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Sequence and Expression of Amphioxus Alkali Myosin Light Chain (*AmphiMLC-alk*) Throughout Development: Implications for Vertebrate Myogenesis

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The lower chordate amphioxus, widely considered the closest living invertebrate relative of the vertebrates, is a key organism for understanding the relationship between gene duplications and evolution of the complex vertebrate body plan. In tetrapod vertebrates, the alkali myosin light chain genes (*MLC-alk*), which code for proteins associated with the globular head of the myosin heavy chain, constitute a large family with stage-, tissue-, and fiber-type-specific expression of different isoforms thought to have arisen by duplication of a single ancestral gene. In protostome invertebrates, e.g., arthropods, molluscs, and nematodes, only one *MLC-alk* gene has been found, but the number of such genes in deuterostome invertebrates and lower vertebrates is unknown. The present report, describing the sequence and expression throughout development of the amphioxus gene for alkali myosin light chain (*AmphiMLC-alk*), thus fills a major gap in understanding the relation between gene duplication and increasing diversity of muscle-cell types. A full-length clone (1 kb) of *AmphiMLC-alk* was isolated from a larval amphioxus cDNA library. It coded for a 149-amino-acid protein most closely related to the vertebrate embryonic form of *MLC-alk*. Southern blot analysis revealed only one copy of *AmphiMLC-alk* and suggested that it is the only *MLC-alk* gene in amphioxus. Northern blot analysis indicated that this gene produces only one transcript, which is expressed at all stages of development and in adults. *In situ* hybridizations showed expression initially in the myotomes of somites 2–5 of neurula embryos and soon thereafter in the myotomes of somite 1 and of newly forming somites progressively added posteriorly. Myotomal expression continues throughout larval development and into the adult stage as the myotomal cells differentiate into striated, mononucleate muscle cells—unlike vertebrate striated muscle cells, those of amphioxus never become multinucleate. In late larvae and adults myotomal expression of *AmphiMLC-alk* is localized along the medial edge of the myotome and at the ends of the cells. This is the first demonstration of intracellular localization of *MLC* transcripts in muscle cells of any animal. Expression of *AmphiMLC-alk* was also detected in smooth muscles as well as in striated muscles not derived from the myotome. These expression data are consistent with the Southern blot analysis in suggesting that there is only one *MLC-alk* gene in amphioxus. Thus, duplication of an ancestral vertebrate *MLC-alk* gene probably occurred after the vertebrate and amphioxus lineages split. We conclude that development of a segmented axial musculature preceded the evolution of multiple *MLC-alk* isoforms, which evidently arose about the time of multinucleation. Since myogenesis in amphioxus is similar to but far simpler than myogenesis in vertebrates at both the structural and gene levels, an understanding of myogenesis in amphioxus can give insights into both the evolutionary history and the detailed mechanisms of vertebrate myogenesis.

INTRODUCTION

Vertebrates are characterized by an extensive duplication of genes thought to be related to an increase in complexity of the vertebrate body plan (Holland, 1992; Holland *et al.*, 1994b). The invertebrate chordate amphioxus, probably the nearest living relative of the vertebrates, has a body plan similar to, but far simpler than, that of vertebrates. For

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example, it has a segmented body musculature and a dorsal nerve cord but lacks limbs and migratory cells such as neural crest cells. Studies of gene families in amphioxus are few, but at least for the insulin, *Hox*, and *Wnt* families there are far fewer gene duplications than in vertebrates (Chan *et al.*, 1990; Holland *et al.*, 1994a,b). Thus developmental genetic studies in amphioxus may provide insights into the evolution and mechanisms of complex developmental processes in vertebrates.

In vertebrates, the best understood differentiative pathway is myogenesis, which has become a model for understanding differentiation of specialized cell types. The emerging picture, however, is complicated both cytologically and genetically. During vertebrate myogenesis, striated muscles derive from the embryonic somites. Each somite becomes compartmentalized into a ventromedial sclerotome and a dorsolateral dermamyotome. After giving off migratory cells which form all limb muscles and some trunk muscles, the dermamyotome divides into a dermatome and a myotome. Myotomal cells (somitic myoblasts) then differentiate into the axial musculature. These myoblasts, initially rounded and mononucleate, elongate until they span the entire length of the myotome. After filaments appearing in the cytoplasm become organized into myofibrils, groups of myoblasts fuse to become multinucleate, striated muscle cells (fibers) (Holtzer *et al.*, 1957; Youn and Malacinski, 1981). Primary fibers form first, later becoming surrounded by smaller secondary fibers. In adults, there are several different fiber types within each muscle.

The increase in cytological complexity during vertebrate myogenesis is paralleled by an increase in molecular complexity. As a muscle develops, several isoforms of contractile proteins are expressed in a developmental series. In general, nonmuscle isoforms and embryonic isoforms appear before the neonatal and adult isoforms. At the 25-somite stage (9.5 days), the myoblasts of the most rostral somites begin to express two myosin heavy chain (*MHC*) genes [embryonic *MHC* (*MHC*$_{emb}$) and ventricular/slow *MHC* (*MHC*β)] and two alkali myosin light chain (*MLC-alk*) genes [embryonic MLC (MLC$_{emb/A}$) and fast skeletal *MLC* (*MLC1*$_f$)] (Lyons *et al.*, 1990). By 10.5 days, the perinatal form *MHC*$_{pn}$ is also expressed (Lyons *et al.*, 1990). As the myotomal cells begin to fuse and become multinucleated, additional isoforms appear. In adults, different isoforms of MHC and MLC-alk characterize the different fiber types within a muscle: MLC1$_f$ and MLC3$_f$ predominate in fast skeletal muscles, while MLC1$_{sa}$ and MLC1$_{sb}$ predominate in slow skeletal muscles (Barton and Buckingham, 1985; Uetsuki *et al.*, 1990). In addition to isoforms in striated muscle, there are also smooth muscle and nonmuscle isoforms of MLC-alk (Barton and Buckingham, 1985; Hailstones and Gunning, 1990).

