Isolation and Characterization of the Gene for Myosin Light Chain Two of *Drosophila melanogaster*

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Abstract. A recombinant lambdaphage DNA clone containing *Drosophila melanogaster* sequences encoding the gene for myosin light chain (MLC) two has been isolated from a library of randomly sheared DNA. The *Drosophila* MLC2 gene is located in region 99E1-3 on the right arm of chromosome 3, several bands removed from the site reported for the other myosin light chain gene at 98B. The MLC2 sequence at 99E1-3 appears to encode all of the isoforms of *Drosophila* MLC2.

The polypeptide encoded at 99E was identified as MLC2 by the following criteria: the in vitro translation product is identical in size to MLC2 isolated from *Drosophila* muscle, and on two-dimensional gels the in vitro translation product can be separated into two or more peptides that co-migrate with isoforms of larval and thoracic MLC2. RNA encoding the polypeptide was detected in embryos only after the onset of muscle differentiation and was also abundant in adult thoracic muscle. The nucleotide sequence of cDNA generated from late embryonic RNA would be translated to yield a protein sequence with multiple regions of homology to vertebrate MLC2. (There are shorter regions of homology to vertebrate MLC1). Like a number of vertebrate muscle proteins, *Drosophila* MLC2 has an acetylated amino-terminus.

THE myosin molecule consists of one pair of heavy chains and two pairs of light chains. In vertebrates myosin light chains (MLCs)[1] have been divided into two classes on the basis of their solubility. One class is soluble in alkali and the other class can be extracted with 5-5'-dithiobis(2-nitrobenzoic acid) (DTNB). The alkali-extractable class contains two polypeptides (MLC1 and MLC3), while the DTNB class consists solely of MLC2 (42). In some types of muscle the DTNB light chains (MLC2) control contraction by regulating the interactions between actin and myosin. In other muscles, contraction is controlled by the troponin–tropomyosin system associated with actin (37, 41). For example, vertebrate smooth muscle has regulatory DTNB myosin chains but skeletal muscle contraction is regulated by the actin-based system (32, 33). In contrast, all of the insect muscles studied so far possess both the myosin-associated and the actin-associated regulation of muscle contraction (12). In this respect *Drosophila* has not been analyzed.

Muscles of *Drosophila melanogaster* resemble vertebrate muscles in that they contain three myosin light chains; however the *Drosophila* polypeptides are significantly larger than those of vertebrate muscles. Takano-Ohmuro et al. (38) have isolated three classes of myosin light chains from purified *Drosophila* myosin and have numbered the classes on the basis of size as estimated by gel migration. The largest light chain, MLC1, appears to be 34,000 D in fibrillar muscle and 31,000 D in tubular muscle. MLC2 appears to be 30,000 D and MLC3 appears to be 20,000 D in both types of muscle. MLC2 has at least two isoforms in each type of muscle and the isoforms of tubular muscle differ slightly from those of fibrillar muscle.

Although *Drosophila* is an especially useful organism for genetic analysis of muscle development, no mutants of the myosin light chains have been identified, and molecular analyses of the genes have only begun. We report here our studies of a gene encoding MLC2. This cloned sequence is from chromosome region 99E1-3. It is a single copy gene that produces at least two transcripts and multiple protein isoforms. The nucleotide sequence indicates that the *Drosophila* MLC2 gene encodes a polypeptide of 24,000 D, although both the polypeptide and the in vitro translation product of MLC2 RNA appear to be 30,000 D when judged by gel migration. The derived protein sequence of *Drosophila* MLC2 has amino acid homologies with regions throughout vertebrate MLC2 sequences, suggesting an evolutionary, and perhaps a functional, relation between the intermediate-sized light chains in these distantly related organisms.

Materials and Methods

Isolation of Drosophila Nucleic Acids

High molecular weight DNA was isolated from embryos or hand-dissected

1. *Abbreviations used in this paper*: DTNB, 5-5' dithiobis(2-nitrobenzoic acid); MLC, myosin light chain.

brains of third instar larvae. Embryos were dechorionated in 50% Clorox, washed with 0.15 M NaCl, and homogenized in a buffer containing 0.25 M sucrose, 50 mM Tris-HCl, and 1 mM EDTA (pH 7.4). The nuclei were pelleted by centrifugation, resuspended in buffer containing 0.15 M NaCl, 0.1 M EDTA, and 0.02 M sodium borate (pH 9.6), and lysed by adding SDS to 0.5%. Nucleic acids were extracted with phenol-chloroform and recovered by ethanol precipitation. The resulting pellet was resuspended in buffer containing 0.15 M NaCl, 0.05 M Tris, and 5 mM EDTA (pH 8.0). The solution was made 0.5% SDS, 0.1 mg/ml proteinase K, and 0.1 mg/ml RNase and incubated at 36°C for 2 h. DNA was extracted with phenol-chloroform and ethanol precipitated. Brains were collected directly into buffer containing 1% SDS, 0.1 M NaCl, 0.1 M EDTA, 0.01 M Tris, (pH 7.9), and 50 μg/ml proteinase K, homogenized and incubated at 37°C for 2 h. The DNA was extracted as described above.

Total RNA was isolated from staged embryos and pupae that were frozen in liquid nitrogen and ground to a fine powder. The powder was dissolved in 100 mM NaCl, 1 mM EDTA, 0.5% DEPC, 10 mM Tris-HCl (pH 7.4), 0.5% SDS and extracted with phenol/chloroform/isoamylalcohol (25:24:1) followed by two chloroform/isoamylalcohol extractions. Nucleic acids were precipitated with ethanol, spun down, and resuspended in water. RNA was precipitated with 2.5 M lithium chloride, pelleted, washed twice with 70% ethanol and resuspended in water. Poly(A)$^+$ RNA was purified on oligo-(dT)-cellulose as described by Storti et al. (35).

Purification of Larval and Thoracic Myosin Light Chains

Myofibrils were prepared from 50 g of third instar larvae (Canton S strain) by the method of Bullard et al. (2) and homogenized in a solution containing 0.3 M sucrose, 0.1 M KCl, 0.01 M potassium phosphate buffer (pH 7.0), 1 mM $MgCl_2$, 1 mM EGTA, and 0.01 M sodium azide. The washed myofibrils were resuspended in 0.1 M KCl, 0.01 M potassium phosphate buffer (pH 7.0), and myosin was extracted in Hasselbach-Schneider-Zebes solution containing 1 M KCl, 0.01 M sodium pyrophosphate, 1 mM $MgCl_2$, and 0.02 M potassium phosphate buffer (pH 6.5). Residue was removed by centrifugation, and actomyosin was then precipitated by dialysis against 0.25 M KCl and removed by centrifugation. The supernatant was dialysed for 12 h against 0.03 M KCl, adjusted to pH 6.5 with $NaHCO_3$, and the precipitated myosin was spun down and resuspended in 2 M KCl (pH 7.0) to a final concentration of 0.5 M KCl.

