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Expression of muscle-related genes and two *MyoD* genes during amphioxus notochord development

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SUMMARY The notochord is one of the diagnostic features of the phylum Chordata. Despite the similarities in the early morphogenetic patterns of the notochords of various chordates, they are strikingly distinct from one another at the histological level. The amphioxus notochord is one example of an evolutionary novelty because it is made up of muscle cells. Our previous expressed sequence tag analysis, targeting messenger RNAs expressed in the adult amphioxus notochord, demonstrated that many muscle-related genes are expressed there. To characterize amphioxus notochord cells and to gain insights into the myogenic program in the notochord, we determined the spatial and temporal expression patterns of these muscle-related genes during amphioxus development. We found that *BbNA1* (notochord actin), *Amphi-Trop I* (troponin I), *Amphi-TPmyosin* (tropomyosin), *Amphi-MHC2* (myosin heavy chain), *Amphi-nMRLC* (notochord-specific myosin regulatory light chain), *Amphi-nTitin/MLCK* (notochord-specific titin/myosin light chain kinase), *Amphi-MLP/CRP3* (muscle LIM protein), and *Amphi-nCalponin* (notochord-specific calponin) are expressed with characteristic patterns in notochord cells, including the central cells, dorsally located cells, and ventrally located cells, suggesting that each notochord cell has a unique molecular architecture that may reflect its function. In addition, we characterized two *MyoD* genes (*Amphi-MyoD1* and *Amphi-MyoD2*) to gain insight into the genetic circuitry governing the formation of the notochord muscle. One of the *MyoD* genes (*Amphi-MyoD2*) is expressed in the central notochord cells, and the coexistence of *Amphi-MyoD2* transcripts along with the *Amphi-MLP/CRP3* transcripts implies the participation of *Amphi-MyoD2* in the myogenic program in the notochord muscle.

INTRODUCTION

The phylum Chordata is a monophyletic group comprising urochordates, cephalochordates, and vertebrates (Turbeville et al. 1994; Wada and Satoh 1994) and is named after one of its defining features, a notochord (Shaeffer 1987). Morphogenesis of the notochord proceeds in a similar fashion in the various groups of chordates (Cloney 1964; Ruppert 1997a). The notochord originates from the archenteron roof, which subsequently separates from the gut, and the notochord cells then become discoidal in shape as they arrange themselves in a single longitudinal series. In addition to this similarity in the early morphogenetic pattern, a T-box containing transcription factor, *Brachyury*, has a crucial role in the differentiation and formation of chordamesodermal tissue in urochordate ascidians (Yasuo and Satoh 1993, 1998; Takahashi et al. 1999) and vertebrates (Herrmann and Kispert 1994; Tada and Smith 2001), and the *Brachyury* cognates of amphioxus is expressed quite similarly to vertebrate *Brachyury* (Holland et al. 1995; Terazawa and Satoh 1997). Recently, the *Brachyury* cognates of urochordate appendicularians, which represent a sister-group of the clade of other urochordates (Wada 1998), have also been characterized (Bassham and Postlethwait 2000; Nishino et al. 2001), and delimitated and distinct expression in the notochord lineage indicates a crucial role of the *Brachyury* in the formation of chordate tail (Nishino and Satoh 2001).

In contrast with the similarities in early morphogenetic pattern and gene expression, there are striking differences among the notochords of various chordates at the histological level, which has led to considerable debate about the phylogenetic implications (Flood et al. 1969; Welsch and Storch 1976; Ruppert 1997a). In particular, the notochord of amphioxus is regarded as an example of an evolutionary novelty, because it is made up of a type of muscle cell from the rostrum to the tip of the tail (Ruppert 1997b). During the

development of the amphioxus notochord, thick and thin myofilaments are formed in the cytoplasm (Conklin 1932; Eakin and Westfall 1962; Stach 1999). Furthermore, the existence of a junction between the notochord cells and the nerve cord (Flood 1966, 1968) and the presence of acetylcholinesterase immunoreactivity at the junction (Flood 1970) suggest that the amphioxus notochord is capable of altering its mechanical properties in response to nervous stimulation.

Although the anatomical details of the amphioxus notochord have been well studied, its embryonic development and molecular architecture remain obscure. Previously, we conducted expressed sequence tag (EST) analysis targeting messenger RNAs expressed in the adult amphioxus notochord (Suzuki and Satoh 2000). Among a set of 257 ESTs analyzed (both 5′ ends and 3′ ends), 11% of genes appeared to be associated with the formation and function of the muscular tissue.

Taking advantage of the EST analysis, the present study was conducted to investigate a basic issue concerning the embryonic development of the amphioxus notochord and its evolution. We characterized the development of notochord cells according to the expression of eight muscle-related genes and addressed whether these muscle-related genes share an expression domain with other muscular tissues or, alternatively, whether they showed notochord-specific expression. We then examined the developmental expression pattern of two *MyoD* genes to gain an insight into genetic circuitry that orchestrates the expression of the muscle-related genes and governs the formation of the notochord muscle.

MATERIALS AND METHODS

Biological materials

Specimens of the Chinese amphioxus, *Branchiostoma belcheri*, were collected in the vicinity of the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China. Naturally spawned eggs and sperm were obtained from adults as described in Yasui et al. (1998). Embryos and larvae were staged according to Hirakow and Kajita (1991, 1994). They were fixed for in situ hybridization analysis and stored in 75% ethanol at −20°C until use. For cDNA library construction, adult specimens were collected in Mikasa-Bay, Mie, Japan.

cDNAs

cDNAs used for this study were previously isolated for EST analysis of the adult amphioxus notochord (Suzuki and Satoh 2000). With regard to *Amphi-MyoD1*, *Amphi-MyoD2*, and *Amphi-MHC2*, full-length cDNA clones were newly isolated as described below.

Library construction and screening

The myotomes were surgically dissected from a dozen adult specimens. Total RNA was extracted by the acid guanidinium thiocyanate-chloroform method. Poly(A)$^+$ RNA was purified using Oligotex beads (Roche Diagnostics, Tokyo, Japan) and converted to double-stranded cDNA containing an *Eco*RI site at the 5′ end and an *Xho* I site at the 3′ end by using a ZAP cDNA synthesis kit (Stratagene, La Jolla, CA, USA). Subsequently, the cDNAs were ligated to Uni-ZAP XR vector (Stratagene). We screened the somite cDNA library with [^{32}P]-random-labeled probes prepared from *BMD1* and *BMD2* cDNA fragments (Araki et al. 1996). Because the expression of *Amphi-MHC1* was detected only in the somitic mesoderm (Urano et al., unpublished observation), the present study attempted to isolate cDNA clones for *MHC* family genes that are expressed in the notochord. Screening of the notochord cDNA library was performed under reduced stringency using *Amphi-MHC1* cDNA as templates. As a result, several cDNA clones that showed sequence similarity to the *Amphi-MHC1* gene were isolated, and in situ hybridization revealed that one of them was expressed in the developing notochord. Hereafter, we designate the corresponding gene *Amphi-MHC2*.