Although the mechanism of excitation–contraction coupling in amphioxus muscle is similar to that of vertebrate skeletal muscle (Benterbusch *et al.*, 1992), the cytology of myogenesis is much simpler in amphioxus. Amphioxus is a predominantly epithelial animal. The somites arise as

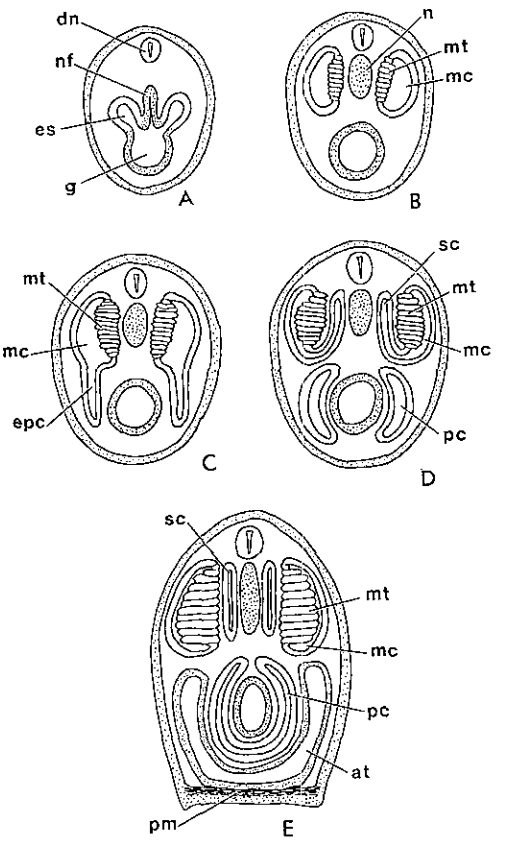


FIG. 1. Diagrammatic cross sections of developmental stages of amphioxus showing major muscles and the relationships between major coeloms. Developmental times given are for *Branchiostoma floridae* raised at 25°C. (A) Early embryo (13 hr). (B) Mid-embryo. (18 hr). (C) Late embryo (26 hr). (D) Larva (2 day). (E) Postmetamorphic juvenile (6 weeks). Abbreviations in alphabetical order are: at, atrial cavity (an ectodermal invagination that is unrelated to the heart); dn, dorsal nerve cord; es, evaginating somite; epc, evaginating perivisceral coelom; g, gut; mc, myocoel; mt, myotome; n, notochord; nf, notochordal fold; pc, perivisceral coelom; pm, pterygeal muscles; sc, sclerocoel.

evaginations from the gut wall that pinch off, resulting in a single-layered epithelium surrounding a cavity, the myocoel. The origin of the somites and their subsequent relations to major coelomic cavities during development are diagrammed in Figs. 1A–1E. The somite does not differentiate into a dermamyotome and no cells migrate away from it. Instead, the large medial compartment of each somite simply is the myotome, which retains its epithelial organization throughout development (Figs. 1B–1E). All of the myotomal cells differentiate in place, becoming the striated muscle cells constituting the segmental muscle blocks running the length of the body. Throughout life, each myotomal muscle cell remains mononucleate, contains but one myofibril, and spans the entire length of the myotome. Thus, even in adult amphioxus, the myotomal muscles are

cytologically comparable to the myotomal muscles in early embryos of vertebrates. According to Flood (1968), adult amphioxus have two types of muscle cells with different fine structure; however, it has not been possible to correlate cell types with fast and slow electrical responses (Guthrie and Banks, 1970). Although no cells wander away from the somite to found muscles elsewhere, several nonmyotomal muscles (both striated and smooth) eventually form in other parts of the body. These include the pterygeal muscles in the floor of the atrial cavity, the notochord, which forms as an endodermal outpocketing that gives rise to cells containing some myofilaments (Flood, 1975), and cells lining some of the coelomic cavities (Holland and Holland, 1990).

The genetics of myogenesis is completely unknown in amphioxus. However, one would expect there to be relatively few myogenic genes. Not only do other amphioxus gene families have few duplications, but invertebrates in general have relatively few isoforms of muscle-specific proteins. Only a single *MLC-alk* gene has been found in each of the following: *Drosophila,* molluscs, and a nematode (Falkenthal *et al.,* 1984; Goodwin *et al.,* 1987; GenBank L03412), and just one isoform of MLC-alk protein was isolated from body wall muscle of an ascidian (Takagi *et al.,* 1986). Thus, it has been suggested that duplication of a single ancestral *MLC-alk* gene occurred at the base of the vertebrates (Barton and Buckingham, 1985). Myogenesis in amphioxus may, therefore, be a system, at once simple and vertebrate-like, that can provide insights into myogenesis in vertebrates in particular and into cell differentiation in general and can also shed light on the larger question of how gene duplications relate to increasing complexity of the vertebrate body plan.

The present paper is concerned with *MLC-alk* (sometimes called *essential MLC*) from amphioxus. *MLC-alk* is one of two types of *myosin light chain,* the other being *regulatory MLC;* both genes code for small proteins associated with the globular head of myosin heavy chain. For studying the myogenic gene cascade in amphioxus, *MLC-alk* is an opportune entry point, because not only are homologs of this gene well-characterized in higher vertebrates and protostomes, but also the expression patterns of several vertebrate isoforms have been determined, and the interactions of *MLC-alk* with upstream regulatory factors have been investigated (Uetsuki *et al.,* 1990; Rosenthal *et al.,* 1990; Fujisawa-Sehara *et al.,* 1992; Grieshammer *et al.,* 1992). Our present purpose is to describe the sequence and expression patterns of the amphioxus gene for *MLC-alk* from early embryology through the adult stage. Our results show that the development of a segmental axial musculature preceded the evolution of multiple MLC-alk isoforms and suggest that duplication of a single ancestral vertebrate *MLC-alk* occurred after the amphioxus and vertebrate lineages split. Thus, both cytologically and genetically, amphioxus myogenesis is similar to, but far simpler than, vertebrate myogenesis, thereby constituting a very favorable system for studying the mechanism of muscle differentiation.

MATERIALS AND METHODS

Amphioxus Collection and Rearing of Larvae

Ripe adults of the Florida amphioxus (*Branchiostoma floridae*) were collected by shovel and sieve from Old Tampa Bay, Florida. Spawning was induced, and the developmental stages were reared at 24°C as described in Holland and Holland (1993).

cDNA Library Construction and Screening

Total RNA was purified from 2- to 4-day (1- to 2-gill slit) larvae of amphioxus by the method of Chomzynski and Sacchi (1987). Poly A(+) RNA was purified from total RNA on Oligo(dT) Dynabeads (Dynal Inc., Great Neck, NY). Double-stranded cDNA was synthesized from mRNA with the Amersham cDNA system Plus kit (US Biochemical-Amersham Arlington Heights, IL) and ligated into the *Eco*RI site of Lambda Zap II (Stratagene Inc., La Jolla, CA) according to the manufacturer's instructions.