Muscle proteins were prepared from thoraces of adult flies of *Drosophila melanogaster*. Thoraces were separated from adult flies and dissected with fine forceps in a drop of Ringer's solution. Muscle tissue from the dissected thoraces was then homogenized by hand in O'Farrell lysis buffer.

Gel Electrophoresis of Proteins

Proteins were routinely separated on 12.5% (wt/wt) acrylamide/0.36% (wt/wt) bis-acrylamide gels (9). Gels were stained with Coomassie Brilliant Blue or silver stained as described by Oakley et al. (20). Radioactively labeled proteins were detected by fluorography with Kodak XAR-5 film. Two-dimensional gels and samples were prepared by the method of O'Farrell (21). Samples were fractionated on a pH gradient of 3.5–10 in the first dimension and separated on 12.5% polyacrylamide gels in the second dimension.

Acetylation of in vitro translation products was blocked by the method of Palmiter (22). Micrococcal nuclease-treated rabbit reticulocyte lysates were treated with 1 mM oxaloacetic acid and 50 U/ml citrate synthase at 20°C for 1.5 h before in vitro translation. Hybrid-selected mRNA and ^{35}[S]methionine were then added to the lysates which were incubated at 37°C for 1 h. Gel-purified larval MLC2 and in vitro translated proteins were labeled with dansyl-chloride by the method of Kato and Sasaki (8) and visualized by UV light (UVSL 25 lamp; UVP Inc., San Gabriel, CA).

Restriction Digests and Electrophoresis of DNA

Restriction endonuclease maps of cloned DNA were generated with single and double digests under conditions recommended by the suppliers. Electrophoresis of the restricted DNA was done in horizontal 1.1% or 0.7% agarose gels buffered with 0.08 M Tris-phosphate. For fragment isolation, the restricted DNA was fractionated on 0.7% low melt agarose gels buffered with 0.04 M Tris-acetate. Fragments were isolated by the Elutip method (Schleicher and Schuell, Inc., Keene, NH) and precipitated with ethanol.

Filter Hybridizations

DNA was transferred to nitrocellulose filters by the method of Southern (34). Filters were baked for 2 h at 70°C and prehybridized in 4× SET–4× Denhardt's solution (1× SET: 0.15 M NaCl, 0.03 M Tris, 2 mM EDTA, pH 8.0) (3, 15) and 100 μg/ml sheared, denatured *E. coli* DNA. After prehybridization, filters were transferred to a fresh solution containing denatured probe, carrier DNA and 4× SET–4× Denhardt's solution.

Hybridization of ^{32}[P]-labeled probes to nitrocellulose-bound DNA was performed overnight at 65°C and the filters were then washed three times with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and 0.5% SDS at 65°C. Hybridized filters were exposed to Kodak XAR-5 film with intensifying screens (10).

For hybridization-selection experiments total RNA from 18–24-h embryos (50 μg/filter) was hybridized to nitrocellulose-bound DNA by the procedure of Riciardi et al. (29). The selected mRNA was translated in micrococcal nuclease-treated rabbit reticulocyte lysates (25) containing 5 μCi ^{35}[S]methionine under the conditions described by Storti et al. (35).

For Northern hybridizations (39), glyoxylated RNA from staged embryos and pupae was separated on 1.5% agarose gels and transferred to Gene Screen Plus filters (NEN Research Products, Boston, MA). Glyoxylated HaeIII-digested φX174 DNA and Hind III-digested lambda DNA were fractionated and visualized by UV light after treatment with alkalai and staining with ethidium bromide. These fragments were used as size markers. Prehybridization and hybridization were done at 60°C in 10% dextran sulfate, 1.0 M NaCl, and 1% SDS overnight. Filters were washed in 1× SSC, 0.1% SDS at 65°C with three changes of the wash solution.

In Situ Hybridization

In situ hybridizations to polytene chromosomes were performed according to Pardue and Dawid (23). Total DNA of lambdaphage Dm11a was nick-translated and DNA (6–7 × 10^7 counts/min) in 2× TNS (1× TNS: 0.15 M NaCl, 0.01 M Tris-HCl, pH 6.8) plus 50 μg/ml *E. coli* DNA was hybridized to polytene chromosome squashes at 67°C for 12 h.

Subcloning

pSP65LC2 and pSP64LC2 were constructed by subcloning a gel-purified Eco RI/Bam HI fragment of Dm11a DNA into pSP65 and pSP64 linearized with Bam HI and Eco RI as described by Melton et al. (16). The ligation mixture was transformed into *E. coli* strain LM 1035 and ampicillin-resistant colonies were selected. The DNA was isolated as described by Maniatis et al. (14).

Isolation of cDNA Clones

A lambda gt10 library containing *Drosophila melanogaster* cDNA (26) was screened for sequences homologous to the MLC2 gene by plaque hybridization. cDNA was subcloned from one lambda cDNA clone, gtFlg, by digesting with EcoRI and ligating the resulting fragment into pBR322. A subclone, pFlg, containing a 760 bp insert which includes the entire coding region of the MLC2 gene was used for subsequent analysis.

M13 subclones were initially generated by digestion of pFlg with EcoRI and ligation of the 760 bp fragment into M13mp19. A procedure based on digestion by Bal31 was used (11) to rapidly sequence the entire insert region. Digestion of the linearized M13Flg subclone with the exonuclease Bal31 resulted in a set of deletions that were subsequently sequenced.

DNA Sequencing

DNA sequencing was done by the dideoxy chain termination method (31) with M13 phage vectors (19).

Results

Isolation and Characterization of the Clone Dm11a

The clone (Dm11a) containing the MLC2 sequences was isolated from a genomic library of *D. melanogaster* DNA consisting of randomly sheared embryonic *Drosophila* DNA inserted into the Charon 4 lambdaphage vector with Eco RI linkers (15). The clone was selected by screening with a ^{32}[P]-cDNA probe highly enriched for sequences complementary to mRNA of mature myotubes. The cDNA was synthesized on oligo-(dT)-fractionated cytoplasmic RNA isolated from mature pulsating *Drosophila* myotubes that had undergone the complete developmental program of the

