Myosin Functional Domains Encoded by Alternative Exons Are Expressed in Specific Thoracic Muscles of *Drosophila*

Gregg A. Hastings and Charles P. Emerson, Jr.

Biology Department, University of Virginia, Charlottesville, Virginia 22901

Abstract. The *Drosophila* 36B muscle myosin heavy chain (MHC) gene has five sets of alternatively spliced exons that encode functionally important domains of the MHC protein and provide a combinatorial potential for expression of as many as 480 MHC isoforms. In this study, in situ hybridization analysis has been used to examine the complexity and muscle specificity of MHC isoform expression in the fibrillar indirect flight muscle (IFM), the tubular direct flight muscles (DFM) and tubular tergal depressor of the trochanter muscle (TDT), and the visceral esophageal muscle in the adult thorax. Our results show that alternative splicing of the MHC gene transcripts is precisely regulated in these thoracic muscles, which express three MHC isoforms. Individual thoracic muscles each express transcripts of only one isoform, as detectable by in situ hybridization. An apparently novel fourth MHC isoform, with sequence homology to the rod but not to the head domain of the 36B MHC, is expressed in two direct flight muscles. These findings form a basis for transgenic experiments designed to analyze the muscle-specific functions of MHC domains encoded by alternative exons.

THE myosin heavy chain (MHC)[1] protein is a major component of thick filaments of the skeletal muscle myofibrillar contractile apparatus. Biochemical and biophysical studies have shown that discrete domains of the MHC protein control its assembly into thick filaments as well as its function in binding to actin and regulatory contractile proteins during the contraction cycle (Harrington and Rodgers, 1984; Warrick and Spudich, 1987). The amino-terminal region forms a globular head that includes domains that function in ATP binding, actin-activated ATP hydrolysis, binding of myosin light chain subunits, and actin binding. The carboxy-terminal region forms an α-helical coiled-coil rod structure involved in dimerization of MHC subunits and in higher order interactions to assemble myosin dimer molecules into thick filaments. The coiled-coil structure of the rod is interrupted by a random coil "hinge" sequence domain that interrupts the coiled-coil structure of the rod and may be a site for molecular force production during contraction (Ueno and Harrington, 1986*a*,*b*).

Although biochemical studies have provided structure/function information on the vertebrate skeletal muscle myosins, molecular genetic studies of myosin in *Drosophila* and the nematode, *Caenorhabditis elegans*, are identifying new functional MHC domains that control the assembly of myosin molecules into thick filaments and specify the contractile functions of myosin isoforms in muscle types with physiologically different contractile properties (Emerson and Bernstein, 1987; Anderson, 1989). *Drosophila* is an organism particularly well suited for genetic studies of myosin function because it has only a single muscle MHC gene in its haploid genome (Bernstein et al., 1983; Rozek and Davidson, 1983). The *Drosophila* 36B muscle MHC gene, however, has the potential to produce a very large array of MHC isoforms through RNA splicing of a complexity of alternative exons. Five sets of alternative exons located in the MHC gene encode variant MHC protein sequences that are in or neighbor functionally important sequence domains of the myosin protein (George et al., 1989). RNA hybridization studies show that the temporal and muscle-specific expression of individual members of these sets of alternative exons is regulated at larval and late pupal stages (Bernstein et al., 1986; George et al., 1989; Kazzaz and Rozek, 1989), suggesting that these MHC alternative exons encode functionally important isoforms of the MHC protein.

A large number of *Drosophila* muscle mutants have been isolated based on screens to identify mutations that specifically disrupt flight. For many of these mutants, it has been determined that the flightless phenotype is the result of mutations that cause structural defects in various myofibrillar protein isoforms that are expressed in indirect flight muscle (IFM) (Mogami and Hotta, 1981; Karlik et al., 1984; Bernstein et al., 1986; Karlik and Fyrberg, 1986; Falkenthal et al., 1987; Chun and Falkenthal, 1988). The specificity of these mutations to IFM function has been surprising con-

G. A. Hastings' present address is National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Rockville, MD 20852. C. P. Emerson's present address is Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111.

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

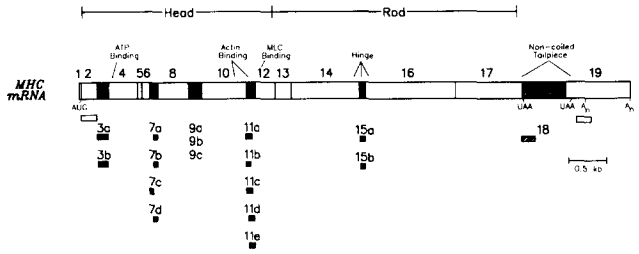


Figure 1. Organization of the *Drosophila* muscle MHC 36B mRNA. The bar, designated MHC mRNA, represents constitutive (*open boxes*), alternative (*solid boxes*), and combinatorial (*hatched box*) exons as determined by George et al. (1989). The constitutive and alternative/combinatorial exons are numbered above and below the mRNA, respectively. The structural and functional domains of the MHC protein are aligned above the mRNA. The translational start codon (*AUG*), stop codons (*UAA*), and polyadenylation sites (A_n) are shown below the mRNA. Open boxes below the mRNA indicate the size and position of the constitutive exon-specific probes for exon 2 and exon 19. Alternative exon probes are represented by the labeled solid boxes. The combinatorial exon 18 probe is shown as the hatched box. The relative sizes and positions of the antisense RNA (exons 2, 3, 11, 15, 18, and 19) and oligonucleotide (exon 7) probes are indicated. Nucleotides covered by each probe are described in Materials and Methods. Probes were not generated from exon 9. *MLC*, myosin light chain. Bar, 0.5 kb.

sidering that *Drosophila* has a very diverse array of muscle types with a wide variety of physiological and structural properties involved in flight behavior. In part, the IFM specificity of some MHC mutations reflects the finding that flight behavior is not an essential muscle function of *Drosophila* and that thick filament assembly in IFM is dosage dependent and requires expression of both MHC alleles. However, in addition to the fibrillar IFM, a large number of other muscles are broadly grouped into tubular and visceral muscle fiber types (Crossley,, 1978; Miller, 1950). Very little is known regarding the myosin isoforms that constitute thick filaments of these *Drosophila* muscles.

To investigate the complexity and muscle specificity of the expression of myosin isoforms in different thoracic muscle fiber types, we have developed exon-specific probes to alternatively spliced exons of the 36B MHC gene. In situ hybridization analyses with these probes reveal that the thoracic muscles express only three MHC isoforms, and specific muscles within the thorax express only one of these MHC isoforms. One isoform (MHC I) is expressed within the IFM. A second isoform (MHC II) is expressed in both the TDT and the DFM #52. Interestingly, a third isoform (MHC III) is expressed in the tubular DFM #51 and the visceral muscle of the esophagus, which contains both thin and tubular fibers. Finally, we identified two direct flight muscles, DFMs #49 and #53, that do not express alternative or common exons encoding the MHC head domain, although these DFMs express exons encoding the rod portion of the MHC molecule. This MHC appears to be a novel muscle-specific MHC isoform. The highly restricted expression of MHC isoforms in different muscles establishes the feasibility of transgenic studies to characterize the structure and function of specialized MHC isoforms and their roles in specifying the contractile properties of physiologically diverse muscle fiber types.

Materials and Methods

Cloning MHC Exons

MHC restriction fragments isolated from plasmid subclones derived from a *Drosophila* Canton S genomic library (Bernstein et al., 1983; George et al., 1989) were ligated either to the pBSKS+ or pBSSK- T3/T7 transcription vector (Stratagene, La Jolla, CA). Each insert was designed to produce a transcript of 90–250 nucleotides thus obviating the necessity for size reduction in alkali before in situ hybridization. The nucleotides included in each subcloned MHC exon are numbered according to George et al. (1989) and are as follows: exon 2, 1958-2128; exon 3a, 2388-2508; exon 3b, 2709-2834; exon 11a, 11239-11319; exon 11b, 11632-11712; exon 11c, 12328-12404; exon 11d, 12970-13043; exon 11e, 10871-10952; exon 15a, 17057-17119; exon 15b, 17476-17533; exon 18, 20300-20473; exon 19, 21318-21519.

Oligonucleotide Probes

Oligonucleotide antisense probes, 50 nucleotides in length, corresponding to the most divergent portions of MHC 36B exon 7a–d were synthesized (Operon Technologies, Inc., Alameda, CA).

7a probe:
(3'GTTCCAGTGACATCGGTCATAGCTACTACGACTCCTCAAGAGGGAGTGGC 5').

