The Role of Evolutionarily Conserved Sequences in Alternative Splicing at the 3′ End of *Drosophila melanogaster* Myosin Heavy Chain RNA

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ABSTRACT

Exon 18 of the muscle myosin heavy chain gene (*Mhc*) of *Drosophila melanogaster* is excluded from larval transcripts but included in most adult transcripts. To identify *cis*-acting elements regulating this alternative RNA splicing, we sequenced the 3′ end of *Mhc* from the distantly related species *D. virilis*. Three noncoding regions are conserved: (1) the nonconsensus splice junctions at either end of exon 18; (2) exon 18 itself; and (3) a 30-nucleotide, pyrimidine-rich sequence located about 40 nt upstream of the 3′ splice site of exon 18. We generated transgenic flies expressing *Mhc* mini-genes designed to test the function of these regions. Improvement of both splice sites of adult-specific exon 18 toward the consensus sequence switches the splicing pattern to include exon 18 in all larval transcripts. Thus nonconsensus splice junctions are critical to stage-specific exclusion of this exon. Deletion of nearly all of exon 18 does not affect stage-specific utilization. However, splicing of transcripts lacking the conserved pyrimidine sequence is severely disrupted in adults. Disruption is not rescued by insertion of a different polypyrimidine tract, suggesting that the conserved pyrimidine-rich sequence interacts with tissue-specific splicing factors to activate utilization of the poor splice sites of exon 18 in adult muscle.

THE insect *Drosophila melanogaster* exhibits a diversity of morphologically, physiologically, and functionally distinct muscle types in its various tissues and at different stages of its life cycle (for review, see Bernstein *et al.* 1993). For example, the indirect flight muscles of the adult are fibrillar in nature and contract at extremely high frequency, whereas larval body wall muscles have less organized myofibrils and contract slowly. Muscle-specific "isoforms" of contractile proteins such as myosin, actin, tropomyosin, and troponin are important to generating the functional differences among Drosophila muscle types (Fyrberg and Beall 1990; Bernstein *et al.* 1993).

Myosin serves as the molecular motor of muscle and the major constituent of thick filaments. The tissue specificity of myosin heavy chain (MHC) isoform expression is important in regulating muscle functional diversity in vertebrates (Schwartz *et al.* 1992) and in Drosophila (Wells *et al.* 1996). In *D. melanogaster*, there is a single muscle *Mhc* gene and alternative RNA splicing generates mRNAs encoding up to 480 MHC isoforms (George *et al.* 1989). *Mhc* primary transcripts contain five sets of exons that are spliced in a mutually exclusive and often tissue-specific manner; only one member from each set is chosen for inclusion in the mature mRNA (George *et al.* 1989; Hastings and Emerson 1991; Kronert *et al.* 1991). In addition, alternative splicing of penultimate exon 18 results in its exclusion from all embryonic and larval muscle *Mhc* mRNAs, but its inclusion in adult indirect flight muscles and other adult muscle mRNAs (Bernstein *et al.* 1986; Rozek and Davidson 1986; Kazzaz and Rozek 1989; Hastings and Emerson 1991). Skip splicing of exon 17 to exon 19 generates a MHC carboxy terminus containing 27 amino acids encoded by exon 19; inclusion of exon 18 introduces a single amino acid codon and a stop signal, producing myosin with a truncated carboxy terminus. The alternative C termini could differentially regulate thick filament assembly properties and/or interaction with other contractile proteins. For instance, because a region near the C terminus of vertebrate myosin interacts with the myofibril organizing protein titin (Houmeida *et al.* 1995), the alternative C termini of Drosophila MHCs could influence myofibril assembly or function through differential interaction with Drosophila titin.

Stringent regulation of *Mhc* RNA alternative splicing is critical to proper functioning of the musculature, because alternative exons are not functionally equivalent (Wells *et al.* 1996). Further, mutations that disrupt alternative splicing of *Mhc* RNA can have severe effects on myosin accumulation (Collier *et al.* 1990; Kronert *et al.* 1991). The choice of alternative exons in *Mhc* transcripts can be regulated in a complex, muscle-specific manner. For example, a mutation at the 5′ splice

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site of exon 9a results in accumulation of partially processed transcripts in the indirect flight muscle because of the inability of this tissue to recognize mutant exon 9a or to substitute either of two alternative exons (9b or 9c; Kronert *et al.* 1991). The jump muscle, however, switches its splicing choice to exon 9b in the mutant. The jump muscle displays plasticity in exon 9 choice, whereas the indirect flight muscle stringently regulates exon 9 selection. These observations highlight both the essential nature of alternative splicing to muscle function and the need to analyze this process *in vivo*, where appropriate muscle-specific splicing factors are present.

We are studying the *cis*-acting signals required for alternative inclusion or exclusion of exon 18 in *Mhc* transcripts. We previously used an *in vitro* system to examine the sequences important in exclusion of exon 18 and showed that weak splice junctions play a key role in this process (Hodges and Bernstein 1992). Because our *in vitro* assay system used undifferentiated embryonic tissue culture cells, it is not amenable to determination of sequences responsible for exon 18 inclusion in adults *in vivo*. We therefore developed a mini-gene construct that permits efficient inclusion of exon 18 in transgenic Drosophila (Hess and Bernstein 1991). Transcription of the mini-gene is controlled by the *Mhc* promoter, which contains the regulatory elements required for correct and efficient stage- and tissue-specific expression. Splicing of exon 18 in the *Mhc* mini-gene transcripts is regulated in the same manner as in endogenous *Mhc* transcripts (Hess and Bernstein 1991). We previously used this system to show that the purine-rich sequence preceding the 3′ splice site and the adjacent 10 nucleotides at the 5′ end of exon 18 are dispensable for regulated splicing of this exon (Hess and Bernstein 1991).

In this study, we cloned and sequenced the 3′ end of the distantly related *D. virilis Mhc* gene and used the sequence conservation to identify potential *cis*-acting elements important for alternative splicing of exon 18. Comparison to the *D. melanogaster Mhc* sequence revealed that there are nonconsensus splice junctions flanking exon 18 in both species. Furthermore, a polypyrimidine tract in intron 17 and much of the noncoding region of exon 18 are conserved between the two species. To examine the function of these conserved regions *in vivo*, we constructed *Mhc* mini-genes containing deletions or substitutions of the areas under study and produced transgenic organisms by *P*-element-mediated germline transformation. We then determined how the mutations affect the RNA splicing patterns in larvae and adults. Our results demonstrate that the conserved sequences within exon 18 are not essential for proper stage-specific splicing of *Mhc* transcripts. However, the nonconsensus splice junctions flanking exon 18 prevent its inclusion in larval muscle mRNAs, consistent with the *in vitro* results. Further, the conserved polypyrimidine tract upstream of exon 18 is required for exon 18 inclusion in adult *Mhc* mRNAs. This suggests that the polypyrimidine tract interacts with adult-specific *trans*-acting factors to mediate recognition of the nonconsensus splice sites of exon 18.

MATERIALS AND METHODS

Preparation and screening of a *D. virilis* genomic DNA library: DNA was isolated from young *D. virilis* adults as described in O'Donnell and Bernstein (1988). Genomic DNA (0.5 μg) was partially digested with *Eco*RI. A genomic DNA library was prepared using a Lambda Zap II/*Eco*RI cloning kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The resulting *D. virilis* library had 1.5×10^6 plaque-forming units (pfu) with >90% of the pfu containing inserts. The library was amplified and 225,000 pfu were screened by low-stringency hybridization at 42° in 20% formamide, 5× Denhardt's solution (1.0 g/liter Ficoll 400, 1.0 g/liter polyvinylpyrrolidone, and 1.0 g/liter bovine serum albumin), 5× SSPE (0.9 m NaCl, 50 mm $NaPO_4$, pH 7.6, 5 mm EDTA), 0.1% sodium dodecyl sulfate, 1 μg/ml poly(A), and 100 μg/ml denatured salmon sperm DNA containing 3×10^5 cpm/ml of antisense RNA probe (3′ end of *D. melanogaster Mhc* cDNA). Eighteen positive plaques were identified and purified. Plasmid clones, obtained by *in vivo* excision, were analyzed by restriction enzyme digestion, followed by Southern blotting and hybridization to *D. melanogaster Mhc* 3′ end clones to identify inserts of interest. Insert DNA was purified from agarose gels using a Gene Clean II kit (BIO 101, Vista, CA) and subcloned prior to sequence analysis. Sequencing of cloned DNA was performed using a Sequenase kit (Amersham Life Sciences, Arlington Heights, IL).

