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

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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/En^R 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 *tbx6* (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 1.6×10^8 bp/haploid (Simmen et al., 1998), whereas the compound ascidian *Botryllus schrosseri* has a comparatively large genome of about 7.3×10^8 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 *As-T2* of *H. roretzi* is expressed around the 32- to ~44-cell stage; at first, very transient expression of the gene is seen in endodermal cells, then distinct and continuous expression is found in muscle precursor cells, and later expression is seen in epidermal cells situated in the distal tip region of the elongating tail (Yasuo et al., 1996). Molecular phylogenetic analysis suggests that *As-T2* is a divergent member of the *Tbx6* subfamily, and ectopic and/or overexpression of *As-T2* has been shown to promote the ectopic expression of *HrMA4* and *HrMHC* in non-muscle 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 hermaphrodite. 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^R construct

The As-T2/En^R 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 p46.21, a version of pPD1.27 which lacks the *C. elegans* sup-7 gene (Fire et al., 1990). p46.21 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 p(−2164)As-T2/*lacZ* with *Hin*dIII/*Hin*cII, and subcloned into the *Hin*dIII/*Sma*I site of pPD46.21. To construct p(−1754 ~ −556)As-T2/*lacZ*, primers Hd1754F and Bh556R were used for PCR amplification. The amplified fragments were digested with *Hin*dIII and *Bam*HI, and ligated with *Hin*dIII/*Bam*HI-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 Hd1454F, Hd1154F, Hd854F and Bh556R. These primer sequences were: Hd1745F 5′-cccaagcttgggAATA-TTTTAAATCGGGAG-3′; Hd1454F 5′-cccaagcttgggGTGATTGAA-TGAAATATA-3′; Hd1154F 5′-cccaagcttgggAGTGGTAAAAGGAA-TATA-3′; Hd854F 5′-cccaagcttgggTAATTGTTTAGTTCCGTT-3′; and Bh556R 5′-cgggatcccgGACAACTGGTTTGTAAAG-3′.

The p(−2164 Δ(−754 ~ −301))As-T2/*lacZ* construct was made by self ligation digesting p(−1754)As-T2/*lacZ* with *Eco*T22I. The p(−301)As-T2/*lacZ* construct was made by digestion of p(−2164)As-T2/*lacZ* with *Hin*dIII/*Eco*T22I, followed by gel purification. To construct p(−230)As-T2/*lacZ*, primers Hd230F and Bh40R were used for PCR amplification. The amplified fragments were digested with *Hin*dIII and *Bam*HI, and ligated with *Hin*dIII/*Bam*HI 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 Hd140F, Hd104F, Hd50F and Bh40R. These primer sequences were: Hd230F 5′-cccaagcttgggACTATCTT-GCTGCGTTTA-3′; Hd140F 5′-cccaagcttgggAGCGAAAGGTT-AAAGTAA-3′; Hd104F 5′-cccaagcttgggAGTGGTAAAAGGAATATA-3′; Hd50F 5′-cccaagcttgggATAGTTTCGGAAGTGAAT-3′; and Bh40R 5′-gggatcccgATGAGACTT ACTAACAAG-3′.

As-T2/*lacZ* constructs without T-binding domains

To construct p(−351)(ΔTd)As-T2/*lacZ*, primers Hd854F, ΔTdR, ΔTdF and Bh40R were used for PCR amplification. The PCR products amplified with primers Hd854F and ΔTdR, and with primers ΔTdF and Bh40R, were used as templates for the second PCR amplification. The second PCR was performed using primers Hd854F and Bh40R. The amplified fragment was digested with *Hin*dIII and *Bam*HI, and ligated with *Hin*dIII/*Bam*HI-digested p46.21. The construct was then linearized with *Sty*I. p(−351)(ΔTp)As-T2/*lacZ* was also constructed in the same manner using primers Hd854F, ΔTpR, ΔTpF and Bh40R. These primers were: ΔTdF 5′-CCATGGTTAATATGACATCTT-CCCTTCAAAAACTCA-3′; ΔTdR 5′-TGAGTTTTTGAAGG-GAAGATGTCATATTAACCATGG-3′; ΔTpF 5′-GACCACACG-TTTGTGTCCCGAATGCAATGAATACTA-3′; and ΔTpR 5′-TAGTATTCATTGCATTCGGGACACAAACGTGTGGTC-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 *Sma*I and *Kpn*I, and ligated in *Eco*RI(blunted)/*Kpn*I-digested pRN3:GFP, which was made in the following manner. pEGFP-