7b probe:
(3'TATGACAGACGAGAGCTTGTTGTAAATGCTGATAGCGTAACAGAGCGTTC 5').

7c probe:
(3'CTACCATAAAGACCCGGTTGTATAACCACTAATAGGGCCGTAAACGGTCC 5').

7d probe:
(3'ATTTCATTGACATGGGTTGTACCTACTGCCACTCCTTAAGGTCGAACGTC 5').

The antisense probe to exon 2 of the cytoplasmic MHC cDNA:
(3'CCTCCTTCATCTAGCGTTGCTAGGCCTCGAGTTCATGGAGAGCCACCTCG 5'),

includes sequences that encode amino acids 58-74 (Kiehart et al., 1989).

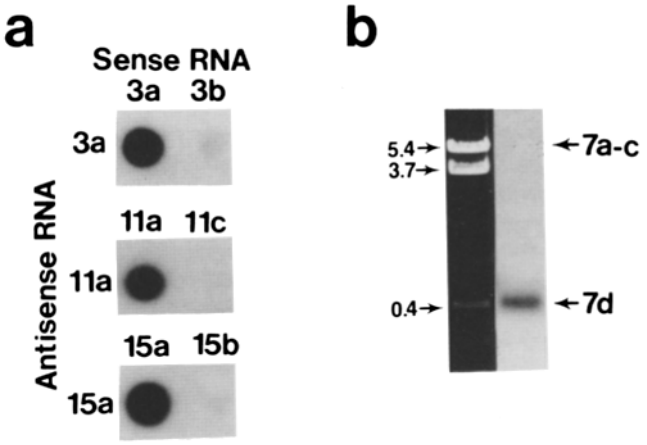


Figure 2. Alternative exon-specific probe cross-hybridization controls. (*a*) Unlabeled sense RNA was transcribed from exons 3a and 3b; 11a and 11c; and 15a and 15b. RNA was dot blotted to nitrocellulose and hybridized with ^{32}P-labeled, antisense RNA from exons 3a, 11a, and 15a. Hybridization occurred between the corresponding sense and antisense RNAs and not to the homologous exon copies. Plasmid DNA, containing exons 7a-d, was digested with PvuII and EcoRI. The DNA was transferred to nitrocellulose and hybridized to the ^{32}P-labeled 7d antisense oligonucleotide probe. The panel on the left shows the ethidium bromide stained agarose gel. At right is an autoradiograph showing signal corresponding only to the exon 7d-containing restriction fragment.

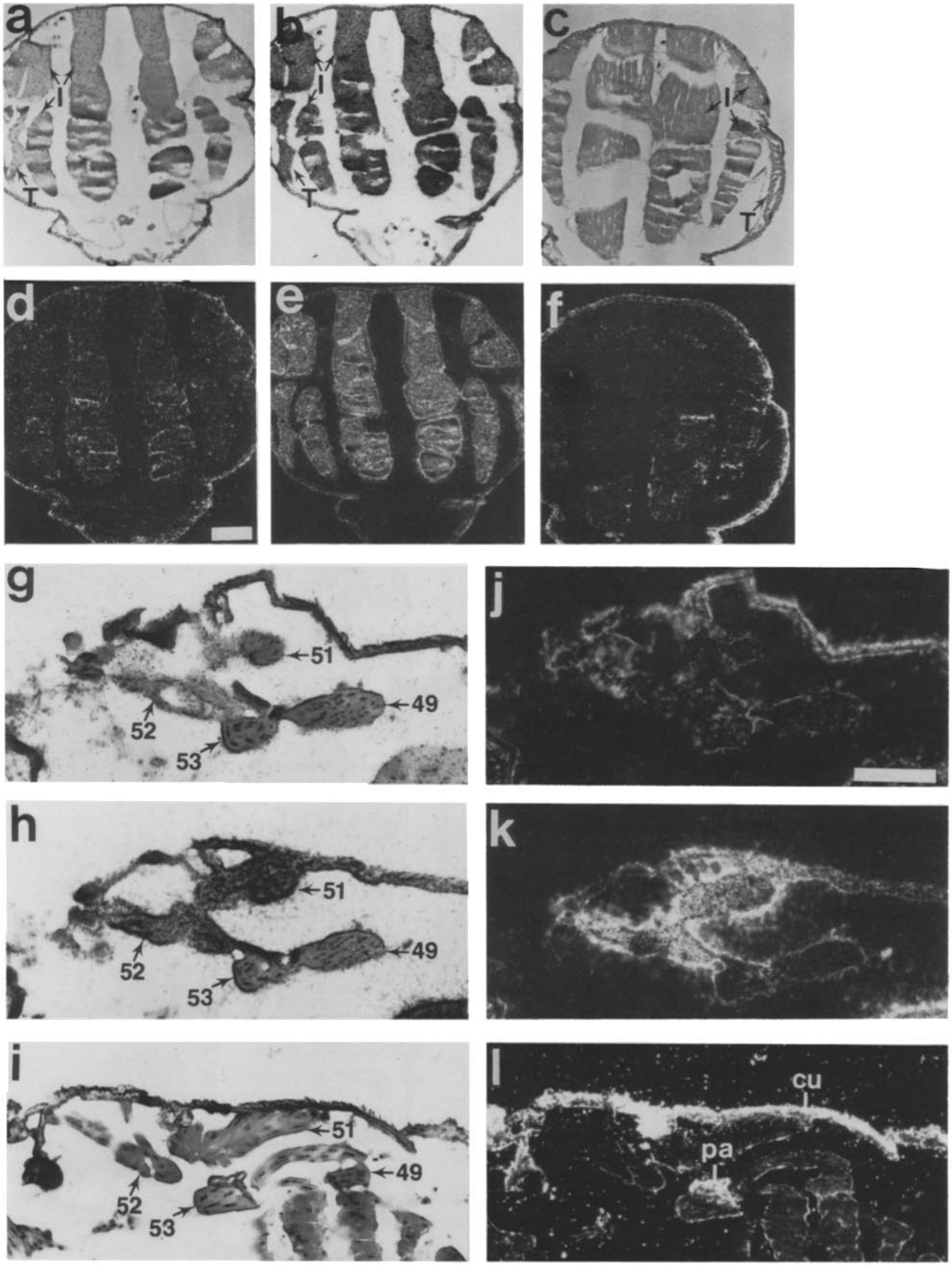
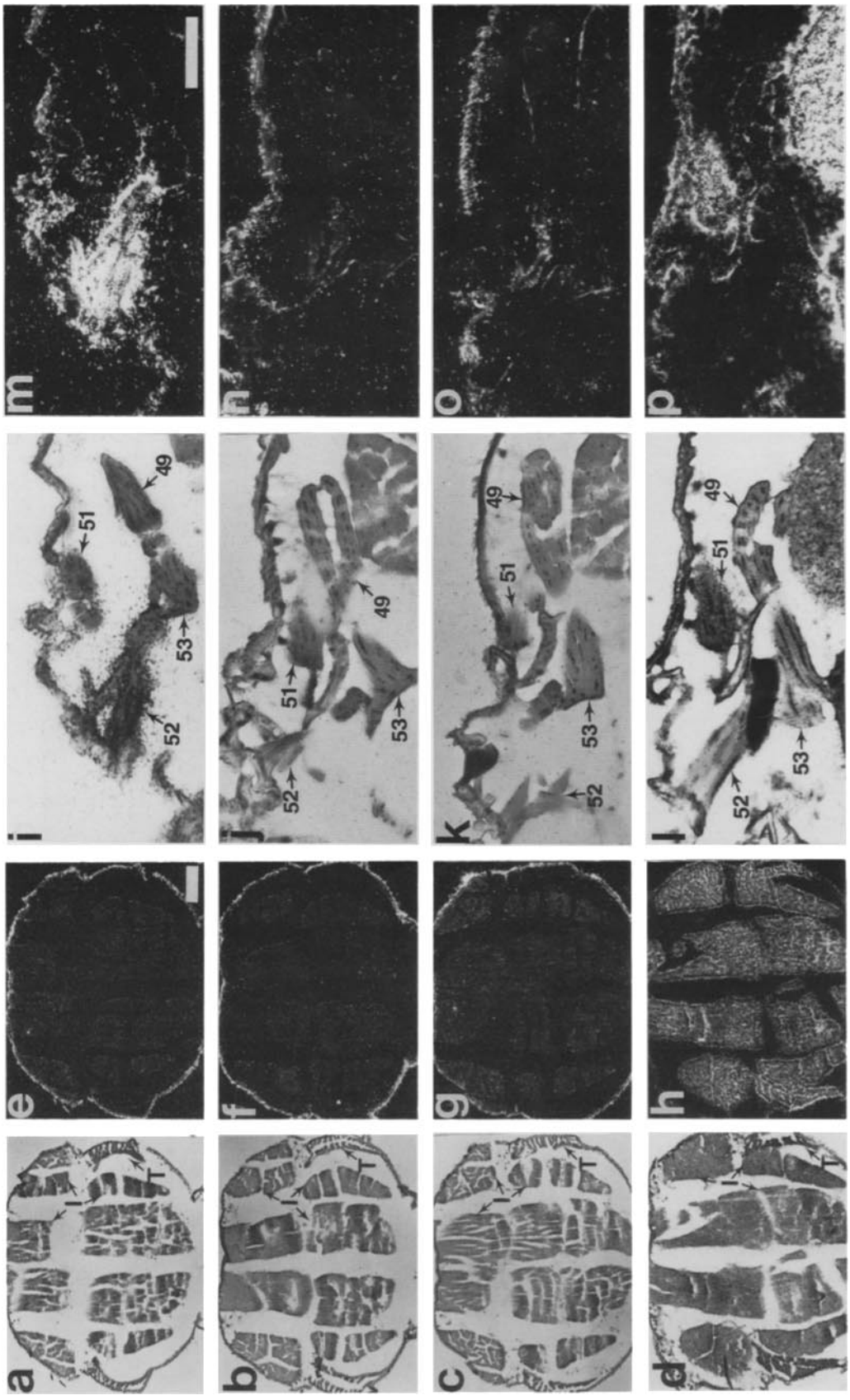


