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An intrinsic genetic program for autonomous differentiation of muscle cells in the ascidian embryo

(ascidian embryos/autonomous muscle cell differentiation/determinants/myogenic factor/actin gene expression)

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ABSTRACT **The B-line presumptive muscle cells of ascidian embryos have extensive potential for self-differentiation dependent on determinants prelocalized in the myoplasm of fertilized eggs. Ascidian larval muscle cells therefore provide an experimental system with which to explore an intrinsic genetic program for autonomous specification of embryonic cells. Experiments with egg fragments suggested that maternal mRNAs are one of the components of muscle determinants. Expression of larval muscle actin genes begins as early as the 32-cell stage, prior to the developmental fate restriction of the cells. The timing of initiation of the actin gene expression proceeds the expression of an ascidian homologue of vertebrate *MyoD* by a few hours. Mutations in the proximal E-box of the 5′ flanking region of the actin genes did not alter the promoter activity for muscle-specific expression of reporter gene. These results, together with results of deletion constructs of fusion genes, suggest that muscle determinants regulate directly, or indirectly via regulatory factors other than MyoD, the transcription of muscle-specific structural genes leading to the terminal differentiation.**

During early embryogenesis in animals, the developmental fate of embryonic cells is specified either autonomously or conditionally (1–4). In the case of autonomous specification, particular maternal information or morphogenetic determinant is prelocalized in a certain region of the egg cytoplasm and is segregated during cleavage to a certain lineage. This information serves to regulate, either directly or indirectly, the transcription of genes that are required for specific functions of cells (1, 5, 6). One of the examples of autonomous embryonic cell specification is the lineage that gives rise to larval tail muscle cells in the ascidian embryo (6, 7).

During embryogenesis of the ascidian *Halocynthia roretzi*, 42 unicellular and striated muscle cells are formed in the larval tail, which are associated with active locomotion of the larva. Lineage analysis has shown that the B4.1 cell pair of the bilaterally symmetrical 8-cell embryo gives rise to 28 muscle cells in the anterior and middle part of the tail, A4.1 pair gives rise to 4 muscle cells in the posterior part of the tail, and the b4.2 pair gives rise to 10 muscle cells at the tip of the tail (Fig. 1; ref. 8). Presumptive muscle cells of the B-line (primary lineage) have extensive potential for self-differentiation or autonomous development, while those of the A- and b-lines (secondary lineage) are unable to differentiate autonomously (9). Even if B4.1 cells are isolated from the 8-cell embryo and division of the isolated cells is arrested with cytochalasin B immediately after isolation, the cells eventually develop a marker of muscle differentiation (10). Therefore, the B-line presumptive cell is a self-sustained system in regard to muscle differentiation.

From the turn of the century, the existence of muscle determinants in so-called myoplasm of ascidian eggs has been suggested by several observations and experiments (11, 12). Recently, convincing evidence for the presence of muscle determinants has been offered by Nishida (13). B4.1 cells isolated manually from *Halocynthia* 8-cell embryos were divided into fragments that were with and without myoplasm. The enucleated myoplasm was fused with nonmuscle lineage a4.2 cells and the fusion products were allowed to develop into partial embryos. Nearly all of the partial embryos produced markers of muscle differentiation, while none of those from the fusion of a4.2 cells with enucleated B4.1 fragments without myoplasm produced such markers.

Therefore, ascidian larval muscle cells provide an experimental system with which to explore an intrinsic genetic program for autonomous specification of embryonic cells. In this system, as shown in Fig. 1*A*, muscle determinants, presumably via zygotic transcription factors, eventually activate muscle-specific structural genes. We describe and discuss here results of recent studies.

Muscle Determinants: Their Molecular Nature and Segregation Mechanisms

Molecular Nature of Muscle Determinants. As mentioned above, recent experiments clearly showed the presence of muscle determinants in the myoplasm of ascidian eggs. Several studies have already been undertaken to elucidate the molecular nature of muscle determinants (6). Jeffery (14) isolated the yellow myoplasm from *Styela plicata* eggs and found that the myoplasm fraction contains at least 15 polypeptides that are undetectable in the other cytoplasmic fraction. However, there were no detectable prevalent mRNAs specific to the myoplasm, as determined by extraction and translation of myoplasmic poly(A)$^+$ RNA in an *in vitro* system (14). Nishikata *et al.* (15) produced monoclonal antibodies that specifically recognize components of the myoplasm of *Ciona intestinalis* eggs. One of the antigens, named myoplasmin–C1, is a single 40-kDa polypeptide of the cortex of the myoplasm. The myoplasmin–C1 is implicated in muscle differentiation, because injection of its antibody into fertilized eggs partially

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Abbreviations: DFL, deep filamentous lattice; PML, plasma membrane lamina; bHLH, basic helix–loop–helix; RT–PCR, reverse transcription–PCR.