N1(Clontech) was digested with *Afl*II, blunted with T4 DNA polymerase, and then digested with *Bgl*II. This fragment containing EGFP and the SV40 polyadenylation signal was ligated in *Not*I(blunted)/*Bgl*II-digsted pBSRN3. To construct HrBra tagged with GFP, primers T7 and KpBraR were used for PCR amplification. The amplified fragment was digested with *Hin*cII and *Kpn*I, and ligated in *Eco*RI(blunted)/*Kpn*I-digested pRN3:GFP. These primers were: T3 5′-AATTAACCCTCACTAAAGGG-3′; KpTbx6R 5′-ggggtacccc-CATTGTCAGTAAATTGCT-3′; T7 5′-GTAATACGACTCACTA-TAGGGC-3′; and KpBraR 5′-ggggtacccCAAGTCTCAAATT-CTGTAA-3′.

HrMA4/*lacZ* and HrMHC/*lacZ* constructs

In the present study, we also used fusion gene constructs containing the muscle-specific actin gene *HrMA4* or myosin heavy chain gene *HrMHC*. HrMA4/*lacZ* and HrMHC/*lacZ* were originally constructed by Satou et al. (Satou et al., 1995) and Araki and Satoh (Araki and Satoh, 1996). To construct p(−216)(ΔT)HrMA4/*lacZ*, primers MAF, dMAR, dMAF and BhMAR were used for PCR amplification. The amplified fragment was digested with *Bam*HI and *Pst*I, and ligated in *Bam*HI/*Pst*I-digested p46.21. To construct p(−132)(ΔT)MHC/*lacZ*, primers pPDFF, dMHR, dMHF and pPDR were used for PCR amplification. The amplified fragment was digested with *Xba*I and *Sma*I, and ligated in *Xba*I/*Sma*I-digested p46.21. These primers were: MAF 5′-AACAGCTATGACCATGAT-3′; dMAR 5′-TACGTGCGAA-CAATTGAGGGGCGGCCTTGGTGTCGT-3′; dMAF 5′-ACGA-CACCAAGGCCGCCCCTCAATTGTTCGCACGTA-3′; BhMAR 5′-cgggatcccgTTCGCTCATGTGAACTGT-3′; pPDFF 5′-GGCTCG-TATGTTGTGTAG-3′; dMHR 5′-AACGTTCATCACCTATCAG-AAAAAAGCGCAACTGCT-3′; dMHF5′-AGCAGTTGCGCTTT-TTTCTGATAGGTGATGAACGTT-3′; and pPDR 5′-ATCGCGG-CTCAGTTCGAG-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 ^{32}P-labeled primer 5′-CTCCGTAAATTGTTTCGTG-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* (Kusakabe 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-En^R, 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/En^R 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/En^R, and the effects of As-T2/En^R 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/μl As-T2/En^R mRNA had a minor effect on morphogenesis and the expression of *HrMA4* (Fig. 1C,F) and *HrMHC* (Fig. 1I,L), 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,I). However, the injection of 0.2 μg/μl As-T2/En^R mRNA suppressed the expression of *HrMA4* (Fig. 1B) and *HrMHC* (Fig. 1H) at the 110-cell stage. The injection of As-T2/En^R 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/En^R mRNA (0.2 μg/μl) and *As-T2* mRNA (0.05 μg/μl) 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/En^R mRNA (0.2 μg/μl) and *As-T2* mRNA (0.2 μg/μl) 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/En^R mRNA (0.2 μg/μl) and *HrBra* mRNA (0.1 μg/μl) 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 (Yasuo 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/En^R 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/En^R mRNA (0.2 μg/μl). 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/En^R 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/En^R mRNA (0.2 μg/μl) (data not shown). Injection of As-T2/En^R 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 8×10^4 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).