DNA was purified from this cDNA library according to Sambrook *et al.* (1989) and used in an anchored polymerase chain reaction to obtain the 739 base pairs (bp) at the 3′-end of an amphioxus *MLC* cDNA of which 203 bp were coding and 536 noncoding. The 3′-end of this partial clone is marked with an arrow in Fig. 2. Primers used were the vector-specific SK primer and a gene-specific primer (5′ GAACTCCTTTTTCAGGCGCTGGAGTTGTTCG 3′) initially designed as a reverse primer to amplify the 5′-end of the *engrailed* gene. The DNA obtained was cloned into the pCR-Script SK(+) plasmid (Stratagene Inc.) and used to probe the cDNA library. Out of 250,000 clones screened, approximately 300 hybridized with the probe. The inserts of 7 clones were excised from the plasmids with *Eco*RI, and the sizes of the inserts were determined by agarose gel electrophoresis.

Southern Blot Analysis

Genomic DNA for Southern blotting was purified by extraction of fresh amphioxus adults with guanidinium isothiocyanate and centrifugation at 35,000*g* (SW 41 rotor) on a 5.7 *M* CsCl cushion (Holland *et al.,* 1995). Ten-microgram samples of DNA were digested with restriction enzymes that did not cut the cDNA and subjected to electrophoresis on an 0.7% agarose gel in 1× TAE buffer at 1 V/cm for 24 hr. The gel was blotted by the methods in Sambrook *et al.* (1989) onto Hybond-N^+ transfer membrane (US Biochemical-Amersham). Stripping of probe for rehybridization was by the manufacturer's instructions. High-stringency hybridization was in 6× SSC, 10× Denhardt's, 0.1% SDS, 100 μg/ml tRNA at 65°C with 1×10^6 cpm/ml probe (specific activity 1×10^9 cpm/μg) labeled with ^{32}P by random priming (Feinberg and Vogelstein, 1983). The probe, consisting of the 739 3′-most bp of the *MLC-alk* cDNA, was the same one used to screen the library. Washes were in 1× SSC,

0.1% SDS, 2 × 20 min at 65°C and 0.1× SSC, 0.2% SDS for 5 min at 65°C. For low-stringency hybridization, the probe included the 5′-most 725 bp, which spanned the entire coding region. Hybridization was as above with the temperature lowered to 55°C. Low-stringency washes were in 1× SSC, 0.1% SDS at 50°C.

Northern Blot Analysis

RNA for Northern blot analysis was purified from larval and adult amphioxus by the method of Chomczynski and Sacchi (1987). Ten micrograms of total RNA was subjected to electrophoresis on a 1.5% agarose gel and blotted onto a Hybond-N$^+$ (US Biochemical-Amersham) according to methods in Sambrook *et al.* (1989). Hybridization conditions were as above for the high-stringency Southern blot.

In Situ Hybridization

Fixation of larvae and whole-mount *in situ* hybridization were by the methods in Holland *et al.* (1992, 1995). The clone for transcription of the antisense riboprobe was the same as for library screening. After *in situ* hybridization, the whole mounts were photographed and then counterstained pink overnight in 1% Ponceau S (CI 27195) in 1% acetic acid, dehydrated in an ethanol series, embedded in Spurr's resin, and sectioned with glass knives; 3.5-μm sections were mounted in immersion oil.

RESULTS

Cloning and Sequence Analysis of MLC-alk from Amphioxus

Screening of the cDNA library from larval amphioxus with a 739-bp partial clone of *MLC-alk* from amphioxus yielded approximately 300 clones hybridizing with varying intensities. Seven of the more strongly hybridizing clones were selected at random, and their insert sizes were analyzed by agarose gel electrophoresis. Two of these clones yielded two bands apiece (555 and 450 bp), and 5 each yielded a single band (1000 bp). To distinguish whether all of these clones represented the same cDNA, we sequenced about 70% of each one. Special attention was given to the 5′-ends of the clones, because vertebrate $MLC1_f$ and $MLC3_f$, being derived from the same gene by differential mRNA-splicing, differ only in that region. Except for a small amount of polymorphism (less than one difference per 200 bp), all of the clones were the same, and we will refer to this single gene as *AmphiMLC-alk* hereafter in this paper. Because of the polymorphism of *AmphiMLC-alk*, there was an *Eco*RI site in the 3′ untranslated region (UTR) of 2 of the 7 clones, which was responsible for the difference in restriction patterns. We chose 2 clones, 1 with each type of restriction pattern, and sequenced both strands completely. Aside from some differences in the third codon position which did not change any amino acids and a few differences in the 3′-UTR, all base sequences were identical.

The base sequence and the deduced amino acid sequence for the clone without the *Eco*RI site are shown in Fig. 2. The deduced 149 amino acids are 33–52% identical to the sequences in a variety of invertebrate and vertebrate MLC-alk proteins (Fig. 3; Table 1). The sequences are compared in detail in the Discussion.

Southern Blot Analysis

A Southern blot of amphioxus genomic DNA under high stringency shows a single band hybridizing with the 739-bp partial *AmphiMLC-alk* clone for each of three enzymes (*Bam*HI, *Bst*XI, and *Hin*dIII) and two bands for *Pst*I (Fig. 4). We thus conclude that there is only one copy of the *AmphiMLC-alk* gene, with the two bands obtained with *Pst*I probably reflecting a *Pst*I site in an intron. An identical pattern was obtained when this blot was stripped and reprobed at low stringency with a probe spanning the entire coding region. Thus, there is only a single *MLC-alk* gene in amphioxus.

Northern Blot Analysis of Expression of AmphiMLC-alk

Northern blot analysis (Fig. 5) reveals that conspicuous expression of *AmphiMLC-alk* begins in embryos and continues throughout the life history of the Florida amphioxus. At all life history stages, the transcript size is approximately 1 kb, in agreement with the 993-bp sequence of the cDNA. In vertebrates, the *MLC1/3* gene gives rise to two transcripts by differential splicing of the mRNA; one transcript exceeds the other by about 120 nucleotides. Our Northern blot should have resolved a difference of this magnitude if two such forms of *MLC-alk* were present in amphioxus. Our failure to observe a second transcript of the amphioxus gene could not have been due to differences in the 5′-UTR, since it is only 20 bp long in *AmphiMLC-alk*. Therefore, there is little doubt that the *AmphiMLC-alk* gene codes for only a single mRNA species, which is expressed at all life history stages.

