified fragment was digested with XbaI and SmaI, and ligated in XbaI/SmaI-digested p46.21. These primers were: MAF 5′-AACAAGCTTATGCCTCATGAT-3′; dMAR 5′-TACGTCGAA-CAATTTGAGGGGCGGCTTGGTCG-3′; dMAF 5′-AGCGCACCAGCCGCTTATGCCTCGAGCTG-3′; BhMAR 5′-ccggatccgTTCGTCGACTGAACTG-3′; pPDF 5′-GGCTCGTTAGTTCGAGCTG-3′; dMHR 5′-GGCTCGTTAGTTCGAGCTG-3′; dMHP 5′-GCTCGTTAGTTCGAGCTG-3′; and pPDR 5′-AATGCGGG-CTCGATTGTCGAG-3′.

Primer extension analysis of As-T2 mRNA

As-T2 mRNA was reverse-transcribed from 1 μg of poly (A) RNA of neurulae by use of a Primer Extension System (Promega) with 32P-labeled primer 5′-CTCGGTAAATTTGTTTTCG-3′. Products from primer extension reactions were separated by electrophoresis on an 8% denaturing polyacrylamide gel and detected with the Fuji BAS system (Fuji Film, Tokyo).

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

Microinjection of fusion constructs and histochemical detection of β-gal activity were performed as described previously (Takahashi et al., 1999).

Whole-mount in situ hybridization

Whole-mount specimens were hybridized in situ at 42°C using DIG-labeled antisense and sense RNA probes, essentially as described by Satou et al. (Satou et al., 1995). The probes were prepared with a DIG RNA-labeling kit (Boehringer Mannheim), and used at a concentration of 1 μg/ml in the hybridization buffer. Probes for HrMA4 (Kasakabe et al., 1991) and HrMHC (Araki and Satoh, 1996) were used.

Histochemical staining for alkaline phosphatase (AP)

Differentiation of endodermal cells was monitored by histochemical detection of AP activity as described by Whittaker and Meedel (Whittaker and Meedel, 1989).

RESULTS

As-T2 is involved in differentiation of muscle cells of Halocynthia embryos

In a previous study, we showed that ectopic and/or overexpression of As-T2 by injection of its in vitro synthesized mRNA into fertilized eggs promoted ectopic expression of muscle-specific structural genes HrMA4 and HrMHC in cells of non-muscle lineages, suggesting involvement of As-T2 in upregulation of the muscle-specific genes in the ascidian embryo (Mitani et al., 1999). Conlon et al. (Conlon et al., 1996) demonstrated that the construct Xbra-EnR, in which the DNA-binding domain of Xbra (the product of the Brachyury gene of Xenopus) is fused to the repressor domain of Drosophila engrailed protein, inhibits transcriptional activation by Xbra and thus blocks Xbra function in Xenopus embryos. Using this strategy to directly investigate the function of As-T2 in Halocynthia embryos, we created an As-T2/EnR construct to disrupt the transcriptional activation mediated by this gene and then examined its effects on muscle cell development. Halocynthia embryos were injected at the one-cell stage with in vitro synthesized mRNA encoding As-T2/EnR, and the effects of As-T2/EnR on the differentiation of embryonic cells were examined at the 110-cell and early tailbud stages. Injected embryos cleaved normally until the beginning of gastrulation, which takes place around the 120-cell stage (Fig. 1). The injection of 0.01 μg/ml As-T2/EnR mRNA had a minor effect on morphogenesis and the expression of HrMA4 (Fig. 1C,F) and HrMHC (Fig. 1L), and tail elongation was slightly affected (Fig. 1F,L). The expression of HrMA4 and HrMHC was evident in eight pairs of B-line muscle cells in 110-cell stage embryos (Fig. 1C,J). However, the injection of 0.2 μg/ml As-T2/EnR mRNA suppressed the expression of HrMA4 (Fig. 1B) and HrMHC (Fig. 1H) at the 110-cell stage. The injection of As-T2/EnR mRNA at this higher concentration also affected the morphogenesis of the tailbud embryo (Fig. 1E,K). By the tailbud stage, however, HrMA4 (Fig. 1E) and HrMHC (Fig. 1K) transcripts appeared to some extent. Each experimental result was confirmed in more than 25 injected embryos.