Figure 3. In situ hybridizations with radiolabeled exon 3a,b probes. Sections were hybridized with exon 3a antisense (*a*, *d*, *g*, and *j*), 3b antisense (*b*, *e*, *h*, and *k*), and 3b sense (*c*, *f*, *i*, and *l*) RNA. IFM (*I*) and TDT (*T*) muscles exhibit silver grains when hybridized to exon 3b antisense RNA (*b* and *e*) but not to 3a antisense (*a* and *d*) or 3b sense RNA (*c* and *f*). Sections containing direct flight muscles showed silver grains over DFM #51 and #52 when hybridized to exon 3b antisense RNA (*h* and *k*). Nonspecific hybridization to the cuticle (*cu*) was seen in all cases and occasionally with the prealar apophysis (*pa*). Bright-field (*a–c*, *g–i*) and dark-field (*d–f*, *j–l*) dorsal views are shown. Anterior is above (*a–f*) and to the right (*g–l*). Bars: (*a–f*) 125 μm and (*g–l*) 60 μm.



Radiolabeling of Sense and Antisense Exon Probes

As cross-hybridization controls, unlabeled sense RNAs were transcribed from linearized plasmids as described (Promega Biotec, Madison, WI) (Melton et al., 1984). Radioactively labeled antisense RNAs for controls were generated by including [^{32}P]UTP, 3000 Ci/mM (all radioisotopes obtained from New England Nuclear, Boston, MA) in parallel reactions. Antisense ^{35}S-labeled RNAs for in situ hybridization reactions were produced by incorporation of ^{35}S-UTP, 1350 Ci/mM. The exon 7d oligonucleotide probe was prepared for control filter hybridization by 5'-end labeling with T4 polynucleotide kinase (Boehringer Mannheim Biochemicals, Indianapolis, IN) with γ [^{32}P]ATP, 3000 Ci/mM. Oligonucleotides for in situ hybridizations were 3'-end labeled (IBI, New Haven, CT) using terminal transferase to incorporate ^{35}S-dATP, 1350 Ci/mM. Average tail lengths of radiolabeled oligonucleotides were 5–10 nucleotides.

Controls for Exon Probe Cross-hybridization

In vitro transcribed sense RNAs of exons 3a/b, 11a/c, and 15a/b were prepared. 100 ng of each RNA were added to 1.4 M formaldehyde, 6× SSC and denatured at 65°C for 15 min. Each sample was loaded into the well of a dot-blot apparatus (Schleicher & Schuell, Keene, NH) and absorbed to nitrocellulose. After a 20× SSC wash, the membrane was baked at 80°C in vacuo for 30 min. Prehybridization of the filter was performed at 65°C for 30 min in 50% deionized formamide, 0.3 M NaCl, 20 mM Tris-Cl, pH 8.0, 5 mM EDTA, 1× Denhardt's, 10 mM DTT, 500 μg/ml yeast tRNA and 10% dextran sulfate. ^{32}P-labeled antisense RNAs were then added to the same solution to a concentration of 1×10^6 cpm/ml. Antisense probes 3a, 11a, and 15a were applied to filters bearing sense RNAs of 3a/b, 11a/c, and 15a/b, respectively. After overnight hybridization at 65°C the filters were washed three times in 4× SSC, 10 mM DTT at room temperature. Nonhybridized RNA was removed by incubation in 20 μg/ml RNase A, 0.5 M NaCl, 10 mM Tris-Cl, pH 8.0 at 37°C for 30 min followed by a wash under the same conditions without RNase A. Stringent washes were done in 2× SSC, 50% formamide (once) and 0.1× SSC (three times) at 50°C, 15 min each. The filters were dried and autoradiography was performed on Eastman Kodak (Rochester, NY) XAR film.

A genomic subclone, gD149 (Bernstein et al., 1983; George et al., 1989), of the region containing exon 7a–d was digested with the enzymes PvuII and EcoRI. 1 μg of this digest was subject to electrophoresis on a 1% agarose gel, which was stained with ethidium bromide, photographed, and the sample transferred to nitrocellulose (Maniatis et al., 1982). The filter was baked at 80°C for 30 min and then prehybridized in 10% deionized formamide, 0.3 M NaCl, 20 mM Tris-Cl, pH 8.0, 5 mM EDTA, 1× Denhardt's, 10 mM DTT, 500 μg/ml yeast tRNA and 10% dextran sulfate at 50°C for 60 min. The ^{32}P-end-labeled 7d oligonucleotide was added to this solution at a concentration of 1×10^6 cpm/ml and incubated at 50°C overnight. The filter was washed in 2× SSC, 10 mM DTT at 50°C for 2 h followed by 0.1× SSC at room temperature for 15 min. The filter was dried and autoradiographed as described above.

Preparation of Tissue Sections for In Situ Hybridizations

1–2-d-old Canton S female flies were etherized before removing the heads, abdomens, wings, pro-, and metathoracic legs with a razor blade. The dissected thoraces were treated briefly with acetone to remove wax and immediately immersed in 4% paraformaldehyde in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) at 4°C. The tissue was fixed overnight and then washed twice in 4°C PBS. In situ hybridization with antisense probes to tissue sections was performed essentially as described by Ingham et al. (1985). Briefly, the thoraces were dehydrated in ethanol, cleared in xylene, and embedded in paraffin. 8-μm sections were collected on slides coated with polylysine. The sections were treated with xylene to remove wax and rehydrated with ethanol and dH_2O. Proteinase K (1 μg/ml in 100 mM Tris-Cl, pH 8.0, 50 mM EDTA) digestion and acetylation with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0, preceded dehydration through ethanol. ^{35}S-labeled RNA (1.3×10^4 cpm/μl) and 3' end labeled ^{35}S-labeled oligonucleotide (4×10^4 cpm/μl) probes were hybridized to sections in the identical hybridization solution used in the control filter hybridizations at 65°C and 50°C, respectively. Coverslips were removed in three washes in 4× SSC, 10 mM DTT at room temperature. RNase A digestion and washes were performed as described above for control filter hybridizations. Autoradiography was performed at 4°C for 3 d. Ehrlich's hematoxylin and eosin were used to stain the sections after development.