Expression of AmphiMLC-alk Revealed by in Situ Hybridization

In situ hybridizations of an *AmphiMLC-alk* antisense riboprobe to embryos, larvae, juveniles, and adults of *Branchiostoma floridae* are shown in Figs. 6A–6P. No hybridization was detected in embryos younger than 13 hr. In 13-hr larvae (5-somite stage), *AmphiMLC-alk* becomes detectable in somites 2–5 and is strongest in somites 3 and 4 (Figs. 6A and 6B). Expression begins in the dorsal half of the somites (Fig. 6B) and is always limited to the medial wall of the somite, which is the myotome, destined to form segmental blocks of striated musculature (Fig. 6C). During the next few hours of development (Figs. 6D and 6E), detect-

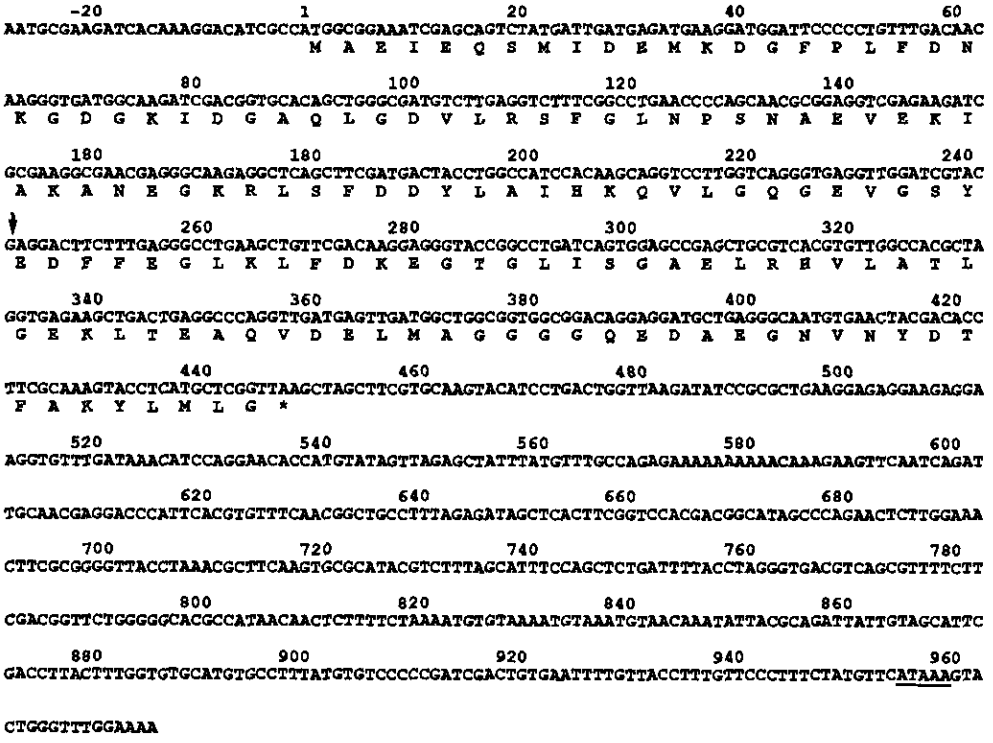


FIG. 2. Base and deduced amino acid sequence (149 amino acids) of the *AmphiMLC-alk* cDNA from *Branchiostoma floridae*. Seven cDNA clones sequenced all had the same 5′-UTR. The polyadenylation signal is underlined. The 739-bp sequence downstream from the arrow was used for library screening, *in situ* hybridizations and in the analysis of the high stringency Southern and Northern blots.

able gene expression extends to the ventral part of each myotome and also anteriorly into the myotome of somite 1 and posteriorly into new segments as they are added posteriorly.

In addition to the myotomal muscles, several nonmyotomal muscles, both striated and smooth, begin expressing *AmphiMLC-alk* during development, although less conspicuously. In the 20-hr embryo, a low level of expression becomes detectable in notochordal cells (Figs. 6F and 6G), which contain some myofibrils (Flood, 1975). A very low level of expression is apparent in some of the coelomic epithelial cells, which contain a few fibrils resembling those in smooth muscle cells (Holland and Holland, 1990). Starting around 30 hr, *AmphiMLC-alk* expression can also be detected in the smooth muscles associated with the forming gill slits (Fig. 6H, arrow) and mouth (Fig. 6I: inset, arrowhead). We did not observe expression above background in nonmuscle cells. However, nonmuscle cells in general contain comparatively low levels of contractile proteins. Furthermore, amphioxus lacks wandering cells such as neural crest and migratory myoblasts which might be expected to express *MLC-alk*. In addition, there are very few fibroblasts (Welsch, 1968), which in mammals express a high level of nonmuscle *MLC-alk* (Hailstones and Gunning, 1990). Thus, in amphioxus one would expect there to be at most an extremely low level of *MLC-alk* expression in such nonmuscle cells as early embryonic cells, dividing cells, and possibly neurons.

The opening of the mouth signals the transition from the embryonic to the larval stage. As the larvae grow, expression of *AmphiMLC-alk* becomes nonuniform throughout the myotome (this pattern can be accentuated by stopping the alkaline phosphatase reaction before completion). Figures 6J–6L show this pattern in myotomes of 10-day larvae. Expression is conspicuous only at the anterior and posterior ends of each myotome and along the medial edge of the myotome next to the notochord (Fig. 6L). This pattern indicates that the mRNA for *AmphiMLC-alk* is being concentrated in cytoplasmic areas where new muscle proteins are presumably being synthesized most intensely. The expression along the medial edge of the myotome might reflect either the addition of new muscle cells or a zone of active elaboration of new muscle proteins within the cytoplasm of existing cells.