Transgene construction: pπMHC 5′3′ (Hess and Bernstein 1991) was used as the starting plasmid for construction of mutant mini-genes. This plasmid contains the *Mhc* transcriptional promoter (454 nt upstream of exon 1, exon 1, intron 1, and part of exon 2) fused to exons 17, 18, and 19 and their associated introns and polyadenylation signals. To prepare the various plasmids, *Mhc* fragments from mutant constructs from *in vitro* splicing studies (Hodges and Bernstein 1992) or other mutated fragments were used to replace the homologous fragments in pπMHC 5′3′. In brief, the *Mhc Sfi*I-*Pfl*MI fragment from the pπMHC 5′3′ plasmid (beginning near the end of exon 17 and ending near the end of intron 18) was replaced with the *Sfi*I-*Pfl*MI fragment from MHC E17-18-19 CAG, MHC E17-18-19 +Int 5, or MHC E17-18-19 +Int 5 CAG plasmids described in Hodges and Bernstein (1992). Restriction enzyme analysis and/or sequencing of the cloning junctions were performed to confirm the identity of each new construct. To eliminate problems associated with G418 selection, both wild-type and mutant mini-genes were transferred from the pπMHC 5′3′ neomycin selection vector into the pCaSpeR vector (Pirrotta 1988). pCaSpeR contains a *white*$^+$ (w^+) gene and allows for selection of transformants by rescue of the white eye phenotype of w^{1118} flies. Each modified *Mhc* insert was isolated from the pπMHC 5′3′ construct by digestion with *Xba*I or *Xba*I and *Kpn*I and inserted into the pCaSpeR or pCaSpeR K vector (kindly provided by Dr. J. Posakony), which was cut with the same enzymes (these two vectors differ by the presence of an *Eco*RI site in pCaSpeR that is replaced with a *Kpn*I site in pCaSpeR K).

We constructed a plasmid to study the effects of deleting most of exon 18; deletions were prepared by cutting exon 18 with *Ava*III and then performing Bal 31 exonuclease digestion (Sambrook *et al.* 1989). *Bam*HI linkers were added, followed by ligation and transformation into *Escherichia coli*. Clones

containing deletions were identified by restriction enzyme analysis and sequencing. Two of these deletion clones, 5 and 32, retained only 23 nt of exon 18 sequence either 5′ or 3′ to the *Bam*HI site, respectively. The *Hin*dIII-*Bam*HI fragment from clone 5 and the *Bam*HI-*Eco*RI fragment from clone 32 were combined to produce MHC 5/32 and this was inserted into the *P*-element vector as described above to yield MHC Δ 450 E18.

To study the polypyrimidine tract in intron 17, we deleted the 22-nt pyrimidine-rich element (5′ TATATTCTTCCCTTT CATATTG 3′) and replaced it with a *Kpn*I site by PCR cloning. A PCR fragment, beginning at the *Hin*dIII site in exon 17 and ending just 5′ to the pyrimidine tract was generated using the following primers: 5′ GAAGCTTGAGCAGCGCGTCC 3′ (which contains a *Hin*dIII site at its 5′ end) and 5′ AGGTAC CACACATTATTCAATAAC 3′ (which has a *Kpn*I site at its 5′ end). A second PCR fragment, beginning 3′ of the pyrimidine tract and extending into exon 18 past the *Pst*I site, was prepared using the following primers: 5′ TGGTACCTCGCG TATGCTCTGCT 3′ (containing a *Kpn*I site at its 5′ end) and 5′ TCTACTGCTCCAGCAGCGCG 3′. The PCR fragments were digested with *Hin*dIII, *Kpn*I, and *Pst*I. They were gel isolated and ligated into pBS/MHC HIII-RI (Hodges and Bernstein 1992), which had been digested with *Hin*dIII and *Pst*I and gel isolated to remove the homologous wild-type fragment. The mutated region from the resulting plasmid was transferred into the *Mhc* mini-gene and pCaSpeR as detailed above. The presence of two *Kpn*I sites in this construct (pCaSpeR ΔPy), one in intron 17 and one in the flanking vector sequence, made it cumbersome to insert oligonucleotides into the *Kpn*I site in the intron. To destroy the site in the vector, two 10-base, complementary oligonucleotides were synthesized and the phosphorylated double-stranded DNA was ligated into pCaSpeR ΔPy plasmid that had been partially digested with *Kpn*I and dephosphorylated. We isolated a clone in which insertion of the annealed oligonucleotide abolished the *Kpn*I site within the vector, leaving the site in intron 17 intact. Subsequently, we experienced difficulty inserting oligonucleotides within the intron because of degradation of the plasmid during *Kpn*I digestion. We therefore designed oligonucleotide inserts to be compatible with a neoschizomer of *Kpn*I, *Acc* 65I. This enzyme produces protruding 5′ ends and did not cause degradation. Oligonucleotides were annealed, phosphorylated, and ligated into the *Acc*65I site. When 5′ GTACTATATTCTTCCCTTTCATATT 3′ was annealed with 5′ GTACAATATGAAAGGGAAGAATATA 3′ and inserted in the sense orientation, it restored the polypyrimidine tract (construct pCaSpeR ΔPyWt+). The antisense orientation yielded pCaSpeR ΔPyWt−, with a purine-rich tract. Another construct, pCaSpeR ΔPyMt+, was prepared from the following oligonucleotides: 5′ GTACCACACCTCCTTTCCCTACACC 3′ and 5′ GTACGGTGTAGGGAAAGGAGGTGTG 3′. This construct contains a pyrimidine tract with the C's and T's, compared to those of wild type, reversed.

Probe preparation and transcript analysis: Constructs used as probes were prepared as follows: (1) Genomic E2/17-18-19 was made by isolation of the *Bam*HI-*Eco*RI fragment from pπMHC 5′3′ and ligation into the pKS vector cut with the same enzymes, (2) E2/17-Int 17 was prepared by deletion of the *Bgl*II-*Eco*RI fragment from genomic E2/17-18-19 followed by treatment with the Klenow fragment (Promega, Madison, WI) and blunt end ligation, (3) E2/17-18-19 cDNA was obtained by replacement of the *Sfi*I-*Eco*RI fragment of genomic E2/17-18-19 with the *Sfi*I-*Eco*RI fragment from the adult cDNA (pKS/AcDNA), (4) E2/17-18 was prepared by deletion of the 3′ half of exon 18 and all of exon 19 by digestion of E2/17-18-19 cDNA with *Nsi*I and *Eco*RI, followed by T4 DNA polymerase (Promega) treatment and religation, and (5) the 1.5-kb 3′ end construct was prepared by isolation of the MHC 1.5-kb *Eco*RI fragment from IID1 (Bernstein *et al.* 1983) and its ligation into the pKS vector, which had been cut with *Eco*RI and dephosphorylated with calf intestinal phosphatase (Promega).

Antisense RNA probes complementary to exon 18 or to exons 17, 18, and 19 were used for Northern and Southern blots and for screening the *D. virilis* library. The exon 18 antisense RNA probe was prepared from pBS/MHC HIII-Pst truncated at the *Bgl*II site in exon 18 and transcribed using T7 RNA polymerase (Stratagene). This probe begins at the 3′ splice site of exon 18 and extends to the *Pst*I site, thus covering 436 nt of this 500-nt exon. The 3′ end cDNA antisense RNA probe containing exons 17, 18, and 19 was generated from pKS/AcDNA by *Hin*dIII digestion and transcription with T3 RNA polymerase (Stratagene). Probes prepared for RNase protection studies were obtained as follows: (1) Plasmids E2/17-Int 17, E2/17-18, and 1.5 3′ end were digested with *Bam*HI and transcribed using T3 RNA polymerase and (2) Int 18-E19 was prepared from pBS/MHC HIII-RI that was cut with *Sna*BI and transcribed with T7 RNA polymerase.

Transcriptions were performed in 30-μl reactions containing 0.5 μg DNA template, 40 mm Tris-HCl (pH 8.0), 8 mm $MgCl_2$, 50 mm NaCl, 2 mm spermidine, 10 mm dithiothreitol, 1 unit Inhibit-ACE (5′-3′, Inc.), 400 μm each of ATP and CTP, 80 μm each of GTP and UTP, 2 μm [^{32}P]UTP and [^{32}P]GTP (20 μCi, 800 Ci/mmol), and 50 units of T3 or T7 RNA polymerase (Stratagene). Reactions were performed for 75 min at room temperature followed by DNase digestion, two phenol-chloroform extractions, one chloroform extraction, and two ethanol precipitations.

Drosophila RNA was prepared from first, second, and early third instar larvae and 1- to 2-day-old adults using a modified version of the procedure of Clemens (1984) as described in Hess and Bernstein (1991). Ten micrograms of total RNA was electrophoresed on a 1.5% agarose, formaldehyde gel according to the protocol of Davis *et al.* (1986), transferred to Gene Screen II membrane (Dupont, NEN Research Products) overnight in 5 mm NaOH, UV crosslinked, and then hybridized to antisense RNA probes using standard procedures (Sambrook *et al.* 1989).

cDNA synthesis of total RNA purified from transformed *D. melanogaster* larvae or adults and amplification by the polymerase chain reaction was performed as described in Hodges and Bernstein (1992) or according to the protocol of Grady and Campbell (1989). Oligonucleotide primers specific for exons 17, 18, and 19 are given in Hodges and Bernstein (1992). The exon 2/17 oligonucleotide (5′ GTTGGTCTG CAGGCATGCAAGCTTGAGCAG 3′) hybridizes to the exon 2/17 junction of transcripts expressed from the *Mhc* mini-gene and was used to specifically amplify the mini-gene transcripts. Cycle sequencing of RT-PCR products was performed on gel-isolated DNA using the CircumVent Thermal Cycle Sequencing kit (New England Biolabs, Beverly, MA) or a test cycle sequencing kit kindly provided by Stratagene. RNase protection experiments were performed with a Ribonuclease Protection Assay Kit (RPA II) as recommended by the manufacturer (Ambion Inc., Austin, TX).