Results

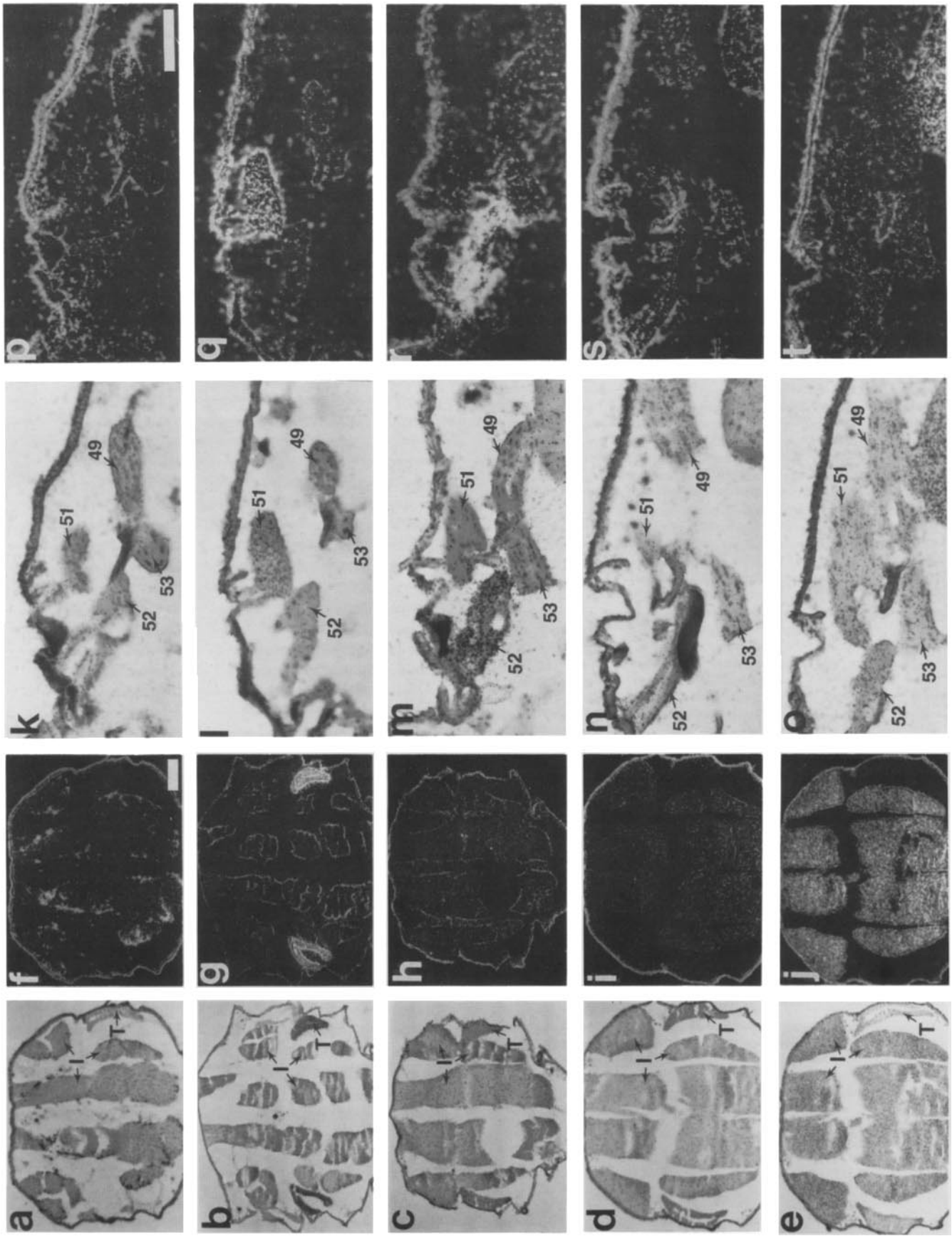
Specificity of Exon-specific Antisense Probes

The processed mRNA transcript of the MHC 36B gene is comprised of one combinatorially spliced exon, exon 18, five sets of alternatively spliced exons, exons 3a/3b, 7a/7b/7c/7d, 9a/9b/9c, 11e/11a/11b/11c/11d, and 15a/15b, as well as 13 common exons (George et al., 1989; Fig. 1). To examine the expression of individual exon members of these alternatively spliced exon sets, we developed exon-specific hybridization probes. The sequence homology between exon members of the alternative exon sets is variable and in some cases high. Control experiments to determine sufficient hybridization stringencies were performed with antisense and sense RNA or DNA probes derived from the most conserved portions of each alternative exon set (Fig. 1). Radiolabeled antisense RNA was transcribed from plasmids of exons 3a, 11a, and 15a. These probes were hybridized to dot blots bearing in vitro transcribed sense RNA from exons 3a and 3b, 11a and 11c, and 15a and 15b (Fig. 2 *a*). Hybridization and wash stringencies were established such that no cross-hybridization was observed between homologous alternative exons. High stringency hybridization conditions for the exon 7d antisense oligonucleotide probe were determined by filter hybridization, which resulted in specific binding to a restriction fragment containing 7d, but not to 7a–c (Fig. 2 *b*). Exons 9a–c were not analyzed in this work since the nucleotide conservation (91% between exons 9a and 9c) of each copy is extremely high. Previous Northern blot analysis of RNA isolated from first-instar larvae and late pupae showed no distinctive expression pattern for any of the copies of exon 9 (George et al., 1989). However, a recent study (Edwards, 1990), indicates that exon-specific antisense oligonucleotide probes to exons 9a–c show muscle-specific in situ hybridization.

Expression of Alternative Exons in Visceral Muscles

Visceral muscles, a third major muscle group, are found in the digestive tract muscles of embryos, larvae, and adults and in body wall muscle (Crossley, 1978; Miller, 1950). The esophagus of the adult thorax, representative of visceral muscles, is composed of both tubular and thin fiber types. Longitudinal sections of the adult thorax containing the esophagus were hybridized with each of the alternative

Figure 4. In situ hybridizations with radiolabeled exon 7a-d probes. ^{35}S-end-labeled oligonucleotide antisense probes. Sections were probed with exons 7a (*a*, *e*, *i*, and *m*), 7 b (*b*, *f*, *j*, and *n*), 7c (*c*, *g*, *k*, and *o*), and 7d (*d*, *h*, *l*, and *p*). The IFM (*I*) and TDT (*T*) showed silver grains when hybridized to 7d (*d* and *h*). DFM #52 and #51 accumulated silver grains when hybridized with 7a (*i* and *m*) and 7d (*l* and *p*), respectively. Bright-field (*a–d*, *i–l*) and dark-field (*e–h*, *m–p*) dorsal views are shown. Anterior is above (*a–d*, *e–h*) and to the right (*i–l*, *m–p*). Bars: (*a–h*) 125 μm and (*i–p*) 60 μm.



thoraces of young adult flies and hybridization of each exon probe was examined in thoracic fibrillar and tubular muscle types. Fig. 3, *a–f* shows low-magnification views of longitudinal sections of the dorsal–proximal region of the adult thorax. This region includes both the IFM and TDT. Exon 3b is expressed in the fibrillar IFM and the tubular TDT. Exon 3a hybridization is not detected at a significant level in either the IFM or the TDT (Fig. 3, *a* and *d*). The positive hybridization signal of 3b was judged by comparing the density of silver grains present over the same muscle in sections hybridized with an exon 3b sense probe. Muscle tissue was identified by hybridization with constitutive exons 2 and 19 probes (data not shown). Fig. 3, *h* and *k* show the localized hybridization of exon 3b to DFMS #51 and #52 (see Lawrence, 1982, for the numbering of direct flight muscles). Exon 3a is not expressed in these DFM. Direct flight muscles #49 and #53 do not show a detectable level of silver grains with either exon 3 probe. Thus, despite the diversity of structure and function between the IFM, TDT, and DFM, all of these thoracic muscle types expressed MHC transcripts containing only alternative exon 3b.

Expression of Alternative Exons 7a–d

Exons 7a-d encode a region of the MHC protein that has evolutionarily divergent sequences that have no known function (Fig. 1) (Warrick and Spudich, 1987). Antisense oligonucleotide probes 50 bases long corresponding to divergent regions of exon 7a-d were hybridized to adult thorax sections. Fig. 4 displays the muscle-specific localization of each alternative of exon 7. Exon 7d is the only exon 7 expressed in muscles of the adult thorax, including IFM, TDT, and DFM #51 (Fig. 4, *d, h, l,* and *p*). Of the remaining exons of the exon 7 set, 7a is expressed in DFM #52 (Fig. 4, *i* and *m*). DFMs #49 and #53 do not express any of these alternative exon 7s (Fig. 4, *i–p*).

Expression of Alternative Exons 11a-e

Several actin binding sites have been mapped to the MHC head region (Fig. 1; Warrick and Spudich, 1987). The amino-terminal half of the 20-kD peptide has two high-affinity actin-binding sites (Sutoh, 1983; Mitchell et al., 1986), whereas the carboxy-terminal end of the adjacent 50-kD tryptic peptide contains a secondary actin binding site covering a portion of exon 10 (Sutoh, 1983). The carboxy-terminal six amino acids of the 39 residue exon 11 are included in the downstream high-affinity actin-binding site (George et al., 1989). Alternative exon 11 antisense RNA probes were hybridized to paraffin sections of adult thorax. Fig. 5 shows the results of these in situ hybridization reactions. Of the five alternative exon 11s, only exon 11e is expressed in the fibrillar IFM (Fig. 5, *e* and *j*). Exon 11b is expressed in the tubular TDT and DFM #51 (Fig. 5, *b, g, l,* and *q*). DFM #52, however, expresses exon 11c (Fig. 5, *m* and *r*). Exons 11a and 11d are undetected in the muscles analyzed in the adult thorax (Fig. 5, *a, f, k, p,* and *d, i, n,* and *s* respectively). No expression of alternative exon 11 was detected in DFMs #49 or #53 (Fig. 5, *k–t*).

