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Alternative RNA Splicing Generates Transcripts Encoding a Thorax-Specific Isoform of *Drosophila melanogaster* Myosin Heavy Chain

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Genomic and cDNA sequencing studies show that transcripts from the muscle myosin heavy-chain (MHC) gene of *Drosophila melanogaster* are alternatively spliced, producing RNAs that encode at least two MHC isoforms with different C termini. Transcripts encoding an MHC isoform with 27 unique C-terminal amino acids accumulate during both larval and adult muscle differentiation. Transcripts for the second isoform encode one unique C-terminal amino acid and accumulate almost exclusively in pupal and adult thoracic segments, the location of the indirect flight muscles. The 3′ splice acceptor site preceding the thorax-specific exon is unusually purine rich and thus may serve as a thorax-specific splicing signal. We suggest that the alternative C termini of these two MHC isoforms control myofilament assembly and may play a role in generating the distinctive myofilament organizations of flight muscle and other muscle types.

Myosin heavy chain (MHC) is the major structural protein of thick filaments in muscle sarcomeres (18). The globular head of the myosin molecule binds actin-containing thin filaments during the process of muscle contraction (18). On hydrolysis of ATP by MHC (54), the myosin head changes conformation, resulting in the movement of thin filaments past thick filaments and muscle contraction (18). Structurally related protein isoforms of striated muscle MHC occur in humans (30), rats (32, 62), mice (60), chickens (47, 59, 61), and the nematode *Caenorhabditis elegans* (23). MHC isoforms accumulate at specific developmental stages and in specialized muscle types (23, 30, 32, 47, 59–62). Isoforms exhibit different ATPase activities or actin-binding abilities (1, 31) that likely contribute to the physiological and functional specializations of various muscle types of these organisms. In nematode body wall muscle, different MHC isoforms assemble in the central zone and polar regions of the thick filament (36).

Human, rat, mouse, chicken, and nematode MHC isoforms are encoded by multigene families (23, 30, 32, 42, 47, 59, 60, 62). These families likely arose by duplication of a primordial MHC gene. Some members of each family have evolved independent modes of transcriptional regulation for expression in selected muscles. MHC isoforms encoded by these genes may be adapted to the specialized functions of the tissues in which they are differentially expressed.

In contrast to other metazoans studied to date, *Drosophila melanogaster* has only a single muscle MHC gene (7, 50). This result is surprising since *D. melanogaster*, like other organisms, has several specialized muscle types with unique physiological and ultrastructural properties (11). For instance, the indirect flight muscles of adults are highly organized, have six thin filaments surrounding each thick filament, and contract asynchronously (many times per nerve impulse). On the other hand, larval body wall muscles are less organized than flight muscle, have 9 to 12 thin filaments surrounding each thick filament, and contract synchronously. These observations, as well as the identification of two biochemically distinct forms of intact *Drosophila* myosin (46), suggest that *D. melanogaster* synthesizes more than one form of muscle MHC.

Alternative splicing patterns of transcripts from a single gene can permit the production of RNAs that encode more than one protein isoform (2, 4, 8, 10, 14, 40, 41, 48, 49, 63). Previously, Rozek and Davidson (50) showed that three size classes of MHC RNA are produced from the *Drosophila* MHC gene. In this study, we examined the stage- and tissue-specific accumulation of the alternative MHC transcripts and whether these transcripts encode MHC protein isoforms that may be crucial for the ultrastructural and physiological differentiation of various *Drosophila* muscle types. We showed that the single *Drosophila* MHC gene encodes at least two MHC proteins which are synthesized using alternatively spliced forms of the MHC transcript. Genomic DNA and cDNA sequencing data indicate that these transcripts differ in their C-terminal coding regions. Transcripts encoding one of these proteins accumulate almost exclusively in the adult thorax. The stage-specific exon of this transcript is preceded by an unusual acceptor splice junction that may only be recognized during adult muscle differentiation.

MATERIALS AND METHODS

RNA isolation, electrophoresis, and blotting. RNA was isolated from intact organisms as described by Fyrberg et al. (17). Briefly, embryos, larvae, pupae, or adults were homogenized in a buffer containing guanidine isothiocyanate and β-mercaptoethanol. After addition of cesium chloride, the homogenate was centrifuged overnight. The RNA band was isolated from the gradient by tube puncture. RNA was ethanol precipitated and dissolved in sterile distilled water. RNA from dissected adults or pupae was isolated by grinding tissue in a Dounce homogenizer in a 1:1 mixture of water-saturated phenol and RNA extraction buffer (0.1 M NaCl, 0.1% sodium dodecyl sulfate, 0.01 M Tris hydrochloride, pH 7.4) at 80°C. An equal volume of chloroform was added, and the homogenate was vortexed and centrifuged. The aqueous layer was re-extracted with phenol-chloroform

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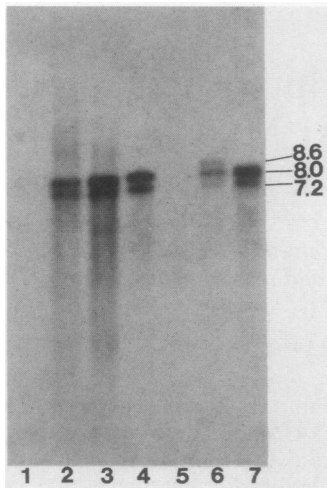