To determine whether these effects were caused by the interference with As-T2-mediated transcriptional activation, we performed co-injection experiments. As shown in Fig. 1O, 110-cell stage embryos that developed from eggs that were co-injected with As-T2/EnR mRNA (0.2 μg/ml) and As-T2 mRNA (0.05 μg/ml) showed HrMHC expression in some of the B-line muscle cells. In addition, the same stage embryos developed from eggs co-injected with As-T2/EnR mRNA (0.2 μg/ml) and As-T2 mRNA (0.2 μg/ml) showed expression of HrMHC not only in B-line muscle cells, but also in some non-muscle lineage cells (Fig. 1P). By contrast, about half of the experimental embryos developed from eggs co-injected with As-T2/EnR mRNA (0.2 μg/ml) and HrBra mRNA (0.1 μg/ml) did not show HrMHC expression, while the other half showed the gene expression in just a few cells (Fig. 1Q).

The timing of initiation of HrMA4 and HrMHC expression, which occurs at the 32-cell stage, precedes that of As-T2 expression in muscle precursor cells, which occurs at the 44-cell stage (Yasu et al., 1996). This suggests that As-T2 is not involved in the process of initiation of the activation of these muscle-specific structural genes. If that is the case, the injection of As-T2/EnR mRNA should not inhibit the initial activation of HrMA4 and HrMHC at the 32- and 64-cell stages. As shown in Fig. 1N, HrMHC expression was evident in 64-cell stage embryos which developed from eggs injected with As-T2/EnR mRNA (0.2 μg/ml). This result was confirmed at the 32-cell stage (data not shown), and similar results were obtained for HrMA4 expression (data not shown).
As-T2 is expressed weakly in endodermal lineage blastomeres around the 32- to ~44-cell stage, and later in TT (tip of the tail) progenitor cells of tailbud embryos. We examined the effects of injection of As-T2/EnR mRNA on the differentiation of these cells. An endodermal cell differentiation marker, AP, was detected histochemically in early tailbud embryos developed from eggs injected with As-T2/EnR mRNA (0.2 μg/μl) (data not shown). Injection of As-T2/EnR mRNA (0.2 μg/μl) did not alter the expression of the TT-specific HrTT-1 gene (data not shown).

All these results, together with the previously reported results (Mitani et al., 1999), strongly suggest that As-T2 is involved in the upregulation of HrMA4 and HrMHC in muscle progenitor cells of Halocynthia embryos, although As-T2 is not involved in the initiation of expression of these muscle-specific genes.

The proximal module of the minimal promoter of As-T2 is involved in muscle-specific expression of the gene

The 2164-bp 5′-flanking region of As-T2 contains the promoter required for expression in two different embryonic regions. The As-T2 gene is expressed primarily in two embryonic regions, muscle cells and TT cells of the tailbud stage embryo. To determine the minimal promoter required for the specific expression of As-T2, we first made a fusion gene construct, p(−2164)As-T2/lacZ, in which 2164 bp of the 5′ flanking region of As-T2 (from −2164 to +40, including the first 33-bp of the coding region; Fig. 2A) was linked with the reporter gene lacZ. We injected linearized pAs-T2/lacZ into Halocynthia eggs about 30 to 90 minutes after insemination. After several trials, we found that injection of 8·10⁴ copies of pAs-T2/lacZ yielded the best results. On average, about half of the eggs injected with fusion constructs cleaved normally and developed to tailbud embryos with normal morphology (Fig. 3). We scored the reporter gene expression only in injected embryos that exhibited normal morphology. Expression of lacZ in cells of the tailbud stage embryos was usually mosaic (Fig. 3), presumably owing to the random incorporation of the constructs into early blastomeres. No endogenous β-gal activity was detected in control non-injected embryos (data not shown).

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**Fig. 1.** Effects of microinjection of As-T2/EnR mRNA on the expression of muscle-specific actin gene (HrMA4) and myosin heavy chain gene (HrMHC), assessed by whole-mount in situ hybridization. (A-F) Expression of HrMA4 and (G-Q) HrMHC. (A,C,G,LO-Q) Embryos around the 110-cell stage, (D,F,J-L) at the early tailbud stage and (M,N) at the 64-cell stage. (A,D,G,J,M) Control embryos; (B,E,H,K,N) embryos developed from eggs injected with 0.2 μg/μl As-T2/EnR mRNA and (C,F,I,L) those injected with 0.01 μg/μl As-T2/EnR mRNA. (O-Q) Co-injection of 0.2 μg/μl As-T2/EnR mRNA with (O) 0.05 μg/μl As-T2, (P) 0.2 μg/μl As-T2 or (Q) 0.1 μg/μl HrBra mRNA. Scale bar: 100 μm.
We examined a total of 91 embryos in this experimental series (Fig. 2B). In this group, 22 embryos (25%) showed the reporter gene expression specifically in both muscle cells and TT cells (Figs 2B, 3A). In addition, 25 embryos showed specific lacZ expression in muscle cells, while in 11 embryos the TT cells expressed lacZ (Fig. 2B). These results suggest that the 2164-bp 5′-flanking region contains the promoter required for the region-specific expression of the As-T2 gene.