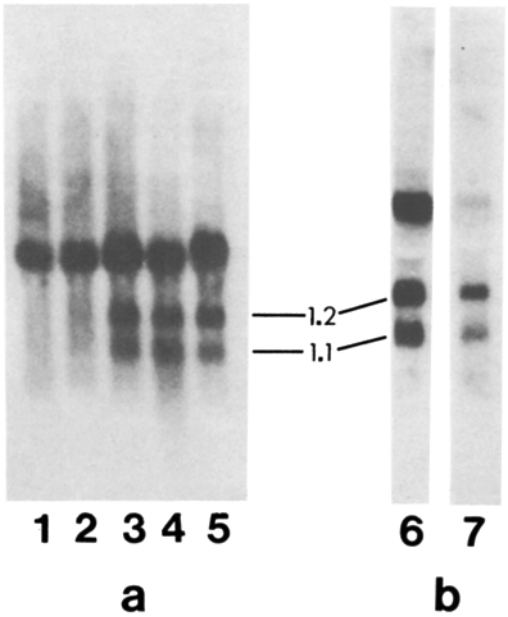

Figure 1. Analysis of RNA homologous to the Dmlla gene shows two developmentally regulated transcripts (1.1 and 1.2 kb) that are expressed only during the stages of muscle synthesis (*a*). A 2.4-kb RNA with some homology to the coding sequences is detected in RNA from all stages examined and serves as a control for the loading and transfer of each gel lane. The 2.4-kb RNA shows very little binding to oligo-(dT)-cellulose (*b*) and thus does not appear to be a typical poly A^- RNA. The 2.4-kb RNA does not yield a translation product in hybrid-selection experiments (see text). Denatured RNA isolated from *Drosophila* embryos of different age ranges and from late-stage pupae was separated on agarose gels and transferred to Gene Screen Plus filters. In *a* the RNA is from (lane *1*) 0–6-; (lane *2*) 6–12-; (lane *3*) 12–18-; (lane *4*) 18–24-h old embryos and (lane *5*) late-stage pupae. In *b* the RNA is (lane *6*) total RNA from pupae and (lane *7*) poly(A)$^+$RNA from 11–20-h-old embryos. Lanes *1–6* have 10 μg of total RNA per lane and lane *7* has 2 μg of oligo-(dT)-fractionated RNA. The filters were probed with single-stranded RNA transcribed from the coding region of Dmlla subcloned into pSP65. Molecular size markers of the poly(A)$^+$RNAs are in kilobases.

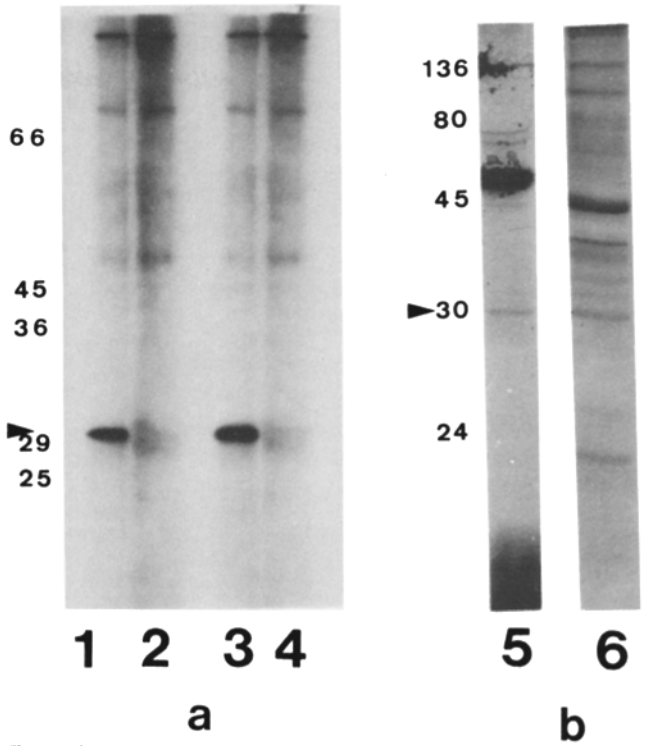

Figure 2. The in vitro translation product of RNA hybrid-selected by Dmlla has an apparent molecular mass of 30,000 D and comigrates with *Drosophila* MLC2 on SDS polyacrylamide gels. (*a*) Autoradiogram of ^{35}S-labeled products of rabbit reticulocyte lysates fractionated on a 12.5% SDS-polyacrylamide gel. (Lane *1*) Lysate containing 1× RNA hybrid-selected by Dmlla (from 25 μg total RNA). (Lane 2) Lysate with no added RNA, showing endogenous products labeled in the lysate. (Lane *3*) Lysate containing 2× RNA hybrid-selected by Dmlla (from 50 μg). (Lane *4*) Lysate with no added RNA. The migration of unlabeled molecular mass markers is noted on the left in daltons. The product of the hybrid-selected RNA (*arrow*) runs just above the 29,000-D marker (carbonic anhydrase) and coincides with the larger band of a doublet of endogenous proteins. (*b*) The hybrid-selected product of Dmlla comigrates with the 30,000-D *Drosophila* MLC. (Lane *5*) Autoradiogram of ^{35}S-labeled products of a rabbit reticulocyte lysate fractionated on a 12.5% polyacrylamide gel. The product of Dmlla RNA is indicated with an arrow. (Lane *6*) Coomassie Blue-stained gel lane of *Drosophila* myofibrillar proteins co-fractionated with the lysate in lane *5*. The heavy band at 30,000 D is MLC2. Marker sizes are indicated to the left of lane *5*.

primary myogenic cell culture system described previously (36). Before the screening procedure, the cDNA had been further enriched for myotube-specific sequences by prehybridization with total cytoplasmic RNA, isolated from undifferentiated cells (Schneider line 2-L), to remove sequences coding for general "housekeeping" proteins.

RNAs Encoded by Dmlla

The method used to select the clone Dmlla implies that the expression of the encoded *Drosophila* gene should follow the developmental pattern of muscle differentiation and our studies confirm this. We have investigated the pattern of expression of transcription of RNA homologous to the coding region of Dmlla DNA by analyzing RNA isolated from different embryonic stages (0–6, 6–12, 12–18, and 18–24 h after egg laying), and from larvae, pupae, and adult thoraces. The RNA was probed with ^{32}[P]-labeled single-stranded antisense RNA transcribed from the coding region of Dmlla DNA subcloned into pSP65 (in some experiments the probe was the isolated coding region of the lambda clone). Hybridization is seen to a pair of developmentally regulated RNAs of 1.1 and 1.2 kb (Fig. 1). These two RNAs are not detected in the earliest embryonic stages, however there is a low level of hybridization to RNA from 6–12-h embryos and much stronger hybridization to RNA from later embryonic stages. This pattern of gene expression correlates well with the timing of muscle development in the embryo (27). In addition, the temporal expression of the Dmlla gene also resembles that of other muscle-specific genes, such as those encoding the *Drosophila* tropomyosins (1). Larvae, pupae, and adult thoraces all synthesize muscle, and all have RNA complementary to Dmlla.