FIG. 1. Accumulation of MHC transcripts during *Drosophila* development. Polyadenylated RNAs extracted from 0- to 4-h-old embryos (lane 1), 16- to 20-h-old embryos (lane 2), first-instar larvae (lane 3), third-instar larvae (lane 4), early-stage pupae (approximately 1 day after pupation) (lane 5), mid-late-stage pupae (after deposition of eye pigments) (lane 6), and fused cultures of embryonic muscle (lane 7) were resolved by denaturing gel electrophoresis and blotted onto nitrocellulose. A nick-translated MHC gene hybridized to transcripts of 7.2 and 8.0 kb which accumulated during the late embryonic stage through the larval instars (lanes 2 to 4). These transcripts were present in cultures of fused embryonic muscle (lane 7) but absent in cultures prior to myoblast fusion (data not shown). No MHC transcripts were detected during the early stages of metamorphosis (lane 5), but three size classes of MHC RNA (7.2, 8.0, and 8.6 kb) accumulated in mid-late-stage pupae (lane 6). The 7.2-kb transcript is a relatively minor component. Reduced amounts of these transcripts were present in adults, and identical results were obtained when total RNA was used in place of polyadenylated RNA (data not shown).

and then chloroform; after addition of NaCl (to a final concentration of 0.2 M) and 2.5 volumes of ethanol, the RNA was precipitated by incubation at −20°C overnight. To prepare RNA from cultured embryonic muscle cells, myoblasts were isolated from gastrulae and cultured as previously described (6). Cultured cells were lysed directly in RNA extraction buffer, and RNA was isolated by phenol-chloroform extraction (5). Polyadenylated RNA was selected by oligo(dT)-cellulose chromatography (5). Twenty micrograms of total RNA or 0.5 μg of polyadenylated RNA was electrophoresed on a formaldehyde-containing agarose gel (33). Gels were blotted to nitrocellulose (33) for hybridization analysis.

DNA probe preparation and hybridization. DNA probes for RNA gel blot hybridization or for screening cDNA libraries were prepared by nick translation (33). Blot hybridizations were performed in hybridization buffer containing 50% formamide as previously detailed (7). To reduce nonspecific DNA binding, the hybridization mixture containing the denatured probe was incubated for several hours with a blank piece of nitrocellulose (R. M. Grainger, personal communication). The mixture was then heated to 70°C for 10 min, chilled on ice, and added to the prehybridized nitrocellulose blot. Autoradiography was for 2 to 5 days at −70°C with a Dupont Cronex Lightning-Plus screen and Kodak XAR-5 film. Larval, pupal, and adult cDNA libraries were prepared in plasmids as previously detailed (19). Additional *Drosophila* cDNA libraries, in the vector λ gt10, were obtained from Donna Lastowski-Perry (University of North Carolina, Chapel Hill) and Barry Yedvobnick (Emory University, Atlanta, Ga.). Libraries were screened by the procedures of Maniatis et al. (33).

DNA sequencing. Fragments of the MHC gene were subcloned into pBR322 or pUC9 for ease of restriction enzyme analysis and end labeling. Blunt-ended or protruding 5′ termini were labeled by the forward kinase method (34). Recessed 3′ termini were labeled by fill in with [α-^{32}P]dNTPs using the large fragment of DNA polymerase I (16). Protruding 3′ termini were labeled using terminal transferase and [α-^{32}P]di-dATP (Amersham 3′ labeling kit). Sequencing was performed by the Maxam and Gilbert protocol (34), except that 0.65 M piperidine was used for DNA cleavage and only a single lyophilization (done overnight) was performed after the DNA cleavage step. Sites that were utilized for labeling and sequencing were always sequenced from another labeled restriction site to ensure that any small restriction fragments were detected.

S1 nuclease mapping. End-labeled DNA fragments were strand separated prior to S1 nuclease mapping. To facilitate this, we cloned a 5.0-kilobase (kb) *Hin*dIII fragment of the MHC gene into pKH47, a derivative of pBR322 with a stretch of T residues inserted at the *Pvu*II site (20). Treatment of this MHC gene subclone with *Sal*I liberates a fragment which contains a portion of the vector linked to 0.2 kb of MHC DNA. The remainder of the vector, containing the oligo(T) stretch, is linked to 4.8 kb of the *Drosophila* DNA which is located 3′ to the *Sal*I site of the noncoding strand. We labeled this plasmid at its *Sal*I sites by the 3′ fill-in method (16) and strand separated the fragment containing the oligo(T) stretch by serial passage over oligo(dA)- and oligo(dT)-cellulose columns (20). The DNA bound to each column was eluted, ethanol precipitated, and hybridized (43) to 20 μg of larval or pupal RNA. After overnight incubation, the samples were diluted in S1 buffer (43), treated with 100 U of S1 nuclease for 40 min at 45°C, and precipitated. Samples were suspended in sequencing dye (34) and electrophoresed on a 4% acrylamide-urea gel (34). Protected fragments were observed by autoradiography.