***P*-element transformation:** *P*-element-mediated germline transformation was performed using a helper *P*-element plasmid as described by Rubin and Spradling (1982). Cesium-chloride-purified pCaSpeR MHC mini-gene DNA (350 μg/ml) and Δ 2-3 helper plasmid (50 μg/ml) were coinjected into embryos homozygous for the white-eyed mutation, w^{1118}. Adults from surviving embryos were mated to w^{1118} flies and transformants with pigmented eyes were identified in the next generation. Each transformant was crossed to a balancer line: *w*; *SM1*, al^2 *Cy* cn^2 sp^2/ *Sco*; *TM2*, emc^2 Ubx^{P130} *ry* e^s/ *MKRS*, *M(3)76A kar* ry^2 *Sb* (kindly provided by Greg Harris) containing

dominant markers for *Curly* (*Cy*), *Ultrabithorax* (*Ubx*), and *Stubble* (*Sb*). Chromosomal linkage and stable balanced insert lines were obtained by appropriate sibling crosses.

RESULTS

Identification of *Mhc* sequence elements conserved between distantly related Drosophila species: To discern potential *cis*-acting elements important to exon 18 alternative splicing in *Drosophila melanogaster*, we identified evolutionarily conserved *Mhc* sequences in distantly related Drosophila species. Conserved nucleotide sequences frequently encode functional motifs or contain *cis*-acting regulatory elements (Kassis *et al.* 1985, 1989; Treier *et al.* 1989; Heberlein and Rubin 1990; Thackeray and Ganetzky 1995). We first determined whether the 3′ ends of *Mhc* transcripts of distantly related species are also alternatively spliced. We compared *D. melanogaster* to *D. ananassae*, *D. simulans*, and *D. virilis* because these species represent a broad range of the evolutionary time scale. Of these species, *D. virilis* and *D. melanogaster* are the most distantly related, having diverged ∼ 60 mya (Beverly and Wilson 1984). We analyzed the pattern of *Mhc* transcript accumulation in larvae and adults of each species by Northern blotting. The expression pattern was very similar for all four species. Each has transcripts of ∼6.6 and 7.1 kb that hybridize specifically to the *D. melanogaster* exon 18 probe in adults but not larvae (Figure 1A). The probe that detects all *D. melanogaster Mhc* transcripts hybridized to transcripts of 6.1 and 6.6 kb that lack exon 18 in larvae of all species as well (Figure 1B). In *D. melanogaster* the two transcripts present in each stage result from use of alternative polyadenylation sites and the presence of similar size transcripts in the other species examined here is likely due to alternative polyadenylation as well. This demonstrates that the developmental stage-specific pattern of muscle *Mhc* gene expression has been conserved over 60 million years.

To ensure that the multiple transcripts are produced from alternative splicing (as in *D. melanogaster*) rather than from multiple genes, we performed a Southern blot on genomic DNA from the most distantly related species, *D. virilis*. We hybridized a blot of *D. virilis* genomic DNA, digested with the restriction enzymes *Eco*RI or *Hin*dIII to an RNA probe containing *D. melanogaster* exons 17, 18, and 19; single 2.5-kb *Eco*RI and 3.25-kb *Hin*dIII fragments were detected (data not shown). A *D. melanogaster* probe, specific to constitutive exons 4, 5, and 6, hybridized to single 4.5-kb *Eco*RI, 5.8-kb *Hin*dIII, and 7.8-kb *Bam*HI fragments. Detection of single DNA fragments generated by digestion with each of these enzymes strongly suggests that, as in *D. melanogaster*, a single muscle *Mhc* gene exists in *D. virilis*.

On the basis of the evidence for conserved alternative splicing of a single *Mhc* transcript in the most distantly related species, we isolated the 3′ end of the *D. virilis Mhc*

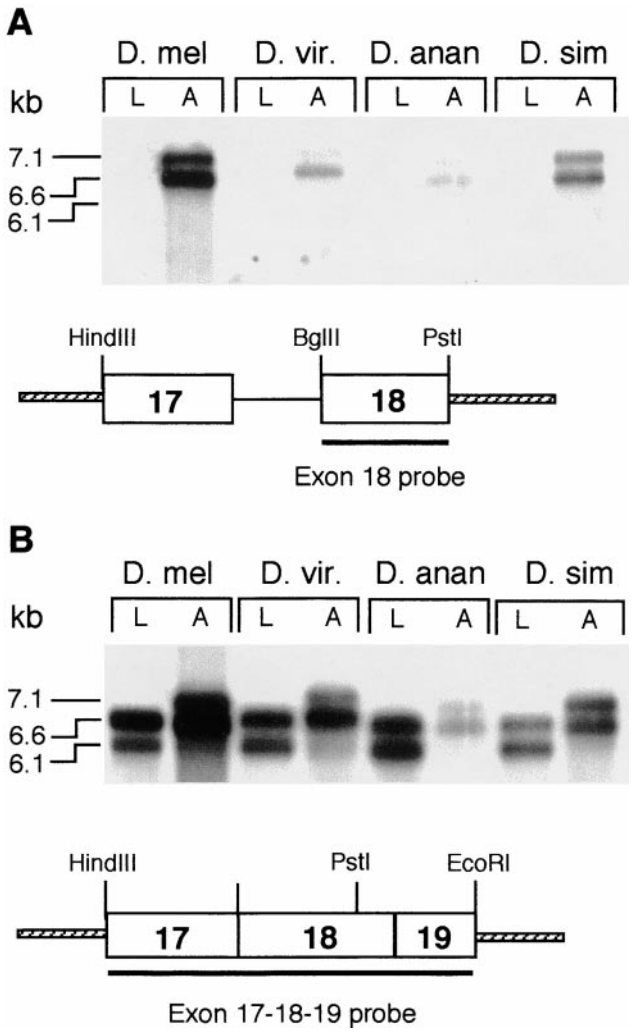

Figure 1.—Cross-species Northern blot. Total RNA isolated from larvae (L) and adults (A) of *D. melanogaster* (D. mel), *D. virilis* (D. vir), *D. ananassae* (D. anan), and *D. simulans* (D. sim) was electrophoresed on a 1.5% agarose, formaldehyde gel and transferred to nylon membrane. (A) Autoradiograph of the blot hybridized to the *D. melanogaster Mhc* exon 18-specific probe. A diagram of the *D. melanogaster Mhc* genomic construct used for preparation of antisense RNA probes is shown beneath the blot. All of exon 18, except for 64 nt 3′ of the *Pst*I site, is present in this plasmid. Exons are represented by open boxes and introns by narrow lines. Hatched boxes represent vector sequences. The probe, indicated by a thick line beneath exon 18, was transcribed from this construct after digestion with *Bgl*II. All four Drosophila species express adult-specific transcripts of ∼6.6 and 7.1 kb. (B) The above blot was stripped and rehybridized with a probe that detects all three 3′ exons. The cDNA plasmid, shown beneath the blot, was digested with *Hin*dIII and used as a template for transcription of antisense RNA probe. The exon 17-18-19 probe (thick line) contains 368 nt of exon 17, all 500 nt of exon 18, and 250 nt of exon 19. Larval transcripts of approximately the same sizes (6.1 and 6.6 kb) are detected in all four species, in addition to the previously detected adult transcripts.

gene from a genomic DNA library using low-stringency hybridization. We obtained several size classes of *D. virilis* inserts containing various contiguous *Eco*RI fragments. All clones included a 2.5-kb *Eco*RI fragment, the size fragment detected on the genomic Southern blot when hybridized to the *D. melanogaster Mhc* exon 17-18-19 antisense RNA probe. Sequence analysis showed that the 2.5-kb *Eco*RI fragment and flanking fragments of 1.4 kb and 0.4 kb correspond to the 3′ end of the *D. melanogaster Mhc* gene.

We compared the sequence of *D. virilis Mhc* DNA to *D. melanogaster* (Bernstein *et al.* 1986; Rozek and Davidson 1986; George *et al.* 1989; Collier *et al.* 1990) and found strong conservation in coding regions and discrete stretches of conservation in noncoding regions (Figure 2). The 160 codons at the end of exon 17 in *D. melanogaster* are conserved, except for one change of a valine in *D. melanogaster* to isoleucine at amino acid position number 1850. Both versions of exon 18 encode a single amino acid, with *D. melanogaster* having an isoleucine and *D. virilis* having an asparagine; this is followed by a stop codon in both species. Transcripts lacking exon 18 use exon 19 to encode carboxy termini containing an extra 27 amino acids in *D. melanogaster*. Identical amino acids are encoded by exon 19 in the two species. We did not obtain the 3′ end *Eco*RI fragment of the *D. virilis* gene that would contain the putative stop codon in exon 19.

There is strong identity between exon 18 of *D. melanogaster* and *D. virilis* in the noncoding regions. Because this exon encodes only a single amino acid, the degree of sequence conservation at the DNA level is surprising. There are two areas of striking identity (Figure 2). A 117-nt region, beginning 69 nt downstream of the *D. melanogaster* 3′ splice site, is 84% identical to *D. virilis*. The 88 nt at the 3′ end of exon 18 are also almost totally conserved between the two species. This includes the exon portion of the 5′ splice site, which is unusual in that this sequence is TTT, rather than the consensus $^{C}/_{A}$AG. There are smaller regions of identity scattered throughout exon 18 as well. Exon 18 differs in size between the two species (*D. melanogaster* is 500 nt whereas *D. virilis* is 663 nt). The extra *D. virilis* sequence maintains the A/T-richness of *D. melanogaster* exon 18 (>66% A/T).