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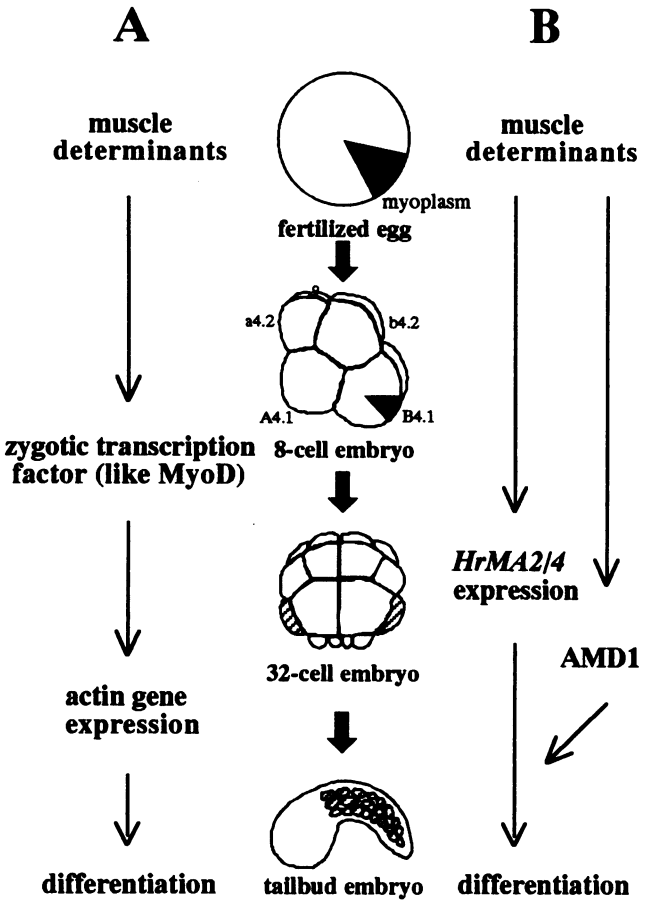

FIG. 1. Diagrammatic representation of the genetic cascade for muscle cell differentiation in ascidian embryos. Muscle cell differentiation in ascidian embryos is triggered by maternal factors or determinants localized in the myoplasm of fertilized eggs. (*A*) A previous working hypothesis. The determinants segregated into the B4.1 line presumptive muscle cells may trigger the expression of genes for zygotic transcriptional factors like vertebrate MyoD, which in turn activates expression of muscle-specific structural genes leading to differentiation of cells. (*B*) A cascade suggested by recent studies. An ascidian homologue (AMD1) of MyoD may be involved in maintenance of differentiation level but not in the upstream of the actin genes (*HrMA2/4*).

blocks the development of muscle-specific acetylcholinesterase activity (15). Isolation and characterization of myoplasmin–C1 cDNA clones suggested that myoplasmin–C1 is a cytoskeletal component of the myoplasm and that it may play a role in anchorage and segregation of the determinants (16). In addition to these studies, it has been shown that UV (ultraviolet) irradiation of fertilized eggs of *Styela clava* suppresses the development of acetylcholinesterase activity (17). The characteristic absorbance of this suppression, estimated with a specific cut-off filter, suggested that the UV-sensitive targets resemble proteins (18).

In spite of such laborious studies, however, the molecular nature, mechanism of segregation, and mode of action of muscle determinants are not fully elucidated yet. This paucity of information is mainly due to the difficulties encountered in obtaining a sufficient amount of intact myoplasm for biochemical and molecular biological analyses and to the absence of an appropriate assay system to examine activity of muscle determinants. To conquer these difficulties, Marikawa *et al.* (19) developed an experimental system that consists of egg fragmentation and fusion of the fragments. As shown in Fig. 2, centrifugation of unfertilized *Ciona savignyi* eggs yielded four types of fragments: a large nucleated red fragment, and small

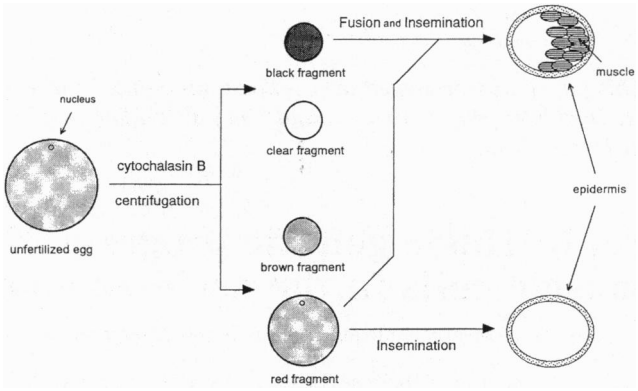

FIG. 2. Schematic diagram illustrating an experimental system to elucidate the molecular nature of muscle determinants. The system includes egg fragmentation and fusion of the fragments. Muscle determinants are separated into the black fragments.

enucleated black, clear, and brown fragments. When inseminated, only red fragments cleave and develop, but they form so-called permanent blastulae in which only epidermal cell differentiation is evident. However, when red fragments are fused with black fragments and fusion products are fertilized, nearly all of the fusion products develop muscle cells (Fig. 2; ref. 19). In addition, the ability of black fragments to promote muscle cell differentiation is evident when the fragments are fused with nonmuscle lineage a4.2 cell. In contrast, clear and brown fragments have no such abilities. Therefore, the muscle determinants appear to be preferentially separated into black fragments.