RESULTS

Developmental expression of the *Drosophila* MHC gene. We examined the accumulation of MHC transcripts during *Drosophila* development. RNA isolated from embryos, larvae, pupae, adults, and cultures of embryonic myoblasts was resolved by electrophoresis on denaturing gels, blotted to nitrocellulose, and probed with a nick-translated MHC gene. MHC RNA began to accumulate during the late embryonic stage and was present throughout the larval stages (Fig. 1). These transcripts were not detectable in an undifferentiated tissue culture cell line or in embryonic myoblasts prior to fusion (data not shown). They were, however, present at high levels in cultures of fusing embryonic myoblasts (Fig. 1), indicating that MHC RNA specifically accumulates in differentiating and mature embryonic muscle cells. During early pupation, when larval structures are histolyzed, MHC transcripts were not detected. They again accumulated during the mid-late pupal stage and persisted at reduced levels in adults. The developmental stages at which MHC transcripts accumulate correspond to the periods of muscle differentiation and maintenance (11, 45). During late embryogenesis, embryonic myoblasts fuse to form myofibers. These fibers increase in size during larval growth. At the mid-late pupal stages, adult muscles differentiate and these muscles are maintained during the adult stage.

Interestingly, different-sized MHC transcripts accumulated at larval and adult myogenesis. During the late embryonic and larval stages, transcripts of 7.2 and 8.0 kb were present. Both of these transcripts also accumulated in cultures of embryonic muscle beginning at the fusion stage of myoblast development. During metamorphosis, the level of the 7.2-kb transcript was greatly reduced compared with that of the 8.0-kb transcript, and a third MHC RNA of 8.6 kb appeared. Rozek and Davidson (50) originally noted the presence of multiple MHC transcripts, and we adopted their molecular weight designations. Our subsequent analysis established that these transcripts encode two different MHC proteins.

Alternative patterns of RNA splicing generate transcripts that code for different isoforms. To locate regions of the MHC gene that are specific to the different-sized MHC RNAs, we isolated and labeled restriction fragments of the gene and probed blots of gels containing larval and pupal RNAs. Since several other genes which generate multiple-sized transcripts produce RNAs which differ at their 3′ or 5′ termini (2, 4, 40, 48, 49, 63), we examined these regions of the MHC gene in our initial analysis. We did not detect any differential hybridization by probing with various restriction fragments near the 5′ end of the gene (D. R. Wassenberg and S. I. Bernstein, unpublished data). However, an *Eco*RI fragment from the 3′ region of the gene (Fig. 2, probe 3) hybridized to the 8.0- and 8.6-kb RNAs but not to the 7.2-kb RNA (Fig. 2B).

To understand the basis of the differential hybridization of the 3′-terminal gene fragment to the MHC transcripts, we sequenced approximately 2.8 kb of DNA at the 3′ end of the gene. Fig. 3 shows the DNA sequence of this portion of the MHC gene. The derived amino acid sequence for the *Drosophila* protein is compared with the amino acid sequences of a rat cardiac MHC (32) and the *C. elegans unc-54* MHC (23). Located at base 484 within the presumptive C-terminal coding region of the *Drosophila* gene is a sequence (GCGGTAAGT) that is similar to the 5′ splice consensus sequence $\frac{C}{A}$AGGT$\frac{A}{G}$AGT (39) and is identical to a 5′ splice junction in the *Drosophila* alcohol dehydrogenase gene (3). To examine whether this sequence is a donor splice junction, which may be involved in the observed transcript heterogeneity, we labeled the cloned gene at an upstream *Sal*I site (base 281). An S1 nuclease protection experiment (see Materials and Methods for our protocol) yielded a protected fragment of 206 bases (Fig. 4), establishing that the observed 5′ splice consensus sequence is utilized as a donor splice site. Since no other protected fragments were observed, this splice junction is utilized in all larval and pupal transcripts. The exon which contains this 5′ donor site is identified as exon 1 in Fig. 2 and 3.

By isolating and sequencing MHC cDNA clones, we determined the location of the splice acceptors and polyadenylation sites downstream of the donor splice site. We screened several larval, pupal, and adult cDNA libraries with the 3′ end of the MHC gene to isolate clones possessing reverse-transcribed copies of the 3′ end of the mRNA. We obtained and sequenced two larval cDNA clones. One of them (280 base pairs [bp]) has a poly(A) tail beginning at nucleotide 2435, 13 bases downstream from the polyadenylation consensus sequence AATAAA (15). Although seven putative polyadenylation consensus sequences were found within the genomic DNA sequence (Fig. 3), only two are apparently functional (see below). The 280-bp cDNA clone contained no splice junctions. However, in the other larval cDNA clone (1.8 kb), the 5′ splice junction of exon 1 (Fig. 2 and 3) is spliced to nucleotide 1691 of the genomic DNA sequence. The 3′ exon beginning at nucleotide 1691 is 745 bp long and encodes 27 C-terminal amino acids followed by a termination codon (Fig. 2 and 3, exon 3).