Sequence determination and similarity search

pBluescript SK(−) phagemid sequences within lambda ZAP vectors were excised using a rapid excision kit (Stratagene). The sequences of each cDNA were completely determined using a BigDye terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM 377 DNA sequencer (Perkin-Elmer, Norwalk, CT, USA). The nucleotide sequences were then used as a query sequence for tBLASTX programs against peptide sequence databases. Most of the cDNAs used in this study yielded significant scores (probability), and we designated cDNAs according to this scoring. Except for *Amphi-nTitin/MLCK*, all the cDNA clones contained putative full open reading frames. Comparison of the deduced amino acid sequence of cDNA clones for *Amphi-nTitin/MLCK* with other *Titin/MLCK* genes showed that this clone lacks the N-terminal half of its gene product. The *Titin/MLCK* family genes encode giant proteins with a molecular weight in the megadalton range, and no full-length cDNA clone for *Amphi-nTitin/MLCK* has yet been characterized.

For *Amphi-MLP/CRP3*, the percent identity with the LIM domain region of other cystein-rich protein (CRP) family genes was calculated using a Clustal W program (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalW/Top.html). This alignment revealed that the amphioxus gene contained a unique conserved motif that is specific to the *MLP/CRP3* subfamily members, and we therefore designated the corresponding gene as *Amphi-MLP/CRP3*. The accession numbers of the GenBank/EMBL/DDBJ databases are as follows: *BbNA1*, AB035660, AB035661, and AB035662; *Amphi-Trop I*, AB035664; *Amphi-TP myosin*, AB035663; *Amphi-MHC2*, AB092419; *Amphi-nMRLC*, AB035665; *Amphi-nTitin/MLCK*, AB092417; *Amphi-MLP/CRP3*, AB035567; *Amphi-nCalponin*, AB092418; *Amphi-MyoD1*, AB092415; and *Amphi-MyoD2*, AB092416.

Whole-mount in situ hybridization

In situ hybridization was carried out according to the method described by Satoh et al. (2001) with a minor modification. Hybridization was performed at 50°C with a digoxigenin (DIG)-

labeled antisense riboprobe (Roche Diagnostics). cDNAs used as templates for the riboprobes were completely sequenced as described above.

Phylogenetic analysis for *MyoD* family genes

A molecular phylogenetic tree was constructed based on the comparison of 110 highly conserved amino acid residues, including the bHLH domain, by the neighbor-joining method (Saitou and Nei 1987). Genetic distances were calculated using PAUP (Swofford 1998) in 100 rounds of heuristic random stepwise additions. Branch stability was assessed by 100 replicate bootstrap resamplings of the alignment data. The accession numbers of the GenBank/EMBL/DDBJ databases for the myogenic regulatory factor (MRF) genes used for this analysis are as follows: chicken *MyoD*, X16189; mouse *MyoD*, M18779; human *MyoD*, X56677; frog *MyoD*, X16106; zebrafish *MyoD*, AF318503; chicken *MYF5*, X75250; mouse *Myf5*, X56182; human *Myf5*, X14894; cow *Myf5*, M95684; zebrafish *Myf5*, AF253470; and sea urchin *SUM1*, AAD33917.

RESULTS AND DISCUSSION

Expression of eight muscle-related genes during notochord development

The amphioxus notochord comprises at least two different types of cell: a central notochord cell and a noncontractile cell, the so-called Müller's cell (Müller 1871). Actually, Müller's cell is a collective name for all noncentral notochord cells eventually located dorsally and ventrally to the central cells (see Fig. 5A) and the function of these cells remains obscure (Ruppert 1997b).

BbNA1

We first describe the expression patterns of genes likely expressed in the central notochord cells that eventually develop myofibrils in their cytoplasm. This group was found to include *BbNA1*, *Amphi-Trop I*, *Amphi-TPmyosin*, *Amphi-MHC2*, *Amphi-nMRLC*, and *Amphi-nTitin/MLCK*, and we consider these genes are likely to have roles in muscle formation and function.

BbNA1 encodes an actin. Comparison of the amino acid sequence deduced from the *BbNA1* cDNA with those of other actins revealed that *BbNA1* encodes an actin that is neither a cytoplasmic type nor a skeletal type, and this gene was therefore designated *BbNA1* (notochord actin of *B. belcheri*) (Suzuki and Satoh 2000).

A weak in situ hybridization signal for *BbNA1* was observed from early neurulae (the N1 stage; Hirakow and Kajita 1994), but the transcript was detected only in somites, not in the notochord (Fig. 1A). At middle neurulae (the N2 stage), the signal of *BbNA1* in somites became stronger, whereas its expression in the notochord was still undetectable (Fig. 1B). In early larva stage (about 24 h after fertilization), the *BbNA1* transcript became evident in the notochord, excluding the anterior-most region (Fig. 1, C and Y), whereas the signal in somites became obscure (Fig. 1C). In 48-h larvae, expression of *BbNA1* was no longer detectable (Fig. 1D).

Amphi-Trop I and Amphi-TPmyosin

Both troponin and tropomyosin are actin-binding proteins, and interaction between these components is essential for muscle contraction (da Silva and Reinach 1991; Farah and Reinach 1995; Tobacman 1996; Gordon et al. 2000). Troponin is composed of three interacting subunits: troponin C (which binds Ca^{2+}), troponin I (which binds to actin and inhibits actomyosin ATPase in a calcium-insensitive manner), and troponin T (which links the troponin complex to tropomyosin). The binding of calcium to troponin C induces a conformational change, modifies the tropomyosin position on the actin filament, allows interaction of actomyosin ATPase with myosin, and consequently initiates muscle contraction.

In accordance with probable closeness of their biochemical functions, most of the expression domains of *Amphi-Trop I* and *Amphi-TPmyosin* were overlapping, and their expression patterns resembled each other. *Amphi-Trop I* transcripts were evident in the somites of the N1-stage neurulae, and a weaker signal was also seen in the newly segmented somites and unsegmented somitic mesoderm (Fig. 1E). During the period when somites were progressively added posteriorly, strongly labeled cells were restricted to the somites (Fig. 1F), and this expression pattern was maintained until the early larva stage (Fig. 1G). In 48-h larvae, the expression in somites was retained only in the anterior region (Fig. 1H). In the notochord, a weak signal relative to that of the somites was seen from the N1 stage to the early larva stage (Fig. 1, E–G).

We were first able to detect the expression of the *Amphi-TPmyosin* at the N1 stage, and the signal first became prominent in the segmented somites, whereas a weak signal was also seen in the unsegmented somitic mesoderm and newly segmented somites (Fig. 1, I and J). Compared with the *Amphi-Trop I* expression, the expression of *Amphi-TPmyosin* was appeared to be down-regulated earlier in the somites (Fig. 1, I–K). Similarly to the *Amphi-Trop I*, the *Amphi-TPmyosin* signal in the notochord was very weak relative to that in the somites throughout development (Fig. 1, I–L). Noticeably, a relatively strong signal was retained at the posterior-most tip of the notochord and in the anterior somitic mesoderm in 48-h larvae (Fig. 1L), and this pattern was similar to that of *Amphi-Trop I* expression (Fig. 1H).