Expression of Alternative Exons 15a and 15b

The alternative exons 15a and 15b encode the hinge domain of the MHC rod (George et al., 1989; Collier et al., 1990). Fig. 6 shows the muscle-specific localized hybridization of exons 15a and 15b antisense RNA probes. Exon 15A hybridizes to the IFM, TDT, and DFM #51, which include both fibrillar and tubular muscles. Other tubular muscles, DFMs #49, #52, and #53, express exon 15b. Muscles of tubular and fibrillar types express exon 15a as well as exons 3b (Fig. 3, *b* and *e*) and 7d (Fig. 4, *d* and *h*).

Expression of Exon 18

Exon 18 encodes a single carboxy-terminal amino acid, whereas if exon 18 is spliced out of MHC transcripts, exon 19 encodes a carboxy terminus with 27 amino acids (Bernstein et al., 1986; George et al. 1989). A previous report by Kazzaz and Rozek (1989) demonstrated expression of exon 18 in adult head muscles and leg muscle in addition to the IFM (Bernstein et al., 1986; George et al., 1989). To characterize exon 18 expression, antisense RNA probes of exon 18 were hybridized to thorax sections. Both fibrillar and tubular muscles of the IFM, TDT (Fig. 7, *a* and *b*), and DFM #51 muscles (Fig. 7, *c* and *d*) hybridize with the exon 18 probe. However exon 18 probes do not hybridize with DFM #49, #52, and #53 (Fig. 7, *c* and *d*).

Expression of Alternative Exons in Visceral Muscles

Visceral muscles, a third major muscle group, are found in the digestive tract muscles of embryos, larvae, and adults and in body wall muscle (Crossley, 1978; Miller, 1950). The esophagus of the adult thorax, representative of visceral muscles, is composed of both tubular and thin fiber types. Longitudinal sections of the adult thorax containing the esophagus were hybridized with each of the alternative exons, as well as constitutive exon 19, probes. Identification of tubular and thin myofibers in the esophagus was provided by hybridization with the exon 19 probe. Fig. 8 shows that only MHC exons 3b, 7a, 11c, 15b, and 19 show detectable hybridization.

Expression of Constitutive MHC Exons in DFMs #49 and #53

An unexpected finding is that probes of the alternatively spliced exons 3, 7, and 11, encoding sequences of the MHC head, do not hybridize detectably with DFMs #49 and #53 (Fig. 3, 4, 5, and 6), whereas these muscles hybridize with the probe of exon 15b, which encodes the hinge domain. To

Figure 5. In situ hybridizations with radiolabeled exon 11a–e probes. Sections were probed with exons 11a (*a* and *f, k,* and *p*), 11b (*b* and *g, l* and *q*), 11c (*c* and *h, m* and *r*), 11d (*d* and *i, n* and *s*) and 11e (*e* and *j, o* and *t*). Silver grains over the IFM (*I*) and TDT (*T*) were observed with exon 11e (*e* and *j*) and 11b (*b* and *g*), respectively. Exon 11b and 11c also hybridize to DFM#51 (*l* and *q*) and DFM #52 (*m* and *r*), respectively. Bright field (*a–e, k–o*) and dark field (*f–j, p–t*) dorsal views are shown. Anterior is above (*a–e, f–j*) and to the right (*k–o, p–t*). Bars: (*a–j*) 125 μm and (*k–t*) 60 μm.

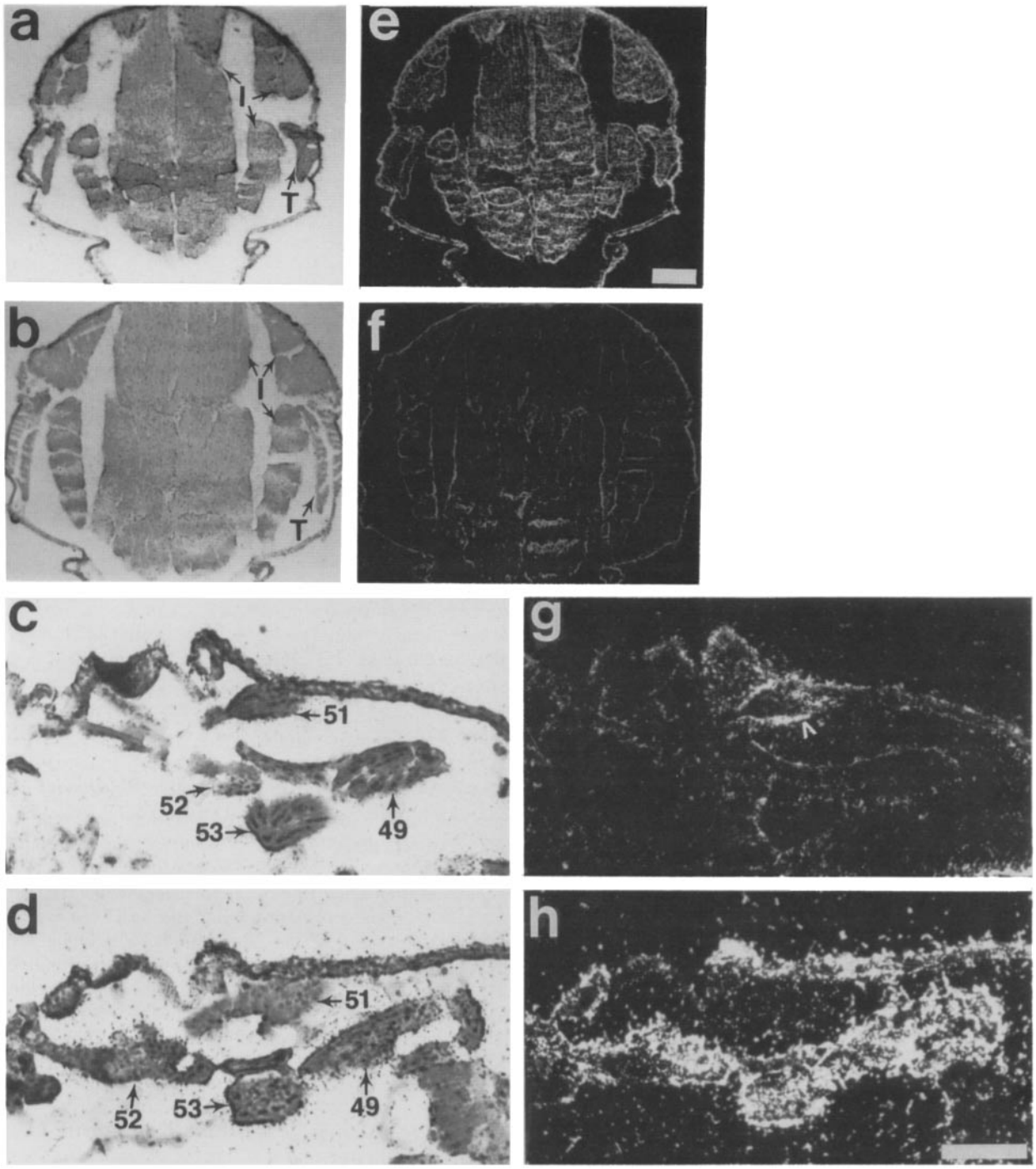


Figure 6. In situ hybridizations with radiolabeled exon 15a,b probes. Sections were probed with exons 15a (*a* and *e*, *c* and *g*) and 15b (*b* and *f*, *d*, and *h*). Silver grains collected over the IFM (*I*) and TDT (*T*) when hybridized with exon 15a (*a* and *e*). DFM #51 showed grains with exon 15a (*c* and *g*). DFM #52, #53 and #49 accumulated grains when hybridized with exon 15b (*d* and *h*). Weak silver grain accumulation over DFM #51 (>) occurs with 15a (*g*). Bright-field (*a–d*) and dark-field (*e–h*) dorsal views are shown. Anterior is above (*a*, *b*, *e*, and *f*) and to the right (*c*, *d*, *g*, and *h*). Bars: (*a*, *b*, *e*, and *f*) 125 μm and 60 μm (*c*, *d*, *g*, and *h*).