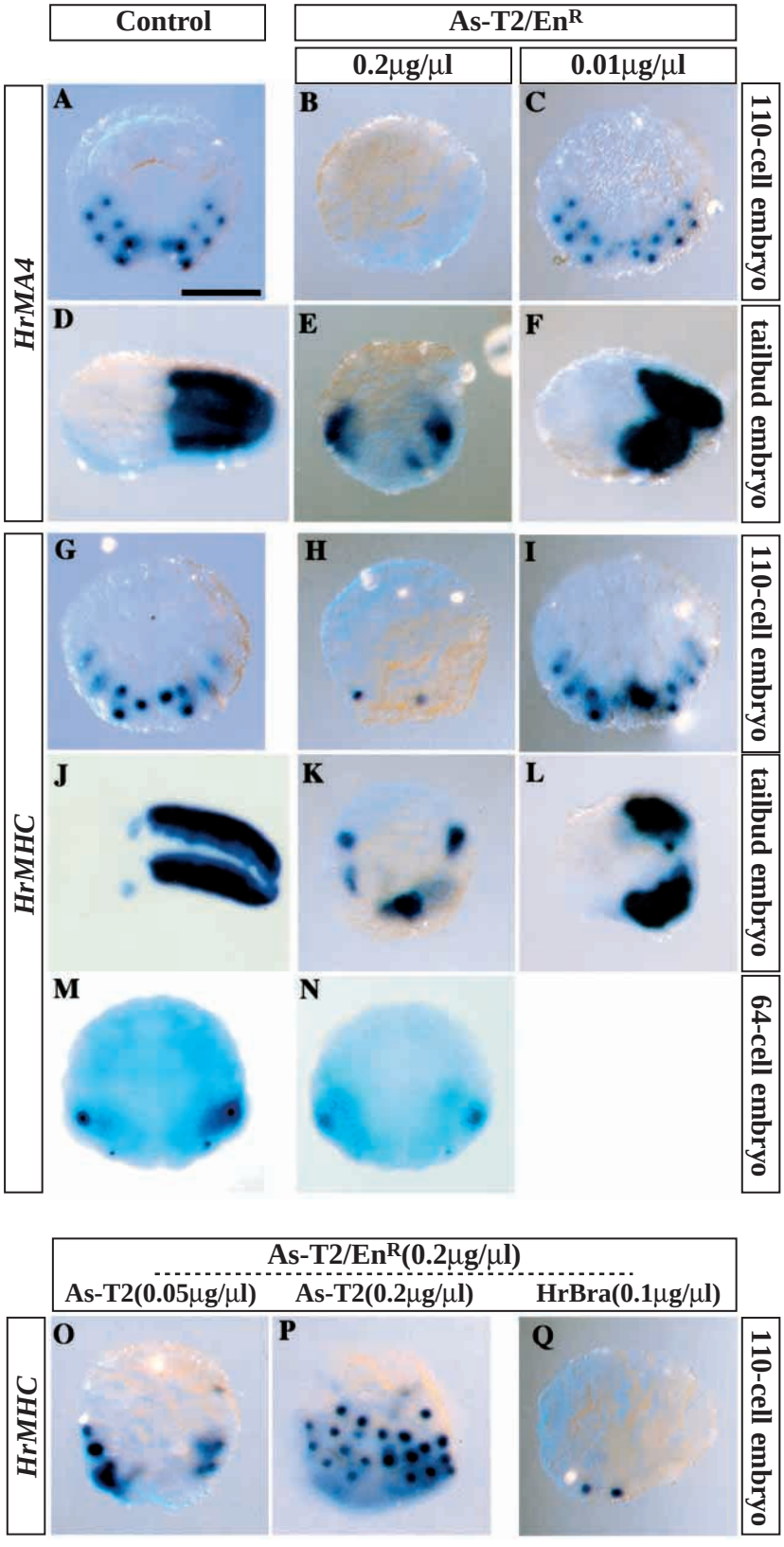

Fig. 1. Effects of microinjection of As-T2/En^R mRNA on the expression of muscle-specific actin gene (*HrMA4*) and myosin heavy chain gene (*HrMHC*), assessed by whole-mount in situ hybridization. (A-F) Expression of *HrMA4* and (G-Q) *HrMHC*. (A-C,G-I,O-Q) Embryos around the 110-cell stage, (D-F,J-L) 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/En^R mRNA and (C,F,I,L) those injected with 0.01 μg/μl As-T2/En^R mRNA. (O-Q) Co-injection of 0.2 μg/μl As-T2/En^R 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.