Amphi-MHC2 (myosin heavy chain), Amphi-nMRLC (notochord-specific myosin regulatory light chain), and Amphi-nTitin/MLCK (notochord-specific titin/myosin light chain kinase)

Myosin constitutes a large superfamily of proteins that share an evolutionarily conserved domain that interacts with actin.

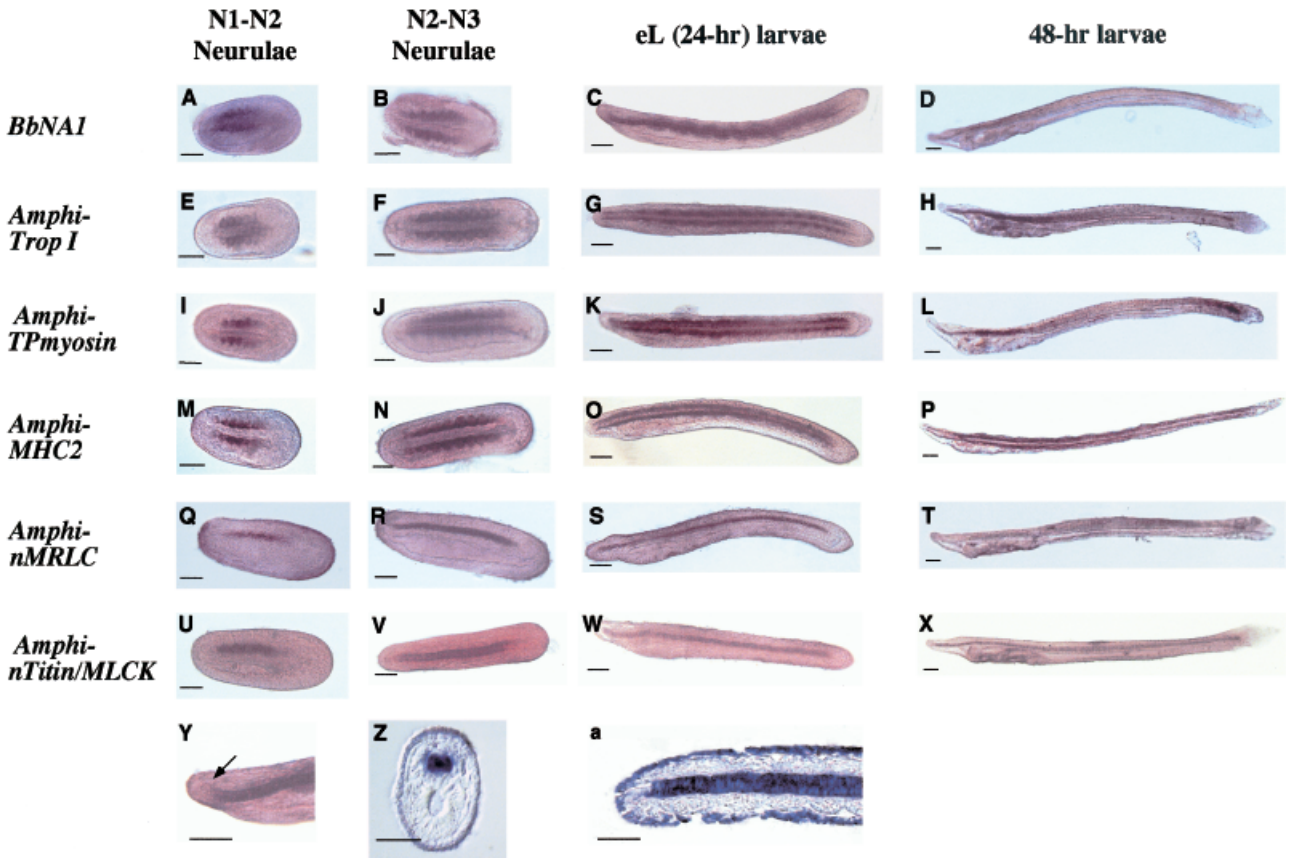

Fig. 1. (A–a) Patterns of gene expression in central notochord cells during neurulation and early larval (eL) development. (A–C), (E–G), (I–K), (M–P) and (V) Dorsal view. (D), (H), (L), (Q–U), and (W–Y) Lateral view. (Z) Transverse view. (a) Horizontal view. (A–D) *BbNa1*. Dorsal view of the N1-stage (A) and N2-stage (B) neurulae showed that distinct *BbNA1* expression was detected only in somites. The expression in the notochord became evident at the eL stage (C), and the signals were no longer detectable in the 48-h larvae (D). (E–H) *Amphi-Trop I*. A distinct signal for *Amphi-Trop I* was seen in the somites of the N1-stage neurula (E) and relatively weaker signal was also seen in the newly segmented somites, unsegmented somitic mesoderm, and notochord during neurulation (F). (G) Dorsal view of the eL stage, showing that this expression pattern was maintained. (H) Lateral view of the 48-h larva. The signal in the somites was seen only in the anterior region and that in the notochord was detected only in the posterior tip. (I–L) *Amphi-TPmyosin*. (I) Dorsal view of the N1-stage neurula. A distinct signal for *Amphi-TPmyosin* expression was first able to detect in the somites. (J) Dorsal view of the N2-stage neurula, showing relatively weaker signal in the newly segmented somites, unsegmented somitic mesoderm, and notochord. This expression pattern was maintained in the eL stage (K). (L) Lateral view of the 48-h larva. The signal was retained in the anterior somites and the posterior tip of the notochord. (M–P) *Amphi-MHC2*. A prominent signal for *Amphi-MHC2* was detected in the somites at the N1-stage (M) and the N2-stage (N) neurulae. The expression in the somites was still prominent in the eL stage (O) and 48-h larvae (P), whereas that in the notochord was considerably lower than that of other genes examined in this study. The signal in the notochord was seen from the N2 stage (N), and this weak signal was retained until 48-h larva (P). (Q–T) *Amphi-nMRLC*. (Q) Lateral view of the N1-stage neurula. The *Amphi-nMRLC* was expressed predominantly in the notochord. The signal was also detected only in the notochord of the N2-stage neurula (R) and eL stage (S). (T) Lateral view of the 48-h larva, showing very weak expression in the notochord. (U–X) *Amphi-nTitin/MLCK*. (U) Lateral view of the N1-stage neurula, showing that the expression was detected only in the notochord. From the N1 stage onward, the signal was seen only in the notochord of the N2-stage neurula (V), the eL stage (W), and 48-h larvae (X). (Y) Magnifications of C showing the loss of *BbNA1* expression in the anterior tip of the notochord (arrow). (Z) A transverse section of the N2-stage neurula showing the *Amphi-nMRLC* expression in the two rows of the central notochord cell precursors. (a) A horizontal section of the eL stage, showing the *Amphi-nMRLC* expression in the two rows of the central notochord cell columns. Note anterior region of the columns are thinner than those of the posterior region, suggesting that an intercalation of the central notochord cell columns may proceed from anterior to posterior manner. Anterior is to the left, except for Z. Scale bars, 50 μm.