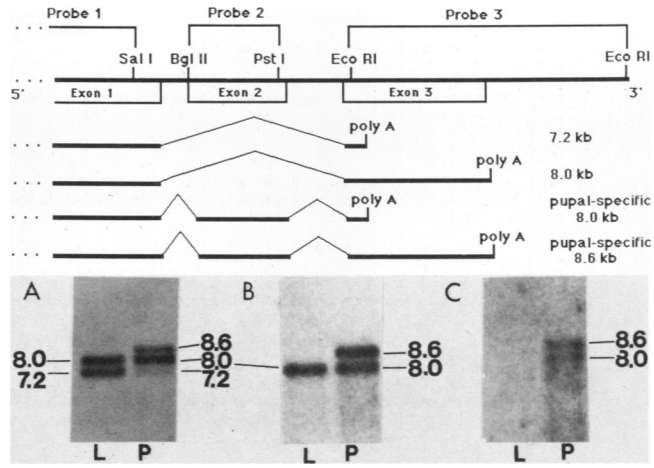

FIG. 2. Probing RNA gel blots with 3′ fragments of the MHC gene revealed differences among the MHC transcripts. MHC gene fragments (top) were nick translated and used to probe gel blots (bottom) of polyadenylated RNAs from larvae (L) and pupae (P). Fragments from the 5′ and central portions of the gene (probe 1) hybridized to larval transcripts of 7.2 and 8.0 kb and to pupal transcripts of 7.2, 8.0, and 8.6 kb (A). The terminal *Eco*RI fragment of the gene (probe 3) hybridized to the larval 8.0-kb and the pupal 8.0- and 8.6-kb transcripts (B). The *Bgl*II-*Pst*I fragment (probe 2) hybridized to the pupal 8.0- and 8.6-kb fragments but to neither larval transcript (C). These results, along with S1 nuclease and DNA sequence analyses (Fig. 3 and 4) and the observations of Rozek and Davidson (50a) concerning alternative polyadenylation sites, have permitted elucidation of the pattern of 3′ processing of MHC transcripts shown in the line drawing. cDNA sequence analysis indicates that exon 2 is 500 bp long, whereas exon 3 is 194 or 745 bp long, depending on the site of polyadenylation. Note that, although probe 3 shares 118 bp of homology with the 7.2-kb transcript, the G+C content of this region is only 12%, which precludes the formation of a stable hybrid under the conditions used.

We isolated eight MHC cDNA clones from adult cDNA libraries. DNA sequence analysis of one clone, along with restriction endonuclease mapping and DNA blot hybridization of the others, revealed that these clones have a 500-bp exon inserted between exons 1 and 3 (Fig. 2 and 3, exon 2). This result agrees with the R-looping studies of Rozek and Davidson (50), which showed that such an exon is included only in the pupa-specific 8.6-kb transcript. Our sequence analysis indicates that insertion of this exon results in a transcript that encodes an MHC isoform with a single, unique C-terminal amino acid (Fig. 3). Exon 3 is apparently not translated in transcripts that contain exon 2. Although all of our adult cDNA clones terminate upstream of the polyadenylation signals in exon 3, this exon apparently provides a polyadenylation site for these RNAs (see below).

To prove that the 8.6-kb transcript contains the exon encoding the single C-terminal amino acid (exon 2), we hybridized a nick translated *Bgl*II-*Pst*I fragment (which contains most of this exon) to an RNA gel blot. This probe (probe 2) hybridized to the pupal 8.6-kb transcript and also to the pupal (but not the larval) 8.0-kb RNA (Fig. 2C). Thus, both the pupal 8.0- and 8.6-kb transcripts contain this exon. The size differences between these pupal transcripts and between the larval 7.2 and 8.0-kb transcripts (which are also