The distal module between −2164 and −1754 of the 5′ flanking region of As-T2 is likely to be responsible for the specific reporter gene expression in TT cells. The 5′ flanking region of As-T2 contained a HincII restriction site at −555, which allowed us to make the fusion construct p(−2164 − −555)As-T2/lacZ (construct b in Fig. 2A). When construct b was injected into fertilized eggs, the reporter gene was expressed mainly in TT cells of the tailbud stage embryos (Figs 2B, 3B). The simplest explanation of this result is that...
the region between –2164 and –555 is responsible for the TT-specific domain of the reporter gene, whereas the proximal 5’-flanking region up to –555 is responsible for the muscle-specific expression of this gene.

To test this possibility, we made several deletion constructs from construct b (Fig. 2A), including p(–1754 ~ –555)As-T2/lacZ (construct c), p(–1454 ~ –555)As-T2/lacZ (construct d), p(–1154 ~ –555)As-T2/lacZ (construct e) and p(–854 ~ –555)As-T2/lacZ (construct f). As summarized in Fig. 2B, constructs c, d and e did not show the reporter gene expression, suggesting that the 5’-flanking sequence between –2164 and –1754 contains a distal module that is responsible for specific expression of As-T2 in TT cells. Because construct f showed the reporter gene expression in both muscle and TT cells, it is likely that the region between –1154 and –854 is involved in the suppression of As-T2 transcription.

The proximal module between –230 and –50 of the 5’-flanking region of As-T2 is responsible for the specific reporter gene expression in muscle cells

When deletion constructs were further examined, it became evident that there was a proximal module responsible for muscle-specific expression of lacZ (Fig. 2). Injection of p(–351)As-T2/lacZ (construct g in Fig. 2A) and p(–301)As-T2/lacZ (construct h) resulted in reporter expression in muscle cells and in some of non-muscle lineage cells, which were usually epidermal cells (but not in TT cells). In addition, 30 out of the 53 embryos that developed from eggs injected with p(–230)As-T2/lacZ (construct i) showed the specific expression of lacZ in muscle cells (Figs 2B, 3C). p(–140)As-T2/lacZ (construct j) and p(–104)As-T2/lacZ (construct k) also showed reporter gene expression in muscle cells (Fig. 2B). However, p(–50)As-T2/lacZ failed to promote reporter expression. These results suggest that a proximal module between –230 and –50 is associated with muscle-specific expression of As-T2.

**Primer extension analysis**

As mentioned above, As-T2 is expressed primarily in embryonic muscle cells and TT cells, and the 5’-flanking region of As-T2 contains two promoter regions that regulate its specific expression: a distal module responsible for its specific expression in TT and a proximal module required for its muscle-specific expression. It is possible that the gene produces two different transcripts by alternative splicing. We therefore examined this possibility by primer extension analysis, and observed only one band in the gel (data not shown). This suggests that a single type of As-T2 mRNA is expressed in *Halocynthia* embryos.

**The muscle-related promoter region contains potential T-protein binding motifs**

Kispert and Herrmann (Kispert and Herrmann, 1993) examined the specific DNA binding of the mouse Brachury (T) protein to DNA fragments which were selected from a mixture of random oligomers. They identified a 20 bp palindrome,

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\text{TG(C)ACACCTAGGTGGA}A
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as a possible consensus sequence to which the Brachury DNA-binding domain binds. This motif was reported in the promoter region of Xenopus eFGF, which is a direct target of Xbra (Casey et al., 1998), and in the promoter region of the sonic hedgehog gene of zebrafish (Müller et al., 1999). In *H. roretzi*, the promoter of *HrBra* contains a 21 bp palindromic-like sequence, TTTGTTACCTAGGTGGA (AA), between –171 and –151 from the transcription start site (+1), and this sequence has been shown to be responsible for the autonomous regulation of *HrBra* transcription (Takahashi et al., 1999).