Members of the Titin/MLCK family are calcium-binding protein-dependent protein kinases that phosphorylate the myosin regulatory light chain. This phosphorylation subsequently increases the actin-activated myosin ATPase activity and thus has a crucial role in muscle contraction. Previous studies have also demonstrated that Titin/MLCK binds

tightly to actomyosin-containing filaments via immunoglobulin like motifs located at the N- and C-terminus, respectively, and plays an important role in sarcomere assembly (Higgins et al. 1994; Maruyama 1997). The amino acid sequence deduced from the *Amphi-nTitin/MLCK* cDNA contains the immunoglobulin-like motif at the C-terminus, as does *Amphi-CAVP* (calcium vector protein) (Takagi and Cox 1990; Urano et al., unpublished data).

The *Amphi-MHC2* signal in the notochord appeared considerably lower than that of *BbNA1* and other myosin-related genes. The signal of *Amphi-MHC2* in the notochord became detectable from the N2 stage (Fig. 1N) and gradually became stronger as development proceeded (Fig. 1, N and O). At the early larva stage, *Amphi-MHC2* was expressed in the central notochord cells (Fig. 1O), and a weak signal was seemed to retain in the notochord of the 48-h larvae (Fig. 1P).

The expression patterns of *Amphi-nMRLC* and *Amphi-nTitin/MLCK* were similar to one another, as were those of *Amphi-Trop I* and *Amphi-TPmyosin*. Unexpectedly, these genes were predominantly expressed in the notochord throughout the developmental stages we observed in this study. This is why we designated these genes as *Amphi-nTitin/MLCK* and *Amphi-nMRLC* (amphioxus notochord-specific *Titin/MLCK* and *MRLC* genes, respectively). In situ hybridization analyses showed that the expressions of *Amphi-nMRLC* and *Amphi-nTitin/MLCK* were initially seen in the notochord of the N1-stage neurula (Fig. 1, Q and U), and thereafter the signal was found in the progressively elongating notochord up to 48-h larvae (Fig. 1, Q–X). The dorsal view of the N2-stage neurula (Fig. 1V) indicates that the signal was intense in the central notochord cells. Moreover, transverse (Fig. 1Z) and horizontal (Fig. 1a) sections make it evident that *Amphi-nMRLC* was expressed in the two rows of the central notochord cell columns.

Because it seemed that the labeled cell columns became aligned in a single file in the early larva stage (Fig. 1a), the two longitudinal cell columns may be intercalated from anterior to posterior manner during neurulation and early larval development. The columnar cell organization of the developing notochord are discussed later together with the development of other cell types.

Amphi-MLP/CRP3 (muscle LIM protein)

Next, we characterized the expression of muscle-related genes in dorsally and ventrally located cells within the notochord. In this study, we found that *Amphi-MLP/CRP3* and *Amphi-nCalponin* were expressed in the noncentral notochord cells.

The LIM domain is a zinc-finger structure that is present in many kinds of proteins and has been shown to be involved in protein–protein interaction (Dawid et al. 1998; Bach 2000). Among the LIM proteins, CRP1, CRP2, CRP3/MLP, and cystein-rich intestinal protein (CRIP) are classified into the group 2 or CRP subfamily by their LIM domain, which is linked to an additional unique conserved motif (Taira et al. 1994; Perez-Alvadoro et al. 1996; Dawid et al. 1998). CRP proteins interact with actin filaments and myofibrils of the Z lines and show distinct expression profiles among a wide variety of organisms, suggesting that they execute in many kinds of myogenesis with conserved biochemical properties (Arber et al. 1997; Louis et al. 1997). In addition to the role of these proteins in the cytoplasm, nuclear-localized MLP is associated with products of bHLH transcription factor genes such as *MyoD*, *MRF4*, and *Myogenin* and acts cooperatively to promote myogenesis in *Drosophila* (Kong et al. 1997). Furthermore, *Drosophila* dMEF2 is able to induce *Mlp* gene expression by binding to consensus sites within the *Mlp* genomic locus, suggesting that *Mlp* is a direct target of dMEF2 (Stronach et al. 1999).

An alignment of deduced amino acid sequences of the LIM domain of *Amphi-MLP/CRP3* with the sequences of cognates from other organisms revealed a conservation of the LIM domain with those of other MLP/CRP3 subfamily (see Fig. 3A). The expression of *Amphi-MLP/CRP3* was observed from the N2-stage neurulae in a pattern suggesting that *Amphi-MLP/CRP3*-positive cells were lining the entire length of the notochord (Fig. 2A). At the N3 stage, the signal was also seen in cells located transversely in the middle region of the notochord, but in the posterior region, where the notochord was newly separated from the archenteron, the signal was still pronounced in the periphery of the notochord (Fig. 2, B and C). This sequential expression pattern continued until the notochord was completely separated from the archenteron in the 48-h larva (data not shown). Like the expression of the above-described genes, the signal intensity of *Amphi-MLP/CRP3* at the anterior-most tip appeared considerably weaker relative to that in the rest of the notochord (Fig. 2, B and C).

The *Amphi-MLP/CRP3* expression observed at the periphery of the notochord indicates that the gene is expressed first in the noncentral notochord cells and subsequently in the central notochord cells. According to the observations of Flood (1970, 1975) and Stach (1999), dorsally and ventrally located cells are smaller than the central notochord cells and are detected as early as in the neurulae of *B. lanceolatum*. In accordance with these observations, a transverse section of the N2-stage neurulae showed that *Amphi-MLP/CRP3*-positive cells are located dorsally and ventrally within the notochord, and no signals are seen in the central notochord cells (Fig. 2D), suggesting that *Amphi-MLP/CRP3* is expressed only in the dorsally and ventrally located cells at the N2 stage. Likewise, a transverse section of the early larva stage showed the expression of *Amphi-MLP/CRP3* in the central cells as well as dorsally and ventrally located cells (Fig. 2E). It seemed that the peripheral expression of *Amphi-MLP/CRP3* disclosed