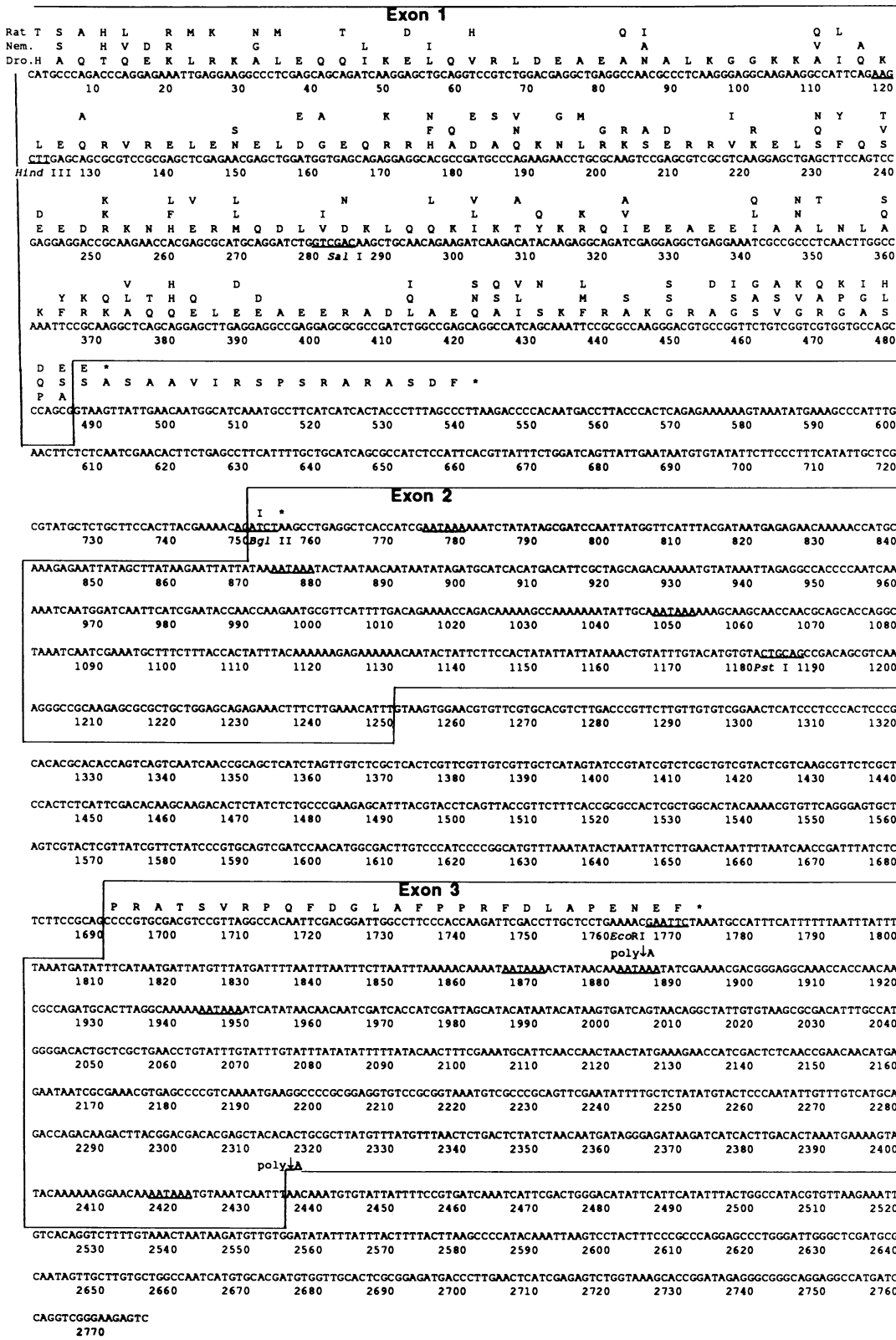

FIG. 3. DNA sequence analysis of the 3′ end of the *Drosophila* MHC gene. The derived amino acid sequence of *Drosophila* MHC is compared with the amino acid sequences of a rat cardiac MHC (32) and *C. elegans unc-54* MHC (23). Only differences from the *Drosophila* sequence are shown. The locations of splice junctions were determined by S1 nuclease mapping (Fig. 4) and cDNA clone sequencing. Exons are boxed, and exon numbers correspond to those in Fig. 2. Important restriction enzyme sites and possible polyadenylation signals (AATAAA) are underlined. Polyadenylation sites are indicated by arrows.

present at the pupal stage) result from use of an alternative polyadenylation site located at base 1884 of our sequence (50a). Transcripts which utilize this alternative polyadenylation site should be approximately 0.6 kb shorter than transcripts which are polyadenylated at base 2435, and this corresponds fairly well with the observed size difference between each pair of transcripts.

All of the sequences obtained from the MHC cDNA clones are colinear with the exons of the sequenced MHC gene, indicating that four unique mature RNAs are generated by alternative splicing and polyadenylation of the primary transcripts from a single gene. The 7.2- and 8.0-kb transcripts, which are present at all stages of muscle differentiation, contain exons 1 and 3. These transcripts encode an MHC protein with 27 unique C-terminal amino acids. The pupa-specific 8.0- and 8.6-kb RNAs contain exons 1, 2, and 3 and encode a protein with a single, unique C-terminal amino acid. The size differences between the 7.2- and 8.0-kb transcripts and between the pupa-specific 8.0- and 8.6-kb transcripts result from use of alternative polyadenylation sites (50a). The alternative splicing patterns of *Drosophila* MHC transcripts have been independently determined by Rozek and Davidson (50a).