In late larvae *AmphiMLC-alk* also starts to be expressed in the striated pterygeal muscles (Figs. 6M and 6N), differentiating in the floor of the atrial cavity (as diagrammed in Fig. 1E). This expression continues into the adult stage. Later larvae and adults of amphioxus are so large that reagents no longer penetrate the myotomal muscles effectively. If the muscle mass of such animals is incised to facilitate entry of the reagents, *AmphiMLC-alk* expression

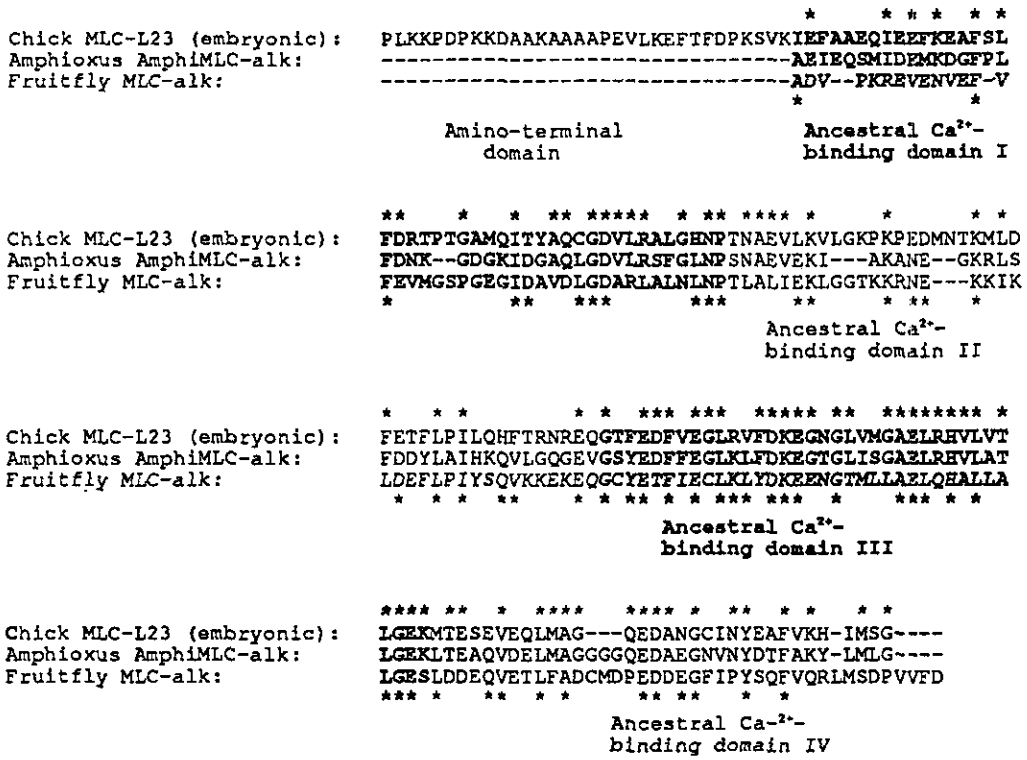


FIG. 3. Alignment of the AmphiMLC-alk amino acid sequence with myosin light chains that are the most similar (Chick MLC-L23) and least similar (Fruitfly MLC-alk). Asterisks above indicate amino acid identities between the amphioxus and the chick proteins, and asterisks below indicate amino acid identities between the amphioxus and the fruitfly proteins. Beneath the sequences the amino-terminal domain (not always present) and the ancestral Ca^{2+}-binding domains I–IV (I and III are in bold type) are indicated.

can be demonstrated in the nearby muscle cells (Fig. 6O). Higher magnification shows the localization of *AmphiMLC-alk* mRNA at the anterior and posterior ends of the myotomal cells (Fig. 6P). In late larvae and adults, striated muscles develop in the velum and lip, but we detected no expression of *AmphiMLC-alk* there. These muscles are small, and failure to detect *AmphiMLC-alk* may be due to low levels of mRNA rather than to the expression of a different, as yet undiscovered, isoform of MLC-alk. In conclusion, *AmphiMLC-alk* is expressed continuously in amphioxus from the early embryo to the adult in the myotomal musculature and at various times in development in other muscles, both striated and smooth.

DISCUSSION

Comparison of MLC-alk between Amphioxus and Other Animals

Figure 3 compares amphioxus AmphiMLC-alk protein with other alkali myosin light chains that are the most similar (Chick MLC-L23) and least similar (fruitfly MLC-alk). Figure 3 also shows the subdivisions of MLC-alk proteins: the amino-terminal domain (not always present) and the ancestral Ca^{2+}-binding domains I–IV. It has been proposed that an originally single Ca^{2+}-binding domain underwent tandem duplications to give a protein with four such domains (Barker *et al.*, 1978; Baba *et al.*, 1984; Collins, 1991). Subsequently, some domains lost Ca^{2+}-binding ability. Thus, calmodulin has four functional domains, while troponin C and parvalbumin each have two and regulatory MLC has only one. It is controversial whether any MLC-alk binds Ca^{2+} (Collins, 1991), although molluscan MLC-alk appears to be required for Ca^{2+}-binding in a complex with myosin heavy chain and the regulatory light chain (Kwon *et al.*, 1990). Ca^{2+}-binding, if present, would probably be within domain III, which is evolutionarily the most conserved (Fig. 3).

Table I compares amino acid identities between AmphiMLC-alk and the alkali myosin light chains of other animals. Compared to vertebrate MLC-alk isoforms, AmphiMLC-alk is more like embryonic MLC-alk of chick (52% identities) and human (51% identities) than like other isoforms (41–48%)—if the amino-terminal domain of the vertebrate sequences is not taken into consideration. Compared to MLC-alk of other invertebrates, AmphiMLC-alk is

TABLE 1

Percentages of Amino Acid Identities in the Four Ancestral Ca^{2+}-Binding Domains of Known Vertebrate and Invertebrate Myosin Light Chains Compared to AmphiMLC-alk

Organism	MLC protein	Tissue	Amino-terminal domain	Amino acid identity	Reference[a]
Chick	MLC-L23	Embryonic	Present	52%	Nabeshima *et al.*, 1988
Chick	$MLC1_v$	Ventricle	Present	48%	Nabeshima *et al.*, 1988
Chick	MLC1	Fast stri.	Present	47%	Nabeshima *et al.*, 1982
Chick	MLC3	Fast stri.	Absent	46%	Nabeshima *et al.*, 1982
Chick	MLC_{nm}	Non-mus.	Absent	46%	Nabeshima *et al.*, 1987
Chick	MLC_s	Smooth mus.	Absent	44%	Nabeshima *et al.*, 1987
Human	$MLC_{emb/A}$	Emb/Atrium	Present	51%	Rotter *et al.*, 1991
Human	MLC_{1f}	Fast stri.	Present	48%	Seidel and Arnold, 1989
Human	MLC_{3f}	Fast stri.	Absent	48%	Seidel and Arnold, 1989
Human	$MLC3_{nm}$	Non-mus.	Absent	47%	Hailstones and Gunning, 1990
Human	$MLC1_{sb}$	Ventricle	Present	46%	Fodor *et al.*, 1989
Human	MLC_{sm}	Smooth mus.	Absent	45%	Lash *et al.*, 1990
Fish	MLC3	Fast stri.	Absent	43%	Dalla Libera *et al.*, 1991
Fish	MLC1	Fast stri.	Present	41%	Dalla Libera *et al.*, 1991
Ascidian	MLC-alk	Smooth mus.[b]	Absent	46%	Takagi *et al.*, 1986
Scallop	MLC-alk	Striated[c]	Absent	44%	Barouch *et al.*, 1991
Fruitfly	MLC-alk	Striated	Absent	33%	Falkenthal *et al.*, 1984

[a] Updated, if necessary, from GenBank.
[b] Body wall.
[c] Adductor muscle.

closer to ascidian MLC-alk (46% identities) than to MLC-alk of nonchordate invertebrates (33–44% identities). This pattern may reflect a difference in contractile systems: amphioxus, ascidian, and vertebrates have an actin-linked system; molluscs have a myosin-linked system; and insects have a combination of the two (Lehman and Szent-Gyorgi, 1975).