We found little sequence identity within introns, with the exception of the 5′ and 3′ splice sites and a pyrimidine-rich sequence in intron 17 (Figure 2). Intron 18 of *D. melanogaster* is 246 nt smaller than *D. virilis*, whereas the size of intron 17 is about the same in the two organisms. The purine-rich nature of the 3′ splice site of exon 18 and the absence of a good consensus branchpoint sequence within 40 nt of the 3′ splice site are unusual features in *D. melanogaster* and they are observed also in *D. virilis*. A polypyrimidine tract (TATATTCTTCCCTT TCATATTGC), beginning at position −56 from the 3′ splice site of exon 18 of *D. melanogaster*, is present in the *D. virilis* sequence at a similar position (overlined in Figure 2). Both species contain a consensus branchpoint sequence just 5′ to this polypyrimidine tract (underlined in Figure 2, with asterisks indicating the adenosine residue that would form the 2′–5′ linkage).

In summary, coding sequences at the 3′ end of the *D. melanogaster* and *D. virilis Mhc* genes are highly conserved, as are large portions of exon 18 and its nonconsensus splice junctions. A pyrimidine-rich sequence upstream of exon 18 is also remarkably conserved. Because the actual sequence, and not just the pyrimidine character of this sequence, is conserved, this might be an element involved in regulation of the alternative splicing of exon 18. The nonconsensus splice junctions of exon 18 and the long stretches of conserved sequences within this exon are also candidates for alternative splicing regulatory sequences.

Alternative splicing of *Mhc* mini-gene transcripts *in vivo*: To test whether conserved elements at the 3′ end of the *Mhc* genes are important to alternative splicing of exon 18 *in vivo*, we made modifications predicted to improve the splice sites, remove competing splice junctions, or inhibit exon 18 inclusion, using our previously developed *Mhc* mini-gene (Hess and Bernstein 1991). The mini-gene contains the *Mhc* promoter and the beginning of the coding region joined in frame to exons 17, 18, and 19 along with their associated introns and polyadenylation signals; it is expressed and regulated *in vivo* in the same manner as the endogenous gene (Hess and Bernstein 1991). Figure 3 diagrams the wild-type CaSpeR *Mhc* mini-gene construct and the expected transcripts in adults and larvae that result from differential use of two polyadenylation sites and adult-specific inclusion of exon 18. We inserted mini-gene constructs into the *D. melanogaster* germline by *P*-element-mediated transformation and determined the resulting splicing pattern of mini-gene transcripts by Northern blotting. We obtained a minimum of three independent lines expressing each construct and mapped each insert to a linkage group by analyzing the segregation of the *white*$^{+}$ gene from marked chromosomes. Most lines were viable as homozygotes; we maintained recessive lethals over one of the balancer chromosomes. We generally present the data on the expression of a single line, although all lines expressing a given construct yielded identical results.

Improvement of both splice sites of exon 18 is required for efficient removal of both introns from larval transcripts: The splice sites of exon 18 in *D. melanogaster* and *D. virilis* do not match the consensus sequences derived for *D. melanogaster* (Mount 1982; Mount *et al.* 1992). As discussed above, the 3′ splice sites for this exon in both species are not pyrimidine rich and lack a consensus branchpoint sequence within 40 nt upstream of the splice site, while the 5′ splice sites lack the 3-base consensus sequence usually found at the 3′ end of an exon. We prepared modified *Mhc* mini-genes

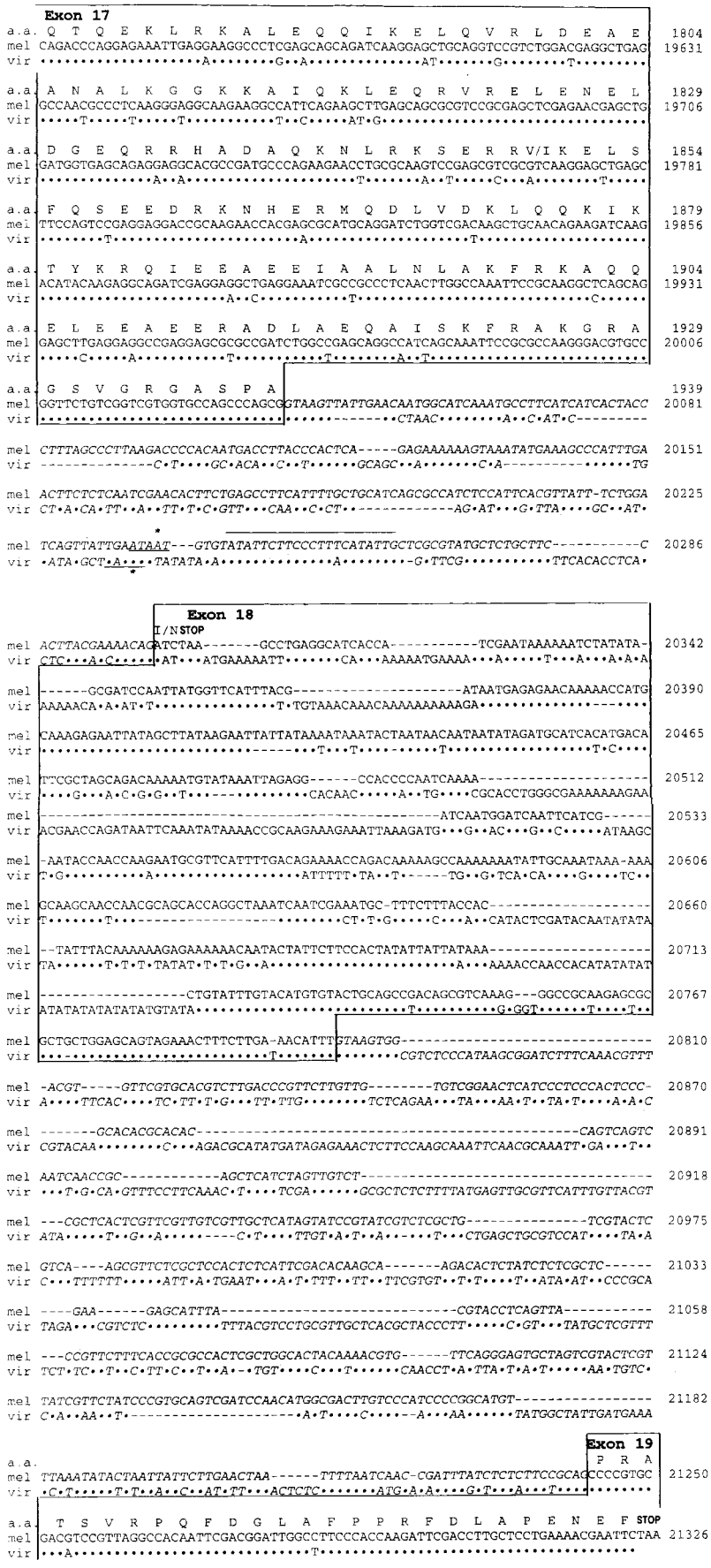

Figure 2.—Sequence comparison of the 3′ end of the *D. virilis* and *D. melanogaster Mhc* genes. The *D. melanogaster* nucleotide sequence (mel) is shown with the *D. virilis* sequence (vir) beneath it. The *D. melanogaster* numbering system is according to George *et al.* (1989). Identities are noted as dots. Alignments were made using the BestFit program (GCG), and dashes indicate gaps introduced by the program to produce the best alignment. Decoded amino acid sequences are shown above the DNA sequences with the two amino acid residues that are unique to *D. virilis* given after a slash. Exons are boxed and labeled as exon 17, 18, or 19. Intron sequences are in italics. The conserved polypyrimidine tract in intron 17 is overlined. The potential branchpoint sequences upstream of these polypyrimidine tracts are underlined, with asterisks indicating the putative adenosine residues that would form the 2′–5′ linkage.