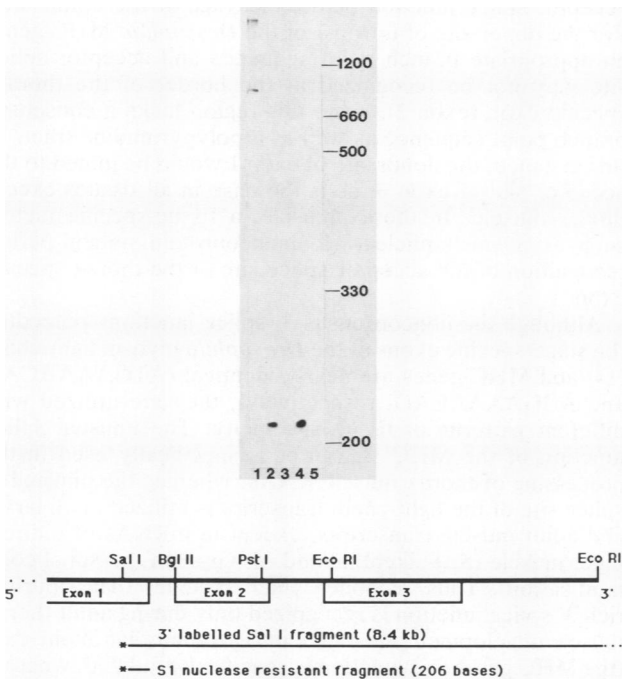

FIG. 4. S1 nuclease analysis of the MHC gene revealed that all transcripts are spliced upstream of the C-terminal protein coding region. The figure is an autoradiograph of a urea-acrylamide gel containing S1-nuclease-treated RNA-DNA hybrids. The DNA was 3′ labeled at an upstream *Sal*I site (Fig. 3), strand separated, and hybridized to larval or pupal RNA. Labeled DNA prior to strand separation was composed of two fragments (8.4 and 0.7 kb; lane 1). After isolation of the strand-separated 8.4-kb fragment, followed by RNA hybridization and S1 nuclease treatment, a single protected fragment of approximately 215 bases was present in both the larval and pupal RNA samples (lanes 2 and 4). No protected fragments were observed when the complementary DNA strand was used as a probe (lanes 3 and 5). By electrophoresing the protected fragment in parallel with a DNA sequence beginning at the *Sal*I site, we determined that the transcripts were spliced 206 bases beyond this site, the precise location of the consensus splice sequence at the border of exon 1 (see Fig. 3 and the text).

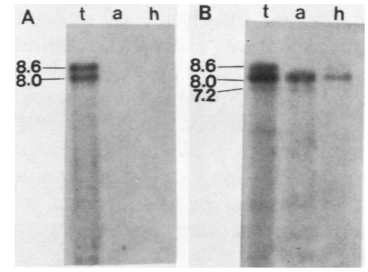

FIG. 5. Tissue-specific accumulation of MHC transcripts. Total RNA isolated from three adult thoraces (t), six heads (h), and six abdomens (a) was electrophoresed on a denaturing gel, blotted, and probed with the pupa-specific exon (probe 2 in Fig. 2) or a genomic clone containing the terminal 12 kb of the MHC gene (A and B, respectively). The pupa-specific 8.0- and 8.6-kb transcripts accumulated almost exclusively in the thoracic segments, whereas the 7.2- and 8.0-kb transcripts accumulated in all regions of the body (B). Prolonged exposures indicate that minor amounts of the pupa-specific transcripts also accumulated in the head (data not shown). Identical results were obtained with pupal tissues (data not shown).

Tissue-specific accumulation of MHC RNA. To examine whether the various MHC transcripts accumulate in a tissue-specific manner, we extracted RNAs from heads, thoraces, and abdomens of pupae and adults. RNA was analyzed by probing gel blots with either the pupa-specific exon (probe 2) or a genomic DNA clone containing a large portion of the MHC gene (probe 1). The pupa-specific exon only hybridized to RNA from thoracic segments (pupa-specific 8.6 and 8.0-kb transcripts), whereas the large genomic fragment hybridized to 7.2- and 8.0-kb transcripts in the head and abdomen and 7.2-, 8.0-, and 8.6-kb transcripts in the thorax (Fig. 5). Long exposures of head RNA revealed the presence of very low levels of the 8.6-kb transcript (data not shown). Our results indicate that the pupa-specific transcripts accumulate almost exclusively in the thoracic segment. Thus, stage- and tissue-specific processing of the MHC transcript results in the accumulation of transcripts encoding a thorax-specific isoform of MHC.

Structure of *Drosophila* MHC. The decoded amino acid sequence of the *Drosophila* MHC gene bears a striking similarity to muscle MHCs of other organisms. Of the 151 amino acids which make up the C-terminal portion of the coiled coil of the intact myosin molecule, 67% are identical to *C. elegans unc-54* MHC (23), and 64% are identical to a rat cardiac muscle MHC (32). The 28-residue repeat structure which maintains the coiled-coil conformation of the intact myosin molecule (35) shows a high degree of amino acid charge conservation among the three species. The encoded 28-residue repeat ends approximately 33 nucleotides (11 amino acids) upstream of the 5′ splice junction of exon 1. The noncoiled "tailpiece" (35) of the protein is highly diverged from those of rats and *C. elegans*. Furthermore, different terminal portions of the tailpiece are encoded by the thorax-specific exon (1 amino acid residue) and the 3′-most exon (27 residues). The tailpieces of each isoform cannot form a stable coiled coil, and both tails are hydrophobic (6 of 12 and 20 of 38 residues are hydrophobic).

DISCUSSION

Stage-specific RNA processing generates transcripts encoding a thorax-specific isoform of MHC. At least four different mature transcripts are produced from the *Drosophila* MHC gene. The transcripts differ by the inclusion or exclusion of

an exon (exon 2) and by choice of polyadenylation site. Transcripts which exclude exon 2 accumulate during both larval and adult muscle differentiation and encode an MHC isoform with 27 unique C-terminal amino acids. Transcripts which include exon 2 encode an MHC isoform with one unique C-terminal amino acid and accumulate only during adult muscle differentiation, almost exclusively in the thoracic region. Thus, tissue-specific alternative RNA splicing generates a transcript encoding an MHC isoform unique to thoracic muscle.

The mechanism of MHC RNA alternative processing. Alternative splicing of *Drosophila* MHC transcripts could result from recognition of stage- or tissue-specific transcript splicing signals. Another possibility is that different-sized, unspliced transcripts are synthesized from the MHC gene, and an extra splice junction is included in longer, unspliced transcripts. This splice junction would be joined to a splice site that is either not utilized (2, 4, 40, 48) or differently spliced (49, 63) in shorter transcripts which lack the extra splice junction. Such different-length unspliced transcripts can be generated as a result of utilization of alternative promoters (2, 4, 40, 63) or multiple polyadenylation signals (48, 49). For the *Drosophila* MHC gene, the synthesis of different-sized transcripts does not affect the pattern of RNA splicing. Although alternative polyadenylation sites are utilized, transcripts that include or exclude the thorax-specific exon (exon 2) are polyadenylated at either of the two identified polyadenylation sites (50a). Furthermore, recent results (Wassenberg and Bernstein, manuscript in preparation) indicate that the same transcription initiation site is used for all MHC transcripts.

