Analysis of Drosophila Paramyosin: Identification of a Novel Isoform Which Is Restricted to a Subset of Adult Muscles

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Abstract. In this report we show that Drosophila melanogaster muscles contain the standard form of the thick filament protein paramyosin, as well as a novel paramyosin isoform, which we call miniparamyosin. We have isolated Drosophila paramyosin using previously established methods. This protein is ≈105 kD and cross-reacts with polyclonal antibodies made against Caenorhabditis elegans or Heliocopris dilloni paramyosin. The Heliocopris antibody also cross-reacts with a ≈55-kD protein which may be miniparamyosin. We have cloned and sequenced cDNAs encoding both Drosophila isoforms. Standard paramyosin has short nonhelical regions at each terminus flanking the expected alpha-helical heptad repeat seen in other paramyosins and in myosin heavy chains. The COOH-terminal 363 amino acids are identical in standard and miniparamyosin. However, the smaller isoform has 114 residues at the NH2 terminus that are unique as compared to the current protein sequence data base. The paramyosin gene is located at chromosome position 66E1. It appears to use two promoters to generate mRNAs that have either of two different 5' coding sequences joined to common 3' exons. Each protein isoform is encoded by two transcripts that differ only in the usage of polyadenylation signals. This results in four size classes of paramyosin mRNA which are expressed in a developmentally regulated pattern consistent with that observed for other muscle-specific RNA's in Drosophila. In situ hybridization to Drosophila tissue sections shows that standard paramyosin is expressed in all larval and adult muscle tissues whereas miniparamyosin is restricted to a subset of the adult musculature. Thus miniparamyosin is a novel muscle-specific protein that likely plays a role in thick filament structure or function in some adult muscles of Drosophila.

The force required to produce muscular movement is generated by contractile proteins that are organized into highly ordered filamentous arrays. Typically, a variety of muscle types exist within an organism, each with different physiological requirements and concomitant differences in ultrastructure. Protein isoforms of each of the structural components of muscle are believed to accommodate these various functional requirements (Epstein and Fishman, 1991). To understand how muscle is fashioned into a biological force generator, it is necessary to identify all of the proteins involved, including the muscle-specific isoforms of each protein, and elucidate the role of specific polypeptide sequences in assembly and function.

Paramyosin is a thick filament protein found in all invertebrates which has no known vertebrate homologue. This protein is a rodlike molecule with high alpha-helical content in which two ≈100 kD monomers interact to form a coiled coil (Cohen and Holmes, 1963; Lowey et al., 1963; McCubbin and Kay, 1968). Dimers assemble in a "gap-overlap" manner, building into a helical-net structure (Bear, 1944; Bear and Selby, 1956) which occupies a portion of the core of a thick filament, directly underlying myosin (Cohen et al., 1971; Szent-Györgyi et al., 1971; Nonomura, 1974; Epstein et al., 1985). Paramyosin filaments are bipolar and interact strongly with myosin, specifically with the alpha-helical rod of light meromyosin and not with heavy meromyosin, suggesting that the paramyosin core provides the foundation for assembly of myosin into the thick filament (Szent-Györgyi et al., 1971; Bennett and Elliott, 1984; Epstein et al., 1977; Harris and Epstein, 1977).

In molluscs, paramyosin may play a role in the specialized function described as catch, in which some muscle types can generate strong isometric contractions for long periods of time, using very little energy (Twarog, 1976; Szent-Györgyi et al., 1971; Cohen et al., 1971). However, paramyosin is present in other invertebrate muscles which do not display catch-like characteristics (Bullard et al., 1973; Waterston et al., 1974; Levine et al., 1983; Hinkel-Aust et al., 1990). Caenorhabditis elegans paramyosin is required for proper assembly and function of the body wall muscle (Waterston et al., 1977), and there is indirect evidence that paramyosin is involved in determining thick filament length and stability (Ikemoto and Kawaguti, 1967; Mackenzie and Epstein, 1980). Nevertheless, the precise function of paramyosin remains unclear.

We have chosen to study paramyosin in the genetically tractable organism Drosophila melanogaster. In this report we describe the purification of Drosophila paramyosin pro-
tein, the cloning of paramyosin cDNAs, and the tissue-specific distribution of paramyosin mRNAs. We show that *Drosophila* produces a standard ~105-kD paramyosin protein and transcripts that encode this protein accumulate in all larval and adult muscle tissues. Surprisingly, *Drosophila* also produces a novel form of paramyosin that is ~55 kD and has a unique NH$_2$-terminal sequence. Transcripts encoding this novel protein, which we call miniparamyosin, are muscle specific and are found in most pupal/adult muscles, but not in larval muscles. The paramyosin isoforms arise as a result of alternative RNA splicing.

**Materials and Methods**

**Purification of Drosophila Paramyosin Protein**

The ethanol precipitation method described by Levine et al. (1982) was modified to isolate *Drosophila melanogaster* paramyosin protein. Approximately 15 g of wild type (Canton S) larval or adult flies were ground to homogeneity in 50 ml of H buffer (0.1 M KCl, 40 mM Tris-HCl, pH 7.3, 10 mM EDTA, 1 mM DTT, 1 mM PMSF) in an ice-cold Waring blender for ~2 min. This material was further homogenized with five to six passes of a motor-driven dounce homogenizer (Eberbach, Ann Arbor, MI). The actomyosin was pelleted by centrifugation at 16,500 g for 10 min at 4°C. The pellet was resuspended in 50 ml of H buffer and centrifuged at 16,500 g for 10 min at 4°C. Reuse of supernatant for 10 min at 4°C. Actomyosin was preferentially denatured by the slow addition of 150 ml of 95% ethanol, 0.5 M DTT to the supernatant while stirring on ice. The denatured actomyosin and precipitated paramyosin were collected by centrifugation at 16,500 g for 30 min at 4°C. The pellet was resuspended in 4 ml of R buffer and then dialyzed overnight against 500 ml of R buffer at 4°C. The dialysate was centrifuged at 16,500 g for 30 min at 4°C, and the actomyosin pellet was discarded. To precipitate the paramyosin, 0.1 N HCl was added in a drop-wise fashion to the slowly stirring supernatant on ice, until the pH reached 5.8. At that point the solution was stirred on ice for 15 min and the precipitated paramyosin was pelleted by centrifugation at 16,500 g for 15 min at 4°C. The paramyosin pellet was subsequently dissolved in 3 ml of R buffer.