The nucleotide sequence of the 5’ flanking region of As-T2 is shown in Fig. 4. We identified two T-protein binding motifs: one (TTTCACACTT) at positions –334 to –325 (Td, distal motif) and the other (AAGTGTGAAC) at positions –253 to –244 (Tp, proximal motif). In addition, there are two E-boxes (CAAATG) between –351 and –50. We next examined whether these T-binding motifs are associated with the expression of As-T2 itself.

The fusion gene construct p(–351)As-T2/lacZ contains the T-protein binding motifs, whereas p(–230)As-T2/lacZ does not (Fig. 5A,C). When either construct was injected into *Halocynthia* fertilized eggs, the reporter gene was expressed in muscle cells (Fig. 5A,E). Although the level of lacZ expression was higher when p(–351)As-T2/lacZ was injected than when p(–230)As-T2/lacZ was injected, this result suggests that the T-binding motifs are not essential for the initiation of As-T2 expression (see also Fig. 2B). However, it is possible that the two potential T-binding motifs are associated with the autoregulatory activation of As-T2. This possibility was examined by co-injection of p(–351)As-T2/lacZ or p(–230)As-T2/lacZ with synthetic As-T2 mRNA. If injection of synthetic As-T2 mRNA causes ectopic expression of As-T2 protein in blastomeres of non-muscle lineages, and the As-T2 protein then binds to the T-binding motifs of the gene and activates expression of the fusion construct, the reporter gene should be expressed ectopically in non-muscle lineages. Expression of As-T2 protein was confirmed by expression of As-T2 mRNA tagged with a sequence encoding GFP (Fig. 5B,F). As shown in Fig. 5G, co-injection of p(–351)As-T2/lacZ and synthetic As-T2 mRNA resulted in ectopic expression of the reporter gene. However, co-injection of p(–230)As-T2/lacZ and synthetic As-T2 mRNA showed the reporter gene expression (Fig. 5C) at the same level as controls (Fig. 5A). Co-injection of p(–230)As-T2/lacZ (Fig. 5D) or p(–351)As-T2/lacZ (Fig. 5H) with *HrBra* mRNA did not cause ectopic expression of lacZ. Furthermore, we examined whether the distal T-binding motif (Td) and the
Fig. 4. (A) Nucleotide sequence of the 5′-flanking region of As-T2, including the regions for its muscle-specific (shown with red letters) and TT-specific (shown with blue letters) expression. A putative Gli protein-binding motif is shown with a yellow underline. The transcription start site is shown as +1, and eleven deduced amino acids are shown in the lower right-hand corner. The purple box indicates the Tp (proximal T-binding motif) and the green box indicates the Td (distal T-binding motif).

(B) Suggested motifs and sequences responsible for the specific expression of As-T2.

(C) Sequences of T protein-binding motifs shared by various T-related genes. Dots indicate conserved nucleotides.
proximal T-binding motif (Tp) are required for the upregulation of the As-T2 gene. As shown in Fig. 6, the deletion of the Td motif from p(−351)As-T2/lacZ had little effect on the ectopic expression of lacZ upon co-injection with As-T2 mRNA (Fig. 6A,B), while deletion of the Tp motif from p(−351)As-T2/lacZ resulted in failure to produce ectopic expression of lacZ when the construct was co-injected with As-T2 mRNA (Fig. 6C,D).

These results suggest that the T-binding motifs, especially the proximal motif of As-T2, are involved in the autoregulatory enhancement of gene expression. This enhancement was promoted by As-T2, but not HrBra, suggesting a specific affinity between the two T-box gene products.

**As-T2 upregulates muscle-specific structural gene expression**

We previously examined the minimal promoter required for muscle-specific expression of HrMA4 and HrMHC. As shown in Fig. 7A, the 103 bp region upstream of HrMA4 is sufficient to yield the correct reporter gene expression in muscle cells (Satou and Satoh, 1996). However, a T-binding motif (AAGTGTAGAA) is present between positions −189 and −180 of HrMA4. This sequence shares seven of the ten nucleotides with the sequence of the As-T2 proximal motif (Fig. 7A).