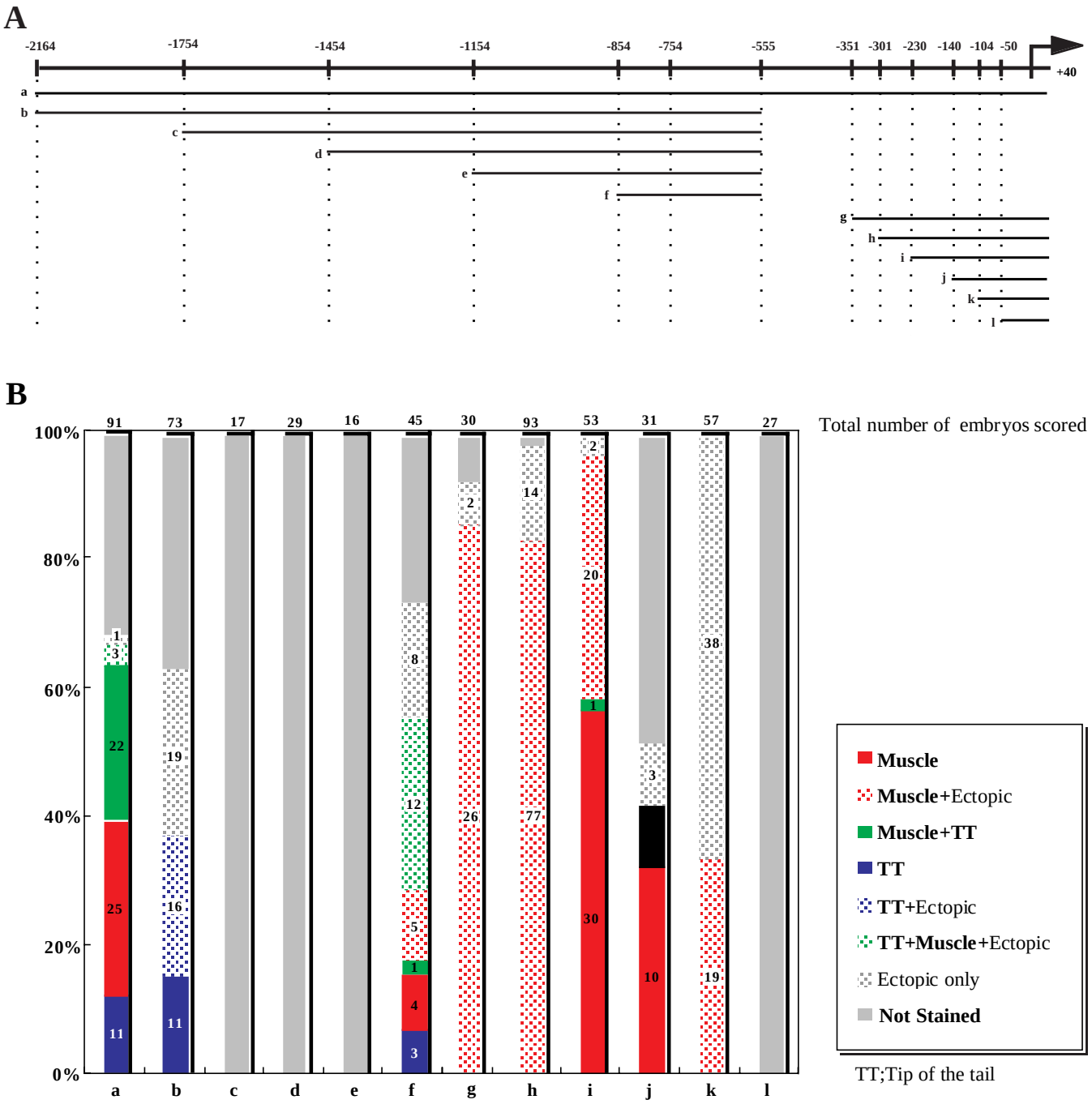

Fig. 2. Minimal promoter for specific expression of *As-T2* in two embryonic regions (muscle and the tip of the tail) of *Halocynthia* embryos. (A) Various deletion constructs examined to determine essential flanking sequences. (B) Frequency of embryos with the reporter gene expression and embryonic regions of expression. Numbers in graphs indicate the number of positive embryos. The deletion constructs indicated at the bottom are the same as those in A.

