

# 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  $\approx 105$  kD and cross-reacts with polyclonal antibodies made against *Caenorhabditis elegans* or *Heliocoprpris dilloni* paramyosin. The *Heliocoprpris* antibody also cross-reacts with a  $\approx 55$ -kD protein which may be miniparamyosin. We have cloned and sequenced cDNA's 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 NH<sub>2</sub> terminus that are unique as com-

pared to the current protein sequence data base. The paramyosin gene is located at chromosome position 66E1. It appears to use two promoters to generate mRNA's 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*.

**T**HE 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 Fischman, 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  $\sim 100$  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 product 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<sub>2</sub>-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 16 g of wild type (Canton S.) larvae 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. Following resuspension in 50 ml of R buffer (0.6 M KCl, 40 mM Tris-HCl, pH 7.3, 10 mM EDTA, 1 mM DTT, 1 mM PMS), the sample was slowly stirred on ice for 30 min. The precipitate was removed by centrifugation at 16,500 g 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 electroblotter 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  $\lambda$ gt11 expression library (provided by Dr. P. Salvaterra, Beckman Research Institute, City of Hope, Duarte, CA) made using poly A<sup>+</sup> RNA isolated from adult heads was screened with an antibody (provided by Dr. B. Bullard, EMBL, Heidelberg, Germany) made against paramyosin protein from *Heliocopriss 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, C2-1, 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 (made using the ExoIII/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; Radnoti 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  $\mu$ l of resuspension buffer (10 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% SDS). This was extracted twice with 300  $\mu$ l of phenol/chloroform (1:1, the phenol is equilibrated with 0.1 M Tris-base and contains 0.01% 8-hydroxy quinoline; Sigma Chemical Co.), then once with chloroform. The RNA was precipitated by adding 30  $\mu$ l 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 cryosections 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 $\times$  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 10 $\times$  digoxigenin-UTP mix (3.5 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 (0.5–1.0  $\mu$ g), 1.0  $\mu$ l 1:10 diluted [ $\alpha$ -<sup>32</sup>P]GTP (stock solution is 800 Ci/mM, 10 mCi/ml), 4.0  $\mu$ l H<sub>2</sub>O (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<sub>2</sub>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. (1984). After precipitation the RNA was dissolved in 20  $\mu$ l of H<sub>2</sub>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, 1 $\times$  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 PBSSM (1 $\times$  PBS [0.13 M NaCl, 0.007 M Na<sub>2</sub>HPO<sub>4</sub>, 0.003 M NaH<sub>2</sub>PO<sub>4</sub>], 10 mM MgCl<sub>2</sub>) at 37°C. Excess RNA probe was removed by treatment with 20  $\mu$ g/ml RNase A made fresh in 1 $\times$  NTE (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 1 $\times$  NTE, 37°C; 15 min at 50°C in 2 $\times$  SSC (20 $\times$  SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 50% formamide, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM DTT; 15 min, at 50°C in 1 $\times$  SSC, 50% formamide, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM DTT; 30 min at 37°C in 0.1 $\times$  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 MgCl<sub>2</sub>, pH 9.5) for 2 min at room temperature. Then 80  $\mu$ l of color solution (4.5  $\mu$ l NBT, 3.5  $\mu$ l 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 were 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 Villafranca 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  $\sim$ 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* protein (data not shown). Unlike the purified protein, the molecular weight of *Drosophila* paramyosin in whole-organism homogenates of both larvae and adults is 105–107 kD. Recently, Vinós et al. (1991) have also purified paramyosin protein from *Drosophila melanogaster*. These authors report the molecular weight of the purified protein to be 107 kD. The smaller size of our purified protein is likely because of proteolysis, since paramyosin is particularly susceptible to

degradation once it is separated from myosin (Levine et al., 1982; Stafford and Yphantis, 1972; Waterston et al., 1974).