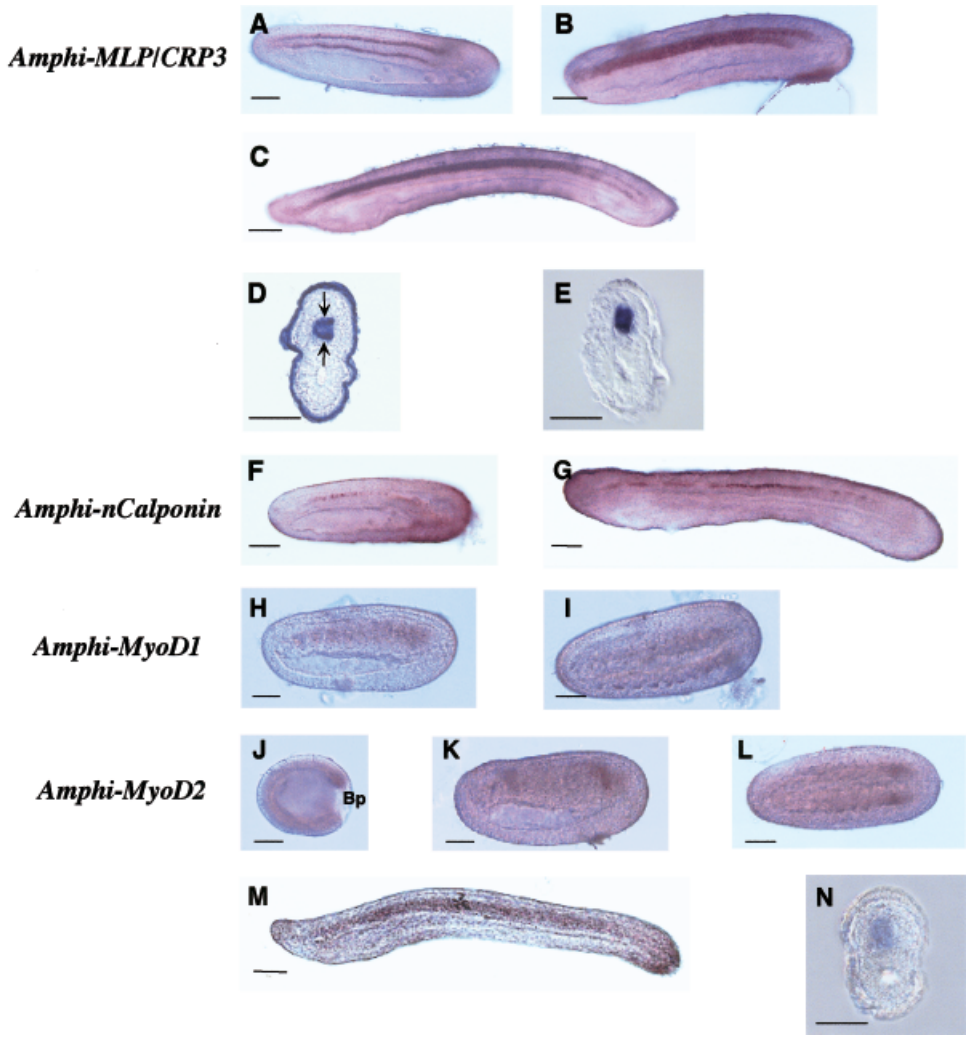

Fig. 2. (A–G) Patterns of gene expression in noncentral notochord cells during neurulation and early larval (eL) development. (A–E) *Amphi-MLP/CRP3*. (A) Lateral view of the late N2-stage neurulae. A signal for *Amphi-MLP/CRP3* was detected as if *Amphi-MLP/CRP3*-positive cells were lining along the entire length of the notochord, suggesting the expression in noncentral notochord cells (dorsally located cells and ventrally located cells). (B) Lateral view of the N3-stage neurula, showing the expression in the central notochord cells, dorsally located cells, and ventrally located cells. Note that the expression in the posterior region of the notochord was still pronounced only in the dorsally located cells and ventrally located cells. This expression pattern was also seen in the eL stage (C) and continued until 48-h larva (data not shown). (D) A transverse section of the N2-stage neurula makes it evident that the *Amphi-MLP/CRP3* was strongly expressed in the dorsally located cells and ventrally located cells (arrows). (E) A transverse section of the N3-stage neurula. In addition to the dorsally located cells and ventrally located cells, the signal was also became detectable in the central cells. (F and G) *Amphi-nCalponin*. (F) Lateral view of the N2-stage neurula. *Amphi-nCalponin*-positive cells were scattered over the dorsal-most region of the notochord, suggesting that the *Amphi-nCalponin* was expressed in the dorsally located cells. (G) Lateral view of the late N3-stage neurula, showing the expression was still maintained in the dorsally located cells. (H–N) Spatial expression of *MyoD*-related genes during amphioxus development. (H and I) *Amphi-MyoD1*. Lateral view (H) and dorsal view (I) of the N2-stage neurulae. Relatively strong signal was seen in the posterior-most unsegmented somitic mesoderm, whereas the signal in the somites became gradually weak as newly segmented somite was added posteriorly. (J–N) Spatial expression pattern of *Amphi-MyoD2*. (J) Late gastrula, showing the *Amphi-MyoD2* expression in presumptive somitic mesoderm. Bp, blastopore. Lateral view (K) and dorsal view (L) of the N2-stage neurulae, showing the *Amphi-MyoD2* expression in developing somites. As in the *Amphi-MyoD1*, an intense signal was seen in the posterior-most unsegmented mesoderm. (M) Lateral view of the eL stage, showing the expression of *Amphi-MyoD2* in the notochord. (N) A transverse section of the eL stage showing the expression in the notochord including the central cells. Scale bars, 50 μm. Anterior is to the left, except for D, E, and N.

the columnar cell organization of the dorsally and ventrally located cells, which appeared to be organized as single rows along the entire length of the notochord. In short, the amphioxus notochord in early development may consist of four rows of longitudinal cell columns, namely, a dorsally and a ventrally located cell column, and two rows of central notochord cell columns.

Amphi-nCalponin

Calponin is a calcium-binding protein that interacts with actin, myosin, tropomyosin, and other calcium-binding proteins. The binding of calponin to actin affects the structure of actin bundles and has a role in the cytoskeletal organization. The interaction between actin and calponin also inhibits actomyosin ATPase and consequently regulates muscle contraction (Winder and Walsh 1993; EL-Mezgueldi 1996; Gusev 2001). In addition to the presence and role of calponin in muscular tissue, calponin immunoreactivity and transcripts are also observed in the postsynaptic side of synapses in the mammalian brain (Represa et al. 1995; Ferhat et al. 1996; Agassandian et al. 2000).

We observed here that the calponin-related gene was expressed exclusively in the notochord, and even there the expression was weak (Fig. 2, F and G). Because calponin transcripts and immunoreactivity have been observed in many kinds of muscular tissue in a wide variety of organisms, the present observation that the calponin-related gene is expressed specifically in the notochord raises the possibility that other genes closely related to this gene exist in the amphioxus genome. We therefore named this gene *Amphi-nCalponin* (amphioxus notochord-specific calponin).

The expression signal of this gene was detectable only from middle neurulae (at the N2 stage) to late neurulae (at the N3 stage). The positive cells were small and scattered over the dorsal-most region of the developing notochord (Fig. 2, F and G), suggesting that the transcript is present in the dorsally located cells. From the early larva stage onward, no obvious signals were seen (data not shown).

Expression of *MyoD* genes in *B. belcheri*

Characterization of the genes

In many organisms, the *MyoD* family transcription factor plays a pivotal role in the determination of the myogenic lineage and in muscle differentiation through activation of the transcription of muscle-related genes by binding to a consensus site, called the E-box (Arnold and Winter 1998; Mckinsey et al. 2001; Rescan 2001).