further examine MHC expression in these muscles, antisense RNA probes of exon 2 of the head and exon 19 of the rod were hybridized to thoracic muscle. Fig. 9 (*a*, *d*, *b*, and *e*) shows that exon 2 does not hybridize with DFMs #49 or #53, although hybridization is detected in DFMs #51 and #52. In contrast, exon 19 probe hybridizes to DFMs #49, #51, #52, and #53. One possible explanation for the absence of hybridization of MHC 36B head exons to DFMs #49 and #53 is that a divergent MHC gene is expressed in these muscles. A cDNA encoding a non-muscle MHC isoform has been cloned and was found to be expressed at very low levels in late pupae (Kiehart et al., 1989). To examine whether this nonmuscle myosin isoform is expressed in DFMs #49 and #53, an antisense oligonucleotide probe of the nonmuscle

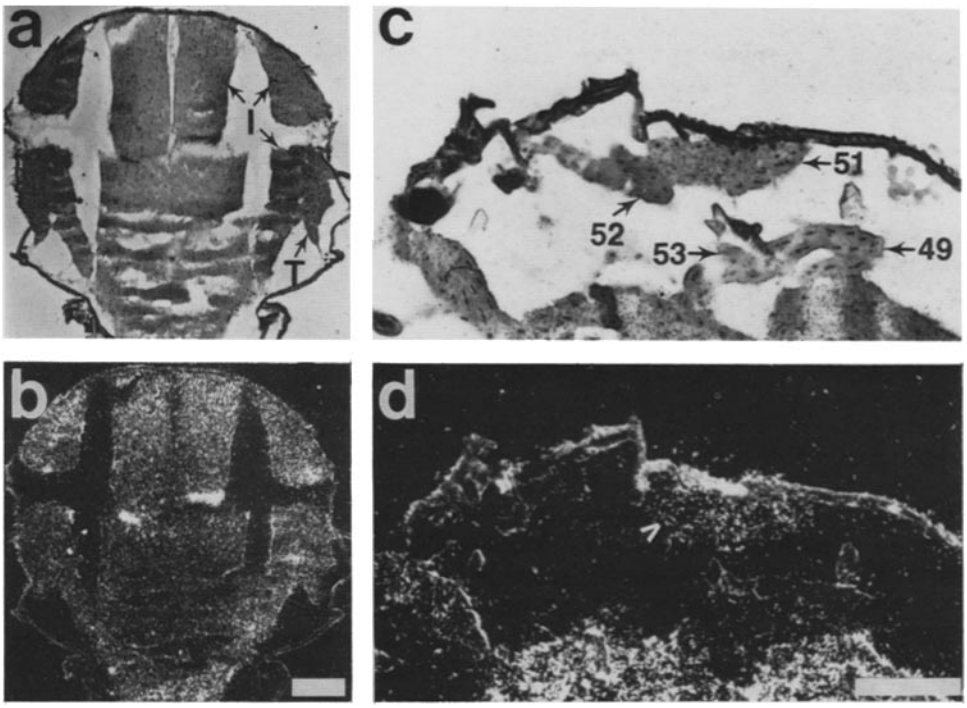


Figure 7. In situ hybridizations with a radiolabeled exon 18 probe. The IFM (*I*) and TDT (*T*) accumulate silver grains (*a* and *b*) as does DFM #51 (*c* and *d*). Weak hybridization to DFM #51 is marked (>). Bright field (*a* and *c*) and dark field (*b* and *d*) dorsal views are shown. Anterior is above (*a* and *b*) and to the right (*c* and *d*). Bars: (*a* and *b*) 125 μm and (*c* and *d*) 60 μm.

MHC sequences corresponding to exon 2 of the MHC 36B gene were hybridized to sections of the thorax (Fig. 9, *c* and *f*). No hybridization was detected in any of DFM, suggesting that the MHC isoforms in DFMs #49 and #53 are not produced by the nonmuscle MHC gene.

Discussion

This paper describes the localized expression of the four sets of alternatively spliced exons and one combinatorially spliced exon of the MHC 36B gene in the adult thoracic muscle of *Drosophila*. The muscle-specific expression of these exons was investigated using in situ hybridization analyses with antisense RNA and DNA probes to sections of adult thorax tissue, which has fibrillar, tubular, and visceral muscle fiber types. Table I summarizes our findings. Surprisingly, for each exon set examined, only one isoform is detectable in each of the muscles of the IFM, TDT, DFM, and esophagus. Only exon 3b of alternative exon 3 (3a/3b) set, located near the ATP-binding domain in the MHC head is expressed in all three muscle fiber types. Exon 7 (7a/7b/7c/7d) displays a more complex expression pattern. Exon 7d is present in fibrillar muscle while both 7a and 7d are expressed in tubular muscles. The visceral muscles of the esophagus express only exon 7d. Exon 11 (11a/11b/11c/11d/11e), which include one of the actin binding domains, is highly muscle-specific in its expression. Only exon 11e is expressed in the IFM, whereas tubular muscles express 11b (TDT, DFM #51) and 11c (DFM #52). The visceral muscle of the esophagus as well as DFM #52 express 11c. Exons 15, encoding the hinge domain in the MHC rod region also shows muscle-specific expression. Exon 15a is expressed in IFM, TDT, and DFM #51. Exon 15b is expressed in DFMs #49, #52, and #53 and the visceral muscle of the esophagus. The penultimate exon 18 and exons 7d and 15a are expressed in the IFM, TDT, and DFM #51. The remainder of the direct flight muscles and visceral muscles analyzed did not hybridize with the exon 18 probe.

The maximum number of MHC isoforms that can be generated by alternative and combinatorial splicing of the 36B MHC gene is 480 (George et al., 1989). Although all of these combinations need not be expressed to account for the usage of each of these individual exons in MHC isoforms, we and others have identified multiple isoforms by cDNA cloning and RNA analysis (George et al., 1989). Using in situ hybridization techniques, as shown in Table I, we have now determined that three different MHC isoforms are expressed in the three major muscle fiber types in the adult thorax (Table I). We note, however, that our analysis does not include analysis of the set of alternative exon 9 (exons 9a/9b/9c). Additional MHC isoforms that include different combinations of exon 9 are expressed and add to the complexity of MHC isoforms in these thoracic muscles (Edwards, 1990).

Our analysis shows that the IFM-specific MHC isoform, designated MHC I, includes alternative exons 3b, 7d, 11e, 15a, and 18. Isoform MHC II, which is expressed in the TDT and DFM #51, differs from MHC I in the substitution of 11e with 11b. A third isoform, MHC III, expressed in DFM #52 and the visceral muscle of the esophagus, differs from MHC I and II by the inclusion of exons 7a, 11c, 15b, but does not include exon 18. We conclude that MHC I, MHC II, and MHC III isoforms are expressed as functional transcripts since each muscle group examined expresses only one predominant MHC isoform with a unique set of alternative exons, as detectable by the in situ hybridization technique. Our data cannot exclude the possibility that other isoforms are expressed at a much lower abundance and have a functional role in myosin filament assembly or function. For instance, the coexpression of a minor mhc A isoform in the nematode regulates thick filament assembly during the development of body wall muscles (Waterston, 1989). Identifi-