**Protein Gels and Western Blots**

One-dimensional SDS-polyacrylamide gels were run according to standard procedures (Sambrook et al., 1989). Two-dimensional protein gel electrophoresis was performed as described by Mogami et al. (1982). Protein gels were transferred to nitrocellulose or nylon membranes using an ABN (Emeryville, CA). Polyblot semi-dry electrobolter as per the manufacturer's recommendations. Western blots were performed using the protocol published in the Promega (Madison, WI) catalogue and applications guide (1988/89).

**Isolation of Drosophila Paramyosin cDNA Clones**

A bacteriophage xgt1 expression library (provided by Dr. P. Salvaterra, Beckman Research Institute, City of Hope, Duarte, CA) was made using poly A$^+$ RNA isolated from adult heads was screened with an antibody (provided by Dr. B. Bullard, EMBL, Heidelberg, Germany) against paramyosin protein from *Heliocoris dilloni* (dung beetle). Approximately 100,000 plaques were screened using the protocol published in the Promega catalogue and applications guide (1988/89). A *Drosophila* embryonic cDNA library made and provided by Dr. N. Brown (Brown and Kafatos, 1988) was screened for clones using a paramyosin cDNA insert isolated from the expression library screen (pPara; see Fig. 2). The full-length clone, C2-L, isolated in this screen was subsequently used as a DNA probe to screen the Salvaterra library for miniparamyosin cDNAs. Hybridization, probe preparation, and DNA library screening were performed by standard protocols (Sambrook et al., 1989).

**Subcloning and Sequencing**

The cDNA clones isolated from the *Drosophila* embryonic library were mapped for positions of restriction endonuclease cleavage using enzymes from Stratagene (La Jolla, CA). Promega, and Boehringer-Mannheim Biochemicals (Indianapolis, IN). cDNA inserts were subcloned using standard methods (Sambrook et al., 1989). For sequencing, both overlapping deletions and miniperiplasmic deletion were sequenced using the ExoII/Mung bean deletion kit from Stratagene) and cDNA sequence-specific primers (synthesized on a 380B instrument: Applied Biosystems, Foster City, CA) were used in conjunction with the Sequenase kit (United States Biochemicals, Cleveland, OH).

**RNA Isolation and Northern Blots**

To isolate RNA from embryonic, larval, and pupal stages, fertilized eggs were collected on grape juice plates (1:1 mixture of grape juice and water. 1.5% agar) for 2-4 h and were aged at 25°C. Adult flies were collected within eight hours post-eclosion. Total cellular RNA was isolated using a slightly modified version of the LiCl precipitation method of Sambrook et al. (1989). Briefly, the aged organisms were ground in 1 ml of lysis buffer (6 M urea, 3 M LiCl) in a 1 ml homogenizer (#411; Radiant Glass Technology, Monrovia, CA). The homogenate was transferred to a 1.5-ml tube, mixed with 0.5 ml of lysis buffer used to wash the homogenizer, and placed on ice for at least 2 h (preferably overnight). The RNA and total cellular debris were pelleted in a microcentrifuge for 10 min at 4°C. The pellet was dissolved in 300 ml of resuspension buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS). This was extracted twice with 300 ml of phenol/ chloroform (1:1, the phenol is equilibrated with 0.1 M Tris-base and contains 0.5% 8-hydroxyquinoline; Sigma Chemical Co.), then once with chloroform. The RNA was precipitated by adding 30 ml of 3 M NaAc, 1 ml of ice cold 100% ethanol and storing at ~70°C for at least 30 min. The RNA was pelleted in a microcentrifuge, washed with 80% ethanol in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA), dried, and resuspended in TE. The optical density, at 260 nm, was measured for each RNA sample and concentrations determined. Northern transfer, hybridization and autoradiography were performed as described by Sambrook et al. (1989).

**In Situ Hybridization**

Biotinylated DNA preparation, salivary gland chromosome squashes, and in situ hybridization to polytene chromosomes were performed as detailed in Ashburner (1989). Preparation of tissue crosssections and hybridization were as described previously (O'Donnell et al., 1989).

**Probe Preparation**

RNA probes labeled with digoxigenin (Boehringer-Mannheim Biochemicals) were prepared from linear DNA templates by mixing 4.0 $\mu$l 5'-transcription buffer (supplied commercially along with the polymerase), 2.0 $\mu$l 100 mM DTT, 1.0 $\mu$l Inhibit-ACE (5 prime 3 prime, West Chester, PA), 2.0 $\mu$l 10x digoxigenin--UTP mix (5.3 mM Dig-UTP, 6.5 mM UTP), 1.0 $\mu$l 10 mM CTP, 1.0 $\mu$l 10 mM ATP, 1.0 $\mu$l 10 mM GTP, 2.0 $\mu$l DNA (0.3-10 ug), 1.0 $\mu$l 1:10 diluted [a32P]GTP (stock solution is 800 Ci/mM, 10 mCi/ml), 0.4 $\mu$l 0.10 (diethyl pyrocarbonate treated) and 1.0 $\mu$l RNA polymerase (T3 or T7, Promega, Madison, WI). The reaction was allowed to proceed at 37°C for 2 h. To remove the unincorporated nucleotides 80 $\mu$l of diethyl pyrocarbonate-treated H$_2$O were added to the reaction and the sample was passed over a prepared RNase-free Sephadex G50 column (5 prime 3 prime). The RNA was size reduced to an average of ~150-200 bases as per Cox et al. (1994). After precipitation the RNA was dissolved in 20 $\mu$l of H$_2$O (diethyl pyrocarbonate treated), then added to 280 $\mu$l of hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 7.5, 1x Denhardt's solution, 500 $\mu$g/ml yeast tRNA, and 10% dextran sulfate).