In amphioxus, *MyoD* has been partially characterized from *B. floridae* as two independent cDNA fragments designated *BMD1* and *BMD2* (Araki et al. 1996). To investigate the spatiotemporal expression pattern of *MyoD* in *B. belcheri*, we isolated full-length cDNAs by screening a *B. belcheri* somite cDNA library using *BMD1* and *BMD2* cDNA fragments as templates for the probe. As a result, several cDNA clones whose deduced amino acid sequences were highly similar to those of other MRFs were isolated, and we renamed the corresponding genes *Amphi-MyoD1* and *Amphi-MyoD2*, respectively. Comparison of the deduced amino acid sequences of *Amphi-MyoD1* and *Amphi-MyoD2* with MRFs of other organisms showed sequence conservation of the bHLH region (DNA binding domain) (Fig. 3B). To infer the phylogenetic relationships among *Amphi-MyoD1*, *Amphi-MyoD2*, and other MRF genes, we constructed a phylogenetic tree by a neighbor-joining method (Saitou and Nei 1987) based on the alignment of 110 amino acid residues, including the bHLH region. As seen in Fig. 4, the tree shows that *Amphi-MyoD1* and *Amphi-MyoD2* constitute an independent clade with a high bootstrap value (95%). Furthermore, the branching pattern of the tree strongly suggests that the vertebrate MRF appeared only after amphioxus had diverged in the lineage leading to the vertebrates, whereas *Amphi-MyoD1* and *Amphi-MyoD2* might have been produced by a gene duplication that occurred in the amphioxus lineage. Comparison between the amino acid sequences deduced for *Amphi-MyoD1* and *Amphi-MyoD2* showed that several distinctive differences were evident even in the bHLH region (Fig. 3B). At the nucleotide level, differences were also evident, particularly in the 5′ and 3′ untranslated regions (data not shown).

Expression of Amphi-MyoD1 and Amphi-MyoD2

The expressions of the *Amphi-MyoD1* and *Amphi-MyoD2* were distinct from one another, both temporally and spatially. The *Amphi-MyoD1* transcript was detected in developing somites of the N2-stage embryo, in which several somites have already been pinched off from the archenteron. The signal intensity in the unsegmented somitic mesoderm was relatively strong, whereas that in the segmented somites gradually became weak as new unsegmented somitic mesoderm was added posteriorly (Fig. 2, H and I).

Amphi-MyoD2 expression first became prominent in presumptive somitic mesoderm of the late gastrula (Fig. 2J). During somatogenesis, a strong signal was seen in the posterior-most unsegmented somitic mesoderm, similarly to the signal of *Amphi-MyoD1* (Fig. 2, K and L). As development proceeded, the signal gradually became weaker until no signal was detected in the somitic mesoderm at the early larva stage. Instead, a distinct signal became evident in the notochord from the late N3 stage (Fig. 2M), and a transverse section (Fig. 2N) makes it evident that *Amphi-MyoD2* is expressed in the central cells of the early larva stage. During larval development, the gene expression persisted in the notochord until early larva stage. In the 48-h larva, the *Amphi-MyoD2* expression was barely detectable anywhere (data not shown).

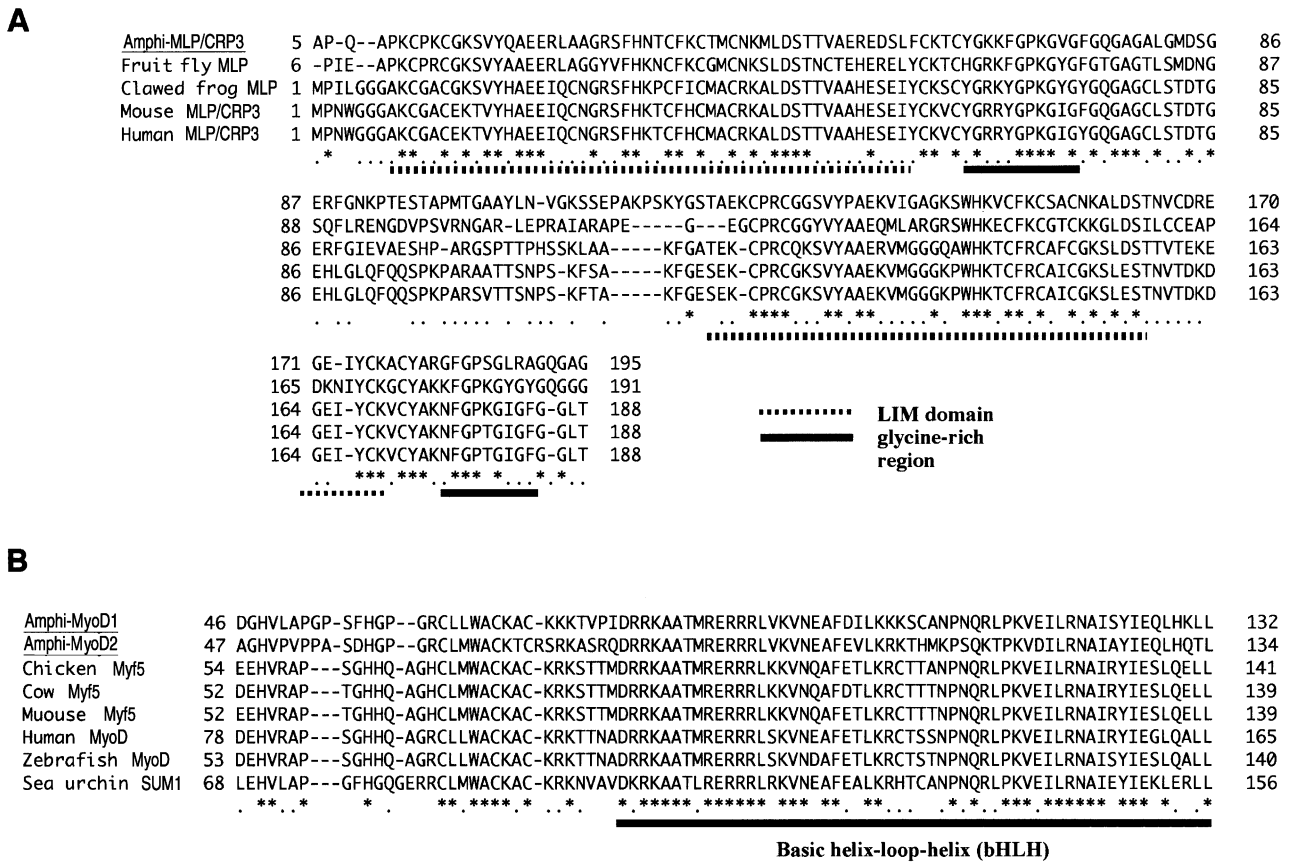

Fig. 3. (A) Comparison of amino acid sequences of MLP/CRP3 class gene products between amphioxus and other organisms. Stars and single dots indicate identical amino acid residues and conserved amino acid substitutions, respectively. This alignment shows that the amphioxus gene product contains both the LIM domains (broken underline) and glycine-rich domains (underline). (B) Alignment of amino acid sequences of Amphi-MyoD1 and Amphi-MyoD2 with those of MRFs of other organisms. Stars and single dots indicate identical amino acid residues and conserved amino acid substitutions, respectively. Amphi-MyoD1 and Amphi-MyoD2 have conserved bHLH regions, although there are several differences between Amphi-MyoD1 and Amphi-MyoD2 both inside and outside of the bHLH domain.