There is a 2.4-kb RNA that is present throughout all developmental stages and appears to share homology with the *Drosophila* MLC2 gene. This RNA is not a typical poly (A)$^+$ RNA; very little of it is retained by oligo-(dT) cellulose (Fig. 1 b, lanes *6*, *7*). Furthermore, the 2.4-kb RNA does not direct the synthesis of a Dmlla-encoded protein. Al-

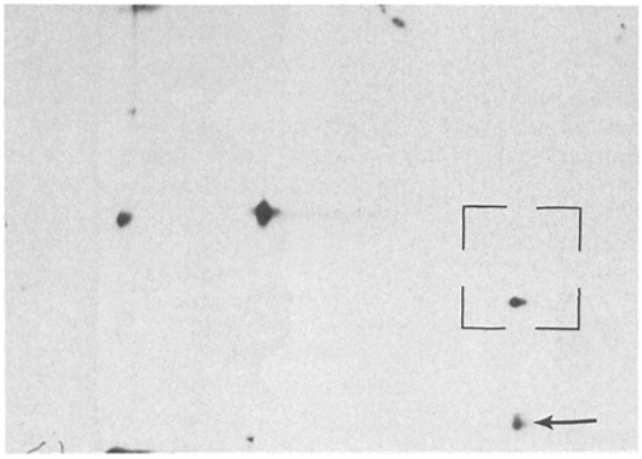

Figure 3. Autoradiogram of the in vitro translation product of Dmlla labeled with [^{35}S]methionine and [^{3}H]alanine and separated on a two-dimensional gel. The spots framed by brackets are the isoforms of the 30,000-D in vitro translation product. The small arrow points to an 18,000-D polypeptide that is an artifact of the in vitro translation system. All other spots are endogenous proteins of the rabbit reticulocyte lysate that are labeled in vitro. RNA was hybrid-selected from late-stage pupae.

though there is abundant hybridization of the 2.4-kb RNA from the earliest embryonic stages to Dmlla, this RNA cannot be translated in vitro to yield a polypeptide. Dmlla hybrid-selects translatable RNA only from stages that have the 1.1- and the 1.2-kb RNAs. These results imply that the 2.4-kb RNA, which is abundant at all stages, is not a messenger RNA. The 2.4-kb RNA is probably not a transcript of the gene on Dmlla: hybridization to this 2.4-kb RNA is more stable when complementary RNA to the MLC2 gene is used to probe Northern blots than when MLC2 cDNA is used as probe. RNA–RNA hybrids are more stable than the corresponding RNA–DNA hybrid, suggesting that Dmlla forms an imperfect cross-hybrid with the 2.4-kb RNA which must be a transcript of another region of the genome.

The two developmentally regulated RNAs that show homology to the Dmlla coding sequence, the 1.1- and the 1.2-kb RNA, are found in all stages that are expected to be synthesizing muscle products. The ratio of these two RNAs is relatively constant at the different stages, suggesting that the two transcripts are both present in the different types of muscle that appear in larvae and adults.

Characterization of the Dmlla Gene Products

The clone Dmlla efficiently hybrid-selects RNA from any muscle-containing tissue. Hybrid-selected RNA can be translated in vitro to yield a 30,000 D polypeptide that co-migrates on SDS polyacrylamide gels with the medium-sized *Drosophila* myosin light chain, MLC2 (Fig. 2). One, or sometimes multiple, smaller polypeptides are seen along with the 30,000-D translation product (Fig. 3); however the smaller polypeptides appear to be artifacts of the reticulocyte in vitro translation system (see Discussion). These smaller polypeptides do not co-migrate with any fibrillar muscle proteins.

On two-dimensional gels *Drosophila* MLC2 can be separated into several isoforms. On some gels the isoforms ap-

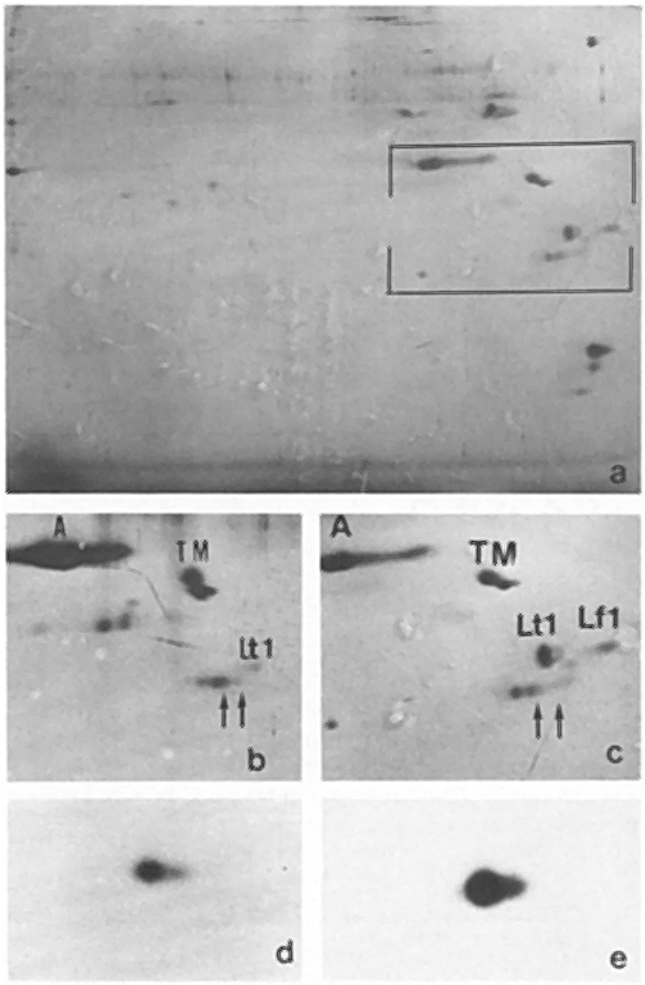

Figure 4. On two-dimensional PAGE gels the *Drosophila* MLC2 shows multiple isoforms in both larval (tubular) and pupal (predominantly fibrillar) muscle. (*a*) Silver-stained myofibrillar proteins purified from adult thoraces and separated in a PAGE gel. The region in the box is enlarged below. (*b*) Silver-stained pattern of muscle proteins purified from third instar larvae (tubular muscle). Shown is an enlargement from a two-dimensional gel in the area of MLC1 and MLC2 (an enlargement corresponding to the area indicated by brackets in both *a* and *d*). *A*, actin; *TM*, tropomyosin; *Ltl*, tubular MLC1. The arrows indicate the two tubular isoforms of MLC2 that co-migrate with the in vitro-synthesized isoforms in Dmlla (shown in the autoradiogram in *d*). (*c*) Silver-stained gel of muscle proteins purified from adult thoraces (primarily fibrillar muscle). This is an enlargement of the boxed region in *a* and is to be compared with the section from the gel of larval proteins in *b*. The under-representation of Lfl in this photograph is due to preferential breakdown of this protein during purification (38). *Lfl*, fibrillar MLC1. The arrows indicate the two isoforms of MLC2 that co-migrate with the in vitro-synthesized isoforms of Dmlla (shown in the autoradiograms in *d* and *e*). (*d*) Enlargement of the MLC2 region of an autoradiogram showing isoforms of MLC2 produced by hybrid-selected RNA from 18–24-h embryos, translated in vitro and separated on a two-dimensional gel. (*e*) Enlargement of the MLC2 region of an autoradiogram showing isoforms of MLC2 translated from late stage pupal RNA treated as in *d*. RNA from both stages produces two isoforms. In neither case is the complete set of isoforms produced by the reticulocyte lysate.