Regarding the HrMHC gene, it has been shown that the 132 bp region upstream of the gene is sufficient for muscle-specific
Functional circuitry of the ascidian As-T2 gene

Fig. 7. Nucleotide sequences of the 5’ flanking region of (A) HrMA4 and (B) HrMHC. There is a T-binding motif in HrMA4 (shown by a red stippled box) and in HrMHC (a pink box). Sequences of T-binding motifs are compared (A) between mouse T consensus, HrMA4 and As-T2 (Tp), and (B) between mouse T consensus, HrMHC and As-T2 (Tp). Black dots represent nucleotides shared by the T consensus, and white dots those shared by As-T2 (Tp).

The 110-cell stage embryos that developed from eggs injected with p(-216)HrMA4/lacZ mRNA (B), p(-216)HrMA4/lacZ with As-T2 mRNA (D), p(-132)HrMHC/lacZ with and without the T-binding motif and p(-132)HrMHC/lacZ with and without the T-binding motif and As-T2 synthetic mRNA.

Fig. 8. Efficiency of T-binding motifs in expression of HrMA4 (A-D) and HrMHC (E-H). (A-D) The reporter gene expression in embryos at the 110-cell stage injected with p(-216)HrMA4/lacZ (A), p(-216)HrMA4/lacZ with As-T2 mRNA (B), p(-216)(ΔT)HrMA4/lacZ (C) and p(-216)(ΔT)HrMA4/lacZ with As-T2 mRNA (D). (E-H) The reporter gene expression in embryos at the 110-cell stage injected with p(-132)HrMHC/lacZ (E), p(-132)HrMHC/lacZ with As-T2 mRNA (F), p(-132)(ΔT)HrMHC/lacZ (G) and p(-132)(ΔT)HrMHC/lacZ with As-T2 mRNA (H). 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.

indicates that HrMA4 expression was upregulated by As-T2. However, when p(-216)(ΔT)HrMA4/lacZ was co-injected with As-T2 mRNA, the reporter gene expression was also upregulated (Fig. 8D).

Similar results were obtained with HrMHC (Fig. 8E-H). The reporter gene expression was downregulated when the T-binding motif was deleted from the p(-132)HrMHC/lacZ reporter expression (Araki and Satoh, 1996). As shown in Fig. 7B, there is a T-binding motif (TTTCACACAG) between positions -64 and -55 of HrMHC. This sequence shares seven of the ten nucleotides with the sequence of the As-T2 proximal motif (Fig. 7B). Therefore, it is likely that these T-binding motifs play a role in the upregulation of HrMA4 and HrMHC by As-T2. This possibility was examined by co-injection of p(-216)HrMA4/lacZ with and without the T-binding motif or p(-132)HrMHC/lacZ with and without the T-binding motif and As-T2 synthetic mRNA.

The 110-cell stage embryos that developed from eggs injected with p(-216)HrMA4/lacZ showed lacZ expression in muscle lineage cells in almost all of the test embryos (Fig. 8A). Injection of p(-216)(ΔT)HrMA4/lacZ resulted in the reduction of not only the number of embryos that exhibited lacZ expression, but also of the number of lacZ-positive blastomeres per embryo (Fig. 8C). When p(-216)HrMA4/lacZ was co-injected with As-T2 mRNA, the expression of lacZ was upregulated dramatically (Fig. 8B). Almost all of the embryos showed lacZ expression in nearly half of the blastomeres. This
(compare Fig. 8G with Fig. 8E). The reporter gene was upregulated and ectopically expressed when p(−132)HrMHC/lacZ was co-injected with As-T2 mRNA (compare Fig. 8F with Fig. 8E). Upregulation and ectopic expression of the reporter gene expression were also evident when p(−132)(ΔT)HrMHC/lacZ was co-injected with As-T2 mRNA (compare Fig. 8H with Fig. 8F).

DISCUSSION

The primary function of As-T2 in Halocynthia embryos

Members of the Tbx6 subfamily are expressed in the paraxial mesoderm and tailbud in embryos of mice (Chapman et al., 1996) and Xenopus (Zhang and King, 1996), and this pattern is also seen in the case of the ascidian Tbx6-related gene As-T2 (Yasuo et al., 1996; Mitani et al., 1999). Knockout of Tbx6 in the mouse resulted in the failure of somite formation and in the induction of a second neural tube (Chapman and Papaioannou, 1998). Ectopic and/or overexpression of As-T2 by injection of in vitro synthesized mRNA into one-cell embryos promotes the ectopic expression of HrMA4 and HrMHC in mainly epidermal cells (Mitani et al., 1999). However, the microinjection of As-T2 mRNA into fertilized eggs does not evoke upregulation of the endodermal cell differentiation marker AP nor of the TT-specific genes HrPost-1 (Takahashi et al., 1997) and HrTT-1 (Hotta et al., 1998). As shown in the present study, the suppression of As-T2-mediated transcriptional activation by injection of As-T2/EnR resulted in downregulation of HrMA4 and HrMHC transcription, but the expression of the endoderm AP and the TT-specific genes was not affected. All of these results strongly suggest that the function of As-T2 is primarily associated with the differentiation of muscle cells. However, because As-T2 is also expressed in the endodermal precursor cells and TT progenitor cells, its function in these types of embryonic cells should be elucidated further in future studies.