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 *Hinc*II 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 expression 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 Brachyury (T) protein to DNA fragments which were selected from a mixture of random oligomers. They identified a 20 bp palindrome,

TG(C)ACACCTAGGTGTGAAA

as a possible consensus sequence to which the Brachyury 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 palindrome-like sequence, TTTGTTACCTAGGTGTGGAAA, 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,E). 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

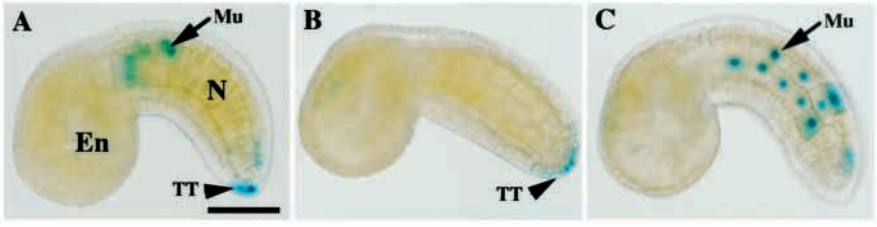

Fig. 3. Expression of *lacZ* in *Halocynthia* tailbud-stage embryos that developed from eggs injected with various deletion constructs of pAs-T2/*lacZ*. (A) Injection of p(–2164)As-T2/*lacZ* resulted in the expression of the reporter gene in muscle cells (Mu; arrow) and TT cells (arrowhead). En, endoderm; N, notochord. Scale bar: 100 μm. (B) Injection of p(–2164 ~ –555)As-T2/*lacZ* resulted in the expression of *lacZ* in TT (tip of the tail) cells. (C) Injection of p(–230)As-T2/*lacZ* resulted in the expression of *lacZ* in muscle cells (Mu).