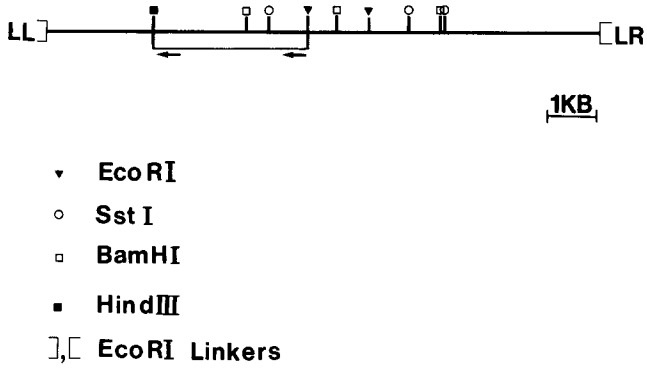

Figure 5. Restriction map of *Drosophila* DNA contained in clone Dmlla. The map was derived by single and double digests with restriction enzymes and by hybridization of in vitro labeled RNA to Southern transfers of restriction digests of the lambdaphage, Dmlla. Restriction sites are indicated by the symbols in the key. LL and LR denote the left and right arms of lambda DNA, respectively. The coding region of the MLC2 gene is indicated by a thin black line and the direction of transcription is indicated by the arrows. There are no Kpn I, Sal I, Xba I or Xho sites in the cloned DNA.

pear as an irregularly shaped elongated spot which has been identified as spots 147, 148, and 149 on the two-dimensional gels of *Drosophila* myofibrillar proteins of Mogami et al. (18). Takano-Ohmuro et al. (38) report that fibrillar muscle contains both more acidic and more basic forms of MLC2 than does tubular muscle. We see similar differences between preparations of larval muscle, which is tubular, and thoracic muscle, which contains predominantly fibrillar muscle (Fig. 4, *b* and *c*). In spite of the differences in the arrays of isoforms found in different muscles, all of the MLC2 isoforms appear to be encoded by the sequence cloned in Dmlla since the genome has only one copy of the sequence (see below). The Dmlla sequence hybrid-selects RNA equally well from late-stage embryos, from pupae, and from adult thoraces. In each case the hybrid-selected RNA is translated by the rabbit reticulocyte cell-free system to yield two isoforms that co-migrate on two-dimensional gels with *Drosophila* MLC2 (Fig. 4, *d* and *e*). In each case, the more basic of the in vitro translation products is the more abundant form. The in vitro translation products do not include the complete array of isoforms, perhaps because the reticulocyte lysate cannot carry out all of the necessary posttranslational modifications (see Discussion).

Characterization of Coding Sequences in Dmlla DNA

Analysis of DNA fragments after restriction enzyme cleavage allowed for the construction of the map shown in Fig. 5. The *Drosophila* DNA cloned in Dmlla is 12.5 kb long. The coding region of the clone was identified by hybridization of end-labeled pupal and 11–20-h embryo poly $(A)^+$ RNA to restriction enzyme-digested Dmlla DNA. The region indicated is the smallest restriction fragment that includes all detectable hybridization. The identification was confirmed by using the 3.5-kb Eco RI/Hind III fragment of

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   ATGGCCGATGAGAAGAAGAAGGTTAAGAAGAAGAAGACCAAGGAAGAGGGTGGTACTTCCGAAACCGCTTCTGAGGCCGCATCCGAGGCA
1  ---------+---------+---------+---------+---------+---------+---------+---------+---------+  90
   MetAlaAspGluLysLysLysValLysLysLysLysThrLysGluGluGlyGlyThrSerGluThrAlaSerGluAlaAlaSerGluAla

   GCAACCCCGGCACCAGCTGCCACTCCTGCCCCGGCCGCATCTGCCACTGGTTCGAAGAGAGCGTCGGGCGGATCCCGTGGCTCCAGGAAG
91 ---------+---------+---------+---------+---------+---------+---------+---------+---------+ 180
   AlaThrProAlaProAlaAlaThrProAlaProAlaAlaSerAlaThrGlySerLysArgAlaSerGlyGlySerArgGlySerArgLys

   TCGAAGCGCGCTGGCTCCTCGGTCTTCTCTGTGTTCTCCCAGAAGCAGATCGCCGAGTTCAAGGAGGCCTTCCAACTCATGGATGCCGAC
181 ---------+---------+---------+---------+---------+---------+---------+---------+---------+ 270
   SerLysArgAlaGlySerSerValPheSerValPheSerGlnLysGlnIleAlaGluPheLysGluAlaPheGlnLeuMetAspAlaAsp

   AAGGACGGTATTATTGGCAAGAACGATCTGCGCGCTGCCTTCGACTCCGTCGGCAAGATCGCCAACGACAAGGAGTTGGACGCCATGCTG
271 ---------+---------+---------+---------+---------+---------+---------+---------+---------+ 360
   LysAspGlyIleIleGlyLysAsnAspLeuArgAlaAlaPheAspSerValGlyLysIleAlaAsnAspLysGluLeuAspAlaMetLeu

   GGCGAGGCCTCGGGTCCGATCAACTTCACCCAGTTGCTGACCCTGTTCGCCAACCGCATGGCCACCTCCGGTGCCAACGATGAAGACGAA
361 ---------+---------+---------+---------+---------+---------+---------+---------+---------+ 450
   GlyGluAlaSerGlyProIleAsnPheThrGlnLeuLeuThrLeuPheAlaAsnArgMetAlaThrSerGlyAlaAsnAspGluAspGlu

   GTTGTTATTGCTGCCTTCAAAACATTCGATAACGATGGTCTCATCGACGGTGACAAATTCCGCGAAATGCTCATGAACTTCGGTGACAAG
451 ---------+---------+---------+---------+---------+---------+---------+---------+---------+ 540
   ValValIleAlaAlaPheLysThrPheAspAsnAspGlyLeuIleAspGlyAspLysPheArgGluMetLeuMetAsnPheGlyAspLys

   TTCACCATGAAGGAGGTTGATGATGCCTACGATCAGATGGTGATCGACGACAAGAACCAGATCGATACCGCCGCCCTGATCGAGATGCTC
541 ---------+---------+---------+---------+---------+---------+---------+---------+---------+ 630
   PheThrMetLysGluValAspAspAlaTyrAspGlnMetValIleAspAspLysAsnGlnIleAspThrAlaAlaLeuIleGluMetLeu