**Post-hybridization Washes and Signal Detection**

After hybridization, the slides were briefly washed twice in PBS (1x PBS (0.13 M NaCl, 0.007 M Na$_2$HPO$_4$, 0.003 M NaH$_2$PO$_4$), 10 mM MgCl$_2$) at 37°C. Excess RNA probe was removed by treatment with 20 $\mu$g/ml RNase A made fresh in 1x TNE (0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 30 min at 37°C. Subsequent washes were as follows: 15 min 1x TNE, 37°C; 15 min at 50°C in 2x SSC (20x SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0) 50% formamide, 10 mM Tris-HCl pH 7.5, 1x EDTA, 10 mM DTT; 15 min, at 50°C in 1x SSC, 50% formamide, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM DTT; 30 min at 37°C in 0.1x SSC, 50% formamide, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM DTT. Finally, the slides were washed briefly twice in PBSS (PBS + 0.1% saponin) (Sigma Chemical Co.). Dilute anti-digoxigenin antibody (1:500 in PBSS) was applied and the slides were incubated with coverslips for 2 h at
room temperature. To remove excess antibody the slides were washed with eight changes of PBSS, 5 min each. Signal was detected by washing twice with buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl2, pH 9.5) for 2 min at room temperature. Then 80 µl of color solution (4.5 µl NBT, 3.5 µl X-phosphate, 1.0 ml buffer 3) was added to each slide. Slides were covered and stored in a moist box at room temperature for up to 24 h. The reaction was stopped by rinsing the slides in TE for 5 min. The preparations were dehydrated through an ethanol series, washed in xylene, and subsequently mounted with Poly-mount (Polysciences, Warrington, PA) and coverslips.

Results

Drosophila melanogaster Has Two Forms of Paramyosin Protein

Our initial studies focused on the isolation and analysis of paramyosin protein from Drosophila melanogaster. Purification was performed using the ethanol precipitation method (Levine et al., 1982) with the addition of the protease inhibitor PMSF to both the homogenization and resuspension buffers. This greatly increased the yield of intact paramyosin protein. Acid precipitation of paramyosin (de Villafraanca and Haines, 1974) was added as the last step in the purification scheme. Our protocol yielded relatively pure Drosophila paramyosin (Fig. 1 A) with an apparent molecular weight of ~98 kD, a value similar to the molecular weights of paramyosin of other insects (Bullard et al., 1973) and molluscs (Olander et al., 1967; McCubbin and Kay, 1968; Woods, 1969).

A polyclonal antibody made against paramyosin from the nematode, Caenorhabditis elegans (kindly provided by R. Barstead and R. Waterston, Washington University School of Medicine, St. Louis, MO) cross-reacts with the Drosophila muscle-specific proteins (#19, 20, and 21 in the nomenclature of Mogami et al., 1982, and data not shown). The isoelectric point of proteins #19, 20, and 21 is ~6.2, in good agreement with that of purified paramyosin. Vinós et al. (1991) also detect these same spots using an antibody prepared against purified Drosophila paramyosin protein. These authors present evidence that one of these paramyosin proteins is post-translationally phosphorylated as is the case for paramyosin in C. elegans (Schriefer and Waterston, 1989) and molluscs (Achazi, 1979).

A polyclonal antibody (provided by B. Bullard) made against the 100-kD paramyosin protein from the dung beetle, Heliocopris dilloni (Bullard et al., 1977), cross-reacts strongly with a Drosophila protein of ~105 kD, providing further evidence that this protein is indeed paramyosin (Fig. 1 C). This antibody also cross-reacts with proteins of 200 kD and ~55 kD (Fig. 1 C). The 200-kD protein is myosin heavy chain (MHC) based upon binding by a polyclonal anti-MHC antibody, and upon the absence of this band in thoraces of MHC-null mutants (data not shown). The ~55-kD protein is weakly cross-reacting with the anti-paramyosin antibody and does not react with the anti-MHC antibody (not shown). In the subsequent text we present evidence that Drosophila has a 55-kD isomorph of paramyosin that has not previously been characterized in any other organism.

Isolation of cDNAs Encoding Drosophila Paramyosins

The antibody made against Heliocopris paramyosin was used to screen a λgt11 expression library made from Drosophila head RNA (kindly provided by P. Salvaterra). Since this anti-

1. Abbreviations used in this paper: AHM, abdominal hypodermal muscles; IFM, indirect flight muscles; MHC, myosin heavy chain; TDT, tergal depressor of the trochanter muscle.

Figure 1. Analysis of D. melanogaster paramyosin protein. (A) Purified Drosophila paramyosin protein isolated from adult flies is shown after gel electrophoresis in two dimensions. (B) Total proteins from the adult thorax separated on a SDS-10% polyacrylamide gel and stained with Coomassie blue. (C) An identical gel as shown in B was transferred to nylon membrane and reacted with a polyclonal antibody made against paramyosin from the dung beetle, Heliocopris dilloni. This antibody crossreacts with MHC (200 kD), standard paramyosin (*) and a 55-kD protein likely to be miniparamyosin (**)
body cross-reacts with MHC (Fig. 1 C), positive clones were rescreened with an anti-MHC antibody (kindly provided by D. Kiehart, Harvard University, Cambridge, MA), and those clones that did not react with the myosin antibody were chosen for further analysis. One such clone contained a 2-kb cDNA insert, which was subcloned and is denoted pPara (Fig. 2 A). DNA sequence obtained from each end of the pPara insert identified open reading frames that have a high degree of amino acid identity to the sequence of Caenorhabditis elegans paramyosin (Kagawa et al., 1989). Based upon its size and sequence, pPara encodes only a portion of standard Drosophila paramyosin.

To isolate full length cDNAs, we used gel-purified pPara insert to probe a cDNA library made from 12–24-h embryos (Brown and Kafatos, 1988). Larval muscle differentiation occurs during late embryogenesis, so it is expected that paramyosin transcripts would be represented in this library. Six paramyosin clones were isolated from ~100,000 primary colonies screened. Extensive restriction mapping and sequence analysis (see Fig. 5) showed that all six cDNAs encode all or a portion of the 105-kD paramyosin protein. Two of the cDNAs represent full-length transcripts and are shown in Fig. 2 A. Both of these cDNA inserts begin at the same 5' nucleotide, share the same consensus for a Drosophila translational start site (Cavener, 1987), and have the same coding potential. The 3'-untranslated regions are identical in both clones up to the last base pair of the DI-1 insert, after which the C2-1 clone continues for 432 bp. The DI-1 and C2-1 clones end 14 and 38 bp, respectively, downstream of consensus signals for polyadenylation. Evidence confirming the differential usage of the two polyadenylation signals is presented below.