Irradiation of black fragments with UV light diminishes the ability of promotion of muscle differentiation of the fragments (20). Effective wave length (250-275 nm) suggests that maternal mRNAs are one of the UV-targets. This was proven by poly(A)$^+$ RNA injection experiment. Black fragments which were first UV-irradiated then injected with poly(A)$^+$ RNA of intact black fragments recover the muscle differentiation-promoting activity. Injection of poly(A)$^+$ RNAs of black fragments into red fragments, however, did not induce muscle differentiation. These results suggest that muscle determinants are comprised of not only maternal mRNAs but also factors other than mRNAs.

At present, we are screening differentially cDNA libraries of black and red fragments to isolate cDNA clones that are specific to black fragments and have functions for muscle differentiation. So far, we have obtained three cDNA clones specific to or enriched in black fragments. Sequencing of these clones revealed two of them to be mitochondrial genes. Since the myoplasm contains many mitochondria, this suggests the adequacy of the method. The third clone is intriguing, because the distribution of transcripts of this gene (named *posterior end mark* or *pem*) marks the posterior end of the developing embryo (S. Yoshida, Y. Marikawa, and N.S., unpublished data). Although the predicted PEM protein showed no similarity to known proteins, overexpression of this gene by microinjection of synthesized *pem* mRNA into fertilized eggs resulted in development of tadpole larvae with deficiency of the anterior-most adhesive organ, dorsal brain, and sensory pigment cells. This result suggests that the gene plays a role in formation of the anterior and dorsal structures of the tadpole larva. However, muscle cells differentiate normally in *pem*-overexpressed embryos. Therefore, *pem* is unlikely to be associated directly with muscle cell specification.

Another maternal molecule has been identified from the *Molgula oculata/Molgula occulta* system. The ascidian *M. oculata* has a conventional tadpole larva, while its sister species, *M. occulta*, exhibits a tailless larva (21). In *M. occulta* the presumptive notochord and muscle cells fail to differen-

tiate and undergo morphogenetic movements leading to tail formation. Therefore the two species provides a novel approach for identifying genes involved in larval development. Swalla *et al.* (22) applied subtractive procedures to identify three urodele (*uro*) genes that are expressed in *M. oculata* but are inactive or downregulated in *M. occulta*. One of the genes, *uro-11* (*Manx*), encodes a zinc-finger protein, which may be a transcriptional factor regulating downstream genes involved in tail formation. Zygotic *Manx* transcripts are expressed transiently between the gastrula and neurula stages, when events leading to tail formation are likely to be determined. The tissues in which *Manx* mRNA is expressed, the presumptive notochord and muscle cells, the neural tube, and the posterior epidermis, are also suggestive of a role in tail formation. *Manx* may be at or near the top of a hierarchy leading to tail formation (22).

Segregation Mechanisms. The myoplasm of ascidian eggs is composed of myoplasmic cytoskeletal domain (23). Myoplasmic cytoskeletal domain consists of two interacting parts, a plasma membrane lamina (PML) and a deep filamentous lattice (DFL). The PML is a network of filaments lying immediately beneath the plasma membrane, and it is attached to the DFL. The PML is thought to interact with the cell-surface components. The DFL is a three-dimensional network of filaments extending throughout the myoplasm and contains embedded pigment granules, mitochondria, and maternal mRNAs (23). A major components of the former is actin filaments, while that of the latter is intermediate filaments (24). Because muscle determinants are preferentially partitioned into black fragments of *C. savignyi* eggs, cytoskeletal components of the fragments were examined (25). Actin filament, a component of PML, was found in all of the fragments. In contrast, intermediate filament, a component of DFL, was highly concentrated in black fragments and excluded from the red, clear, and brown fragments. In addition, pigment granules and mitochondria, both of which are embedded in DFL, were also concentrated in black fragments. The distribution of muscle determinants among the egg fragments therefore coincides with that of DFL. This supports a notion that muscle determinants are associated with DFL rather than with PML.

Expression of an Ascidian Homologue (*AMD1*) of Vertebrate *MyoD*

Vertebrate myogenic regulatory genes, *MyoD*, *myogenin*, *Myf-5*, and *MRF-4* are capable of initiating myogenesis when artificially expressed in a variety of vertebrate cells (26, 27). Gene knock-out studies have revealed that the expression of these genes is essential for myogenesis (28, 29). They encode proteins that belong to a group known as the basic helix–loop–helix (bHLH) family. The members bind to the E-box motif (CANNTG) of the promoter/enhancer regions of many muscle-specific genes (30). They bind to the motifs as heterodimers with E2A proteins (31). An ascidian homologue of vertebrate myogenic bHLH proteins is interesting with respect to muscle determinants, and therefore the homologue, designated *AMD1*, was characterized (32).

The *AMD1* gene is single copy. We could obtain only one gene for myogenic bHLH protein from the ascidian genome, although there may be other, divergent myogenic bHLH genes. The *AMD1* gene contains four exons and is transcribed into at least two distinct mRNAs, which differ in their 3′ untranslated regions (32). Not only sequences of the myogenic regulatory genes but also their functions are conserved among vertebrates and invertebrates. The sea urchin myogenic factor 1 (SUM-1), for example, shows strong myogenic activity when expressed in murine 10T½ fibroblasts (33). AMD1 is also functionally conserved. When expressed in chicken primary skin fibroblasts, AMD1 is able to activate chicken myosin light chain enhancer at levels comparable to the activity of chicken myogenin (I.A., Y. Nabeshima, and N.S., unpublished data).