The proximal module of the minimal promoter of As-T2 is involved in muscle-specific expression of the gene

The 5′ flanking sequence of As-T2 contains at least two distinct promoter modules for cell-type-specific gene expression: a distal module between −2164 and −1754 of its 5′ flanking region is required for expression in TT cells and a proximal module between −230 and −50 of the 5′ flanking region is required for expression in muscle cells. Studies of the minimal promoters responsible for specific embryonic expression of HrMA4 (Hikosaka et al., 1994; Satou and Satoh, 1996), HrMHC (Araki and Satoh, 1996), HrBra (Takahashi et al., 1999) and Ci-Bra (Corbo et al., 1997) demonstrated that the minimal promoters of the ascidian genes are usually located within 300-500 bp upstream of their transcription start sites. As As-T2 contains distal and proximal modules and the latter is associated with muscle-specific gene expression, it is conceivable that the proximal module required for muscle development evolved first and then a second module for the expression of As-T2 in non-muscle cells was added distal to the primary module. The evolution of multiple promoter modules for the same gene may provide us with insight into how genes are co-opted to generate novel morphologies.

However, the present analysis of As-T2-mediated tissue-specific transcription control is not complete, and the control mechanism appears to be quite complex. For example, it is likely that the region between −1154 and −854 is involved in the suppression of As-T2 transcription. In addition, more comprehensive analysis is required to understand the detailed mechanisms involved in the muscle-specific expression of As-T2, although we could not detect in the proximal module any consensus binding motif for known transcription factors.

As-T2 and genetic cascade for muscle cell differentiation in ascidian embryos

In ascidian embryos, whole-mount in situ hybridization signals first appear unambiguously in embryonic nuclei, and then they become distributed throughout the cytoplasm. Taking advantage of this feature, the exact temporal and spatial patterns of transcriptional activation can be studied (e.g. Yasuo and Satoh, 1993; Satou et al., 1995). For example, for genes associated with muscle differentiation (HrMA4 and HrMHC), transcripts begin to appear at the 32-cell stage. This early expression of muscle-specific structural genes is also true in the case of tropomyosin, myosin light chain and creatine kinase (Y. Satou and N. S., unpublished). By contrast, the expression of As-T2 starts around the 32- to ~44-cell stage (Yasuo et al., 1996) and that of the ascidian MyoD homolog HrMD1 first becomes evident at the 64-cell stage (see Satoh et al., 1996). This suggests that the transcription factors As-T2 and HrMD1 are not likely to be involved in the initiation of transcriptional activation of HrMA4 and HrMHC. This was confirmed by the present study: the injection of As-T2/EnR did not block the transcription initiation of HrMA4 and HrMHC (Fig. 1N).

However, as shown in the previous studies and the present

![Fig. 9. Possible functional circuitry of As-T2 associated with muscle differentiation in Halocynthia embryos (see text for details).](image-url)
study, overexpression of As-T2 upregulates the transcription activity of HrMA4 and HrMHC, and suppression of As-T2 downregulates HrMA4 and HrMHC transcription. In addition, co-injection of As-T2 mRNA with HrMA4/lacZ or HrMHC/lacZ promotes ectopic expression of lacZ in non-muscle lineage cells. These results strongly suggest that As-T2 is involved in upregulation and/or maintenance of the transcription activity of HrMA4 and HrMHC.

Injection of As-T2/EnR suppressed the transcriptional activation of HrMA4 and HrMHC in dose-dependent manner. Almost complete suppression of HrMA4 and HrMHC transcription by As-T2/EnR was observed at the 110-cell stage. However, by the tailbud stage, embryos developed from eggs injected with As-T2/EnR showed detectable levels of HrMA4 and HrMHC transcripts. This result may indicate that the amount of As-T2/EnR protein gradually diminished as development proceeded, and thus the inhibitory activity of As-T2/EnR on the transcriptional activity of the muscle-specific structural genes decreased. In other words, continuous activity of As-T2 transcription appears to be required for maintenance of the transcriptional activity of these muscle-specific structural genes.