Sequence analysis shows that none of the six cDNAs encode a 55-kD protein as is observed in Fig. 1 C. Furthermore, hybridization to Northern blots of total cellular RNA indicates that transcripts with the potential to encode the 55-kD paramyosin are not present during embryonic development; such transcripts share 3' but not 5' sequences with transcripts that produce the 105-kD paramyosin protein (see below). By screening the adult head cDNA library from which pPara was cloned, we isolated a full-length cDNA clone (mPM, Fig. 2 A) which encodes the 55-kD isoform of Drosophila paramyosin.

**A Single Paramyosin Gene Produces Four mRNA Size Classes**

The developmental time course of paramyosin gene expression was determined by using radiolabeled antisense RNA to probe Northern blots of total cellular RNA from staged Drosophila (Fig. 3). During embryogenesis, paramyosin transcripts of 3.2 and 3.6 kb are first detected at 10 to 12 h post-oviposition, with the expression levels increasing to a maximum at 16 to 20 h (Fig. 3 A). This pattern of expression is consistent with the observed time course of larval muscle differentiation and growth (Poulson, 1950; Crossley, 1978; Bate, 1990) as well as the early developmental expression of the Drosophila MHC gene (Rozeck and Davidson, 1983; Bernstein et al., 1986) and the troponin I gene (Barbas et al., 1991).

Paramyosin gene expression in larval, pupal, and adult stages occurs during the periods of larval muscle growth and during differentiation and maintenance of adult muscle tissues (Fig. 3 B). In addition to the 3.2- and 3.6-kb paramyosin mRNAs, very low levels of two new transcripts (2.0 and 2.4 kb) are detected during the late larval stages. As is the case for Drosophila MHC and troponin I transcripts, virtually no paramyosin mRNA accumulates during pupariation. This is the beginning of larval muscle histolysis, and adult muscles are not yet being formed (Crossley, 1978). Paramyosin mRNAs accumulate at maximal levels during mid to late pupal stages, and expression is maintained into adulthood. All four paramyosin mRNAs are detected during pupal and adult stages.

Fig. 4 shows that the four size classes of paramyosin mRNAs can be grouped into two pairs based upon their cod-
Figure 3. Developmental expression of the Drosophila paramyosin gene. Total cellular RNA was isolated from various developmental stages of the wild-type Drosophila strain Canton S. grown at 25°C. RNA (5 μg/lane) was separated by electrophoresis through a 1.0% agarose gel containing formaldehyde, blotted to nylon membrane and probed with radiolabeled antisense transcripts made from the full-length cDNA clone, C2-1. (A) Paramyosin RNA accumulation during embryogenesis, with h post-oviposition indicated. (B) Paramyosin RNA accumulation in larvae, pupae and adults: first instar larvae (48-h post-oviposition), second instar larvae (72-h post-oviposition), third instar larvae (96-h post-oviposition), early pupae (12-24 h post-pupariation), red eye pupae (=75 h post-pupariation), pharate adults (=100-h post-pupariation), 1-d-old adults, head RNA (from 2-4-d-old adults). Paramyosin mRNA size classes of 2.0, 2.4, 3.2, and 3.6 kb are shown (arrows). Approximately equal amounts of RNA are present in all lanes as detected by ethidium bromide staining (not shown).
Figure 4. Northern blots of *Drosophila* RNA probed with various portions of the paramyosin and miniparamyosin cDNAs. The positions of the probes used for each blot are shown at the bottom of the figure. Antisense RNA probes labeled with $^{32}$P were hybridized to total cellular RNA from the stages shown. Probe 1 contains 425 bp of the 5' sequence unique to the miniparamyosin cDNA, mPM. Probe 2 has 780 bp of sequence specific to the standard paramyosin cDNA, C2-1. The pattern of hybridization of these two probes indicates that the 2.0- and 2.4-kb mRNAs are probably using a different promoter than the 3.2- and 3.6-kb transcripts. Probe 3 is derived from C2-1, and is complementary to the last 30 bp of coding sequence and all sequences 3' of this position. This probe hybridizes to all four mRNA size classes. Probe 4 contains sequences distal to the first polyadenylation signal and hybridizes only to the 3.6- and 2.4-kb transcripts. Thus, all four transcripts have common 3' sequences and use one of two different polyadenylation sites.

codon. On a genomic Southern blot (not shown), this 1.6-kb fragment is the only band that hybridizes to probe 3 (Fig. 4) supporting the single gene hypothesis. Lastly, we have recently isolated genomic clones that contain most of the *Drosophila* paramyosin gene (Becker et al., unpublished data). The sequences unique to the 5' end of miniparamyosin are located just upstream of the common exon sequences and are within an intron for the standard paramyosin transcriptional unit. It thus appears that only a single paramyosin gene exists per haploid genome in *Drosophila*.

Structure of *Drosophila* Paramyosin and Miniparamyosin Protein Isoforms

Fig. 5A presents the entire sequence of the cDNA insert of clone C2-1 which is 3,624 bp in length. The only long open reading frame in this clone encodes a protein of 879 amino acids with a molecular weight of 102,337 daltons, and pI of 5.39. Posttranslational modifications of the paramyosin protein are observed in molluscs (Achazi, 1979) and in nematode (Schriefer and Waterston, 1989) and may account for the difference in the observed (105 kD) and predicted size of the *Drosophila* protein. The discrepancy between the observed (6.25) and the predicted isoelectric point likely results from the tendency of paramyosin to precipitate out of solution as it approaches its pI under isoelectric focusing conditions (H. Epstein, personal communication).