Since neither the pattern of 3′ end formation nor the production of transcripts with alternative 5′ ends can be implicated in alternative splicing of *Drosophila* MHC transcripts, it appears that identical primary transcripts from the MHC gene can be processed to yield different mature RNAs. At least four other genes, the chicken cardiac muscle troponin T gene (10), the rat skeletal muscle troponin T gene (8), the bovine preprotachykinin gene (41), and the *Drosophila* alkali myosin light-chain gene (14), produce a single form of primary transcript which can be alternatively processed. Like the MHC gene, the alternative transcripts from each of these genes accumulate in a tissue-specific fashion. The mechanism of tissue-specific alternative splicing of identical primary transcripts is presently unknown.

Since a single MHC primary transcript can be spliced via two different pathways, resulting in the inclusion or exclusion of the thorax-specific exon, there must be tissue-specific recognition of splicing signals within this transcript. The acceptor splice junction that precedes the thorax-specific exon is unusual and may therefore play a role in this process. At this splice junction, the region immediately upstream of the consensus sequence NYAG (39) is deficient in pyrimidines (only one of the six upstream bases is a pyrimidine), whereas most 3′ splice junctions have a polypyrimidine tract in this region (39). Recent studies indicate that the polypyrimidine tract is essential for splicing of β-globin transcripts (52). Furthermore, an adenovirus 3′ splice junction with six pyrimidines within the 11 nucleotides upstream of the NYAG sequence is a less efficient splicing substrate than junctions which contain 8 or 9 pyrimidines in this region (55). Although *Drosophila* acceptor splice junctions are not as pyrimidine rich as mammalian 3′ splice sites (25), the presence of a *Drosophila* acceptor sequence nearly devoid of pyrimidines is extremely rare (25). Of 39 *Drosophila* acceptor splice junctions cataloged by Keller and Noon (25), only one (that preceding the alternatively spliced exon of the alkali myosin light-chain gene) is as pyrimidine deficient as the junction preceding the thorax-specific exon of the MHC gene.

The 3′ splice junction of the thorax-specific exon also lacks a consensus branch point sequence. Branch point sequences, which are found 20 to 50 bases upstream of acceptor splice junctions, have been implicated in RNA intron excision (28, 53). The 5′ end of the intron is joined to the 2′ OH group of the adenosine residue within the branch point sequence; this is followed by cleavage at the acceptor splice site and ligation of the two exons (28, 53). A consensus branch point sequence ($\frac{C}{T}T\frac{A}{G}A\frac{T}{C}$; 25) is not readily apparent in the region preceding the thorax-specific exon, although three such sequences (TTAAT, CTAAT, and CTAAT) are found within 55 bases of the 3′ splice junction that is utilized during all periods of muscle development (exon 3). Thus, an unusual branch point sequence may be implicated in recognition of the thorax-specific 3′ splice junction.

The decision to include the thorax-specific exon may depend on recognition of its unusual 3′ splice junction by the splicing machinery. The scanning model (58) of RNA splicing suggests that RNA sequences located 3′ to a donor splice junction are scanned for an appropriate branch point and acceptor splice junction before cleavage at the donor site. For the donor site of intron 1 of the *Drosophila* MHC gene, an appropriate branch point sequence and acceptor splice site may not be recognized at the border of the thorax-specific exon (exon 2), since this region lacks a consensus branch point sequence as well as a polypyrimidine tract. In this instance, the donor site of exon 1 would be joined to the acceptor site of exon 3, as is the case in all tissues except thorax muscle. In thorax muscle, a tissue-specific factor, such as a small nuclear ribonucleoprotein, might permit recognition of the acceptor splice site of the thorax-specific exon.

Although the nonconsensus 3′ splice junctions preceding the stage-specific exons of the *Drosophila* myosin light-chain (14) and MHC genes are nearly identical (ATGAAAACAG and ACGAAAACAG, respectively), they are utilized with different patterns of tissue specificity. The unusual splice junction of the MHC transcript is specifically used in the processing of thorax muscle RNAs, whereas the unusual 3′ splice site of the light-chain transcript is utilized in all larval and adult muscle transcripts, except in mRNAs of indirect flight muscle (S. Falkenthal and M. Graham, personal communication). Thus, a model which specifies that a purine-rich 3′ splice junction is recognized only during adult thorax muscle development must take into account that in one case (the MHC gene) this junction is specifically utilized, whereas in another case (the myosin light-chain gene) this signal is specifically ignored. This observation suggests that these nonconsensus 3′ splice junctions may be of secondary importance in the choice of whether to include or exclude these exons in thoracic muscle.