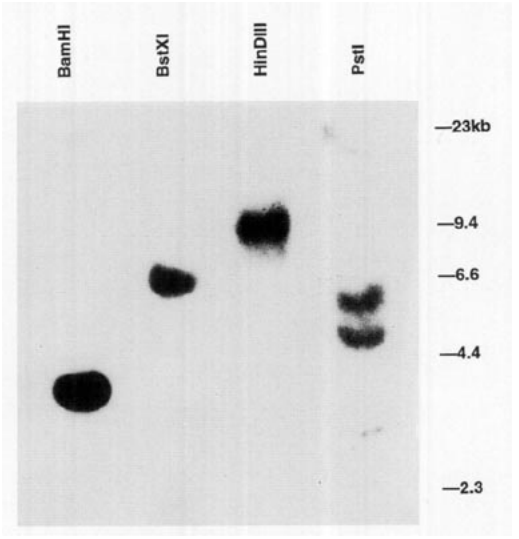


FIG. 4. Southern blot of amphioxus genomic DNA of *Branchiostoma floridae* cut with the indicated restriction enzymes and probed with the 750-bp 3′-end of the *AmphiMLC-alk* cDNA under highly stringent conditions. An identical pattern was obtained when the blot was stripped and reprobed at low stringency with a 725-bp clone spanning the coding region of *AmphiMLC-alk*.

Possible History of MLC-alk Genes during Animal Evolution

Four lines of evidence suggest that there is a single *AmphiMLC-alk* gene in amphioxus. First, only one gene is revealed by low stringency Southern blot analysis with a probe including the entire coding region. Second, Northern blot analysis reveals only one mRNA transcript expressed throughout the life history. Third, an *AmphiMLC-alk* riboprobe hybridizes with mRNA in both smooth and striated muscles of amphioxus at all stages of development and in the adult. Our failure to observe expression above back-

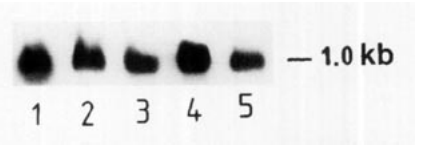
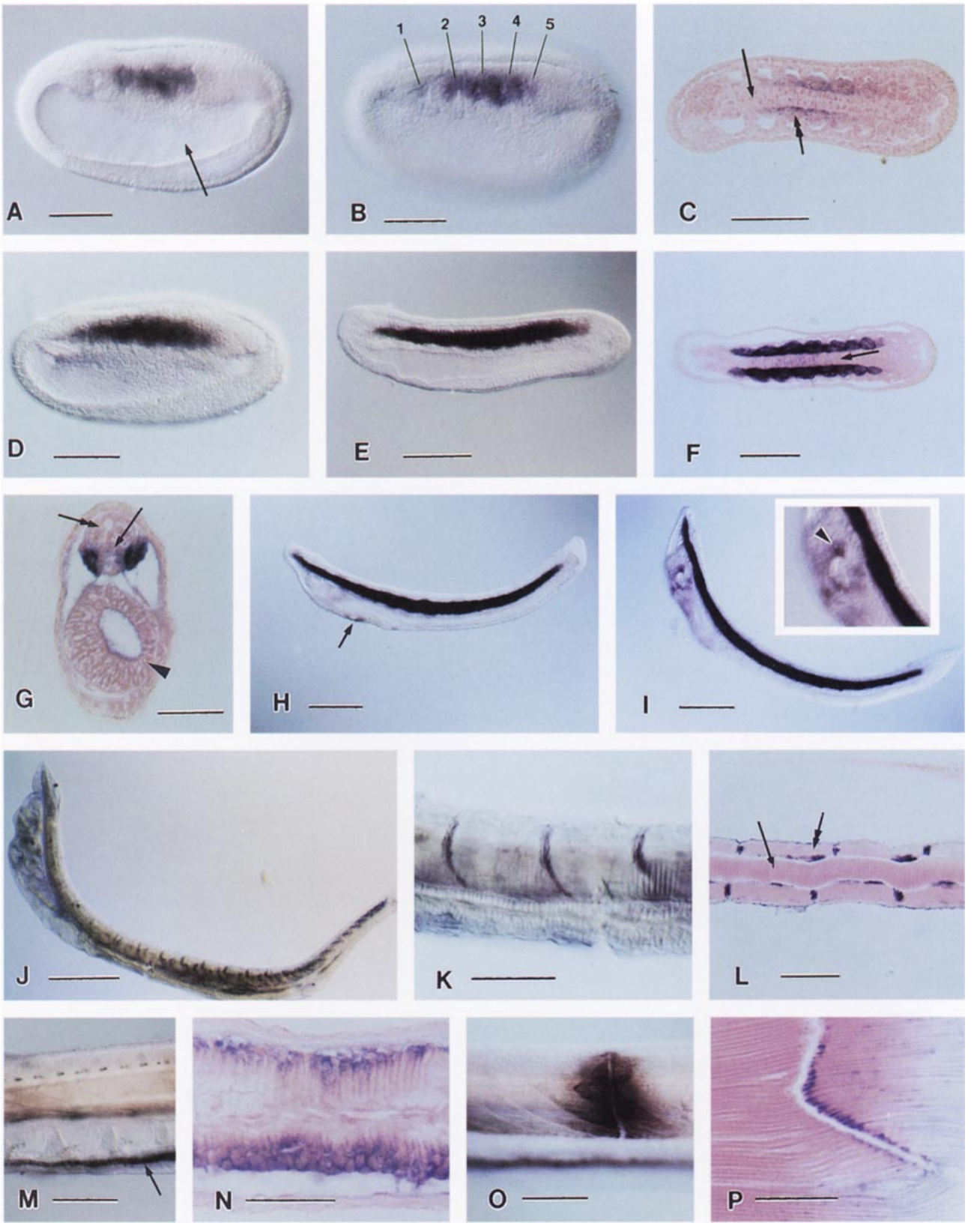