The Chou-Fasman (1978a,b) secondary structure program predicts that the NH$_2$ terminus and COOH terminus of the *Drosophila* paramyosin protein form random coil structures, while the remaining portion of the protein is predicted to form an alpha helix with 91% helical nature (Fig. 6). This value is very close to the helical content of paramyosins from *Drosophila* (Vin6s et al., 1991), and the dung beetle (Bullard et al., 1973) and molluscs (Cohen and Szent-Györgyi, 1957) as measured by circular dichroism. In *Drosophila* paramyosin the ratio of charged to apolar amino acids is 1.2, which is typical of proteins with an extended rod conformation (Co-
Figure 5. Sequence of the Drosophila paramyosin isoforms deduced from the nucleic acid sequences. (A) The DNA sequence of clone C2-1 (Fig. 2 A) encodes standard paramyosin. A portion of this sequence has recently been reported by Vinós et al. (1991). (B) The DNA sequence of clone mPM encodes miniparamyosin. Although both strands of the clones were sequenced only the coding strand is shown.
Figure 6. Comparison of the Drosophila paramyosin amino acid sequence to that of all other known paramyosin sequences. The paramyosin sequence of Drosophila melanogaster (Dm) is compared with paramyosin of two nematodes, Caenorhabditis elegans (Ce) (Kagawa et al., 1989) and Dirofilaria immitis (Di) (Limberger and McReynolds, 1991), and one trematode, Schistosoma mansoni (Sm) (Laclette et al., 1991). All paramyosin proteins have predicted non-helical NH2 and COOH termini (the first 32 residues and the last 24 residues in the sequence). Alpha-helical regions are aligned into heptad repeats, where hydrophobic amino acids predominantly occupy the first and fourth positions of the repeat (Fig. 6). This motif forms a hydrophobic face along the length of the molecule which provides the basis for a coiled-coil interaction (Crick, 1955). Drosophila paramyosin displays the 28 residue charge repeat first described for the rod portion of MHC (McClachlan and Karin, 1982) and found in other paramyosins (Cohen et al., 1987; Kagawa et al., 1989; Limberger and McReynolds, 1990). Local distortions in the helix occur at four positions throughout the MHC rod and at five positions in paramyosin. At these locations an extra amino acid or "skip residue" is added to the 28 residue...
repeat, and is thought to relieve some of the superhelical strain on the molecule (McLachlan and Karn, 1982, 1983). The positions of skip residues in Drosophila paramyosin are conserved with all other published paramyosin sequences (Fig. 6).

Drosophila paramyosin shares a high degree of amino acid identity to paramyosin sequences from other species. Nematoda paramyosins from Caenorhabditis elegans and Dirofilaria immitis are 92% identical with one another, and both of these proteins share 49% amino acid identity and are 79% conserved with Drosophila paramyosin. The other known paramyosin sequence is from the platyhelminth Schistosoma mansoni. This protein shares 33% identity and is 75% conserved with the Drosophila protein.

The mPM cDNA (Fig. 5 B) encodes a 477 residue protein, which we call miniparamyosin. This protein has a predicted molecular weight of 54,887 daltons and a pI of 7.83. The 363 COOH-terminal amino acids of miniparamyosin are identical to those found in standard paramyosin, having the heptad repeat, the 28-residue charge repeat and the conserved skip residues. The NH2-terminal 114 amino acids are unique in comparison to all other paramyosins. This portion of the protein does not have either an extended region of heptad repeat or the 28-residue charge repeat. Only two small regions, of approximately 20 residues each, are predicted to have an alpha-helical secondary structure (Fig. 2 B), yet the ratio of charged to apolar amino acids is 1.1, suggesting that this region may have an extended rod conformation. A search of the current protein and nucleic acid data bases reveals that the NH2-terminus of miniparamyosin does not have significant similarity to any peptide region with an ascribed function.

Both Paramyosin and Miniparamyosin mRNA's Accumulate in Muscle Tissues

The tissue-specific distribution of paramyosin mRNAs was determined by probing cryosections of larvae, pupae, and adults with digoxigenin-labeled antisense RNA's. Standard paramyosin mRNAs are detected in larval body wall muscles (data not shown) as well as in all muscle types of the adult (Fig. 7 C, E, and G) including the musculature associated with the sexual organs and viscera (not shown). Miniparamyosin transcripts are not detected in larval muscle by in situ hybridization (data not shown), which correlates with the very low levels of these transcripts observed on Northern blots of larval RNA (Fig. 3). Miniparamyosin transcripts are detected in all of the adult musculature except for the indirect flight muscles (IFM) (Fig. 7 D and F) and a set of temporary abdominal muscles (Fig. 7 H).

Since both standard paramyosin and miniparamyosin probes yield approximately equal signals in the tergal depressor of the trochanter muscle (TDT) (Fig. 7, E and F), we can use this as a baseline for comparison of relative hybridization levels. In all other tissues which express both isoforms, the amount of signal detected by the standard paramyosin probe is higher than the signal derived from the miniparamyosin probe. Thus the various proboscis, leg, and abdominal body wall muscles accumulate less miniparamyosin transcript relative to standard paramyosin transcript than in the TDT. In the IFM the intensity of signal from the standard paramyosin probe is much less relative to standard paramyosin in other muscle types and is also very low compared to MHC RNA levels in the IFM (Fig. 7, B and C). No miniparamyosin RNA is observed in the IFM (Fig. 7, D and F). However, based upon the relative differences in signal intensity observed with the standard paramyosin and miniparamyosin probes in other adult muscles, it is possible that miniparamyosin is present but not detectable in the IFM. In contrast, the temporary abdominal hypodermal muscles (AHM) produce a high level of signal from both the MHC and standard paramyosin probes, even though no signal is detected using the miniparamyosin probe. This may be due to the unique ontogeny of these muscles (see Discussion). Our data indicate that, aside from the IFM, miniparamyosin expression occurs in all the permanent muscles of the adult.

Discussion

In this report we show that, like other invertebrates, Drosophila melanogaster has a standard paramyosin protein which is ~102 kD. Surprisingly, Drosophila also has a novel ~55-kD isoform of paramyosin, that we call miniparamyosin. The newly discovered isoform has 363 COOH-terminal amino acids in common with standard paramyosin, and 114 NH2-terminal residues which are unique as compared to the current protein sequence data base. Standard paramyosin has been well characterized in several invertebrates (Weisell and Szent-Györgyi, 1975; Bullard et al., 1973; Waterston et al., 1977; Kagawa et al., 1989; Limberger and McReynolds, 1990; Vinöös et al., 1991), and there is some evidence for limited heterogeneity of this molecule (Walker and Stewart, 1975; Costello and Govind, 1984). However, this is the first report of a miniparamyosin protein that differs so substantially in size and sequence from the standard form.