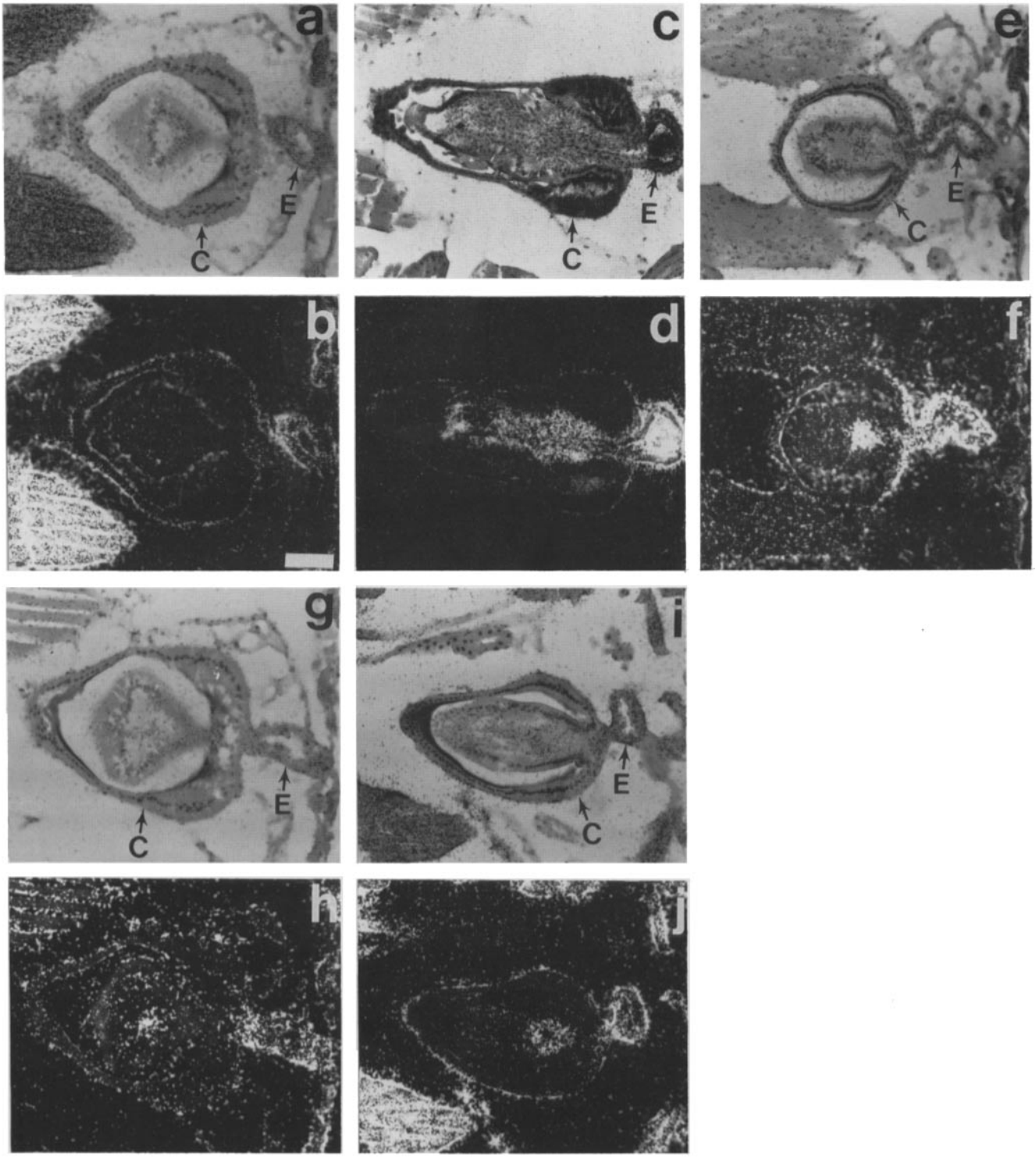


Figure 8. In situ hybridization of the thoracic visceral muscle with radiolabeled MHC 36B antisense RNA and DNA probes. Silver grains accumulate over the esophagus (*E*) when hybridized with 3b (*a* and *b*), 7a (*c* and *d*), 11c (*e* and *f*), 15b (*g* and *h*), and 19 (*i* and *j*) but not with any other alternative exons (data not shown). Bright-field (*a*, *c*, *e*, *g*, and *i*) and dark-field (*b*, *d*, *f*, *h*, and *j*) dorsal views are shown. Anterior is to the right in all panels. Bar, 125 μm.

cation of such minor isoforms of *Drosophila* MHC likely will require development of isoform-specific antibodies.

Myosin Isoform Expression and Muscle Fiber Type

The amino acid sequence divergence between exons within alternative exon sets and the location of amino acid sequences encoded by alterative exons within important functional domains of the MHC protein had previously suggested that these MHC isoforms have a functional role in the specialized contractile properties of the different fiber types of the *Drosophila* thorax (George et al., 1989). The results of this study, which demonstrate the restricted and muscle-specific expression of MHC isoforms in thoracic muscles, strongly

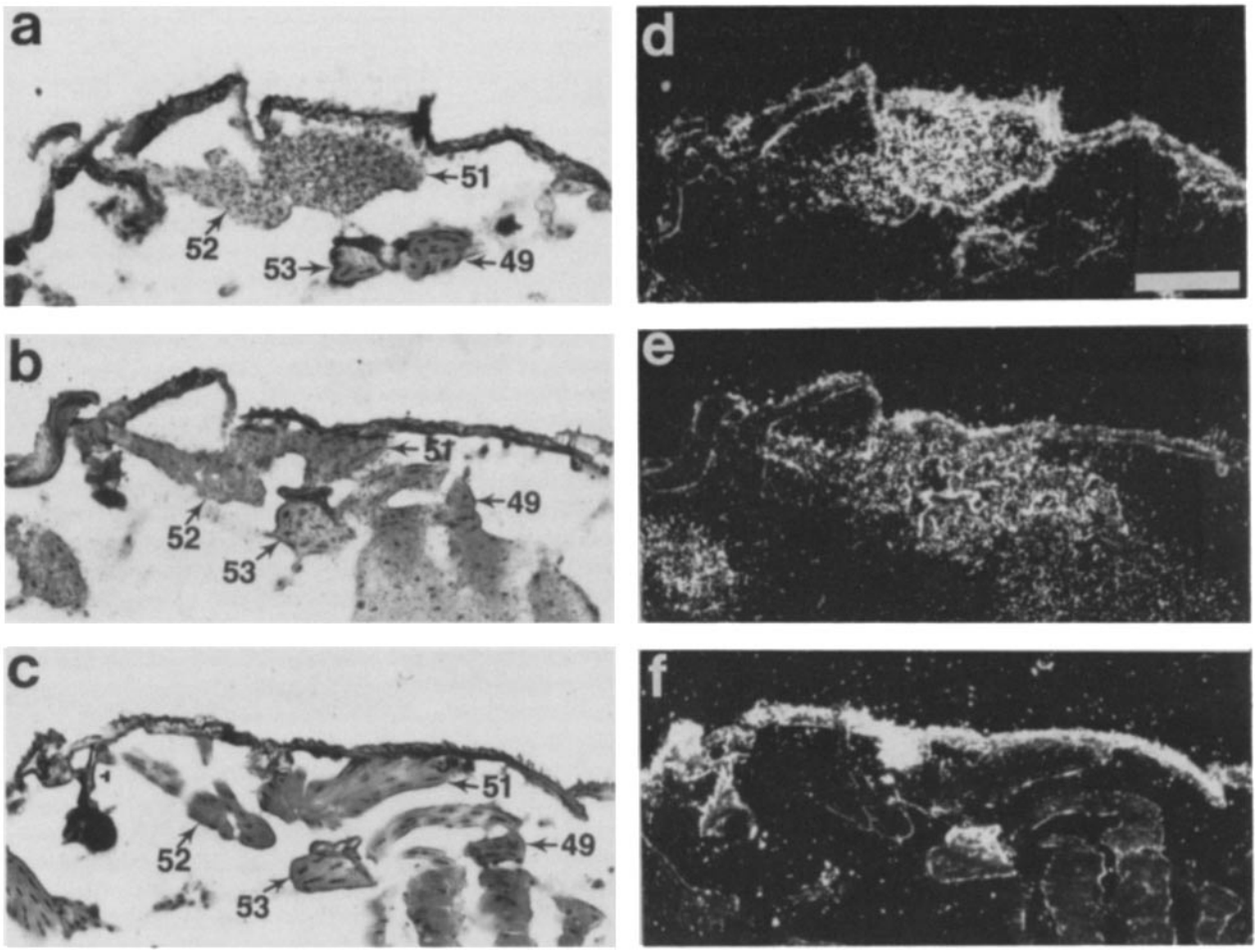


Figure 9. In situ hybridization of thoracic muscle with constitutive muscle and cytoplasmic MHC exons. Direct flight muscle-containing paraffin sections were hybridized with ^{35}S-labeled antisense RNA probes from the constitutive exons 2 and 19 of the muscle-specific MHC 36B gene. Also, an oligonucleotide corresponding to the MHC 36B exon 2 region (Kiehart et al., 1989) was generated from the cytoplasmic MHC cDNA sequence and hybridized to direct flight muscle-containing paraffin sections. Bright-field (*a–c*) and dark-field (*d–f*) dorsal views are shown. Hybridization of the muscle-specific MHC probe of the head region (exon 2) to direct flight muscles resulted in silver grains over DFM #51 and 52 but not to DFM #49 and 53 (*a* and *b*). The rod region probe of the muscle-specific MHC gene (exon 19) hybridized to DFM #51, #52, #49, and #53 (b and e). The ^{35}S-end-labeled cytoplasmic MHC oligonucleotide was hybridized to DFM. Only background levels of silver grains were detected over the DFM (*c* and *f*). Anterior is to the right in all panels. Bar, 100 μm.