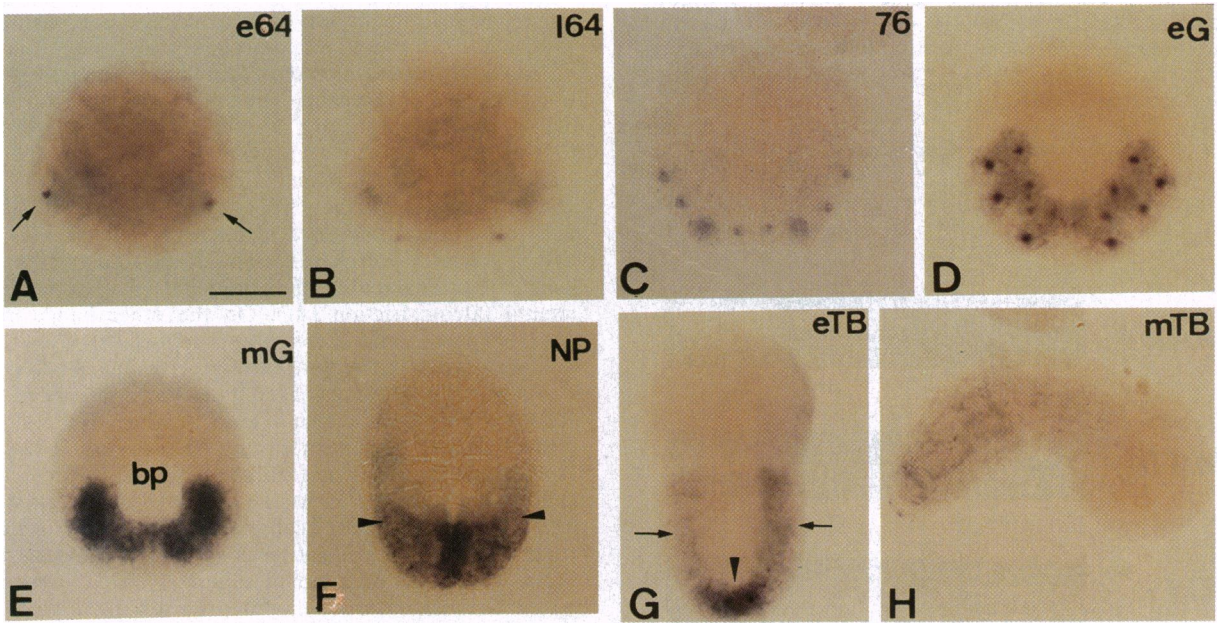

FIG. 3. Expression of *AMD1* in ascidian embryos, as revealed by whole-mount *in situ* hybridization with a digoxigenin-labeled antisense probe. (*A*) An early 64-cell stage embryo viewed from the vegetal pole (future dorsal side), showing first detection of hybridization signals in the B-line primordial muscle cells (arrows). (*B*) A late 64-cell stage embryo, the vegetal pole view. Hybridization signals are seen in pairs of B-line primordial muscle cells. (*C*) A 76-cell stage embryo viewed from the vegetal pole. (*D*) An early gastrula, the vegetal pole view. Hybridization signals are seen in the B-line muscle cells. (*E*) A mid-gastrula, the vegetal side view. Intense signals are found in all B-line muscle cells. bp, Blastopore. (*F*) A neural-plate stage embryo, the dorsal side view. Signals are evident not only in the B-line muscle cells but also in A- and b-line muscle cells (arrowheads). (*G*) An early tailbud embryo, dorsal side view. At this satge, signals in the B-line muscle cells are weak (arrows), while intense signals are seen in the A- and b-line cells (arrowhead). (*H*) Lateral view of a mid tailbud embryo, showing decrease in the intensity of hybridization signals. (Bar = 100 μm.)

As shown in Fig. 3, *in situ* hybridization of whole-mount specimens revealed that zygotic transcript of *AMD1* was first detected in the primary lineage (B-line) primordial muscle cells (B7.4 cells) around the 64-cell stage (Fig. 3 *A* and *B*). As development proceeds, transcripts of *AMD1* became evident in the primary lineage muscle cells in embryos at the 76-cell (Fig. 3*C*) and early gastrula stages (Fig. 3*D*). Expression level of *AMD1* in this lineage, however, decreased at the neurula and later stages. At the early tailbud stage, hybridization signals were weak in the primary lineage muscle cells (Fig. 3*G*). Zygotic expression of *AMD1* in the secondary lineage muscle cells was undetectable in embryos at early stages up to the early gastrula stage (Fig. 3 *A–D*). However, *AMD1* expression was evident at the neural plate stage (Fig. 3*F*). At the early tailbud stage, signals in the secondary lineage cells were stronger than those in the primary lineage cells (Fig. 3*G*). Hybridization signals became less intense at the mid-tailbud stage (Fig. 3*H*). They became undetectable by the late tailbud stage (data not shown).