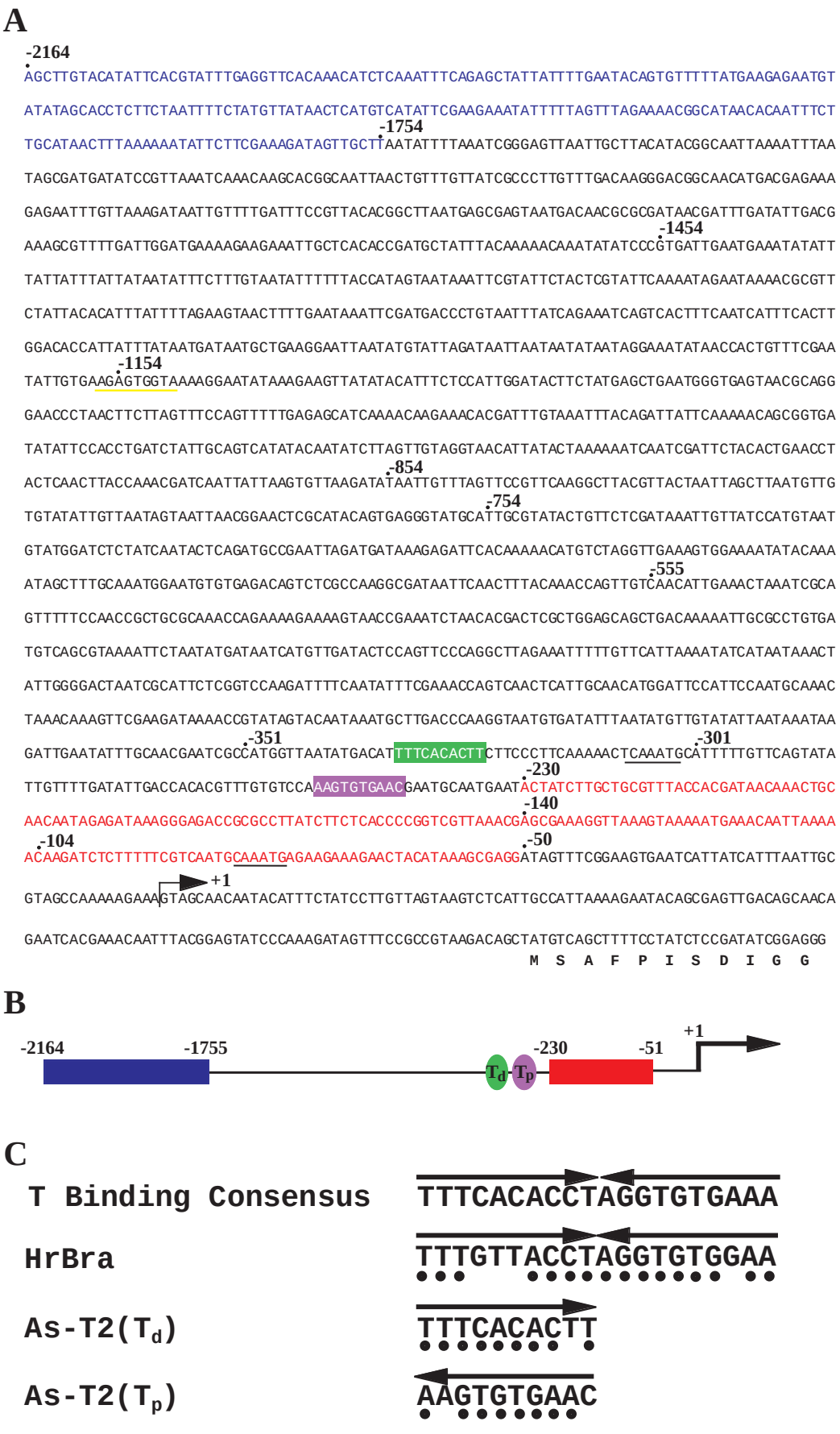

Fig. 4. (A) Nucleotide sequence of the 5′-flanking region of *As-T2*, including the regions for its muscle-specific (shown with red letters) and TT-specific (shown with blue letters) expression. A putative Gli protein-binding motif is shown with a yellow underline. The transcription start site is shown 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.