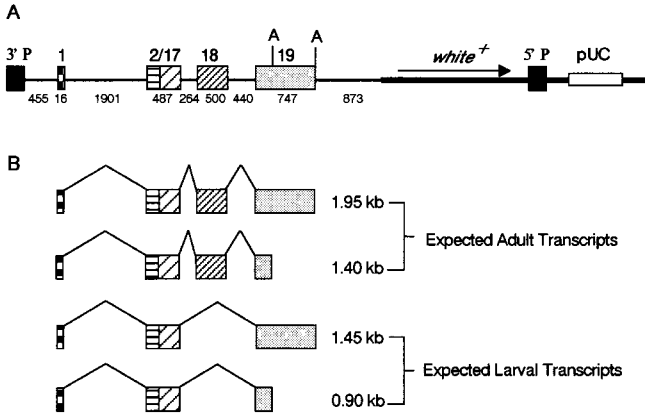

Figure 3.—(A) Diagram of the wild-type CaSpeR *Mhc* mini-gene used for germline transformations. Exons are represented by boxes that are stippled or filled with horizontal or diagonal lines and the corresponding number of the exon is shown above the box. The 5′ end of exon 2 and 3′ end of exon 17 are fused to give exon 2/17. Alternative polyadenylation sites are shown by two A's in exon 19. Regions absent from the spliced mRNA are represented by narrow lines. Lengths (nt) are given below. Solid boxes represent the 5′ and 3′ long terminal repeats of the *P* element (5′P, 3′P). The vector also contains the *white*$^+$ gene (thicker solid line) and pUC sequences (open box). The arrow shows the direction of transcription of the *white*$^+$ gene. (B) Diagram of expected mini-gene transcripts that result from inclusion or exclusion of exon 18 and alternative polyadenylation within exon 19. Lines connecting the exons represent the splicing patterns utilized to produce the size transcripts noted on the right.

to test whether these nonconsensus splice junctions are critical to regulating exon 18 inclusion and/or exclusion *in vivo*. Figure 4A shows the mini-genes, while Figure 4, B and C, depicts a Northern blot with RNA from lines expressing each construct probed with exon 18 (to determine exon 18 inclusion) and with the 3′ end of the *Mhc* cDNA (to show all mini-gene transcripts). For the wild-type mini-gene, we detect the predicted 1.4- and 1.95-kb transcripts with the exon 18-specific probe in adults, but observed no transcripts containing exon 18 in larvae (Figure 4B, WT). The 3′ end cDNA probe detects transcripts lacking exon 18 (0.9 and 1.45 kb) in wild-type larvae (Figure 4C). These data confirm our previous observations (Hess and Bernstein 1991) that this mini-gene contains the necessary *cis*-acting signals for correct stage-specific alternative splicing.

To determine if the skip splicing that generates wild-type larval *Mhc* transcripts is due to the weak 3′ splice site of exon 18 we inserted a 29-nt sequence containing a consensus branchpoint and 3′ splice site from constitutively spliced intron 5 of the *Mhc* gene into the intron 17/exon 18 junction (Figure 4A, +Int 5). If this junction is the key element in exon 18 exclusion, this change would result in exon 18 inclusion in larval mini-gene transcripts. However, the predominant larval RNA species from this MHC +Int 5 mini-gene lacks exon 18 and contains exon 17 spliced to exon 19, as is observed in wild-type transcripts (Figure 4C, 0.9- and 1.45-kb bands). Only small amounts of exon 18-containing transcripts accumulate in transformed larvae (Figure 4B).

To test whether the nonconsensus terminal 3 nucleotides of exon 18 are important to exon 18 alternative splicing we replaced the TTT sequence of the mini-gene with CAG, thus creating a perfect 5′ splice site consensus (Figure 4A, CAG). If the weak 5′ splice site of exon 18 prevents efficient removal of intron 17 and intron 18 from larval mini-gene transcripts, this change should promote exon 18 inclusion. Our results indicate that conversion of the 5′ splice site of exon 18 to the consensus sequence does not promote production of larval transcripts that include exon 18 (Figure 4B, CAG). Larval transcripts contain exons 17 and 19 and lack exon 18 as in wild-type larvae (Figure 4C, CAG). These larval transcripts are about 50 nt longer than those generated from the wild-type mini-gene, apparently from activation of a cryptic splice site. We conclude that a consensus 5′ splice site for exon 18 is not sufficient to permit correct and efficient inclusion of this exon in larval tissue.

Because improvement of either 5′ or 3′ splice site of exon 18 alone did not promote efficient and correct inclusion of exon 18 in larval transcripts, we prepared and tested a construct containing both improvements (Figure 4A, +Int 5 CAG). Transformed larvae expressing MHC +Int 5 CAG generate large amounts of mini-gene mRNAs containing exon 18, as shown by the Northern blot hybridized to the exon 18-specific probe (Figure 4B, +Int 5 CAG). Transcripts containing all three exons are the only mini-gene transcripts detected in both larvae and adults; the skip splicing products (exon 17 spliced to exon 19), typically present in larvae, are eliminated (Figure 4C, +Int 5 CAG). We took several steps to confirm the Northern blotting results (data not shown). RNase protection analysis with a cDNA probe containing MHC exons 2, 17, and 18 yielded full protection by RNA from +Int 5 CAG larvae, whereas smaller protected fragments (expected from hybridization to transcripts in which exon 17 was skip spliced to exon 19) were absent; these smaller protected fragments were the major species detected in RNA purified from WT, CAG, or Int 5 larvae. Further, RT/PCR of the transformed mini-gene transcripts with an exon 2/17 and exon 18 primer set generated DNA of the expected size for splicing of exon 17 to exon 18 in adults expressing the WT, CAG, +Int 5, and +Int 5 CAG mini-genes. The same size PCR product was generated from +Int 5 CAG larval RNA but PCR products were not detected with larval RNA from the other transgene lines when electrophoretic gels were stained with ethidium bromide. We used an exon 18-specific probe against a Southern blot of these PCR products and demonstrated that exon 17 was efficiently spliced to exon 18 in MHC +Int 5 CAG larval mRNAs, whereas larval mini-gene transcripts from MHC +Int 5 and MHC CAG lines generated less than

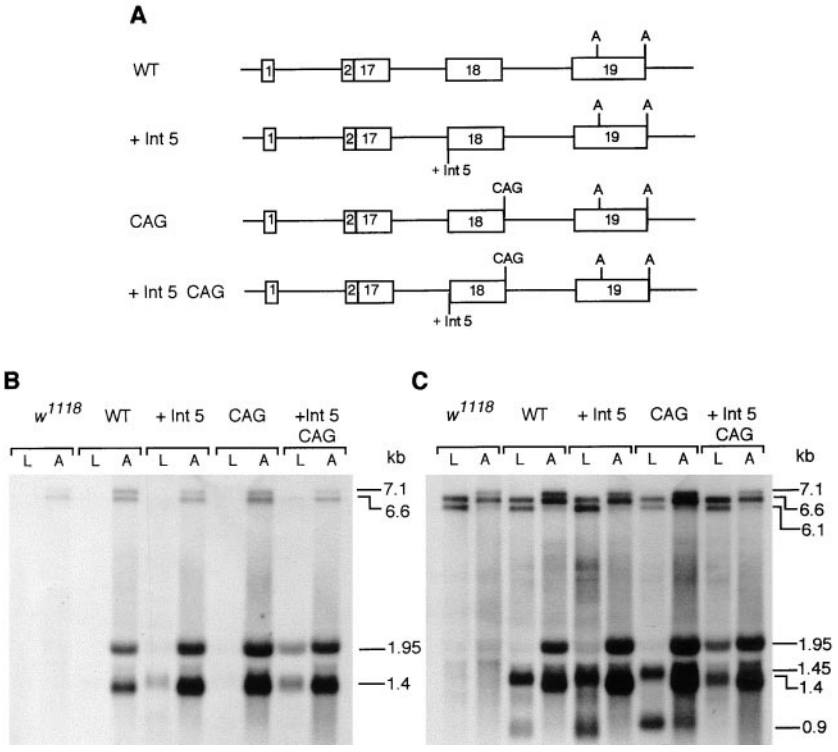

Figure 4.—Northern blot of total RNA from transformants expressing *Mhc* mini-genes in which either or both splice sites of exon 18 have been improved. RNA was isolated from larvae (L) or adults (A) of the following lines: the parental line, w^{1118}; the positive control line, 103-33 (WT); and lines that were obtained by transformation of w^{1118} embryos with pCaSpeR MHC +Int 5 (+Int 5), pCaSpeR MHC CAG (CAG), or pCaSpeR MHC +Int 5 CAG (+Int 5 CAG) mini-genes. A 29-nt sequence from intron 5 of the *Mhc* gene that contains an improved branchpoint and pyrimidine tract has been inserted at the exact intron 17-exon 18 junction in the MHC +Int 5 mini-gene. The TTT sequence at the 5′ splice site of exon 18 was changed to CAG in the MHC CAG mini-gene. The MHC +Int 5 CAG mini-gene contains both changes. Bands of 6.1, 6.6, and 7.1 kb are endogenous *Mhc* transcripts that result from exclusion or inclusion of exon 18 and the use of two polyadenylation sites. (A) Mini-gene maps. Expected transcript sizes for wild-type splicing of the mini-gene transcripts are diagramed in Figure 3. (B) Northern blot probed with a 436-nt antisense RNA specific for exon 18. The expected mini-gene transcripts of 1.95 and 1.4 kb are detected in adults but not larvae of wild-type transformants (WT). Transcripts of the same size are detected in adults expressing the mutated transgenes but similar size adult-specific transcripts are now detected in some larval RNA lanes as well. The 1.4-kb transcript (containing exon 18 and terminated at the first polyadenylation site) is detected at low levels in transformants with the mini-gene containing the intron 5 insertion (+Int 5). The CAG 5′ splice site change has little effect by itself (CAG) but in combination with the intron 5 mutation results in detection of both exon 18-specific transcripts in larvae (+Int 5 CAG). (C) The above blot was stripped and reprobed with antisense RNA complementary to exons 17, 18, and 19, which detects all transcripts in larvae and adults. The majority of mini-gene transcripts containing the single splice site mutations exclude exon 18 in larval RNA (+Int 5 or CAG). However, mini-gene transcripts containing both the Int 5 and CAG mutations are spliced in the adult mode in both larvae and adults with complete elimination of the 0.9-kb skip splicing transcript (+Int 5 CAG).

10% as much of this PCR product. We conclude that precise and efficient inclusion of exon 18 into *Mhc* mini-gene transcripts in larvae occurs when both flanking splice sites are converted to match splice site consensus sequences.