It has been noticed that the differentiation pattern of the secondary lineage differs from that of the primary lineage in several ways (6). In addition to difference in the specification pattern, the timing of appearance of differentiation markers in the secondary lineage is delayed compared with that in the primary lineage. Acetylcholinesterase activity is initially observed in the primary lineage at the neurula stage, whereas in the secondary lineage is it first detected at the middle of the tailbud stage. The delayed expression of *AMD1* in the secondary lineage may cause this difference.

A reverse transcription–polymerase chain reaction (RT–PCR) assay revealed that a small amount of maternal transcripts of the *AMD1* gene are detectable in fertilized eggs and in early embryos (Fig. 4). Appearance of zygotic *AMD1* transcripts was first detected at the 64-cell stage (Fig. 4), a few hours later than the detection of zygotic transcripts of actin gene *HrMA4*. At present it is uncertain whether fertilized eggs contain maternal AMD1 proteins. However, as discussed later, together with the result that mutations in the proximal E-box of the 5′ upstream of *HrMA4* did not diminish the muscle-specific expression of reporter gene, it is unlikely that zygotic expression of *AMD1* promotes the specification of embryonic cells into muscle. AMD1 may function to maintain the differentiation state by enhancing the expression of muscle-specific structural genes.

Control of Muscle Actin Gene Expression

Vertebrates contain two distinct types of actin, which are encoded by a small gene family. The cytoskeletal-type (or cytoplasmic-type) actins, which are expressed in nonmuscle cells, and the muscle-type actins, which are expressed in muscle cells, are distinguishable by amino acid usage at diagnostic positions (34). Although invertebrates have muscle and cytoskeletal actin genes, the amino acid usage of the muscle actins resembles that of vertebrate cytoskeletal-type actins (34). Interestingly, ascidian larval muscle actins are of vertebrate muscle-type actin (35, 36). Therefore, it should be pointed out that vertebrate muscle-type actins appeared during the emergence of chordates (37, 38).

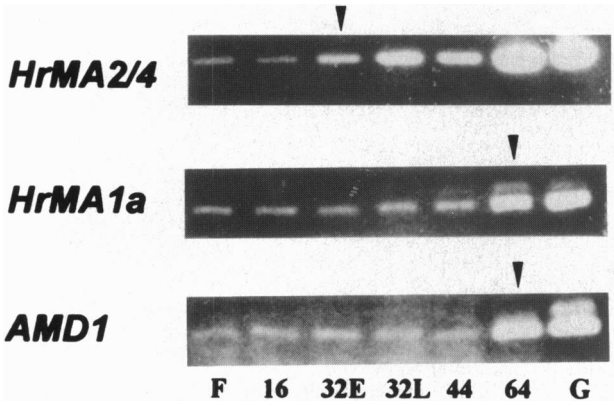

FIG. 4. Temporal expression of *HrMA2/4*, *HrMA1a*, and *AMD1* genes. Quantitative RT–PCR assay of RNAs prepared from fertilized eggs (lane F), 16-cell satge embryos (16), early 32-cell stage embryos (32E), late 32-cell stage embryos (32L), 44-cell stage embryos (44), 64-cell stage embryos (64) and gastrulae (G). Zygotic transcripts of *HrMA2/4*, *HrMA1a*, and *AMD1* genes are evident at the 32-cell, 64-cell, and 64-cell stage, respectively (arrowheads).

The Organization of Muscle Actin Genes in the Ascidian Genome. Southern blot analysis suggested that an ascidian has 10–15 actin genes in the genome (39). Indeed, Beach and Jeffery (39) distinguished four different cDNA clones that encode the same muscle actin expressed during *Styela clava* embryogenesis. In *H. roretzi* five muscle actin genes (*HrMA2*, *HrMA4a*, *HrMA4b*, *HrMA5*, and *HrMA6*) form a cluster (*HrMA2/4* cluster) within a 30-kb region of the genome (36). The five genes are oriented in the same direction. The *HrMA4a*, *HrMA4b*, and *HrMA2* encode an identical protein. The *HrMA4a*, *HrMA4b*, and *HrMA2* genes consist of three exons, and these genes have intron–exon splice junctions at the same positions, which are identical with those of vertebrate muscle-type actins. High stringency northern blot analysis suggested that the genes are expressed exclusively in muscle cells of tailbud embryos but not in adult body-wall and heart muscles (38).

In addition to the *HrMA2/4* cluster, the genome of *H. roretzi* contains a pair of muscle actin genes, *HrMA1a* and *HrMA1b* (*HrMA1* pair; ref. 40). The two genes are linked in a head-to-head arrangement on opposite strands and share a 340-bp 5′ flanking sequence containing two symmetrically located TATA boxes (see Fig. 6; ref. 40). Since single copy muscle actin genes occur in vertebrates, an ancestral muscle actin gene probably duplicated in ascidians. The tandem cluster of *HrMA2/4* gene and the bidirectional promoter of the *HrMA1* pair could expedite utilization of muscle-specific trans-acting factors. The organization of genes in the genome may play an important role in the synthesis of a large amount of actins associated with rapid larval development.