What molecules are involved in the initiation of transcriptional activation of HrMA4 and HrMHC? Recently, Nishida and Sawada (Nishida and Sawada, 2001) isolated and characterized the macho-1 gene from Halocynthia eggs. This gene encodes a zinc-finger nuclear protein, and its mRNA is localized to the myoplasm eggs. Because depletion of macho-1 mRNA results specifically in the loss of B-line muscle cells and because injection of in vitro synthesized macho-1 mRNA causes ectopic muscle formation in non-muscle lineage cells, it is highly likely that macho-1 is an initiator of the musclespecific structural gene expression. We searched for possible Gli protein-binding motifs within the 5′ flanking region of As-T2. Although there is a sequence (AGAGTGGT; 6/9 base match) that resembles the Gli-binding motif (TGGGTGGTC; T2) of Ciona intestinalis or Ciona intestinalis, we could not find any Gli-binding motif within the proximal module between −230 and −50 that is required for the muscle-specific expression of As-T2.

In addition, Erives and Levine (Erives and Levine, 2000) have shown that a maternal mRNA of a T-box gene (CiVegTR) of Ciona intestinalis is localized to the myoplasm, and that CiVegTR protein binds to the T-box protein-binding motif of the 5′ flanking region of the snail gene of Ciona embryos. Although it should be clarified whether Ciona snail controls the muscle-specific structural gene expression, CiVegTR is also a candidate for the muscle determinate molecule.

There are two T-protein binding motifs (Tp and Td) within the proximal minimal promoter of As-T2. Co-injection of As-T2/lacZ and As-T2 mRNA, and co-injection of As-T2/lacZ lacking either Tp or Td and As-T2 mRNA, indicates that the T-binding motifs are involved in the autoregulatory transcriptional activity of As-T2. T-binding motifs are also present in the promoter regions of both HrMA4 and HrMHC. Co-injection of HrMA4/lacZ or HrMHC/lacZ with As-T2 mRNA promotes ectopic and/or overexpression of lacZ, suggesting that As-T2 protein binds to the T-protein binding motifs of HrMA4 and HrMHC to upregulate the genes. However, co-injection of p(216)(ΔT)HrMA4/lacZ or p(−132)(ΔT)HrMHC/lacZ with As-T2 mRNA also promotes ectopic expression of lacZ. This suggests that As-T2 may upregulate other co-factor gene(s) that in turn upregulate HrMA4 and HrMHC. Myogenic bHLH genes, including HrMD1, are candidates for the co-factor genes. The DNA-binding capacities of and transcriptional modulation by different T-box proteins are rather complex (Sinha et al., 2000). Future studies should address the issue of co-operative activity of As-T2 with other T-box proteins in ascidian muscle cell differentiation.

In conclusion, we propose a genetic cascade for muscle differentiation in the ascidian embryo, which is summarized in Fig. 9. First, maternally transcribed mRNAs of the muscle determinant gene(s) like macho-1 is/are localized in the myoplasm. After fertilization, this maternal mRNA is translated and then segregated into the B-line muscle cells to directly or indirectly activate the muscle-specific structural genes, including HrMA4 and HrMHC. This process is evident by the 32-cell stage when transcripts of both HrMA4 and HrMHC can be detected by whole-mount in situ hybridization. Second, the determinant protein(s) or some other transcriptional factors directly or indirectly activate As-T2 and HrMD1. Once transcription of As-T2 is activated, its translated protein binds to the T-protein-binding motif of the gene to upregulate itself via an autoregulatory loop. Third, As-T2 protein binds to the T-binding motif of the 5′ flanking regions of HrMA4 and HrMHC to upregulate the transcriptional activity of these genes. As-T2 may also upregulate other co-factor genes (or HrMD1), which are also involved in the maintenance of the transcriptional activity of HrMA4 and HrMHC. Thus, the transcription of HrMA4 and HrMHC genes is controlled by two phases of regulation: initiation and maintenance. As-T2 is involved in the latter phase of regulation or maintenance. Although more details of this mechanism will be clarified in the near future, this model delineates a genetic cascade for regulation of muscle cell differentiation in Halocynthia embryos.

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