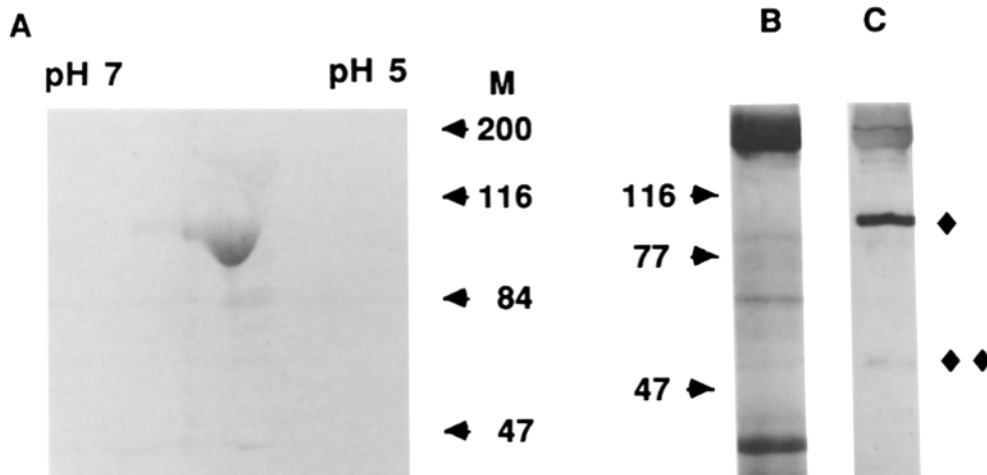
Purified paramyosin protein migrates at an isoelectric point of  $\sim$ 6.25 on a two-dimensional gel (Fig. 1 A). On a Western blot of a two-dimensional gel containing whole adult fly homogenate, the antibody to *C. elegans* paramyosin reacts with a triad of closely spaced *Drosophila* muscle-specific 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  $\sim$ 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, *Helicoverpa dilloni* (Bullard et al., 1977), cross-reacts strongly with a *Drosophila* protein of  $\sim$ 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  $\sim$ 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  $\sim$ 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 isoform of paramyosin that has not previously been characterized in any other organism.

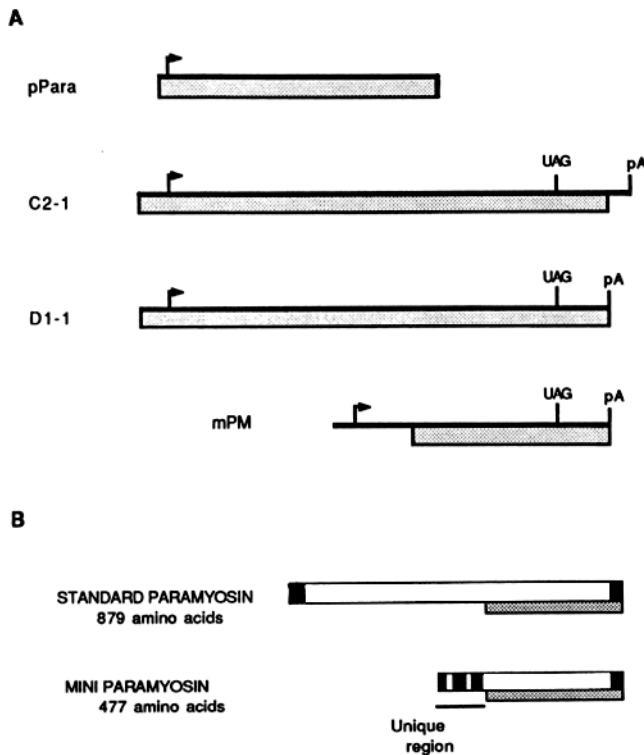
### Isolation of cDNA's Encoding *Drosophila* Paramyosins

The antibody made against *Helicoverpa* paramyosin was used to screen a  $\lambda$ gt11 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, *Helicoverpa dilloni*. This antibody crossreacts with MHC (200 kD), standard paramyosin (◆) and a 55-kD protein likely to be miniparamyosin (◇◇).