FIG. 5. Northern blot analysis of *AmphiMLC-alk* expression in *Branchiostoma floridae*. 10 μg of RNA was isolated from (1) 26- to 28-hr embryos, (2) 2-day larvae, (3) 1- to 2-week larvae, (4) 6-week juveniles (5 m*M* long), and (5) adults (2–3 cm long).



ground in nonmuscle cells, such as dividing cells or neurons [amphioxus has a fundamentally epithelial organization and fibroblasts and mesenchymal cells are uncommon (Welsch, 1968)], is probably due to a very low level of mRNA rather than to the presence of a second isoform of *AmphiMLC-alk*, since in vertebrates smooth muscle *MLC-alk* is also expressed in many or all nonmuscle cells (Takano-Ohmuro *et al.*, 1985; Hailstones and Gunning, 1990). A series of monoclonal antibodies raised against the AmphiMLC-alk protein and tested against cleavage stages of early embryos could help settle this point. Fourth, in other invertebrates (clams, fruitfly, and *Caenorhabditis*), only one *MLC-alk* gene has been found. Similarly, only one MLC protein has been found in ascidians, although only smooth body wall muscles have so far been assayed (Takagi *et al.*, 1986). In sum, the common ancestor of amphioxus and the vertebrates most likely had only one *MLC-alk* gene.

Schemes proposed for the evolutionary history of the vertebrate *MLC-alk* genes all agree that a single ancestral gene first duplicated early in vertebrate evolution. However, they differ in the order of gene duplications. It has been proposed that an ancestral gene like either the nonmuscle *MLC* (Hailstones and Gunning, 1990) or the fast-striated *MLC3* (Barton and Buckingham, 1985) duplicated to give rise to smooth muscle/nonmuscle *MLC* on the one hand and to *MLC1/3* on the other. Subsequently, the *MLC1/3* gene duplicated to give rise to the ancestor of the ventricular *MLC* and embryonic *MLC* forms. Thus, in their opinion, embryonic *MLC-alk* arose very late in vertebrate evolution. There are difficulties with such a scheme: the argument of Barton and Buckingham (1985) was based on the assumption, which they themselves later showed to be incorrect (Barton *et al.*, 1988), that the chick lacks an embryonic *MLC-alk* gene; moreover, the comparisons of Hailstones and Gunning (1990) were based on a limited data set (chiefly human MLC sequences). Collins (1991) proposed a similar phylogenetic scheme based on MLCs of a variety of organisms, but did not speculate as to the nature of the ancestral vertebrate *MLC*. However, he did propose that the embryonic/atrial and ventricular *MLC* genes diverged relatively early—soon after the divergence of the fast skeletal and ancestral embryonic/atrial *MLC* genes.

Consistent with these schemes is our finding of only one *MLC-alk* gene in amphioxus, suggesting that duplications began after the amphioxus and vertebrate lineages diverged. However, because the amino acid sequence of AmphiMLC-alk is most like that of vertebrate embryonic MLC-alk, we think that the ancestral vertebrate MLC was more like an embryonic form of MLC minus the amino-terminal domain. Thus, we propose that early in vertebrate evolution the embryonic *MLC* gene duplicated to give rise to the nonmuscle/smooth muscle *MLC*. Subsequently, the amino-terminal domain was added to the embryonic *MLC*, which later duplicated again to give rise to the fast skeletal *MLC* genes. This scenario is consistent with both *AmphiMLC-alk* and the embryonic chick isoform *MLC-L23* being expressed in developing smooth and striated muscles (Takano-Ohmuro *et al.*, 1985) as well as in nonmuscle tissue (Ta-

FIG. 6. Expression of *AmphiMLC-alk* during the life history of *Branchiostoma floridae*, from early embryo through adult. All whole mounts, frontal sections, and longitudinal sections are oriented with the anterior end of the animal toward the left. (A) Whole mount of 13-hr embryo in midsagittal focus showing the gut (arrow); there is conspicuous expression of *AmphiMLC-alk* in myotomes of the forming somites. Scale bar, 30 μm. (B) Whole mount of previous embryo in parasagittal focus showing the myocoels of the five most anterior somites (1–5); *AmphiMLC-alk* expression is detectable in the second through the fifth somites. Scale bar, 30 μm. (C) Frontal section of a 14-hr embryo at the level of the notochord (single arrow) and somites; some of the somitic myotomes (tandem arrow) are expressing *AmphiMLC-alk*. Scale bar, 50 μm. (D) Whole mount of a 16-hr embryo with conspicuous *AmphiMLC-alk* expression in the myotomes, including the first one. Scale bar, 30 μm. (E) Whole mount of a 20-hr embryo with conspicuous *AmphiMLC-alk* expression in the myotomes. Scale bar, 50 μm. (F) Frontal section through the preceding embryo; *AmphiMLC-alk* expression is conspicuous in the myotomes and is also detectable in some cells of the notochord (arrow). Scale bar, 50 μm. (G) Cross-section of a 20-hr embryo showing the dorsal nerve cord (tandem arrow), notochord (single arrow), and gut (arrowhead); *AmphiMLC-alk* expression is conspicuous in the myotomes and is also detectable in some cells of the notochord. Scale bar, 20 μm. (H) Whole mount of a 30-hr embryo with *AmphiMLC-alk* transcripts conspicuous in the myotomes and detectable in smooth muscles associated with the beginnings of the first gill slit (arrow). Scale bar, 100 μm. (I) Whole mount of a 36-hr larva with *AmphiMLC-alk* transcripts in the myotomes and smooth muscles associated with the mouth. Scale bar, 100 μm. Inset, enlargement showing conspicuous *AmphiMLC-alk* expression in smooth muscle (arrowhead) at the anterior corner of the mouth. (J) Whole mount of a 10-day larva with *AmphiMLC-alk* most strongly expressed at either end of each myotome. Scale bar, 100 μm. (K) Enlargement of part of the previous larva. Scale bar, 30 μm. (L) Frontal section through the previous larva at the level of the notochord (single arrow) and myotomes (tandem arrow); in each myotome, transcripts of *AmphiMLC-alk* are concentrated along the medial border as well as at the anterior and posterior ends. Scale bar, 20 μm. (M) Whole mount of a 30-day (late metamorphic) larva cut to facilitate entry of reagents. Transcripts of *AmphiMLC-alk* are in myotomes near cut surfaces (top right) and also in the pterygeal muscles (arrow). Endogenous pigment cells are scattered along the dorsal nerve cord. Scale bar, 100 μm. (N) Frontal section through the previous larva at the level of the pterygial muscles, which show moderate expression of *AmphiMLC-alk*. Scale bar, 20 μm. (O) Whole mount of a 30-day (late metamorphic) larva in parasagittal focus. The body wall has been cut part way through to facilitate entry of reagents. Transcripts of *AmphiMLC-alk* are visible only in the myotomal muscles that have been cut into. The pterygeal muscles are out of focus along the bottom. Scale bar, 100 μm. (P) Longitudinal section through parts of two myotomes of a 6-week young adult; the muscle cells of the right hand myotome, which was transected before *in situ* hybridization, show conspicuous levels of *AmphiMLC-alk* transcripts at their anterior extremity. Scale bar, 20 μm.