Timing of Initiation of Actin Gene Expression. Reexamination of timing of the gene expression by whole-mount *in situ* hybridization revealed that zygotic transcripts of *HrMA4a* are first evident in B6.2 (the progenitor of B7.4) at the 32-cell stage, in B7.8 at the 64-cell stage, and in B7.5 around the 76-cell stage, respectively (41). Because the developmental fate restriction to give rise to muscle occurs in B7.4 at the 64-cell stage, this result suggests that the transcription of this gene in the B7.4-sublineage is initiated prior to the developmental fate restriction (41). On the other hand, *HrMA1a* transcripts were first detected at the 64-cell stage.

The presence of *HrMA4a* transcripts in the 32-cell embryos was confirmed by means of RT–PCR (41). A result of further RT–PCR analyses is shown in Fig. 4. As in the case of *AMD1*, a small amount of maternal transcripts of both *HrMA4a* and *HrMA1* are detectable in fertilized eggs and in early embryos (Fig. 4). Appearance of zygotic *HrMA4a* transcripts was first detected at the early 32-cell stage (Fig. 4), confirming the results of *in situ* hybridization of whole-mount specimens.

An advantage of ascidian embryos as an experimental system to elucidate molecular mechanisms underlying cellular differentiation is the complete description of cell lineage up to the gastrula stage. We can identify every blastomere of early embryos. In most cases of *in situ* hybridization with whole-mount specimens, signals are first detected in the nucleus of certain blastomeres, then the signals distribute over the entire cytoplasm, as shown, for example, in Fig. 3. This situation enables us to judge unambiguously which blastomeres of early embryos express the gene and which do not. Thus, we could assess the timing of gene expression with respect to that of

developmental fate restriction as well as the developmental potentials of blastomeres.

Control of *HrMA4a* Expression. The 5′ flanking sequences of the five *HrMA2/4* cluster genes resemble each other. As shown in Fig. 5, the 5′ upstream region close to the transcription start site of *HrMA4a* contains several consensus sequences, which include a TATA box at −30, an E-box at −71, a CArG box at −116, and a cluster of three E-boxes between −150 and −190 (36). pHrMA4a-Z is a recombinant plasmid in which about 1.4 kb of the 5′ upstream region of *HrMA4a* is fused with the coding sequence of a bacterial gene for β-galactosidase (*lacZ*). When this construct was introduced into fertilized eggs, the expression of the reporter gene was evident in muscle cells of the larvae (42, 43). About 60% of injected embryos expressed the reporter gene in muscle cells (Fig. 5). In addition, when deletion constructs of the 5′ upstream region fused with *lacZ* were microinjected into fertilized eggs, the reporter gene was also expressed in muscle cells of tailbud embryos (Fig. 5*A*; ref. 43). Analyses of the deletion constructs suggested that the 103-bp upstream region is sufficient for the appropriate spatial expression of the gene (Fig. 5*A*). Frequencies of embryos with ectopic reporter gene expression in the case of 103-bp deletion constructs are comparable to those of pHrMA4a-Z. However, the reporter gene is not expressed in the case of 61-bp upstream region (Fig. 5*A*). Therefore, it is likely that rather short sequences between nucleotides −103 and −61 from the transcription start site are associated with the muscle-specific expression of *HrMA4a*.

The reporter gene was also expressed in larval muscle cells when pHrMA4a-Z was injected into *Ciona savignyi* eggs (42). We have been investigating muscle determinants using *Ciona* eggs, while we have used *Halocynthia* embryos to investigate control mechanisms of muscle-specific gene expression. Although *Ciona* and *Halocynthia* are representative of the two major subgroups of ascidians, the genetic circuitry underlying muscle differentiation seems to be conserved between the two species.

There is an E-box sequence at −71 of *HrMA4a* (Fig. 5). We examined significance of this motif for muscle-specific expression of the reporter gene. As shown in Fig. 5*B*, mutations in the proximal E-box sequence did not diminish the muscle-specific expression of the reporter gene, although frequency of embryos with β-galactosidase activity decreased to about two-third of the control (Fig. 5*B*; ref. 43). Therefore, it is unlikely that AMD1 is required for and is closely associated with muscle-specific expression of *HrMA4a*.

Control of *HrMA1a* and *HrMA1b* Expression. As shown in Fig. 6, the transcription initiation sites of *HrMA1a* and *HrMA1b* genes are only 340 bp apart and a TATA box is located at −30 in each promoter (40). Nucleotide sequences of the 5′ untranslated region and untranscribed region up to the TATA boxes are highly conserved between the two genes, whereas the nucleotide sequence between two TATA boxes showed no distinct symmetry except for the presence of two CArG box-like sequences (44) around position −80 (Fig. 6). One E-box sequence and one MEF-2 binding site (45) are located in the middle of the 5′ flanking region of the genes (Fig. 6). A previous study showed that when constructs in which the shared upstream region of *HrMA1* pair fused with *lacZ* in either direction were microinjected into eggs, the reporter gene was expressed in muscle cells of the larval tail, suggesting a bidirectional promoter that regulates muscle-specific transcription of the *HrMA1* pair (40). The results shown in Fig. 6 confirm the bidirectional promoter activity of the 5′ flanking region shared by the two genes.