**Figure 2.** Structure of *Drosophila* paramyosin. (A) cDNAs isolated from embryonic and adult cDNA libraries. The adult cDNA clone pPara was isolated in an initial screen using an antibody prepared against paramyosin protein from the dung beetle, *Helicoverpa diloni*. The insert is 2 kb and encodes a portion of standard paramyosin. The full-length embryonic clones C2-1 (3.6 kb) and D1-1 (3.2 kb) encode the entire standard paramyosin protein. The only difference between C2-1 and D1-1 is the position at which polyadenylation occurs. The cDNA clone designated mPM encodes the entire miniparamyosin protein. As aligned, identical sequences are marked by a stippled box. (B) Diagrammatic representation of the two paramyosin protein isoforms found in *Drosophila melanogaster*. Amino acid sequences generated from cDNA sequence data were analyzed with the Chou and Fasman algorithm (Chou and Fasman, 1978a,b). The nonhelical regions are shown as black boxes. All other regions are predicted to be highly alpha helical in nature. The larger protein is 879 residues with a predicted molecular weight of 102,337 daltons, and a pI of 5.39. The smaller protein is 477 residues with a predicted molecular weight of 54,889 daltons, and a pI of 7.83. The COOH-terminal 363 amino acids are identical in both proteins and are underlined by stippled boxes. The NH<sub>2</sub>-terminal 114 residues of the smaller protein are not conserved compared to paramyosins of other species and are unique when compared to the current protein data bases.

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 D1-1 insert, after which the C2-1 clone continues for 432 bp. The D1-1 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.

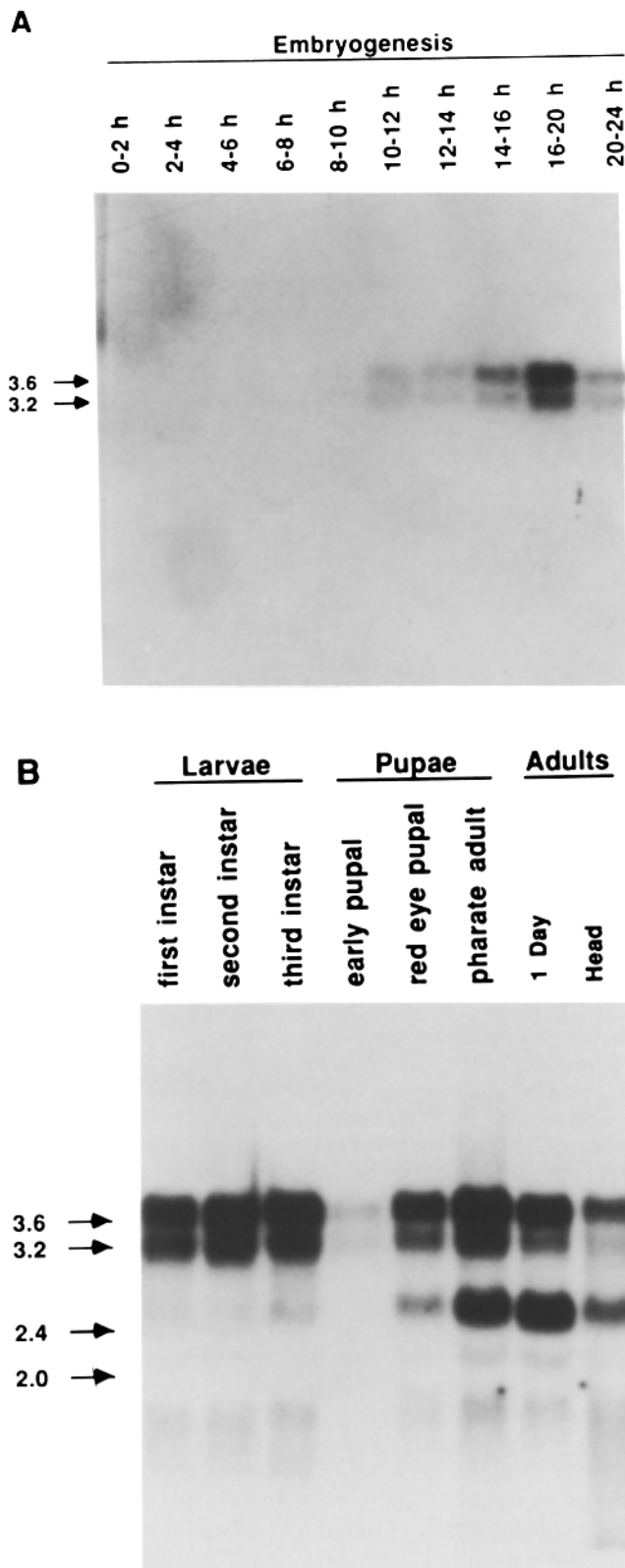
Sequence analysis shows that none of the six cDNAs encode a 55-kD protein as is observed in Fig. 1 C. Furthermore, hybridization to Northern blots of total cellular RNA indicates that transcripts with the potential to encode the 55-kD paramyosin are not present during embryonic development; such transcripts 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 (Rozek 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) Para-