   ACCGGCAAGGGTGAAGAGGAGGAGGAGGAGGCCGCCTAA
631 ---------+---------+---------+--------- 669
   ThrGlyLysGlyGluGluGluGluGluAlaAlaEnd
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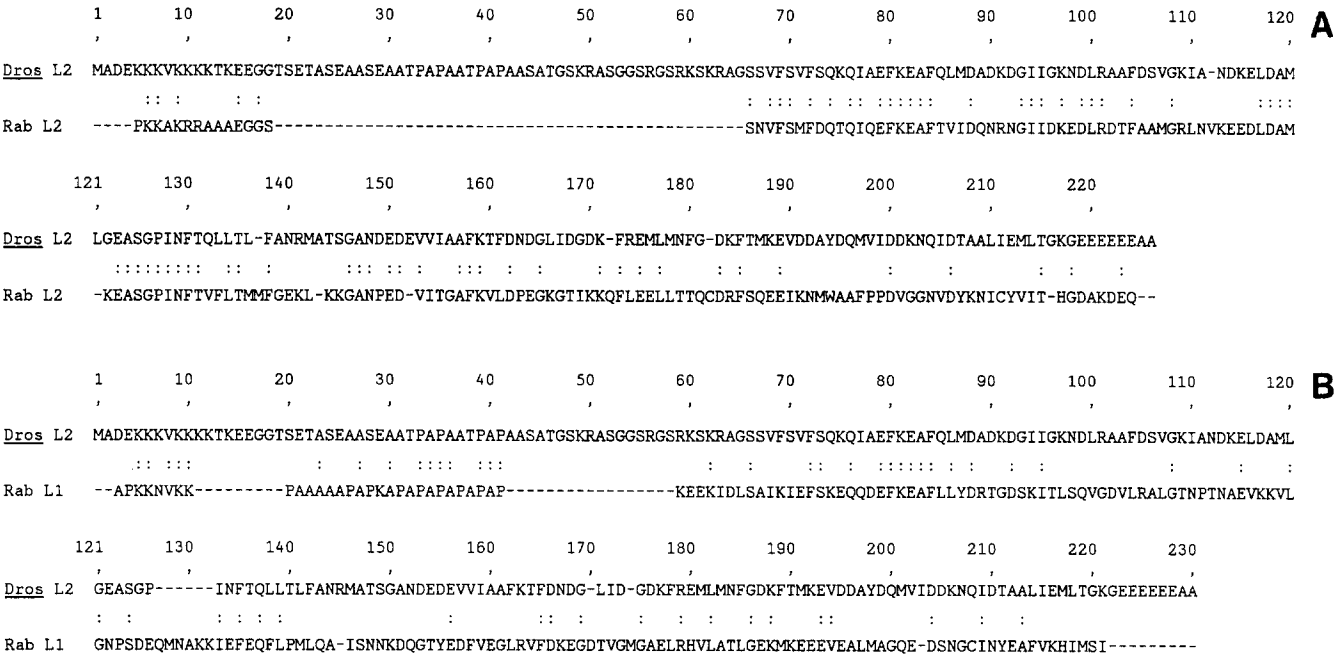
Figure 6. Predicted amino acid sequence of the insert in pFlg. The nucleotide sequence was determined as described in Materials and Methods.



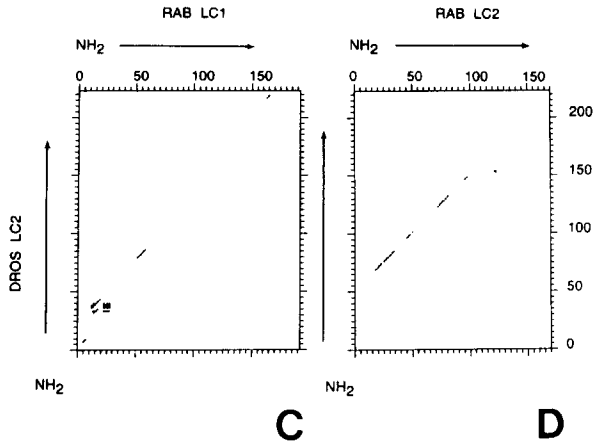


Figure 7. Comparison of the predicted amino acid sequence of *Drosophila* MLC2 with the amino acid sequences of rabbit MLC2 and rabbit MLC1. (*A*) The *Drosophila* sequence was aligned with the rabbit MLC2 sequence, using the ALIGN program with the default stringency. (*B*) The alignment of the *Drosophila* sequence with rabbit MLC1 using the same method. (*C*) Comparison of the *Drosophila* protein with rabbit MLC1 in which amino acids are compared in stretches of eight and a positive score is noted by a dot if five of the eight amino acids match. (*D*) Comparison of the *Drosophila* protein with rabbit MLC2, scoring matches of five out of eight amino acids.

the clone to hybrid-select RNA for in vitro translation. The products of this translation were the MLC2 isoforms.

The orientation of transcription of the MLC2 gene was determined by subcloning the 4-kb Eco RI/Bam HI fragment containing the coding sequences into two vectors, pSP64 and pSP65, which contain polylinker sequences oriented in opposite directions and a promoter for the SP6 polymerase adjacent to the site at which the *Drosophila* DNA was inserted (16). The two resulting plasmids, pSP64LC2 and pSP65LC2, were each cleaved at the restriction site on the end of the MLC2 insert farthest from the SP6 promoter. The two linearized plasmids were then used as templates for the synthesis of single-stranded RNA transcribed from the SP6 promoter. Only the RNA transcribed from pSP65LC2 hybridized to pupal RNA, indicating that only this strand is complementary to mRNA. The direction of transcription is as indicated by the arrow on the map in Fig. 5.

Nucleotide Sequence Analysis of cDNAs

We have isolated cDNA clones that are homologous to the cloned chromosomal gene in Dmlla in order to analyze the encoded protein sequence. A late embryo cDNA library (26) was screened with a probe containing the 3.5-kb Eco RI/Hind III fragment of Dmlla. One clone with a large insert, pFlg, was used for sequence analysis. The insert of pFlg is 760 nucleotides long and contains the entire coding region of the MLC2 gene as well as some of the 5′ and 3′ untranslated flanking sequences. While sequence analysis was underway, Parker et al. (24) reported the nucleotide sequence of this MLC2 gene and of two late pupal cDNA sequences from the chromosomal gene. The nucleotide sequence of the coding region that we have obtained from the embryonic clone is in complete agreement with that of the pupal cDNA reported by Parker et al., indicating that at least one mRNA is used in both stages. Since the sequence of our embryonic cDNA is identical to that of pupal cDNA, we have ascertained the complete nucleotide sequence of our clone in only one direction.

Analysis of the cDNA sequence revealed an open reading frame starting with an ATG codon and, as reported by Parker et al. (24), this reading frame is 666 nucleotides long (Fig. 6). Comparison of the predicted amino acid sequence of the

Drosophila protein with vertebrate MLC2s shows stretches of considerable homology throughout these polypeptides (Fig. 7), as previously noted by Parker et al. The *Drosophila* protein is ~50 amino acids longer than the vertebrate proteins. The additional residues lie predominantly at the amino-terminal end, however the protein begins with a lysine-rich segment that shows homology to the amino-terminal regions of both vertebrate MLC1 and MLC2. There are other regions of homology between the *Drosophila* sequence and vertebrate MLC1, as there are between the MLC1 and MLC2 sequences of vertebrates, but the homology with MLC2 is clearly more significant.