Sequences required for the muscle-specific expression of the *HrMA1* pair were further analyzed. As shown in Fig. 6, mutations in the E-box sequence did not diminish the muscle-specific expression of the reporter gene, although frequency of embryos with β-galactosidase activity decreased to some extent (Fig. 6). Therefore, as in the case of the clustered actin-genes, it is unlikely that AMD1 is required for muscle-specific expression of *HrMA1a* and *HrMA1b*.

The promoter activity of deletion constructs of *HrMA1a* and *HrMA1b* was also examined. A deletion construct of 190-bp upstream region of *HrMA1a* and that of 139-bp upstream region of *HrMA1b* lack the MEF-2-like binding site (Fig. 6). When the deletion constructs were microinjected into fertilized eggs, the reporter gene was expressed in muscle cells of tailbud embryos (Fig. 6). A deletion construct of 85-bp upstream region of *HrMA1a* and that of 89-bp upstream region of *HrMA1b* lack the CArG box-like binding site (Fig. 6). When

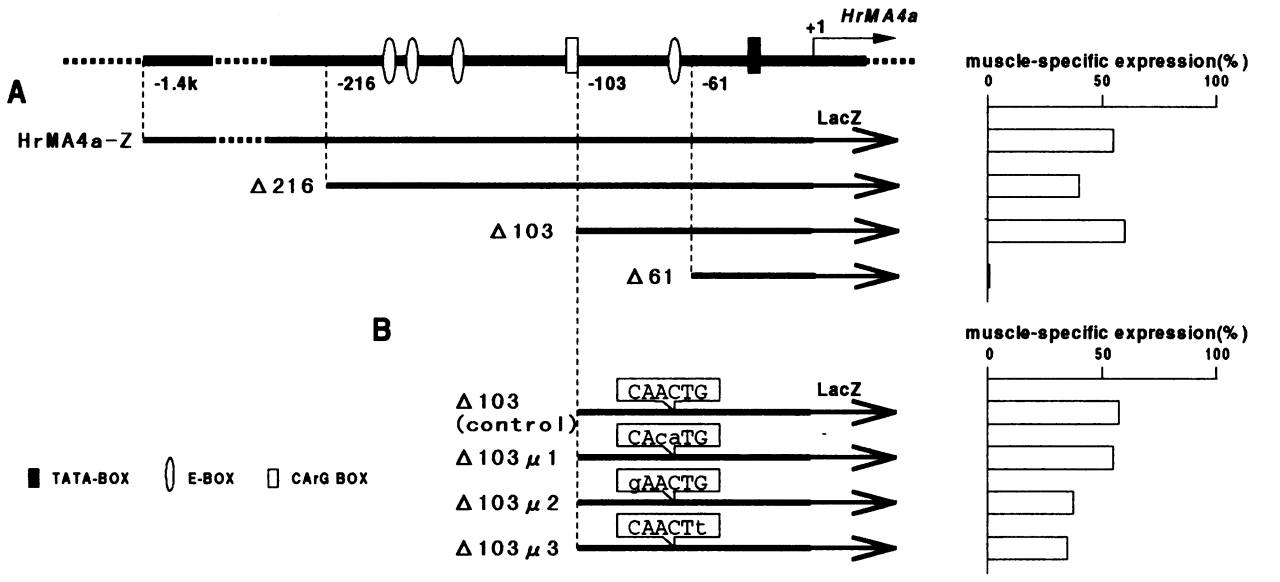

FIG. 5. Expression of the reporter gene showing the promoter activity of the 5′ upstream region of *HrMA4a*. (*Upper*) Diagrammatic representation of consensus sequences in the 5′ upstream region of *HrMA4a*. (*A*) Muscle-specific expression of the reporter gene in embryos that developed from eggs injected with pHrMA4a-Z and its deletion constructs. The promoter activity is shown by frequencies (%) of embryos exhibiting the reporter gene expression. (*B*) Muscle-specific expression of the reporter gene in embryos that developed from eggs injected with pHrMA4a-Z(Δ 103) and its mutations in the E-box sequences.