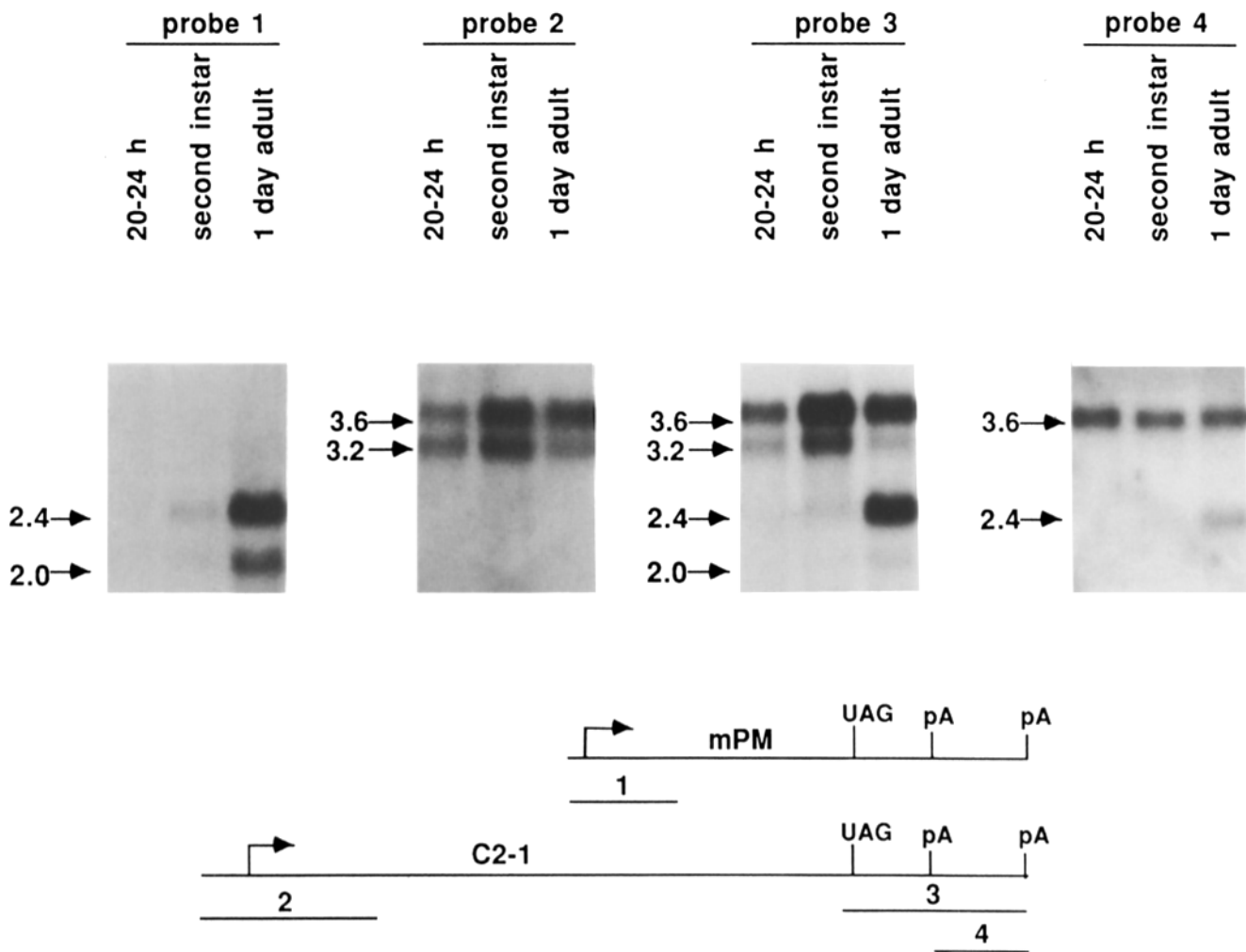
myosin RNA accumulation in larvae, pupae and adults: first instar larvae (48-h post-oviposition), second instar larvae (72-h post-oviposition), third instar larvae (96-h post-oviposition), early pupae (12–24-h post-pupariation), red eye pupae (≈75-h post-pupariation), pharate adults (≈100-h post-pupariation), 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).