A study by Deutscher et al. (12) suggests a testable mechanism for alternative selection of *Drosophila* MHC exons that depends on selective repression of the 5′ splice junction of the thorax-specific exon. They found that a 400-base region of the adenovirus E3 transcription unit represses use of a particular 5′ splice site, perhaps by formation of an RNA stem-loop structure which prevents recognition of this splice site by the splicing machinery (55). Such a 5′ splice repressor sequence, or a repressor molecule, may inhibit use of the 5′ splice site of the thorax-specific exon of the MHC gene. Since the unusual 3′ acceptor site of

the thorax-specific exon lacks a consensus splice junction and branch point sequence, repression of splicing at the thorax-specific 5′ splice junction would prevent inclusion of this exon in mature transcripts. In our model, muscles that utilize the thorax-specific exon produce an inhibitor of the 5′ splice site repressor (which could disrupt the stem-loop structure) or do not synthesize the repressor molecule. In these muscles, the 5′ donor site of the thorax-specific exon is recognized and spliced to the 3′-terminal exon (exon 3). Under these conditions, the only 3′ acceptor available to the 5′ donor site of exon 1 is the unusual thorax-specific splice junction. Use of this junction will result in inclusion of the thorax-specific exon in the mature transcript. Evidence for the utilization of a cryptic 3′ splice site on recognition of a downstream 5′ donor site comes from a study of a β-globin gene point mutation (57). The point mutation produces a 5′ donor site within the intron of this gene. Splicing of the donor to the acceptor site of the downstream exon results in activation of an upstream cryptic 3′ acceptor site which is spliced to the upstream exon. Thus, an additional exon is included in the mature β-globin transcript. Based on our model, we predict that MHC transcripts already spliced at the thorax-specific 5′ splice junction will be processed at the unusual thorax-specific 3′ splice junction in all tissues. If, on the other hand, these transcripts are only spliced at the thorax-specific 3′ splice junction in thoraces, then the sequence of the thorax-specific 3′ splice junction is the primary signal for tissue-specific splicing of this transcript. To test these possibilities, an MHC gene that lacks the 3′-most intron can be constructed in vitro and introduced into the *Drosophila* germ line via P-element-mediated transformation (51, 56).

Function of alternative C termini. Tissue-specific splicing of *Drosophila* MHC RNA yields transcripts encoding MHCs with alternative C termini. Divergence in C termini also occurs in MHC isoforms of rats (32) and chickens (24, 61). The use of alternative splicing to generate this diversity in *Drosophila* MHC isoforms suggests that C-terminal variation is important and not merely the result of nonfunctional evolutionary divergence (24). Some evidence indicates that the C terminus of MHC plays a role in thick-filament assembly. Mutations which delete this region of the *C. elegans unc-54* MHC protein result in high turnover of MHC, possibly owing to poor thick-filament assembly (13). The C-terminal region of *Acanthamoeba* non-muscle MHC is crucial for assembly of thick filaments. Monoclonal antibodies which bind to this portion of the protein inhibit assembly and disassemble preformed filaments, resulting in severe reduction of ATPase activity (26, 27). Phosphorylation of amino acid residues within the C-terminal region of *Acanthamoeba* and *Dictyostelium* non-muscle MHCs inhibits thick-filament assembly (9, 29, 44). Although it is unknown whether the *Drosophila* MHC tailpieces can be phosphorylated, perhaps the aggregation of these hydrophobic regions increases the rate of dimer formation and subsequent thick-filament assembly.

The thorax-specific isoform of *Drosophila* MHC is probably an important component of the indirect flight muscle, the major muscle type of the adult thorax. This muscle is unusual compared with other *Drosophila* muscles (11) in that it has relatively short sarcomeres and a high thick-thin filament ratio (six thin filaments surrounding each thick filament). The thorax-specific form of MHC may play a key role in defining these parameters since the assembly of thin filaments into the contractile apparatus is dependent on the type of myosin present within the thick filament (21, 22). Hayashi et al. (21, 22) reported that the number of thin filaments which assemble around each thick filament in an in vitro assembly system is different when the thick filament is composed of lobster myosin (12 thin filaments per orbital) compared with when it is composed of rabbit myosin (6 thin filaments per orbital); these thick-thin filament ratios reflect the in vivo organization of muscles of these organisms. Based on these in vitro results, the known thick-thin filament ratio differences between indirect flight muscle and other *Drosophila* muscles, and the observed differences in the *Drosophila* MHC isoforms, it appears possible that the C-terminal region of MHC is involved in the assembly of thin filaments around each thick filament. Alternatively, other as yet undetected regions of MHC isoform variability or myosin light-chain heterogeneity may be involved in this assembly process.

We have recently identified several mutations within the *Drosophila* MHC gene which, when homozygous, are lethal early in development (38). The presence of a single mutant allele results in a dominant flightless phenotype (38). None of these mutations are within the C-terminal coding region of the MHC gene. It is likely that mutations that affect the thorax-specific exon or splice junction are not lethal when homozygous since the thorax is largely comprised of flight muscles that are not essential for viability (37). We can now screen for nonlethal flightless alleles of the MHC gene in an attempt to isolate mutations that will indicate the function of the thorax-specific C terminus. In addition, we should be able to construct mutant MHC genes in vitro and integrate these constructs into MHC-null genomes via P-element-mediated transformation (51, 56). This will allow us to test how the structure of the MHC C terminus affects the assembly of the contractile apparatus and should permit us to determine the regions of the gene that act as signals for alternative RNA splicing.

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