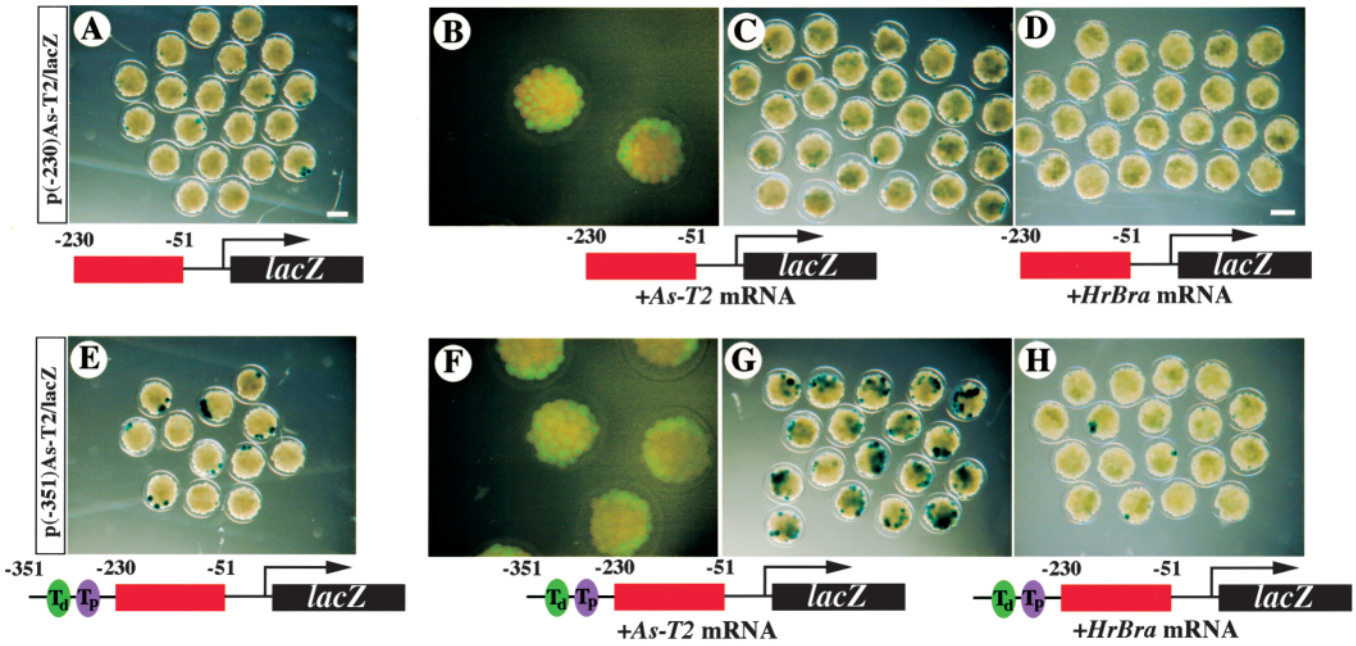

Fig. 5. Two T-binding motifs and autoregulation of *As-T2*. (A-D) Expression of *lacZ* in the 110-cell stage embryos when p(–230)As-T2/*lacZ* was injected into fertilized eggs without (A) or with *As-T2* mRNA (B,C) or *HrBra* mRNA (D). (B) *As-T2* mRNA was tagged with a sequence encoding GFP, and the expression of GFP in embryos showed the proper translation of *As-T2* mRNA. (E-H) Expression of *lacZ* in the 110-cell stage embryos when p(–351)As-T2/*lacZ* was injected into fertilized eggs alone (E) or with *As-T2* mRNA (F,G) or *HrBra* mRNA (H). (F) GFP expression confirming proper translation of injected *As-T2* mRNA. The red box indicates the putative minimal promoter of As-T2; and Td indicates the distal and Tp the proximal T-binding motif. 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: 100 μm.

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

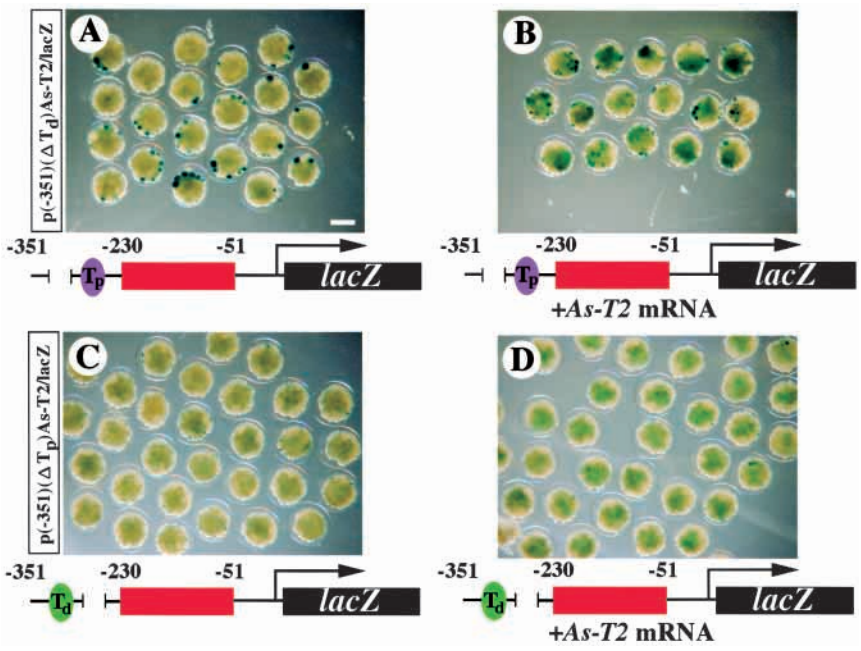

Fig. 6. Requirement of the distal and proximal T-binding motifs of *As-T2* for its upregulation assessed by the reporter gene expression. (A,B) p(–351)(ΔTd)As-T2/*lacZ* was injected solely (A) or with *As-T2* mRNA (B). (C,D) p(–351)(ΔTp)As-T2/*lacZ* injected solely (C) or with *As-T2* mRNA (D). Injected 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.

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).