One segment of the *Drosophila* sequence that shows notable homology to both the vertebrate MLC1 and MLC2 is the region of the presumed calcium-binding site. The homology was first detected when we used a fragment of a rabbit MLC2 cDNA clone to probe restriction fragments of the *Drosophila* clone. The rabbit sequence, encoding amino acids 26–37 of rabbit skeletal muscle MLC2 (28), hybridized with the *Drosophila* sequence at relatively high stringency. Sequence analysis showed that this 36-nucleotide fragment had >75% homology with the *Drosophila* sequence encoding amino acids 77–88:

```
ATCGCCGAGTTCAAGGAGGCCTTCCAACTCATGGAT  Drosophila
|||   ||||||||||||||||||    ||||  |||
ATCCAGGAGTTCAAGGAGGCCTTCACCGTCATCGAT  rabbit
```

The amino acid homology in this region is also >75%. This region is also the site of one of the major regions of homology between the *Drosophila* sequence and rabbit MLC1 (amino acids 48–59). In the two vertebrate MLCs the homology is at a site thought to have once been a calcium binding site. In each case the greatest homology is in the residues of the adjacent alpha helix.

The molecular mass of the protein predicted from the nucleotide sequence is 24,000 D. In contrast, both purified *Drosophila* MLC2 and the in vitro translation product of RNA hybrid-selected by the cloned MLC2 gene migrate at 30,000 D on SDS-polyacrylamide gels (Fig. 2, lanes *5* and *6*).

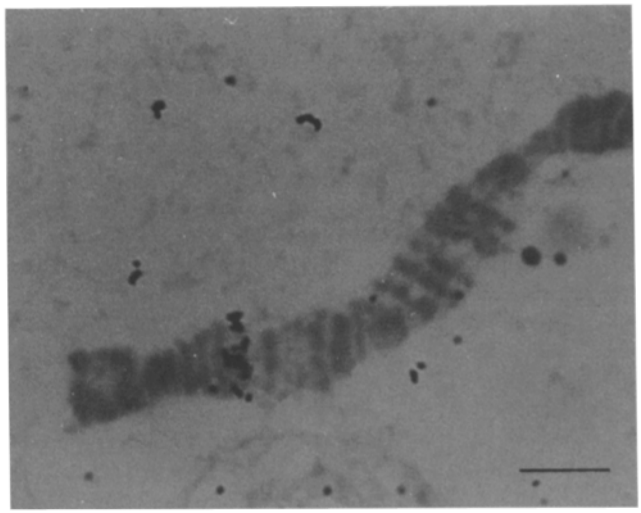


Figure 8. In situ hybridization to polytene chromosomes localizes the MLC2 gene to region 99E1-3. Polytene chromosomes from salivary glands of *Drosophila melanogaster* third instar larvae of the genotype gtXII/gtl were hybridized in situ with a nick-translated ^{3}H probe from the entire chimeric phage of clone Dmlla. 19 d of exposure. Bar, 10 μm.

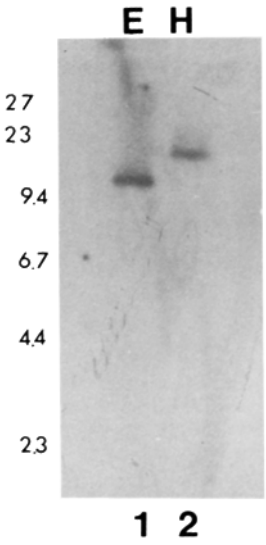


Figure 9. Whole genomic analysis of the MLC2 gene shows hybridization to only one fragment of *Drosophila* genomic DNA. High molecular mass DNA from Canton S embryos was digested to completion with (lane *1*) Eco RI or (lane *2*) Hind III, separated in agarose gels, and transferred to nitrocellulose filters. Filters were hybridized with a gel-purified ^{32}P-labeled 3.5-kb Eco RI/Hind III restriction fragment of clone Dmlla which carries most of the coding region of the *Drosophila* MLC2 gene. Hybridization and washing were done under highly stringent conditions. Molecular size standards are indicated at the left.

The Genomic Localization of MLC Genes

The DNA of Dmlla hybridizes to region 99E1–3 of polytene chromosomes (Fig. 8). The autoradiogram shown in Fig. 8 was exposed for 19 d. Even after 490 d of exposure we have seen no evidence of hybridization to other sites. Since similar experiments with clones carrying α-tubulin genes show cross-hybridization to the sites of the other α-tubulin genes after much shorter exposure times (17), we conclude that there are no closely related MLC2 genes outside the 99E1–3 region. Hybridization to Southern transfers of restriction enzyme-cleaved genomic DNA also indicates that the MLC2 gene does not belong to a large multigene family. The 3.5-kb Eco RI/Hind III fragment carrying most of the MLC2 coding region hybridizes to only one restriction fragment of DNA from the Canton S stock after digestion with any restriction enzyme that does not cleave within the gene (Fig. 9). This is evidence that there is only one copy of this restriction fragment in the genome.

Amino-Terminal Acetylation of Drosophila MLC2

Acetylation of amino-terminal ends is characteristic of vertebrate myosin light chains as well as many other vertebrate proteins. We find acetylation of the amino-terminal ends of both gel-purified larval MLC2 and the in vitro translation product of RNA selected by Dmlla. Both the larval protein and the in vitro translation product show only a little fluorescent labeling with dansyl-chloride, a dye that binds specifically to free amino groups. However, when the reticulocyte lysate used for the in vitro translation was treated with oxalacetic acid and citrate synthase before translation, thus preventing acetylation by the reticulocyte lysate (22), the in vitro translation products showed significantly increased fluorescent labeling by dansyl-chloride. Densitometer tracings of photographic negatives and autoradiograms allowed comparison of dansyl-chloride labeling with the amount of in vitro translation product detected by [^{35}S]methionine incorporation. Results were (units of fluorescence per units of

[^{35}S]methionine incorporation): treated 0.35/1.29 = 0.2; untreated 0.20/1.65 = 0.12. Although the residual dansyl-chloride labeling of the in vivo proteins and the untreated translation products might indicate that there is a low level of unacetylated products, it seems more likely that reactions of other free amino groups with dansyl-chloride, at least in part, may be responsible for the small amount of fluorescence observed both in the in vitro reaction and on isolated MLC2 (data not shown).