Most of exon 18 can be deleted without affecting tissue-specific regulation of *Mhc* transcript splicing: Having determined a possible mechanism whereby exon 18 is excluded from larval *Mhc* transcripts (nonconsensus splice sites), we turned our attention to studying sequences that might promote exon 18 inclusion in adults. The evolutionary conservation of much of the noncoding sequence within exon 18 suggests that regulatory elements reside within this exon. A number of sequences conforming to known *cis*-acting regulatory sites for splicing are present in exon 18 (see discussion for details). We therefore analyzed the *in vivo* expression of a *D. melanogaster Mhc* mini-gene with a deletion of 450 of the 500 nt of exon 18 (Figure 5A, Δ 450 E18). Approximately 25 nt at each splice junction of exon 18 remain in this construct. While there is only a 50-nt difference in the size of transcripts that include *vs.* exclude this shortened exon, they can be differentiated using an exon 18 probe. As in wild-type transgenic flies, exon 18-containing mini-gene transcripts are present in Δ 450 E18 adults but absent in larvae (Figure 5B); normal skip splicing occurs in Δ 450 E18 larvae (Figure 5C). RNase protection studies, RT/PCR amplification using an exon 17-exon 19 primer set, and DNA sequencing of PCR products confirmed that the 450-nt deletion did not alter proper stage-specific splicing (data not shown). RT/PCR yielded a single band in larval transformants, of the size expected when exon 17 is spliced to exon 19. RNA of Δ 450 E18 adults produced a RT/PCR band ~50 nt larger than the larval product, which is the size expected for inclusion of the shortened exon 18 in mini-gene mRNA. Sequencing of this product confirmed that the correct 5′ and 3′ splice sites of shortened exon 18 are utilized for splicing to the flanking exons in adult mini-gene transcripts from the Δ 450 E18 lines. Our results demonstrate that few, if any, of the exon 18 sequences conserved between the two Drosophila species are required for exon 18 inclusion in adult transcripts or for exclusion in larval mRNA.

The distant and conserved polypyrimidine tract within intron 17 is essential for inclusion of exon 18 in adult

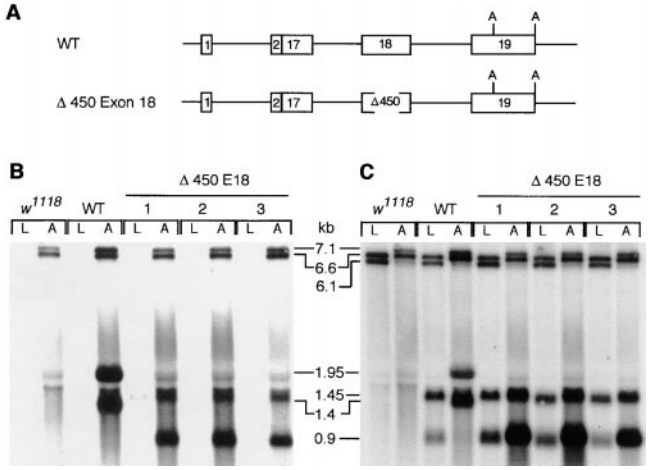

Figure 5.—Northern blot of total RNA from transformants expressing an *Mhc* mini-gene that has most of exon 18 deleted. RNA was isolated from larvae (L) or adults (A) of the following lines: the parental line, w^{1118}; the positive control line, 103-33 (WT), expressing a wild-type mini-gene; and three independent lines (1, 2, and 3) obtained by transformation of w^{1118} embryos with the pCaSpeR MHC Δ 450 E18 mini-gene. Bands of 6.1, 6.6, and 7.1 kb are endogenous *Mhc* transcripts that result from exclusion or inclusion of exon 18 and the use of two polyadenylation sites. (A) Diagrams of the wild-type and exon 18 deletion mini-genes. The internal 450 nt of exon 18 were removed by Bal 31 digestion, leaving about 25 nt of exon 18 sequence remaining at either splice site. Expected wild-type mini-gene transcripts are diagrammed in Figure 3. Because exon 18 in the deletion mutant is about 50 nt in length, transcripts resulting from inclusion of this exon will be only about 50 nt larger than those that exclude it. (B) Northern blot of RNA from larvae and adults of the parental and transformed lines hybridized to the 436-nt exon 18-specific probe. Two transcripts of the sizes expected for correct splicing of exon 18 hybridize to the exon 18 probe in adult RNA from the wild-type transformant and from all three exon 18 deletion lines. Low stringency hybridization conditions were used for this blot because of the small size of the exon 18 sequence in the deletion lines available for hybridization to the exon 18 probe. (C) The blot in B was stripped and probed with antisense RNA complementary to exons 17, 18, and 19. Comparison of the larval and adult RNA lanes indicates that the adult mini-gene transcripts are slightly larger than the larval transcripts in the exon 18 deletion lines because of inclusion of exon 18. This was borne out by RT-PCR and DNA sequencing (see text). Stage-specific exclusion/inclusion of exon 18 is not affected by deletion of most of exon 18.

transcripts: The polypyrimidine tract and branchpoint consensus sequences that are conserved between *D. virilis* and *D. melanogaster* may serve as critical elements for splicing of exon 17 to exon 18 in adults. Polypyrimidine tracts are typically found within 40 nt of the 3′ splice site and are often contiguous with branchpoint consensus sequences (Mount *et al.* 1992). However, more distant branchpoints, such as the putative branchpoint associated with the conserved pyrimidine-rich sequence in intron 17, are efficiently utilized if they are followed by long polypyrimidine tracts (Reed 1989). To test whether the intron 17 polypyrimidine tract is required for exon 18 inclusion in adults, we prepared a *P*-element mini-gene construct in which the core 22 nt of the conserved polypyrimidine sequence are replaced by a *Kpn*I site (Figure 6A, ΔPy). We were careful to maintain the integrity of the putative branchpoint that is located 5′ to the conserved pyrimidine tract. In the ΔPy mini-gene this branchpoint consensus sequence is positioned 43 nt upstream of the 3′ splice site of exon 18 instead of 61 nt in the wild-type gene. The pyrimidine deletion did not affect the normal skip splicing of mini-gene transcripts in ΔPy larvae (Figure 6C, ΔPy). Hybridization of an exon 18-specific probe to a Northern blot of RNA from transformants expressing the ΔPy mini-gene shows that the deletion dramatically reduces exon 18 inclusion in adult mRNA (Figure 6B, ΔPy). Comparison of the ΔPy adult lane with the w^{1118} adult lane shows that background hybridization to ribosomal RNAs produces much of the observed signal, and comparison to the wild-type *Mhc* construct indicates that the remaining signals are not the appropriate sizes to correspond to the normal inclusion of exon 18, suggesting cryptic splice site activation. RNase protection studies, using a hybrid exon 2/17-exon 18 RNA probe, verified that adult ΔPy mini-gene transcripts containing exon 18 were greatly decreased relative to wild-type levels (>10-fold); we confirmed this by RT/PCR using the exon 2/17-exon 18 primer set (data not shown). ΔPy adults generate mini-gene transcripts slightly larger than the 0.9- and 1.4-kb mRNAs produced in larvae. We did not determine the precise nature of the cryptic splicing, but it is clear that exon 18 is excluded from the majority of these transcripts. To ensure that the failure to include exon 18 in adult mini-gene transcripts containing the pyrimidine tract deletion was not due to the introduction of the *Kpn*I site into this construct, we reinserted the polypyrimidine tract into the engineered *Kpn*I site (Figure 6A, ΔPyWt+). This restored the adult-specific inclusion of exon 18 (Figure 6B, ΔPyWt+), verifying that the pyrimidine tract is critical to this process.

We next sought to determine whether altering the spacing of the intron elements might have disrupted exon 18 inclusion in adult mini-gene transcripts that lack the polypyrimidine tract. We inserted the wild-type polypyrimidine stretch in the opposite orientation at the *Kpn*I site of the ΔPy construct to yield construct ΔPyWt−, which now contains a purine-rich, rather than a pyrimidine-rich, sequence (Figure 6A). This maintains the appropriate spacing between the putative branchpoint and 3′ splice sites. Rather than rescue exon 18 inclusion, this alteration was more effective than the pyrimidine deletion at eliminating exon 18 from adult transcripts (Figure 6B, ΔPyWt−).