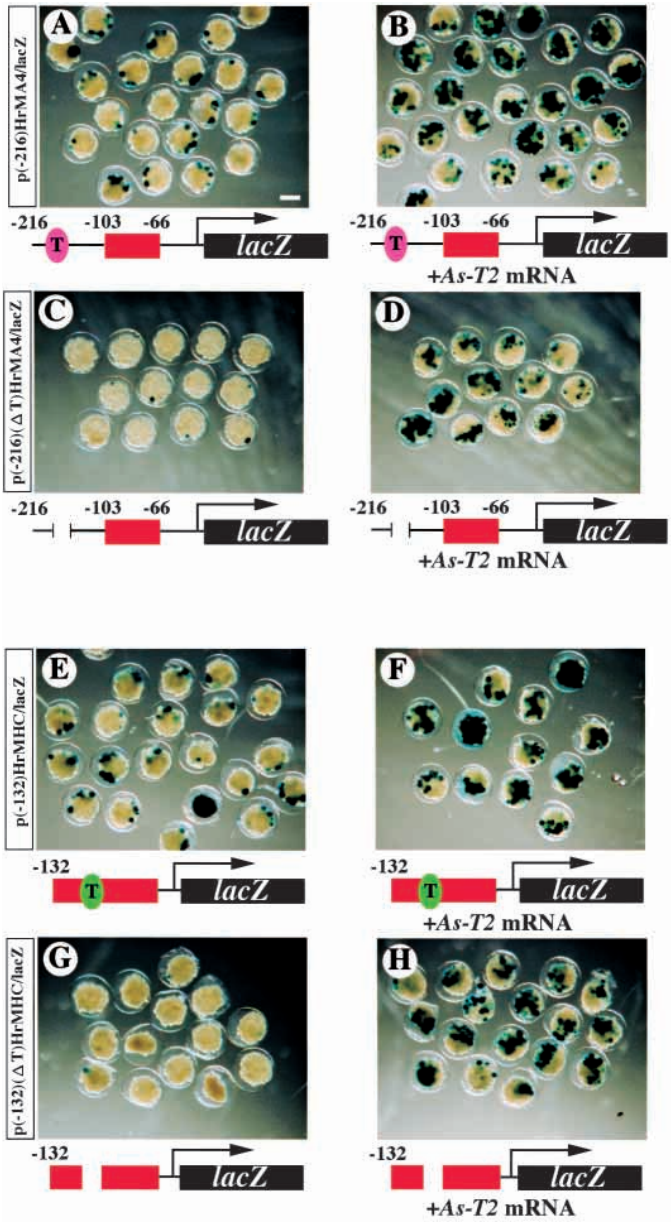

Fig. 8. Efficiency of T-binding motifs in expression of *HrMA4* (A-D) and *HrMHC* (E-H). (A-D) The reporter gene expression in embryos at the 110-cell stage injected with p(–216)HrMA4/*lacZ* (A), p(–216)HrMA4/*lacZ* with *As-T2* mRNA (B), p(–216)(ΔT)HrMA4/*lacZ* (C) and p(–216)(ΔT)HrMA4/*lacZ* with *As-T2*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

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 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*

(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/En^R 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/En^R did not block the transcription initiation of *HrMA4* and *HrMHC* (Fig. 1N). However, as shown in the previous studies and the present

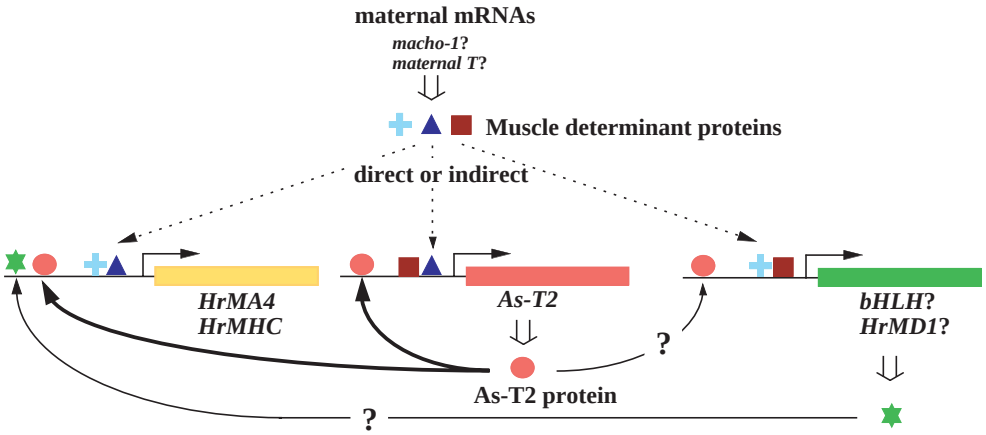

Fig. 9. Possible functional circuitry of *As-T2* associated with muscle differentiation in *Halocynthia* embryos (see text for details).

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/En^R suppressed the transcriptional activation of *HrMA4* and *HrMHC* in dose-dependent manner. Almost complete suppression of *HrMA4* and *HrMHC* transcription by As-T2/En^R was observed at the 110-cell stage. However, by the tailbud stage, embryos developed from eggs injected with As-T2/En^R showed detectable levels of *HrMA4* and *HrMHC* transcripts. This result may indicate that the amount of As-T2/En^R protein gradually diminished as development proceeded, and thus the inhibitory activity of As-T2/En^R 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 muscle-specific 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; Alexandre et al., 1996; Sasaki et al., 1997) around 1154 bp upstream of the putative transcription start site, 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 determinant 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 autoregulative 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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