supports this conclusion. In considering possible functional roles of the different expressed MHC isoforms, it is of interest to review the physiologically different muscles of the thorax. The largest muscle fibers of *Drosophila* are the fibrillar IFM in the late pupal and adult thorax. The IFM, which expresses MHC I isoform, are characterized by large mitochondria, interspersed nuclei and low (6:1) thin-to-thick myofilament ratios. Contractions which generate wing beat frequencies of up to 300 cycles per second are produced in the IFM (Pringle, 1967). Unlike other insect muscle types, the IFM are able to be stretch-activated and sustain continuous asynchronous contraction (greater than one wingbeat per nerve stimulus). Contraction velocity of the IFM fibers, as determined by the measurement of the rate constant of delayed tension, is also high compared to other insect muscle (Peckham et al., 1990). Each of these characteristics contrasts sharply with the tubular fiber type which is utilized by a wide range of skeletal muscles in *Drosophila* (Miller, 1950; Crossley, 1978). Tubular fibers comprise the majority of the adult skeletal muscle in the legs, thorax, and head. Tubular fibers are distinguished from IFM fibers by axial columns of nuclei and high (10-12:1) thin-to-thick myofilament ratios. The TDT, which expresses MHC II, is the large jump muscle of the mesothoracic leg and is the best characterized of the tubular muscles. Contractile properties of the TDT include synchronous contraction and a small rate con-

Table I. Expression of MHC and Actin Isoforms in Adult Thoracic Muscles

Muscle	MHC isoform	Exons	Actin isoform
IFM	I	3b 7d 11e 15a 18	Act88F*
TDT	II	3b 7d 11b 15a 18	Act79B‡
DFM #51	II	3b 7d 11b 15a 18	Act79B‡
DFM #52	III	3b 7a 11c 15b	—§
Esophagus	III	3b 7a 11c 15b	—§
DFM #49, #53	IV	x‖ x x 15b	—§

* Fyrberg et al., 1983.
‡ Courchesne-Smith and Tobin, 1989.
§ Unknown actin isoforms.
‖ Lack of MHC alternative exons.

stant of delayed tension (Pringle, 1967; Peckham et al., 1990). The muscle-specific expression of two different MHC isoforms in the IFM (MHC I) and TDT (MHC II) is one important characteristic that may distinguish the contractile properties of these muscles. In addition to the differential expression of MHC I and MHC II in the IFM and TDT muscles, these muscles express different actin isoforms (Table I) (Fyrberg et al., 1983; Courchesne-Smith and Tobin, 1989). Therefore, a reasonable possibility is that, at least in part, the myofibrillar structure and contractile characteristics of muscle fiber type reflect the interactions of a unique set of muscle protein isoforms expressed in each muscle fiber type. Consistent with this view, studies of *Drosophila* actin, arthrin, myosin light chain, troponin H, and tropomyosin gene expression have identified IFM-specific isoforms (Fyrberg et al., 1983; Ball et al., 1987; Karlik and Fyrberg, 1986; Falkenthal et al., 1987; Bullard et al., 1988; and Hanke and Storti, 1988). Further analysis of the sets of muscle protein isoforms expressed in different tubular muscles will be needed to ascertain whether each tubular muscle contains its own special complement of myofibrillar protein isoforms. The localization of MHC I to IFM, MHC II to the TDT and DFM #51, and MHC III to DFM #52 and esophagus shows that at least three MHC isoforms can be utilized by functionally different muscles to perform flight, jump, and digestive functions.

Functional Aspects of MHC Alternative Exons

Exon 11 encodes one of the actin binding domains likely important in MHC head/actin interactions during the contraction cycle. Alternative exon 11e is expressed in the IFM, whereas alternative exons 11b and 11c each are expressed in two other tubular muscles. The localized expression of exon 11e in IFM is underscored by its more extreme sequence divergence when compared with the other alternative exon 11s (George et al., 1989). The muscle-specific expression of alternative exons 11e, 11b, and 11c, which encode an actin binding domain, is correlated with the muscle-specific expression of actin isoforms. *Drosophila* has six genes encoding actin isoforms that are expressed according to stage and tissue-specific regulation (Fyrberg et al., 1980). Act88F, which is IFM specific (Fyrberg et al., 1983), is coexpressed with the MHC I isoform, which has the alternative exon 11e actin binding domain. Act79B is coexpressed with the MHC II isoform in the TDT and DFM #51 (Courchesne-Smith and Tobin, 1989). This suggests that MHC isoforms with specific alternative exon 11's specify function in coordination with specific actin isoforms during crossbridge interactions. Since *C. elegans* mhc B mutations in this actin binding sequence region, encoded by exons 11 of the *Drosophila* gene, disrupt assembly, it is also possible that alternative exon 11s regulate the muscle-specific assembly of *Drosophila* myosin isoforms into thick filaments (Bejsovec and Anderson, 1990).

Exon 15a is expressed in the IFM and TDT, two muscles with comparatively high contractile velocity (George et al., 1989; Collier et al., 1990). In contrast, exon 15b is expressed in the slower embryonic, larval, and visceral muscles. One model of force generation during contraction proposes that the myosin head region forms crossbridges with thin filament actins, leading to conformational changes in the head and tension development centered in the hinge portion of the myosin rod (Huxley and Kress, 1985). The observation that rodless myosin heads move along actin filaments in vitro supports this model (Hynes et al., 1987; Toyoshima et al., 1987; Kishino and Yanagida, 1988). An alternative, although not necessarily mutually exclusive model, couples a random coil conformational transition in the hinge with force generation (Ueno and Harrington, 1986*a*,*b*). Since alternative exons 15a and 15b both encode sequences that interrupt the rod's coiled-coil structure, it is possible that their presence within the hinge domain could affect contraction either by acting as a point of elastic tension or as the main force generator. Measurements of shortening rate and isometric force of *Drosophila* muscle fibers containing myosin isoforms with the different exons 15 may provide further evidence for the importance of the hinge domain.

Confirmation of the functional nature of MHC isoforms will be aided by further analysis of muscle-defective mutants. Several homozygous viable dominant flightless MHC mutations have been studied (O'Donnell et al., 1989; Chun and Falkenthal, 1988). Mhc^{10} flies have nonfunctional IFM and TDT. Sequence analysis of the mutant MHC^{10} allele reveals a defective 3′ splice-acceptor site at exon 15a (Collier et al., 1990). Our finding that both IFM and TDT normally express exon 15a is consistent with the IFM and TDT phenotypes of this mutant.

Our findings that the individual thoracic muscles express only one abundant MHC isoform opens the possibility for transgenic studies to investigate the roles of specific exon domains in muscle fiber type function. Mutant strains carrying the Mhc^{10} mutation will provide a unique host for constructing transgenic strains for these studies. The Mhc^{10} mutants do not express MHC mRNA in the IFM and TDT muscles, so expression and function of MHC transgenes in muscles of the Mhc^{10} mutant can be studied in the absence of mutant MHC RNA and protein. For instance, introduction of an expression vector containing the MHC I isoform cDNA into the germ line should rescue flight and normal myofibrillar organization in the IFM. Characterization of wing speed, jumping ability, ATPase activity, actin binding, and thick filament assembly of MHC isoforms introduced as transgenes into this mutant should provide valuable functional and structural information.

Implications of Isoform MHC IV

The finding that two direct flight muscles, DFMs #49 and #53, do not express head alternative exons was unexpected. It was also surprising that these muscles do not express constitutive exons that encode MHC head sequences, although rod exons, 15b and 19 were detected. Since expression of the cytoplasmic MHC gene was not detected in these muscles, we suggest that these muscles express a second, previously uncharacterized muscle myosin. This putative myosin would maintain a high degree of sequence conservation with the MHC 36B rod region but diverge considerably with the head region. The cloning of many myosin, dynein and kinesin-like genes suggest the existence of families of force-generating proteins (for a review see Vale and Goldstein, 1990). Previous chromosomal in situ hybridization studies (Bernstein et al., 1983) did not support this possibility because only the 36B locus was detected with a muscle MHC gene probe. Genomic Southern blot analysis with whole gene and exon-

specific MHC 36B probes have failed to identify a second muscle myosin homologue (Bernstein et al., 1983; our unpublished results). Another possibility is that MHC IV is produced by a trans-splicing mechanism similar to that in *C. elegans* (Bektesh and Hirsh, 1988) and trypanosomes (Freistadt, 1988; Layden and Rosen, 1988). The trans splicing of head exons from an unlinked gene would provide an alternative means for the fine tuning of MHC isoforms required by highly specialized muscles. Molecular cloning and sequencing of the MHC IV cDNA or gene will shed light on this possibility.

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