Finally, we tested whether the actual sequence of the conserved polypyrimidine tract is important for exon 18 inclusion or if the pyrimidine-rich nature is sufficient. We inserted an oligonucleotide at the *Kpn*I site of the ΔPy mini-gene that is the same length and pyrimidine

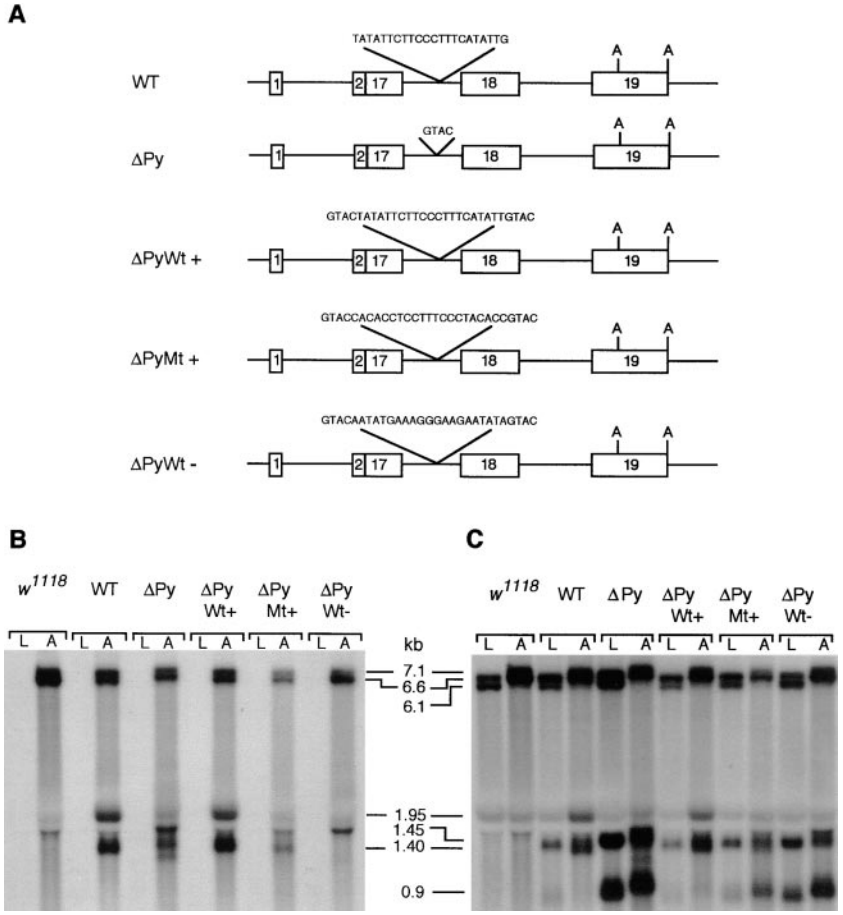

Figure 6.—Northern blot of total RNA from transformants with *Mhc* mini-genes containing mutations in the conserved polypyrimidine tract of intron 17. RNA was isolated from larvae (L) or adults (A) of the following lines: the parental line, w^{1118}; the positive control line, 103-33 (WT); and lines obtained by transformation of w^{1118} embryos with mini-genes depicted in A. Expected wild-type transcript sizes are diagrammed in Figure 3. Bands of 6.1, 6.6, and 7.1 kb are endogenous *Mhc* transcripts that result from exclusion or inclusion of exon 18 and the use of two polyadenylation sites. (A) Diagram of the *Mhc* region of the mini-genes used for transformation (not to scale). The wild-type pyrimidine sequence in intron 17 is shown in WT. The conserved polypyrimidine tract is deleted and replaced with a *Kpn*I site in ΔPy. The wild-type pyrimidine tract (flanked by *Kpn*I sites) is restored in ΔPyWt+, replaced with a pyrimidine tract in which all C and T residues are interchanged in ΔPyMt+, and replaced with a purine-rich sequence because of insertion of the wild-type pyrimidine tract in the opposite orientation in ΔPyWt−. (B) Northern blot probed with a 436-nt antisense RNA specific for exon 18. The WT and ΔPyWt+ adult lanes contain the expected mini-gene transcripts of 1.95 and 1.4 kb but no signal in the larval lanes. The signal for these transcripts is greatly reduced in the ΔPy and ΔPyMt+ lanes and totally eliminated by the ΔPyWt− change. The only hybridization detected in the ΔPyWt− lane, other than the endogenous *Mhc* transcripts near the top of the blot, is due to nonspecific hybridization to ribosomal RNA. This is detected also in all other adult lanes including the w^{1118} line, which lacks a mini-gene. Thus, deletion or replacement of the pyrimidine tract disrupts exon 18 inclusion in adults. This is rescued by reinsertion of the wild-type pyrimidine tract, but not by a different pyrimidine tract. (C) The blot in B was stripped and reprobed with antisense RNA complementary to exons 17, 18, and 19. All lanes with larval RNA from transformed lines contain the expected 1.45- and 0.9-kb mini-gene transcripts indicating that skip splicing of exon 18 is not affected by the mutations. However, mRNA from ΔPy, ΔPyMt+, and ΔPyWt− adults contains transcripts slightly larger than the larval transcripts. For example, there are strong signals in the 0.9-kb region in adult lanes from these transformants; as these bands were not detected in blot B, they lack exon 18 and must result from activation of skip splicing in adults. The results show that the conserved pyrimidine sequence is essential to inclusion of exon 18 in adults, but is not required for its exclusion in larvae.

content as the wild-type polypyrimidine tract. However, every C was replaced by a T and vice versa (Figure 6A, ΔPyMt+). This construct yielded extremely low levels of exon 18 inclusion in adults (well below 10% of wild type; Figure 6B, ΔPyMt+), indicating that the specific conserved sequence, rather than the pyrimidine content, is critical to exon 18 inclusion. *In vivo* data thus identify the conserved polypyrimidine tract as an element essential for inclusion of exon 18, and a potential candidate for the site of interaction of adult-specific *trans*-acting splicing factors.

DISCUSSION

Alternative RNA splicing is a common mechanism used for generating muscle-specific isoforms of myofibrillar components (for review, see Hodges and Bernstein 1994). Our analysis of *Mhc* transcript accumulation in various Drosophila species indicates that alternative inclusion of exon 18 is evolutionarily conserved, suggesting that alternate C termini are essential to stage-specific functions of MHC isoforms. Sequence comparisons of *D. virilis* and *D. melanogaster Mhc* genes defined conserved elements that could regulate alternative splicing, and we tested the function of the conserved sequences through *in vivo* mutational analysis. Our approach identified *cis*-acting elements that control stage- and tissue-specific splicing of this exon. Exclusion of exon 18 in larval muscles results from failure to recognize its nonconsensus splice sites. Inclusion of exon 18 in adults is critically dependent upon a distant polypyrimidine tract upstream of the exon. It is noteworthy that Miedema *et al.* (1994) sequenced the *Mhc* gene of *D. hydei* and their data indicate the same elements are conserved in that species, strengthening our contention that these sequences are functionally significant.

We discuss our results below in the context of other alternatively spliced transcripts and with regard to our previous analysis of exon 18 splicing in an *in vitro* extract from Drosophila Kc cells (Hodges and Bernstein 1992).

Most of exon 18 is dispensable for correct stage- and muscle-specific splicing of *Mhc* mini-gene transcripts: The conserved elements within exon 18 are candidates for splicing activation "enhancer" sequences similar to those that occur within exons of a number of alternatively spliced transcripts (Sun *et al.* 1993; Tian and Maniatis 1993; Watakabe *et al.* 1993; Staknis and Reed 1994; Tanaka *et al.* 1994; Tian and Kole 1995; Coulter *et al.* 1997). Splicing of alternative exons in muscle-specific transcripts can be mediated by such enhancer elements (Xu *et al.* 1993; Ramchatesingh *et al.* 1995; Ryan and Cooper 1996). Two commonly encountered enhancer elements are the purine-rich GAR (R: A or G) and the A/C-rich ACE sequences (Xu *et al.* 1993; Coulter *et al.* 1997). Both GAR and ACE elements mediate splicing via interaction with SR proteins (Lynch and Maniatis 1995, 1996; Ramchatesingh *et al.* 1995; Wang *et al.* 1995; Hertel *et al.* 1996).

A number of GAR-like and ACE-like elements occur in the exon 18 sequences that are conserved between *D. melanogaster* and *D. virilis.* For instance, a long ACE flanked by three GAR repeats is found at the 5′ end of exon 18 (*D. melanogaster* nucleotides 20370–20416). Purine-rich elements, as well as a sequence nearly identical to a binding site for the SR protein SC35 (Tacke and Manley 1995), occur at the 3′ end of exon 18 (*D. melanogaster* nucleotides 20747–20786). Interestingly, the sequence GCTGGAG, which overlaps the 5′ end of the potential SC35 binding site, is a six out of seven nucleotide match to the GCTTGAG sequence that is important for splicing of exon 5 of muscle-specific cardiac troponin T transcripts (Cooper 1992).

The correct and efficient inclusion of exon 18 in mRNA from transgenic adults carrying the MHC Δ 450 E18 construct suggests that no activating sequences reside in the 450 nt of exon 18 that were deleted. The existence of positive-acting elements within exon 18 could not be assessed in our previous *in vitro* studies, because this exon was excluded in spliced transcripts (Hodges and Bernstein 1992). Our current *in vivo* results indicate that putative positive elements, including several ACE/GAR sequences, are not critical to stage-specific alternative RNA splicing. Possible regulatory elements that remain at the 5′ and 3′ ends of exon 18 in MHC Δ 450 E18 are brought into closer proximity by the large deletion. This change, along with the smaller size of the deleted exon (Figure 5), might alleviate the requirement for other enhancer elements. Our previous *in vivo* splicing results, however, suggest that the very 5′ end of exon 18 is not essential, because *Mhc* mini-gene transcripts with a deletion of 10 nt beginning 3 nt downstream of the 3′ splice site of exon 18 were spliced in the correct stage-specific manner (Hess and Bernstein 1991).