Drosophila Paramyosin Isoforms Accumulate at Different Levels in a Muscle-specific Manner

Transcripts encoding standard Drosophila paramyosin are expressed in a pattern that matches the developmental time course of muscle differentiation and growth, and are detectable in all muscle tissues (Figs. 3, A and B and 7 C). Miniparamyosin transcripts are muscle specific as well, and are detected in all adult muscles except the IFM and the temporary AHM (Fig. 7 D). The AHM derive from the fusion of pupal myoblasts with existing larval muscle cells which have lost their contractile apparatus (Crossley, 1978). Following fusion the reconstruction of the AHM during mid-pupation produces muscles that have the same structure and supercontractile properties as do the muscles of the larval body wall. The AHM aid in adult eclosion from the pupal case, subsequently help to inflate the wings, and then degenerate within the first two days of adulthood. Since standard paramyosin mRNAs are found at high levels in these muscles in red eye pupae, but miniparamyosin transcripts are not detected (Fig. 7, G and H), it is possible that the pre-existing larval nuclei direct the adult nuclei to express the larval muscle gene repertoire. A similar situation occurs when nonmuscle nuclei are stimulated to express muscle genes after being fused with differentiated muscle cells in vitro (Blau et al., 1985).

In the IFM, the other tissue in which miniparamyosin transcripts are not detectable, only low levels of the standard paramyosin mRNAs are observed (Fig. 7, E and F). Previous studies have shown that relatively small amounts of the
102-kD paramyosin protein accumulate in Drosophila IFM (Beinbrech et al., 1985; Vinós et al., 1991).

High levels of both standard paramyosin and miniparamyosin mRNAs are found in the TDT (Fig. 7, E and F). This may be a requirement for the unique physiology of this muscle, which generates tension slowly followed by a quick release (Chapman, 1982). Protein data from Peckham et al. (1990) agree with our in situ hybridization results, in that a 55-kD protein (likely to be miniparamyosin) is found in the TDT at levels approximately equivalent to that of standard paramyosin; the 55-kD protein is not present in the IFM, although low levels of standard paramyosin are.

**Paramyosin Protein Levels Correlate with Thick Filament Structure**

The low level of paramyosin in Drosophila IFM correlates well with the apparently hollow nature of thick filaments in this muscle (Beinbrech et al., 1985; O'Donnell and Bernstein, 1988). A direct relationship between increasing paramyosin content and increased density of thick filament cores can be made for the IFM filaments of other insects as well (Bullard et al., 1973; Reedy et al., 1973; Ashurst and Cullen, 1977; Beinbrech et al., 1985, 1988; Hinkel-Aust et al., 1990). The amount of paramyosin in thick filaments may also reflect its distribution within the filaments. In molluscan muscles and nematode body wall muscles paramyosin appears to be present throughout the length of the thick filament, and these filaments have electron-dense cores (Szent-Györgyi et al., 1971; Waterston et al., 1977; Epstein et al., 1985). However, paramyosin is apparently restricted to the central M line region of thick filaments in nematode pharyngeal muscles (Waterston et al., 1977). Thick filaments in these muscles appear to be hollow in the polar region. By analogy, the Drosophila TDT and other tubular muscles have paramyosin protein throughout their thick filaments (Vinós et al., 1991), while “hollow” IFM thick filaments may have standard paramyosin localized to the M line.

**Possible Functions of the Drosophila Paramyosin Isoforms**

Based upon the COOH-terminal sequence identity shared with standard paramyosin, it is probable that miniparamyosin is also a thick filament component. Miniparamyosin may be the Drosophila analog of one of the “core” proteins that underlies paramyosin in C. elegans thick filaments (Epstein et al., 1985, 1988). Alternatively, miniparamyosin could be a part of the Drosophila paramyosin filament. Paramyosin isoforms might form heterodimers or may be spatially separated, like MHCs A and B in thick filaments of C. elegans body wall muscle (MHC A is localized to the central, bipolar portion of the thick filament while MHC B is present in the polar regions) (Mackenzie et al., 1978). Determining the location of the Drosophila paramyosin isoforms should eventually be possible using antibodies made to peptides specific to each isoform. It will also be interesting to know if miniparamyosin is present in other organisms. Muscle-specific proteins in the 50- to 60-kD range have been identified but not well characterized in other invertebrate species (Costello and Govind, 1984; Epstein et al., 1988).

As in the case for the myosin molecule (Kuczmarski and Spudich, 1980; Winkelman and Bullard, 1977; Beinbrech, 1977; Bárány and Bárány, 1980), phosphorylation of the paramyosin isoforms may modulate the role that these proteins have in assembly and/or function of thick filaments. Paramyosin from C. elegans is phosphorylated at serine residues in the NH2-terminal non-alpha helical portion of the molecule (Schiere and Waterston, 1989), and the standard form of Drosophila paramyosin is phosphorylated (Vinós et al., 1991). Both isoforms of Drosophila paramyosin have high numbers of serine residues in their NH2 termini, and these may serve as phosphorylation sites.

Currently, there are no mutations at the Drosophila paramyosin locus. However, mutations in the C. elegans paramyosin gene have been isolated (Brenner, 1974; Waterston et al., 1977), and mutant analysis indicates that single amino acid changes in the paramyosin molecule can disrupt body wall muscle structure and function (Gengyo-Ando and Kagawa, 1991). Similar studies in Drosophila, coupled with introduction of in vitro engineered paramyosin genes into the Drosophila germine (Rubin and Spradling, 1982), should pinpoint sequences required for proper thick filament assembly and muscular function for both paramyosin and miniparamyosin. This approach will also provide the opportunity to determine whether expression of one paramyosin isoform is sufficient to rescue muscle defects in paramyosin/miniparamyosin null mutants.