kano-Ohmuro *et al.*, 1985). Similarly, ascidian *MLC-alk* is expressed in smooth muscle (there is still a need for a study of isoforms expressed in larval ascidians) and is equally identical to embryonic MLC and MLC1 proteins of chick and human.

The above scheme would gain strong support if only non-muscle and embryonic isoforms of *MLC-alk* were found in lower vertebrates. Unfortunately, to date, the *MLC-alk* genes have not been studied in agnathan vertebrates and knowledge of such genes in fishes (sensu lato) is relatively incomplete (Dalla Libera *et al.*, 1991; Crockford and Johnston, 1993; Johnston and Horne, 1994). In sum, our results are consistent with the view that a single ancestral *MLC* gene was present at the base of the vertebrates (Barton and Buckingham, 1985; Collins, 1991) and duplications of this gene did not commence until after the origin of the vertebrates.

Intracellular Localization of mRNA

During early development of amphioxus, transcripts of *AmphiMLC-alk* are evenly distributed throughout the cytoplasm of each myotomal muscle cell; however, in late larvae and adults, transcripts become localized at the anterior and posterior ends of the cells. There is currently much interest in mechanisms for the intracellular distribution of mRNA in animal cells, including muscles. There is good evidence that the 3′-UTR directs specific mRNAs to their appropriate cytoplasmic compartments (Kislauskis *et al.*, 1993). Other aspects of this translocation are less certain: there is a limited diffusion model (Russell and Dix, 1992) and also data suggesting cytoplasmic transport of mRNAs along cytoskeletal elements (Ainger *et al.*, 1993).

Information on the ordered distribution of mRNAs in muscle is largely limited to transcripts of *MHC* in vertebrate striated muscle, in which they tend to be concentrated in the perinuclear cytoplasm and areas of rapid growth (reviewed by Russell and Dix, 1992). Our data for *AmphiMLC-alk* is the first demonstration of intracellular localization of *MLC* transcripts in muscle cells of any animal. The translocation of *AmphiMLC-alk* mRNA in muscle cells of later larvae of amphioxus might be a favorable system for more detailed study, because they enter the cytoplasm from only one nucleus per cell, and their concentrations at the cytoplasmic extremities are high and well defined.

Developmental Changes in Spatial Expression of MLC-alk Compared between Amphioxus and Vertebrates

In amphioxus embryos, detectable expression of *AmphiMLC-alk* begins at the 4- to 5-somite stage in the dorsal half of the myotome in somites 2–5, but not in somite 1. In contrast, in 4-somite mouse embryos (8.5 days) expression of *MLC* and *MHC* is undetectable. At the next stage examined (25 somites = 9.5 days) embryonic *MLC-alk* is expressed deep within the myotome of rostral somites, subsequently spreading to their dorsal and medial edges (Lyons *et al.*, 1990). Because the stages between 4 and 25 somites were not examined, it is not known whether the initial level of expression was identical in all of the rostral somites. By 14.5 days, transcripts of *MLC1* and two myogenic factors (*MyoD* and *myogenin*) are evenly distributed in the myotomes, a pattern similar to the distribution of *AmphiMLC-alk* transcripts in late embryos and early larvae of amphioxus. Interestingly, in mice transgenic for a reporter gene driven by the *MLC1* promoter, there is a rostrocaudal gradient in reporter gene expression. At the 25-somite stage, expression increases from somites 1 to 6 and then decreases to zero by about somite 22 (Grieshammer *et al.*, 1992). Similarly, in 5-somite amphioxus embryos there is a weak gradient of expression of *AmphiMLC-alk* in the somites; however, this gradient, unlike the gradient of the *MLC1* transgene expression, is transitory. For mouse embryos, it was suggested that the endogenous *MLC1* gene and the reporter gene were not expressed in the same rostrocaudal gradient due to the absence of some important but unknown regulatory elements in the transgene construct. It would be interesting to compare sequences upstream and downstream of the transcribed region of *AmphiMLC-alk* with those of the *MLC1* locus in vertebrates to see if additional regulatory elements can be identified in the latter.

In amphioxus, the retention of a single isoform of MLC-alk throughout development correlates with the cytological simplicity of myogenesis. Development of the myotomal musculature in amphioxus parallels that of vertebrates up to the point of myoblast fusion. Correspondingly, the embryonic MLC-alk of vertebrates is the dominant form of MLC-alk in embryonic mammalian skeletal muscle up to the time of myoblast fusion. Afterward, other forms of MLC-alk begin to appear, and the embryonic form eventually becomes limited to the atrial muscle of the heart. Thus, the development of vertebrate skeletal muscles would appear to be an instance of ontogeny recapitulating phylogeny. In all likelihood, the common ancestor of amphioxus and the vertebrates had a myotomal musculature similar to that of modern amphioxus, in which the muscle cells remain mononucleate throughout life and spanned the length of the myotomes. Amphioxus appears to have retained the ancestral form of myogenesis, whereas vertebrates have elaborated on this inheritance by the fusion of muscle cells and the evolution of a multiplicity of isoforms of MLC-alk and other proteins of the contractile apparatus. Therefore, developing muscles in amphioxus could be a very useful model for understanding the early and most fundamental stages of vertebrate myogenesis.

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92-21622. The sequence of *AmphiMLC-alk* has been deposited in GenBank, Accession number V22529.

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