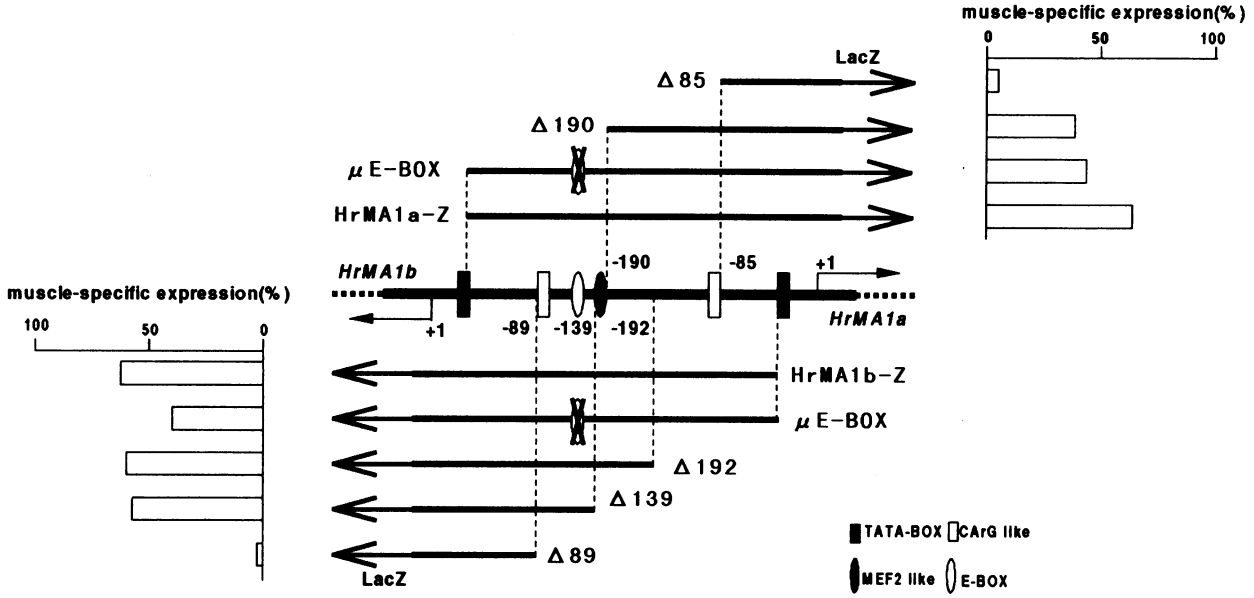

FIG. 6. Expression of the reporter gene showing the promoter activity of the 5′ upstream region of *HrMA1a* and *HrMA1b*. (*Middle*) Diagrammatic representation of consensus sequences in the bidirectional promoter region of *HrMA1a* and *HrMA1b*. (*Top*) Muscle-specific expression of the reporter gene in embryos that developed from eggs injected with pHrMA1a-Z, a mutation in the E-box, and deletion constructs. The promoter activity is shown by frequencies (%) of embryos exhibiting the reporter gene expression. (*Bottom*) Muscle-specific expression of the reporter gene in embryos that developed from eggs injected with pHrMA1b-Z, a mutation in the E-box, and deletion constructs.

these deletion constructs were microinjected into fertilized eggs, the reporter gene expression was not detected in most of the injected embryos (Fig. 6). Therefore, it is likely that rather short sequences including the CArG box-like sequence are essential for the muscle-specific expression of *HrMA1a* and *HrMA1b*.

In addition to actin genes, we have already examined the control of expression of a gene (*HrMHC1*) for myosin heavy chain of *H. roretzi* embryos (46, 47). An complete *HrMHC1* cDNA sequence suggested that the amino acid sequence of HrMHC1 resembles that of myosin heavy chain of vertebrate skeletal and cardiac muscles (47). The *HrMHC1* is expressed in the same manner as the *HrMA4a* gene; namely, the zygotic transcripts are evident in B-line muscle precursor cells as early as the 32-cell stage (41). Therefore, the timing of transcription initiation of the muscle-specific genes proceeds fate restriction in the primary muscle lineage. A fusion gene containing 132 bp upstream of the 5′-end of *HrMHC1* fused with *lacZ* was microinjected into fertilized *H. roretzi* eggs (47). The reporter gene was eventually expressed only in muscle cells of tailbud embryos. Point mutations inserted into the upstream region suggested that cis-regulatory elements between positions −60 and −80 of *HrMHC1* is critical for the promoter activity of the gene (47).

Discussion

As described in this review, muscle cell differentiation in ascidian embryos is triggered by maternal factors or determinants localized in the myoplasm. Maternal mRNAs are one of the important components of the factors. The determinants are segregated into the B-line presumptive muscle cells by trapping by the deep filamentous lattice, a component of myoplasmic cytoskeletal domain. Zygotic expression of an ascidian homologue (*AMD1*) of vertebrate *MyoD* initiates a few hours later than that of muscle actin genes. Mutations of the E-box sequence in the 5′ flanking region of actin genes did not alter drastically the muscle-specific expression of reporter gene, suggesting that zygotic AMD1 is not determinant itself. AMD1 may be responsible for maintenance of differentiation state.

All animal groups appear to adapt a common molecular mechanisms for muscle cell differentiation. However, it also appears that certain animal groups adopt their own systems during evolution. In ascidian embryos, zygotic transcripts of muscle actin and myosin heavy chain in presumptive muscle cells occur before developmental fate restriction of the blastomeres. It is an important question whether this pattern of very early expression of the tissue-specific structural genes is common to other animals.

The present and previous studies demonstrated that rather short upstream sequences of the 5′ flanking region of muscle-specific structural genes are responsible for the tissue-specific expression of the genes. The ascidian embryo has several advantages as an experimental system to study the genetic circuitry underlying specification of embryonic cells and morphogenesis (6, 48). Biochemical approaches to identify developmentally important transcriptional factors may be possible with ascidians. *H. roretzi* are cultured for marketing in Japan. They produce large numbers of eggs, and materials could be obtained for transcriptional factor research. Together with identification of muscle determinants using egg fragments as well as the *Molgula* system, the genetic circuitry involved in the autonomous specification of embryonic cells would be disclosed in ascidian embryos.

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