A columnar cell organization of the developing amphioxus notochord

We presented here the developmental expression pattern of eight different muscle-related genes and two *MyoD* genes. The muscle-related genes were originally isolated by EST analysis targeting messenger RNAs of the adult notochord (Suzuki and Satoh 2000). Anatomical and histological details of the adult amphioxus notochord have been studied, but details of its embryonic development are largely unknown. The 10 genes studied here showed various expression patterns, and some genes appeared to be expressed in a particular type of cell, which provided us with an opportunity to observe the cell organization of the developing notochord in whole-mounted embryos and larvae and revealed a columnar cell organization of the notochord. Namely, precursors of the central notochord cells were aligned first as two rows, and these two rows of cell columns were lain between dorsally and ventrally located cell columns. Subsequently, the two central columns became intercalated to form a single column. Because four types of presumptive notochord cells are distinguishable as early as from the gastrula stage (Conklin 1932), these four types of cells are likely to coincide with those that constitute the four rows of cell columns demonstrated by the present study. Moreover, our observation that the *Amphi-MLP/CRP3*-positive dorsally and ventrally located cells were detectable immediately after they were separated from the archenteron implies that the fate of these cells is specified as soon as, or before, they separate from the archenteron.

Characterization of notochord cells based on their gene expression

As summarized in Fig. 5A, three kinds of notochord cell identified in the present study express specific set of genes that may reflect their function. In the central notochord cells, many muscle-related genes such as *BbNA1*, *Amphi-Trop I*, *Amphi-TPmyosin*, *Amphi-MHC2*, *Amphi-nTitin*/

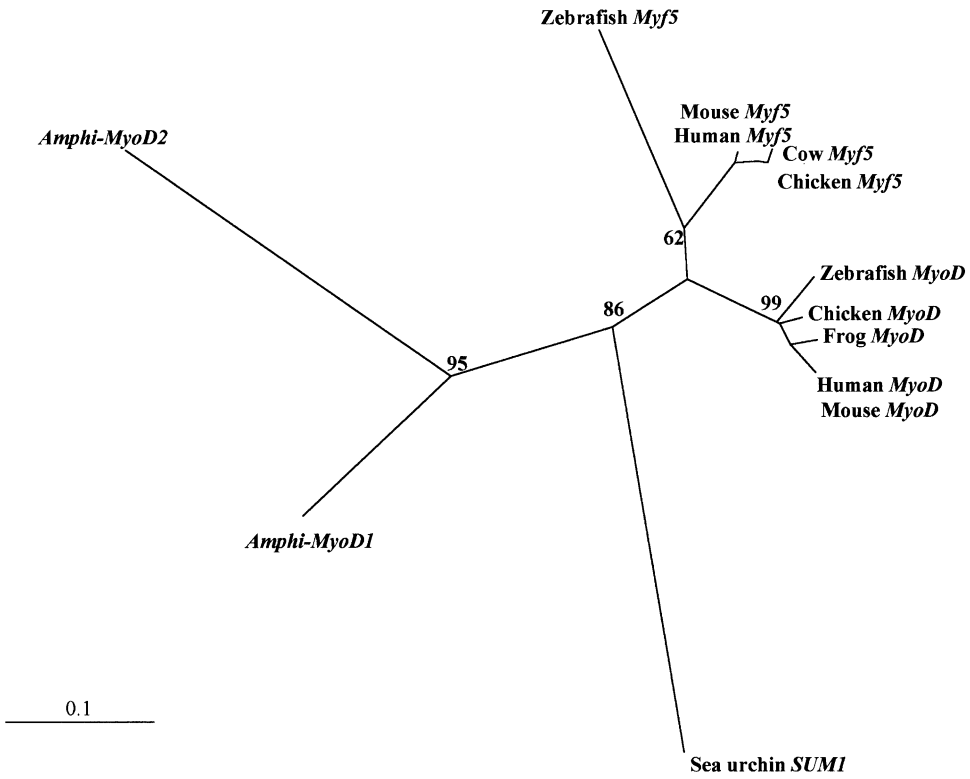

Fig. 4. A molecular phylogenetic tree constructed by the neighbor-joining method (Saitou and Nei 1987) using aligned sequences of 110 amino acid residues, including the bHLH domain. The branch length is proportional to the number of amino acid substitutions, and the scale bar indicates 0.1 amino acid substitutions per position. The numerals at each node indicate the percent confidence value based on the 100 replicate bootstrap resamplings of the alignment data. The tree shows that *Amphi-MyoD1* and *Amphi-MyoD2* constitute an independent clade and that they might have appeared by gene duplication in the amphioxus lineage. The branching pattern of the tree also indicates that the vertebrate MRF genes were appeared after the emergence of the vertebrates.

MLCK, and *Amphi-nMRLC* were expressed. Among them, *BbNA1*, *Amphi-Trop I*, and *Amphi-TPmyosin* are thought to have a role in the formation and function of the contractile myofibrils. Because the expression of *BfMA1* (muscle-type actin gene of *B. floridae*) is detected only in the somatic muscle and that of *BfCA1* (cytoplasmic-type actin gene of *B. floridae*) is seen in most of the mesendodermal derivatives, including the notochord (Kusakabe et al. 1999), *BbNA1* products may constitute a major component of the myofibrils in the notochord (Suzuki and Satoh 2000). In addition, the existence of transcripts encoding putative actin-binding proteins such as *Amphi-Trop I* and *Amphi-TPmyosin* suggests that the contraction of the notochord muscle may be triggered by conformational changes of these actin-binding proteins in a calcium-dependent manner. However, this notion does not necessarily mean that myosin-linked regulation is not involved in the contraction of the notochord muscle. In any case, the contraction of amphioxus notochord muscles may be entirely due to motor commands via motoneurons. In fact, innervation of the notochord occurs at bud-like projections of notochordal tissue, and the cellular processes of the dorsal Müller's cells enter the projection (Welsch 1968; Flood 1975). We therefore emphasize that the *Amphi-nCalponin* transcript is detected in the dorsally located cells. Because calponin is involved in the maintenance of synaptic plasticity by remodeling actin filaments in the postsynaptic side of the mammalian brain (Agassandian et al. 2000), the product of *Amphi-nCalponin* is likely to regulate the formation of the cellular processes that extend toward the nerve cord by modifying the cytoskeletal organization. Assuming that the dorsally located cells receive neurotransmitters from the motoneurons, they may produce changes in the membrane permeability and act as a relay station for excitation impulses to the notochord muscle, as proposed by Welsch (1968) and Webb (1973).