ing potential. The 5' non-coding and coding sequences present in the mPM and C2-1 cDNAs are unique to each clone (Fig. 5). Using these specific 5' regions as templates, we made radiolabeled antisense RNA to probe Northern blots of total cellular RNA. The mPM probe hybridizes only to the 2.0- and 2.4-kb transcripts (Fig. 4, *probe 1*), whereas, the C2-1 specific probe hybridizes only to the 3.2- and 3.6-kb mRNA's (Fig. 4, *probe 2*). Overexposures of these blots reveal no cross hybridization to any other bands, indicating that the 5' ends of the two paramyosin cDNA clones are unique (data not shown). Based upon the hybridization evidence and cDNA sequence analysis, the 3.2- and 3.6-kb mRNAs encode the 105-kD paramyosin protein, and the 2.0- and 2.4-kb transcripts encode the 55-kD paramyosin isoform.

The size difference between the transcripts in each pair results from the differential use of polyadenylation signals. Both mPM and D1-1 have a poly-A stretch starting 14 bases downstream of the most 5' AATAAA sequence. cDNA clone C2-1 continues for 432 bp beyond the first polyadenylation site to a second site. Northern blots probed with antisense RNA specific to the entire 3' untranslated region of clone C2-1, show hybridization to all four paramyosin mRNA size classes (Fig. 4, *probe 3*). However, when a similar blot is probed with antisense RNA specific to the 432 nucleotide extension present exclusively in clone C2-1, only the 3.6- and 2.4-kb transcripts hybridize (Fig. 4, *probe 4*). Thus, the 2.0- and 3.2-kb transcripts use the more 5' signal for polyadenylation and the 2.4- and 3.6-kb mRNAs use the 3' polyadenylation signal.

All of the data presented above are consistent with the four paramyosin transcripts being derived from a single paramyosin gene, with expression initiated from two different promoters. This would be followed by splicing of the unique 5' sequences to common 3' exons. However, another possibility is that two genes that are nearly identical at their 3' end encode the two paramyosin isoforms. Three pieces of evidence support the former hypothesis. First, in situ hybridization to polytene chromosomes with a biotin labeled C2-1 probe detects only a single band at position 66E1 on the left arm of chromosome 3 (data not shown). Secondly, Fenerjian et al. (1989) previously identified two open reading frames at this site which encode a portion of a myosin-like protein. Probes from both open reading frames hybridize to adult mRNA's of 2.4, 3.2, and 3.6 kb. These two open reading frames are the 3' exons common to both mPM and C2-1 (C. Swimmer, personal communication; Becker et al., unpublished data). The published restriction map of this genomic region shows that a 1.6-kb BamHI fragment has its centromere-distal end within the adjacent chorion gene cluster and its proximal end 41 bp upstream of the paramyosin stop

myosin 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 cDNA's. The positions of the probes used for each blot are shown at the bottom of the figure. Antisense RNA probes labeled with  $^{32}\text{P}$  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. 5 A 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 pro-

tein 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<sub>2</sub> 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* (Vinós 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-







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 NH<sub>2</sub>-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 NH<sub>2</sub>-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 RNAs. 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 com-

pared 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.