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Figure 7. In situ hybridization to parasagittal cryosections of late-stage pupae shows the tissue-specific distribution of paramyosin and miniparamyosin transcripts. RNA probes labeled with digoxigenin-UTP were hybridized to sections of wild-type (Canton S.) pupae. In all cases anterior is to the right and dorsal is up. (A) Sense-strand control probe specific to the 5′ unique sequences in mPM (template is probe 1 in Fig. 4). No hybridization signal is detected. Identical results were obtained when sense-strand probes specific for standard paramyosin (probe 2, Fig. 4) and all myosin heavy chain (MHC) transcripts were used (not shown). (B) Antisense probe complementary to exons 4, 5, and 6 in MHC transcripts detects signal in all muscle types. (C, E, and G) Antisense probe specific to standard paramyosin (probe 2, Fig. 4). (D, F, and H) Antisense probe specific to miniparamyosin (probe 1, Fig. 4). (A–D) Whole pupae. (E and F) Magnification of the thoracic section. (G and H) Magnification of the abdominal region. IFM and temporary AHM hybridize to antisense probes from MHC and paramyosin, but not miniparamyosin. Proboscis (P), jump (TDT), leg (L), and intra-abdominal muscles (IM) hybridize to all three antisense probes.
References

Achazi, R. K. 1979. Phosphorylation of molluscan paramyosin. Pflügers Arch. 379:197-201.

Ashburner, M. 1989. Drosophila: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 434 pp.

Ashburner, D. E., and M. J. Cullen. 1977. The structure of fibrillar flight muscle. In Insect flight muscle. R. T. Tregear, editor. North-Holland, Amsterdam. 9-14.

Bárdy, M., and K. Bárdy. 1980. Phosphorylation of the myofibrillar proteins. Annu. Rev. Physiol. 42:275-292.

Barbas, J. A., J. Galceran, I. Krah-Jentgens, J. L. de la Pompa, I. Canaal, O. Pongs, and A. Ferrus. 1991. Troponin I is encoded in the haplolethal region of the Shaker-like complex of Drosophila. Genes & Dev. 5:132-140.

Bates, M. 1990. The embryonic development of larval muscles in Drosophila. Development. 110:791-804.

Bear, R. S. 1944. X-ray diffraction studies on protein fibers - II. Feather rachis, porcine quill tip and clam muscle. J. Am. Chem. Soc. 66:2043-2050.

Bear, R. S., and C. C. Selby. 1956. The structure of paramyosin fibrils according to X-ray diffraction. J. Biophys. Biochem. Cytol. 2:55-69.

Beinbrech, G. 1977. Phosphorylation and the influence on the ATPase of the myosin of insect flight muscle. In Insect flight muscle. R. T. Tregear, editor. North-Holland, Amsterdam. 291.

Beinbrech, G., F. T. Ashton, and F. A. Pepe. 1988. Invertebrate myosin filament: subfilament arrangement in the wall of tubular filaments of insect flight muscles. J. Mol. Biol. 201:557-565.

Blau, H. M., U. Muller, and W. Sasse. 1985. Paramyosin content and thick filament structure in insect muscles. Cell Tissue Res. 241:607-614.

Bennett, P. M., and A. Elliott. 1984. “Splicing” of paramyosin filaments. J. Mol. Biol. 175:103-109.

Bernstein, S. I., C. J. Hansen, K. D. Becker, D. R. Wassenberg II, E. S. Roche, J. J. Donady, and C. P. Emerson, Jr. 1986. Alternative RNA splicing generates transcripts encoding a thorax-specific isoform of Drosophila melanogaster myosin heavy chain. Mol. Biol. Cell. 6:251-251.

Cavener, D. R. 1987. Comparison of the consensus sequence flanking translation start sites in Drosophila and vertebrates. Nucleic Acids Res. 15:67-71.

Chapman, R. F. 1982. Locomotion. In The insect, structure and function. Harwood Academic Publishers, Cambridge, Massachusetts. 169-196.

Chou, P. Y., and G. D. Fasman. 1978a. Empirical predictions of protein configuration. Annu. Rev. Biochem. 47:251-276.

Chou, P. Y., and G. D. Fasman. 1978b. Prediction of secondary structure of proteins from their amino acid sequence. Advan. Enzymol. 47:45-148.

Cohen, C., and K. Holmes. 1963. X-ray diffraction evidence for alpha-helical coiled-coils in native muscle. J. Mol. Biol. 6:423-432.

Cohen, C., D. A. Lanar, and D. A. Parry. 1987. Amino acid sequence and structural repeats in schistosome paramyosin match those of myosin. Bioessays. 7:1-16.

Cohen, C., and D. A. Parry. 1986. alpha-Helical coiled-coils—a widespread motif in proteins. Trends Biochem. Sci. 11:245-248.

Cohen, C., and D. A. Parry. 1990. alpha-Helical coiled coils and bundles: how to design an alpha-helical protein. Protein Struct. Funct. Gen. 7:1-15.

Coh, A., and G. Szent-Györgyi. 1957. Optical rotation and helical polypeptide chain configuration in alpha-proteins. J. Amer. Chem. Soc. 79:243.

Cohen, C., and D. A. Parry. 1980. alpha-Helical coiled-coils and bundles: how to design an alpha-helical protein. Protein Struct. Funct. Gen. 7:1-15.

Costello, W. J., and C. K. Govind. 1984. Contractile proteins of fast and slow insect flight muscles. Dev. Biol. 104:434-440.

Fenner, M. J., C. Martinez-Cruzado, C. Swimmer, D. King, and F. C. Kafatos. 1980. Evolution of the autonomously controlled cluster in Drosophila. II. Chorion gene expression and sequence comparisons of the s16 and s19 genes in evolutionarily distant species. J. Mol. Evol. 29:108-125.

Gengyo-Ando, K., and H. Kagawa. 1991. Single charge change on the helical surface of the paramyosin rod dramatically disrupts thick filament assembly in Caenorhabditis elegans. J. Mol. Biol. 219:429-441.

Hinkel-Aust, S., P. Hinkel, and C. Beinbrech. 1987. Elongating effect of tropomyosin A on the thick filaments in the long-sarcomere muscle of the horse-shoe crab. Proc. Japan Acad. 63:974-979.

Kagawa, H., K. Gengyo, A. M. McLachlan, S. Brenner, and J. K. 1989. Paramyosin gene (unc-15) in Caenorhabditis elegans: nucleotide sequence and models for thick filament structure. J. Mol. Biol. 207:311-333.