Discussion

Our initial identification of the MLC2 gene cloned in Dmlla was based on its homology to a relatively abundant myotube RNA. This RNA could be translated in vitro to produce a polypeptide apparently identical in size and charge to isoforms of a protein that has been identified as a *Drosophila* myosin light chain on the basis of its association with myosin heavy chain (38). In *Drosophila* (38), both fibrillar and tubular muscle contain a myosin light chain with an apparent molecular mass of 30,000 D that has been designated MLC2 because it is intermediate in size between the other myosin light chains, MLC1 (apparent molecular mass 34,000 D in fibrillar muscle, and 31,000 D in tubular muscle) and MLC3 (apparent molecular mass 20,000 D in both types of muscle). Further evidence for the identification of the cloned *Drosophila* gene was ascertained from the derived amino acid sequence of the gene. We have sequenced a cDNA clone prepared from embryonic RNA. The sequence is identical to the sequence of pupal cDNA clones from the same region recently reported by Parker et al. (24), indicating that the same sequence is used in the different developmental stages. As noted by those authors, the sequence has numerous short regions of homology to vertebrate MLC2 scattered along the polypeptide. The homologies are stronger in the amino-terminal half of the vertebrate peptide and include, but are not limited to, the presumed calcium-binding site of these molecules. The *Drosophila* MLC2 does differ from the known vertebrate MLC2 polypeptides in length. When amino acid sequences are aligned, the *Drosophila* sequence has an amino-terminal insertion of some 50 amino acids. The most amino-terminal segment of the *Drosophila* MLC2 is, however, a lysine-rich segment with homology to both rabbit MLC1 and MLC2. Parker et al. (24) have pointed out that, within the extra 50 amino acids, the *Drosophila* MLC2 sequence contains a segment of 13 amino acids with a 10 amino acid homology to chick MLC1. There is a region of similar homology (9 of 13 amino acids) between *Drosophila* MLC2 amino acids 77–88 and rabbit MLC1 amino acids 48–59. This is part of a site thought to have been a calcium-binding site in an ancestral protein. Still, the bulk of the sequence homology is seen between the intermediate-sized *Drosophila* MLC and the intermediate-sized vertebrate MLC. For both vertebrates and *Drosophila*, these polypeptides were named MLC2 on the basis of their size relative to the other myosin light chains in the same species. The sequences now suggest that the name MLC2 is also appropriate in terms of evolutionary relationship and probably also in terms of function.

It is interesting to note that the molecular mass of the MLC2 protein, as derived from the nucleotide sequence, is only 24,000 D yet MLC2 migrates on polyacrylamide gels as a 30,000-D protein (see also Fig. 6). RNA encoding MLC2 is translated in vitro by rabbit reticulocyte lysates to give a product that co-migrates exactly with purified MLC2 in our experiments. This translation product has been estimated as 26,000 D by Parker et al. (24), although it runs well above the 25,000-D marker on their gels. The large discrepancy between the predicted molecular mass and the actual migration of the protein on SDS polyacrylamide gels may be due to tertiary structure or possibly aggregation. Variability in the electrophoretic migration of the myosin light chains has been noted in a number of studies on vertebrate muscle proteins (6, 13) and may reflect an aspect of their structure.

On two-dimensional gels the in vitro translation products of MLC2 mRNA are seen to contain multiple isoforms that co-migrate exactly with isoforms of the polypeptide identified by Takano-Ohmuro et al. (38) as MLC2. These isoforms are spots 147, 148, and 149 on the two-dimensional gels of Mogami et al. (18), which are frequently used as the standard reference for *Drosophila* myofibrillar proteins. Parker et al. (24) identify the product of in vitro translation as spot 181, apparently on the basis of its molecular mass as estimated by gel migration. Spot 181 migrates significantly ahead of both MLC2 and the in vitro translation product of Dmlla when these proteins are fractionated together in a gel.

A second polypeptide is also synthesized in vitro by RNA hybrid-selected by the MLC2 coding region. This polypeptide is smaller (18,000 D) and has the same isoelectric focusing point as MLC2, but, unlike the 30,000-D in vitro translation product, it does not co-migrate with any fibrillar muscle protein on two-dimensional gels. Parker et al. (24) have also noted the synthesis of a smaller polypeptide in cell-free translations. These authors suggest that this protein is identical to spot 184, again apparently on the basis of gel migration in one-dimensional gels. However, we find that the smaller translation product does not co-migrate with spot 184 on two-dimensional gels. As mentioned earlier, the 30,000- and 18,000-D in vitro translation products have identical isoelectric points, whereas spot 184 is somewhat more acidic than spots 147, 148, and 149, the spots with which the 30,000-D polypeptide isoforms co-migrate.

There is strong evidence that this second polypeptide is an artifact of the in vitro translation system. The 18,000-D protein is not always synthesized in vitro and occasionally multiple smaller polypeptides with the same isoelectric focusing point as the 30,000-D protein are observed. The synthesis of extra polypeptides varies with the rabbit reticulocyte lysate used. These products could result either from premature termination of translation or from preferential breakdown in the lysate.

We detect no differences between the nucleotide sequences of embryonic MLC2 cDNAs and the sequence reported for pupal MLC2 cDNA (24). This result suggests that the MLC2 isoforms encoded by the pupal and embryonic hybrid-selected RNAs are identical in amino acid sequence and probably arise through posttranslational modifications.

Our experiments show that *Drosophila* MLC2 resembles vertebrate light chains in having a blocked amino-terminal end. Apparently this posttranslational modification of the *Drosophila* MLC2 can be accomplished by the rabbit reticulocyte lysate since dansyl-chloride binding to the amino-terminal ends of the in vitro translation products is quite low unless acetylation is blocked in the lysate. This evidence that the reticulocyte lysate can perform at least one ap-

propriate posttranslational modification of the *Drosophila* MLC2 raises the question of whether the two isoforms of MLC2 seen in our in vitro translation experiments represent different primary translation products or different posttranslational modifications. Takano-Ohmuro et al. (38) have isolated two isoforms of MLC2 from *Drosophila* tubular muscle and two forms from fibrillar muscle. All of the isoforms have an apparent mass of 30,000 D but differ slightly in pI. The authors report that preliminary studies of partial proteolytic digests suggested that the MLC2 isoforms are produced by posttranslational modification in vivo. The cDNA sequence results strongly support this suggestion. The coding region of our embryonic cDNA is identical to the coding region of the pupal cDNAs (24), although Takano-Ohmuro et al. (38) report that there are different isoforms in these stages. If there is posttranslational modification in vivo, the rabbit reticulocyte lysate must be able to perform some similar modifications in our in vitro translations. On the other hand, since hybrid-selections do not yield the exact set of isoforms seen in vivo, there must be posttranslational modifications that the rabbit reticulocyte lysate cannot make.

Our experiments indicate that the *Drosophila* MLC2 gene is not a member of a dispersed multigene family such as the actins or the tubulins (5, 7, 17, 30, 40). The clone Dmlla hybridizes to a single site, at 99E1-3 on chromosome 3, even after extremely long autoradiographic exposure times. In contrast, the additional members of the α- and β-tubulin multigene families were readily detected after much shorter exposures in similar experiments when one member of the gene family was used as the hybridization probe (17; Valgeirsdottir, K., D. Mischke, and M. L. Pardue, unpublished observations). Thus, any genes with significant homology to the Dmlla coding sequence must lie in the 99E1-3 region. Since restriction maps and nucleotide sequences do not indicate the presence of other MLC2 genes in 99E1-3, we conclude that MLC2 is encoded by a single gene in *D. melanogaster*. Our experiments show no homology of the MLC2 sequences with those of the *Drosophila* MLC3 gene at 98B (4). Although both 98B and 99E are close to the end of the right arm of chromosome 3, the two genes must be separated by several hundred kilobases of DNA. In vertebrates, each molecule of myosin has one pair of alkalai light chains (MLC1 and MLC3) and one pair of MLC2 chains, suggesting that there is coordinate control of expression of myosin light chains. If the same situation holds in *Drosophila*, the myosin light chain genes are another example of coordinately expressed Drosophila genes that are not clustered at a single chromosomal site and hence must be regulated over some distance.

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