Except for *Amphi-MLP/CRP3*, none of the genes studied here was expressed in the ventrally located cells, so the present results give no important insights into their possible functions. Although they share some molecular characteristics with the dorsally located cells and their developmental pattern proceeded similarly to that of the dorsal ones, the ventrally located cells of the adult differ in

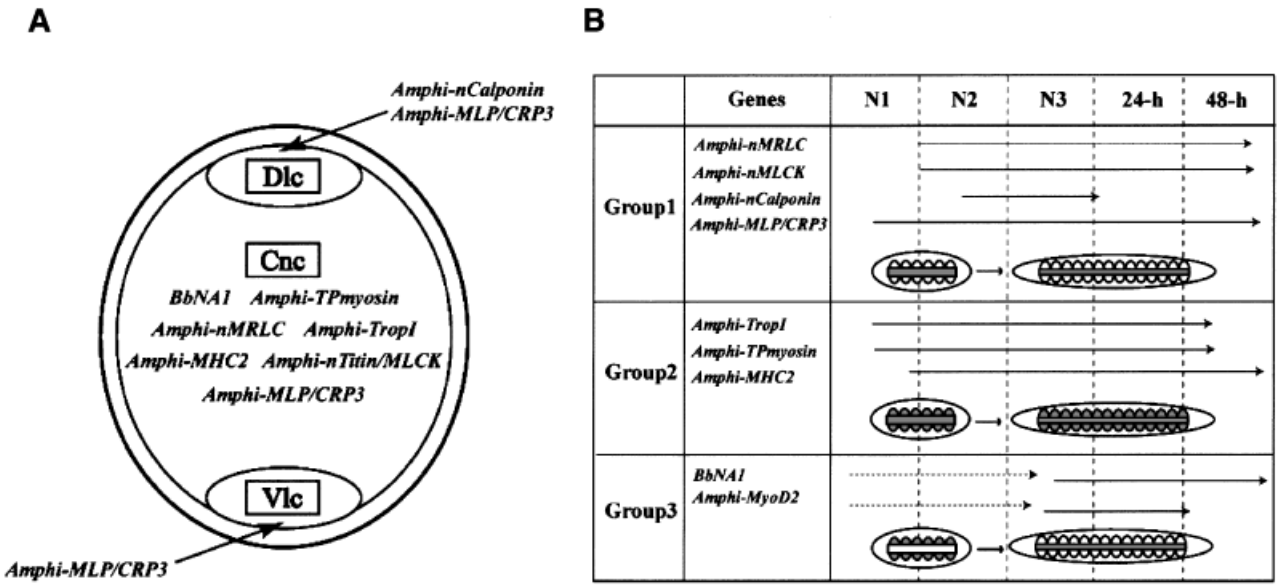

Fig. 5. (A) Diagrammatic representation of three notochord cells and expression patterns of eight muscle-related genes in relation to their inferred functions. Cnc, central notochord cell; Dlc, dorsally located cell; Vlc, ventrally located cell. (B) Schematic representation of expression profiles of eight muscle-related genes and *MyoD*-related gene. Arrows indicate gene expression in the notochord, whereas broken arrows indicate gene expression in the somites. Note that *Amphi-MyoD2* expression in the notochord is initiated after all group 1 and 2 genes are expressed (see text for details).

appearance from the dorsal ones (Flood 1975). It is therefore likely that the ventrally located cells have a specialized function of their own.

Possible involvement of the *MyoD*-related gene in the differentiation of the notochord muscle

As summarized in Fig. 5B, the 10 different genes examined here can be assigned to three groups based on their spatiotemporal expression patterns. Group 1 genes are expressed predominantly in the notochord throughout development and include *Amphi-nMRLC*, *Amphi-nTitin/MLCK*, *Amphi-nCalponin*, and *Amphi-MLP/CRP3*. Group 2 genes include *Amphi-MHC2*, *Amphi-Trop I*, and *Amphi-TPmyosin* and are simultaneously expressed in both the somites and the notochord. The Group 3 genes are *BbNA1* and *Amphi-MyoD2* and are first expressed in the somites; later, the somite expression disappears and the expression in the notochord becomes evident. Obvious expression of *Amphi-MyoD2*, but not *Amphi-MyoD1*, is detectable in the notochord. The fact that one of the *MyoD* genes (*Amphi-MyoD2*) is expressed in the developing notochord implies that the expression of the above-described muscle-related genes may be controlled by *Amphi-MyoD2*. Comparison of the temporal expression profiles (Fig. 5B), however, shows that all group 1 and 2 genes are expressed earlier than the *Amphi-MyoD2* gene, suggesting that the initiation of their expression might not be under the regulation of *Amphi-MyoD2*. Even so, the coexistence of *Amphi-MLP/CRP3* transcripts and *Amphi-MyoD2* transcripts in the central notochord cells in the neurulae implies that Amphi-MyoD2 is likely to form an MLP/CRP3–Amphi-MyoD2-E protein complex to participate in the myogenic program, as occurs in other organisms (Kong et al. 1997). Indeed, the initiation of *BbNA1* expression in the central notochord cells is lagged behind that of *Amphi-MyoD2* and *Amphi-MLP/CRP3* expression (Fig. 5B). Therefore, *Amphi-MyoD2* may be seen as a candidate regulator relevant to the formation of the myofibrils in the central notochord cells. In fact, the *Amphi-MyoD1* expression in the presomitic and segmented mesoderm is reminiscent of the pattern in vertebrates, whereas *Amphi-MyoD2* is expressed in the notochord after transcripts of *Amphi-MyoD1* and *Amphi-MyoD2* have disappeared from the somitic mesoderm.

Because two *MyoD*-related genes are likely to have become duplicated in the amphioxus lineage (Fig. 4) and because a gene duplication is thought to produce a redundant gene that may be able to diverge and subsequently be coopted for new functions (Ohno 1970; Holland et al. 1994; Holland 1998), our observations may be interpreted to mean that *Amphi-MyoD1* has taken over the ancestral role in the differentiation of the somitic muscle, whereas *Amphi-MyoD2* has acquired a new expression domain in the notochord. To support this notion, the amphioxus cognate of *Pax3/7* family gene (*Amphi-Pax3/7*) is also expressed in the developing notochord (Holland et al. 1999), whereas there is no comparable expression in vertebrates (Epstein 2000) or urochordate

ascidians (Wada et al. 1996). Interestingly, *Pax3* is a key regulator of vertebrate myogenesis and is required for the regulation of *MyoD* expression (Tajbakhsh et al. 1997; Maroto et al. 1997; Heanue et al. 1999).

Identification of the *cis*-regulatory elements of the *MyoD*-related genes and other muscle-related genes may give insights into the genetic program for the differentiation of amphioxus notochord cells and may also provide important clues about the role of gene duplication in the appearance of novel cell types.

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