Kuczmerski, E. R., and J. A. Spudich. 1980. Regulation of myosin self-assembling: phosphorylation. J. Biol. Chem. 255:1161-1166.

Kuczmarski, E. R., and L. A. Spudich. 1980. Regulation of myosin self-assembling: phosphorylation. J. Biol. Chem. 255:1161-1166.

Mackenzie Jr., J. M., F. Schachat, and H. F. Epstein. 1978. Immunocytological localization of two myosins within the same muscle cells in Caenorhabditis elegans. Cell. 15:413-419.

McLachlan, A. D., and K. A. Wingate. 1983. Structure and paramyosin content of tarantula thick filament structures. J. Cell Biol. 97:186-195.

McLemore, R. J., and L. A. McKearn. 1990. Filarial paramyosin: cDNA sequence from Dirofilaria immitis and Onchocerca volvulus. Mol. Biochem. Parasitol. 38:271-280.

McCollum, W. J., and C. Webster. 1985. Plasticity of the differentiated state. Science (Wash. DC). 230:738-766.

Mackenzie, J. M., and H. F. Epstein. 1980. Paramyosin is necessary for determination of nematode thick filament length in vivo. Cell. 22:747-755.

Mackenzie Jr., J. M., F. Schachat, and H. F. Epstein. 1978. Immunocytological localization of two myosins within the same muscle cells in Caenorhabditis elegans. Cell. 15:413-419.

McCubbin, W. D., and C. M. Kay. 1968. The subunit structure of fibrous muscle proteins as determined by osmometry. Biochim. Biophys. Acta 44:287-296.

Lowey, S., J. Kucera, and A. Holzter. 1963. On the structure of the paramyosin molecule. J. Mol. Biol. 7:234-244.

Mackenzie, J. M., and H. F. Epstein. 1980. Paramyosin is necessary for determination of nematode thick filament length in vivo. Cell. 22:747-755.

Mackenzie Jr., J. M., F. Schachat, and H. F. Epstein. 1978. Immunocytological localization of two myosins within the same muscle cells in Caenorhabditis elegans. Cell. 15:413-419.

McCubbin, W. D., and C. M. Kay. 1968. The subunit structure of fibrous muscle proteins as determined by osmometry. Biochim. Biophys. Acta 44:239-248.

O'Donnell, P. T., and S. I. Bernstein. 1988. Molecular and ultrastructural defects in a Drosophila myosin heavy chain mutant: differences in muscle function produced by similar thick filament abnormalities. J. Cell Biol. 107:2601-2612.

O'Donnell, P. T., V. L. Collier, K. Mogami, and S. I. Bernstein. 1989. Ultrastructural and molecular analysis of homozygous- viable Drosophila melanogaster muscle mutants indicate there is a complex pattern of myosin heavy chain isoform distribution. Genes & Dev. 3:1223-1246.

Olander, J. M., F. E. Emerson, and A. Holtzer. 1967. On the dissociation and association of the polypeptide chains of tropomyosin and paramyosin. J. Amer. Chem. Soc. 89:3058-3059.

Peckham, M., J. E. Molloy, J. C. Sparrow, and D. C. S. White. 1990. Physiological properties of the dorsal longitudinal flight muscle and the tergal depressor of the thoracic muscle of Drosophila melanogaster. J. Muscle Res. Cell Motility 11:203-215.

Poulsen, D. F. 1950. Histogenesis, organogenesis and differentiation in the embryo of Drosophila melanogaster meigen. In Biology of Drosophila. M. De-
Reedy, M. K., G. F. Bahr, and D. A. Fischman. 1973. How many myosins per cross-bridge? I. Flight muscle myofibrils from the blowfly, Sarcophaga bullata. Cold Spring Harbor Symp. Quant. Biol. 37:397-422.

Rozek, C. E., and N. Davidson. 1983. Drosophila has one myosin heavy chain gene with three developmentally regulated transcripts. Cell. 32:32-34.

Rubin, G. M., and A. C. Spradling. 1982. Genetic transformation of Drosophila with transposable element vectors. Science (Wash. DC). 218:348-353.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 1,500 pp.

Schriefer, L. A., and R. H. Waterston 1989. Phosphorylation of the N-terminal region of Caenorhabditis elegans paramyosin. J. Mol. Biol. 207:451-454.

Stafford, W. F., and D. A. Yphantis. 1972. Existence and inhibition of hydrolytic enzymes attacking paramyosin in myofibrillar extracts of Mercenaria mercenaria. Biochem. Biophys. Res. Commun. 49:848-854.

Szent-Györgyi, A. G., C. Cohen, and J. Kendrick-Jones. 1971. Paramyosin and the filaments of molluscan "catch" muscles. II. Native filaments: Isolation and characterization. J. Mol. Biol. 56:239-258.

Twarog, B. M. 1976. Aspects of smooth muscle function in molluscan catch muscle. Current Topics Physiol. 56:829-838.

de Vilafranca, G. W., and V. E. Haines. 1974. Paramyosin from arthropod cross-striated muscle. Comp. Biochem. Physiol. 47B:9-26.

Vinós, J., A. Domingo, R. Marco, and M. Cervera. 1991. Identification and characterization of Drosophila melanogaster paramyosin. J. Mol. Biol. 220:687-700.

Walker, I. D., and M. Stewart. 1975. Paramyosin: chemical evidence for chain heterogeneity. FEBS (Fed. Eur. Biol. Sci.) Lett. 58:16-18.

Waterston, R. H., H. F. Epstein, and S. Brenner. 1974. Paramyosin of Caenorhabditis elegans. J. Mol. Biol. 90:285-290.

Waterston, R. H., R. M. Fishpool, and S. Brenner. 1977. Mutants affecting paramyosin in Caenorhabditis elegans. J. Mol. Biol. 177:679-697.

Weisel, J. W., and A. W. Szent-Györgyi. 1975. The coiled-coil structure: identity of the two chains of Mercenaria paramyosin. J. Mol. Biol. 98:665-673.

Winkelman, L., and B. Bullard. 1977. Phosphorylation of a light subunit of locust myosin. In Insect flight muscle. R. T. Tregear, editor. North-Holland, Amsterdam. 285-289.

Woods, E. F. 1969. Subunit structure of oyster paramyosin. Biochem. J. 113:39-47.