Negative-acting elements located in exons also play a role in alternative splicing (Streuli and Saito 1989; Graham *et al.* 1992). It is possible that conserved elements within exon 18 of the *Mhc* gene inhibit exon 18 recognition in larvae. Our previous *in vitro* splicing studies implied the absence of inhibitory elements within the internal 450 nt of exon 18, because Kc cell nuclear extracts exclude exon 18 from mature products and this pattern is maintained in a deletion construct that removes portions of this exon (Hodges and Bernstein 1992). In our current work, the failure to detect inclusion of exon 18 in larval mRNA expressed from the MHC Δ 450 E18 mini-gene *in vivo* further shows that inhibitory sequences important for larval muscle cell exclusion of this exon do not reside in the deleted region.

An emerging theme in alternative splicing regulation is the involvement of both inhibitory and activating elements within exons and introns; splice site selection in a particular environment would thus depend upon the relative abundance or activity of both constitutive and cell/tissue-specific factors that interact with these elements (see Grabowski 1998 for review). For instance, an alternative exon of human FGFR-2 transcripts contains a G-rich sequence that inhibits splicing of this exon in the context of normal weak 5′ and 3′ splice sites (Del Gatto and Breathnach 1995). The downstream intron contains two sequence elements that activate inclusion of the exon but are not required when the exon inhibitory sequence is deleted or when the 5′ and 3′ splice sites are improved toward the consensus sequence. Complexity is also demonstrated by alternative splicing of mouse NCAM exon 18 (Tacke and Goridis 1991). Whereas a small deletion in NCAM exon 18 decreased the efficiency of splicing and eliminated regulation of this exon, a larger, overlapping deletion restored the regulation. While this result is difficult to interpret, it is possible that a series of negative and positive regulators was removed by the larger deletion, masking the effect of deletion of a single element. Given the conservation of sequence and the presence of multiple enhancer-like elements in *Mhc* exon 18, it is possible that both negative and positive elements are involved in splicing of this alternative exon. The large deletion in MHC Δ 450 E18 could have removed some of these, leaving behind elements that permit appropriate stage-specific splicing regulation.

Based on our current study, the extensive conservation of the noncoding region of exon 18 between *D. virilis* and *D. melanogaster* is not critical for proper regulation of alternative splicing, because regulation is retained in the MHC Δ 450 E18 transcripts. This is the case for at least one other set of alternative exons in Drosophila *Mhc.* Standiford *et al.* (1997) recently showed that splicing of the exon 11 series is not regu-

lated by exonic sequences, despite the conserved sequence and position of these exons between *D. virilis* and *D. melanogaster*. While the sequences in exon 11 encode conserved MHC isoforms, the conserved noncoding sequences in exon 18 may be necessary for functions such as RNA transport, localization, or translation because they are in the 3′ untranslated region.

Nonconsensus splice junctions are required for exon 18 exclusion in larvae: Comparison of results from the single and double splice site mutants (CAG, +Int 5, and +Int 5 CAG) suggests that simultaneous recognition of both splice sites is required for exon 18 inclusion. This is consistent with the exon definition model of splicing proposed by Berget and colleagues (Robberson *et al.* 1990; Talerico and Berget 1990; Berget 1995). Jumaa and Nielsen (1997) recently demonstrated a similar role for suboptimal 5′ and 3′ splice sites in alternative splicing of the RNA for SR family member SRp20. The SRp20 protein is proposed to promote selection of alternative exon 4 of its own RNA by enhancing recognition of this exon's weak 3′ splice site; interestingly, the SR protein ASF/SF2 antagonizes this effect, possibly by inhibiting recognition of the weak 5′ splice site of the exon.

While our results for construct CAG show that improvement of only the 5′ splice site of exon 18 is not sufficient to allow exon 18 inclusion, similar experiments with pre-protachykinin RNA (Nasim *et al.* 1990; Grabowski *et al.* 1991; Kuo *et al.* 1991) and the alternative exon 11 series of Drosophila *Mhc* transcripts (Standiford *et al.* 1997) yield different results. In these two cases, substitution of a consensus 5′ splice site for a nonconsensus sequence results in inclusion of a normally skipped internal exon. In contrast, improvement of only the 5′ splice site of exon 18 results in activation of a cryptic splicing pathway, suggesting that the splicing machinery is still unable to recognize the weak 3′ splice site of exon 18.

In an extensive series of *in vitro* experiments, we demonstrated that the failure to recognize either splice junction of exon 18 in Kc cells is not a result of splice junction competition, *i.e.*, that the failure to include exon 18 is not simply because the 5′ splice site of exon 17 and the 3′ splice site of exon 19 outcompete the 5′ and 3′ splice sites of exon 18 (Hodges and Bernstein 1992). We performed similar experiments *in vivo* using splicing constructs in which intron 17 or intron 18 had been deleted and showed that the deletion alone did not promote efficient removal of the remaining intron (D. Hodges, R. M. Cripps and S. I. Bernstein, unpublished results). These data support a model of splice site activation, rather than splice site competition.

Replacing both of the nonconsensus splice junctions of exon 18 with consensus sequences is sufficient to completely switch the splicing pattern of larval *Mhc* RNA to that seen in adults, *i.e.*, exon 18 inclusion. The requirement that both splice sites be switched for efficient inclusion indicates that this process is dependent on recognition of signals at both ends of exon 18. This confirms and extends our *in vitro* analyses, where Kc cell extracts only included exon 18 when both splice junctions agreed with the consensus splicing signals (Hodges and Bernstein 1992). The *in vivo* study is a critical test of the importance of the suboptimal splice junctions for regulation in muscle cells, because all of the constituents required for muscle-specific splicing likely are not present in Kc cell extracts. The similarity between splicing of both wild-type and mutant *Mhc* transcripts in nonmuscle Kc extracts and in larvae suggests that inclusion of exon 18 in modified larval mini-gene transcripts is mediated by binding of constitutive splicing factors to the improved splice sites. This supports the hypothesis that failure to include exon 18 in wild-type larval mRNA is due to the absence of adult-specific factors that promote use of the weak exon 18 splice sites, but does not eliminate the possibility that splicing inhibitors play a role in this regulation as well.

The distant polypyrimidine tract in intron 17 is essential for inclusion of exon 18 in adult mini-gene mRNAs: In vertebrates a functional polypyrimidine tract is located between the branchpoint and the 3′ splice site and contains at least five consecutive uridines or nine consecutive pyrimidines (Roscigno *et al.* 1993). Small introns that are frequently present in Drosophila pre-mRNAs also appear to require pyrimidine-rich intron sequences located between the 5′ splice site and the branchpoint (Kennedy and Berget 1997). Neither a branchpoint consensus sequence nor a polypyrimidine tract is present at the conserved distance from the 3′ splice site of exon 18 in Drosophila *Mhc*. Previous studies showed that branchpoints located beyond the conserved distance from the 3′ splice site, such as the putative branchpoint in intron 17, require nearby distal polypyrimidine tracts for efficient splicing (Reed 1989). In agreement with this, our *in vivo* analysis of ΔPy and ΔPyWt− mini-gene pre-mRNA splicing shows that the distant intron 17 polypyrimidine tract is required for adult-specific inclusion of exon 18 in mature mRNAs. In contrast, however, we find that a sequence with pyrimidine content (ΔPyMt+) identical to that of the wild-type polypyrimidine tract cannot rescue exon 18 inclusion, indicating that the sequence itself, not simply the pyrimidine content, is critical. The conserved polypyrimidine tract in intron 17 therefore appears to serve a unique role in regulating exon 18 inclusion. We propose that adult-specific factors bind to the wild-type polypyrimidine tract in adult muscles and assist in the simultaneous identification of both nonconsensus splice sites of exon 18 by the splicing machinery. This would result in recognition of exon 18 as a *bona fide* exon and the subsequent removal of both introns.

Several proteins are known to bind polypyrimidine tracts in introns and positively or negatively influence intron removal. $U2AF^{65}$ is a constitutive splicing factor

that binds the pyrimidine tract of introns at the early (E) step of spliceosome assembly before catalytic step I of splicing occurs (Michaud and Reed 1993) and facilitates binding of U2 snRNP to the branchpoint (Ruskin *et al.* 1988). It can bind to a variety of pyrimidine-rich tracts, but interacts most strongly with a uridine-rich sequence containing two or three interspersed cytidines at frequent intervals (Singh *et al.* 1995). The conserved intron 17 pyrimidine tract is somewhat different from the U2AF^{65} consensus binding sequence, containing fewer continuous pyrimidines and a lower U/C ratio. Binding of U2AF^{65} to the conserved pyrimidine tract could require an additional factor present only in adult muscle cells. It is also possible that a unique protein recognizes the polypyrimidine stretch in intron 17 and acts to stimulate splicing. At least two factors, PTB and Sxl, are quite selective in binding to specific polypyrimidine tracts compared to U2AF^{65} (Singh *et al.* 1995). Both can act as negative regulators of splicing by blocking U2AF^{65} binding (Valcarcel *et al.* 1993; Singh *et al.* 1995; Ashiya and Grabowski 1997).

Our identification of a conserved polypyrimidine tract in intron 17 and its requirement for exon 18 inclusion suggest that recognition and incorporation of exon 18 into mRNA is positively regulated in adult muscle. A unique factor might bind the conserved polypyrimidine tract and promote recognition of the nonconsensus splice sites of exon 18 by the splicing apparatus. Future work will be aimed at identifying *trans*-acting factors that interact with this important *cis*-acting element.

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