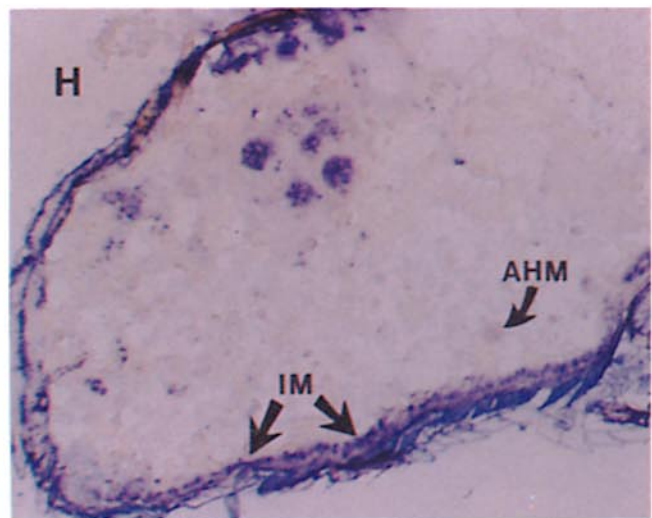
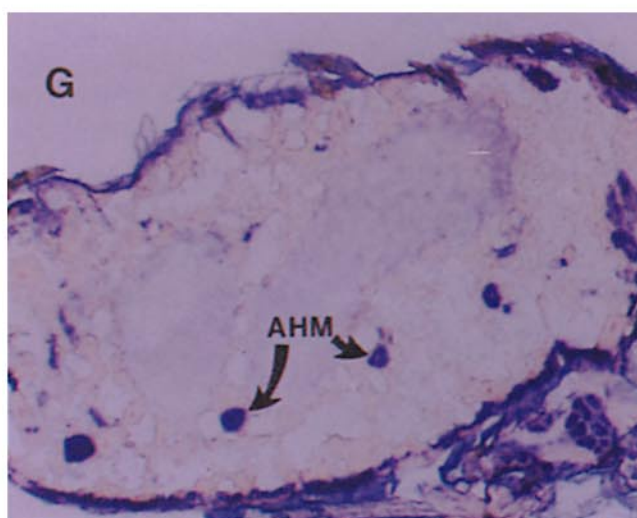
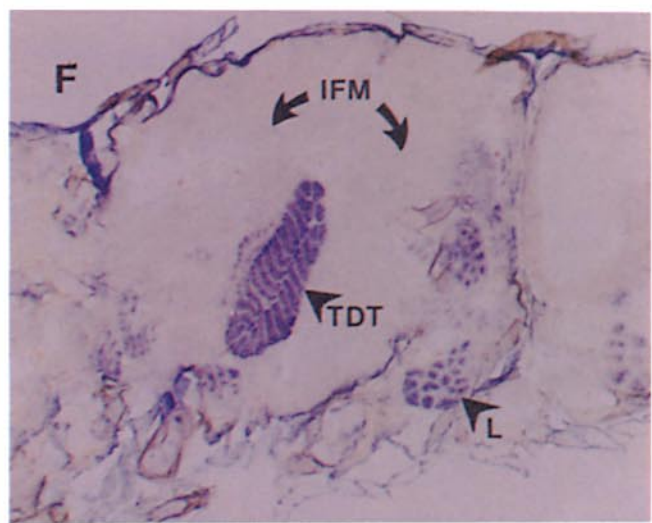
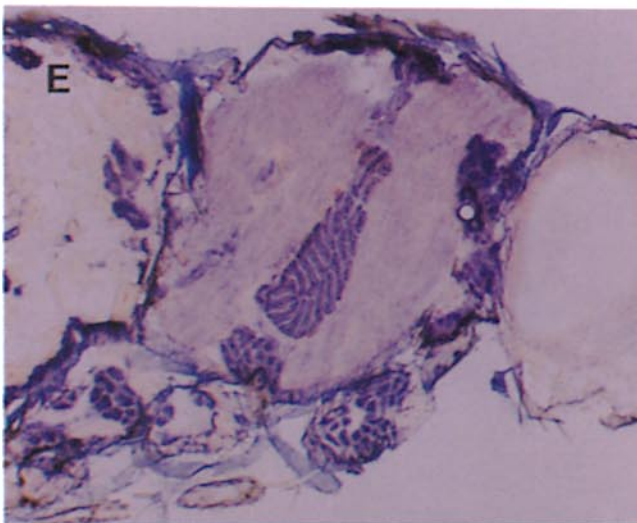
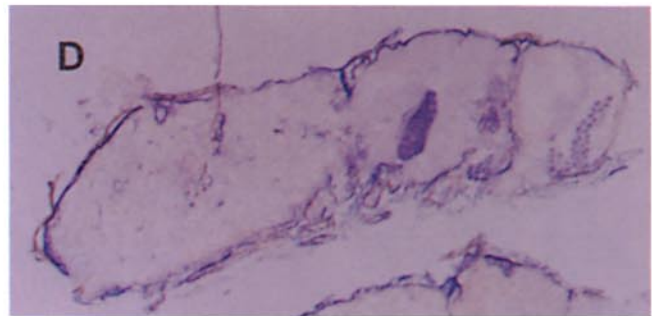
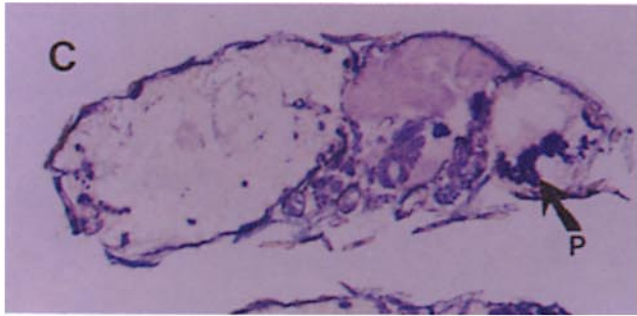
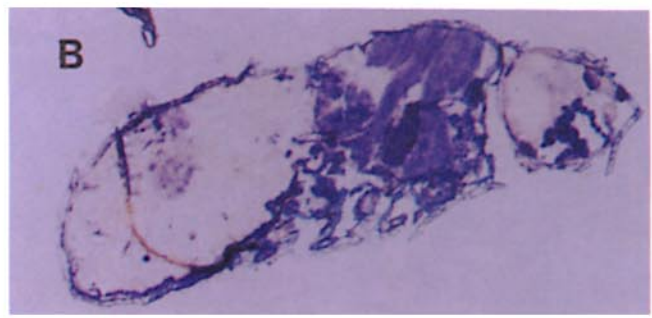
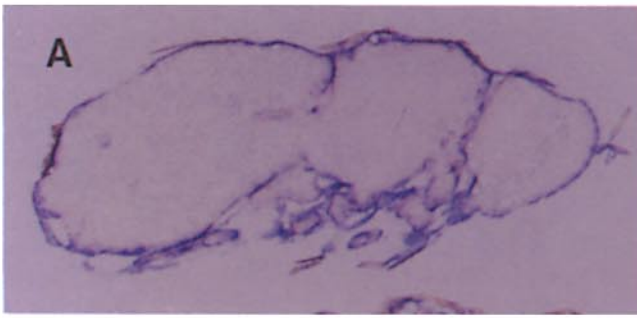
#### **Discussion**

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 NH<sub>2</sub>-terminal residues which are unique as compared to the current protein sequence data base. Standard paramyosin has been well characterized in several invertebrates (Weisel and Szent-Györgyi, 1975; Bullard et al., 1973; Waterston et al., 1977; Kagawa et al., 1989; Limberger and McReynolds, 1990; Vinós 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 mRNA's 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; Ashhurst 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 sepa-

rated, 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 NH<sub>2</sub>-terminal non-alpha helical portion of the molecule (Schriefer 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 NH<sub>2</sub> 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* germline (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.

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