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Muscle-Specific Splicing Enhancers Regulate Inclusion of the Cardiac Troponin T Alternative Exon in Embryonic Skeletal Muscle

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The alternative exon 5 of the striated muscle-specific cardiac troponin T (cTNT) gene is included in mRNA from embryonic skeletal and cardiac muscle and excluded in mRNA from the adult. The embryonic splicing pattern is reproduced in primary skeletal muscle cultures for both the endogenous gene and transiently transfected minigenes, whereas in nonmuscle cell lines, minigenes express a default exon skipping pattern. Using this experimental system, we previously showed that a purine-rich splicing enhancer in the alternative exon functions as a constitutive splicing element but not as a target for factors regulating cell-specific splicing. In this study, we identify four intron elements, one located upstream and three located downstream of the alternative exon, which act in a positive manner to mediate the embryonic splicing pattern of exon inclusion. Synergistic interactions between at least three of the four elements are necessary and sufficient to regulate splicing of a heterologous alternative exon and heterologous splice sites. Mutations in these elements prevent activation of exon inclusion in muscle cells but do not affect the default level of exon inclusion in nonmuscle cells. Therefore, these elements function as muscle-specific splicing enhancers (MSEs) and are the first muscle-specific positive-acting splicing elements to be described. One MSE located downstream from the alternative exon is conserved in the rat and chicken cTNT genes. A related sequence is found in a third muscle-specific gene, that encoding skeletal troponin T, downstream from an alternative exon with a developmental pattern of alternative splicing similar to that of rat and chicken cTNT. Therefore, the MSEs identified in the cTNT gene may play a role in developmentally regulated alternative splicing in a number of different genes.

A large number of genes are remarkable for their ability to generate multiple mRNAs via pre-mRNA alternative splicing (27, 46). Alternative splicing is often regulated according to cell-specific pathways (such as cell type, developmental stage, or sex) to generate mRNAs that differ in protein coding potential, stability, or translation efficiency. Genetic and biochemical studies have established several paradigms for regulated splicing in *Drosophila melanogaster* (27); however, little is known regarding the *cis* elements or *trans*-acting factors that mediate cell-specific regulation in vertebrates.

The *cis* elements required for the regulation of alternative splicing in vetebrates have been investigated primarily by transient transfection of cloned genes into homologous and heterologous cell types (22, 46). In heterologous cells, which presumably lack appropriate regulatory factors, an alternatively spliced pre-mRNA is spliced according to a default pathway determined by the efficiency with which the constitutive splicing machinery recognizes the constitutive splicing signals. Work from many laboratories has demonstrated that the default splicing pathway is determined by a balance of several pre-mRNA features, such as splice site strength, exon size, presence of splicing enhancers or repressors in exons and introns, relative strength of competing splice sites, and secondary structure. These features render a pre-mRNA permissive to undergo regulated selection of more than one splicing pathway. Most of the investigations to identify *cis* elements controlling alternative splicing have defined the pre-mRNA sequences that contribute to default splicing pathways. Only a few *cis* elements have been demonstrated to be likely targets for *trans*-acting factors that mediate cell-specific splicing events (5, 20, 24, 30, 56).

Components of the constitutive splicing machinery have been demonstrated to affect alternative splice site choice, suggesting that modulation of the nuclear concentrations of constitutive splicing factors may regulate some alternative splicing events (37). For example, a family of serine-arginine-rich (SR) proteins has been shown to mediate both constitutive and regulatory splicing activities (19, 34, 63, 64). A role for SR proteins in regulated splicing is supported by the demonstration that individual SR proteins show tissue-specific distribution (61, 64) and by the recently demonstrated correlation between transitions in SR protein isoforms and alternative splicing of CD44 and CD45 during T-cell activation (53). The regulatory activity of some SR proteins is antagonized by the ubiquitously expressed protein heterogeneous nuclear ribonucleoprotein (hnRNP) A1, leading to the suggestion that a balance of these proteins modulates alternative splice site selection (38, 39).

The observation that several different regulatory programs for alternative splicing run concurrently within individual cells makes it unlikely that all regulated splicing in vertebrates is mediated by changes in the nuclear concentrations of constitutive splicing factors. During skeletal muscle development, for example, inclusion of embryo-specific alternative exons of the fast skeletal troponin T (sTNT) and cardiac troponin T (cTNT) genes decreases while inclusion of exon 2 of the AMP deaminase 1 gene and dystrophin exon 78 increases (3, 6, 42, 43). It is likely that, as in *D. melanogaster*, alternative splicing of some vertebrate genes is regulated by programs that utilize distinct sets of *cis* elements and *trans*-acting factors.

We are using the chicken cTNT gene as an experimental

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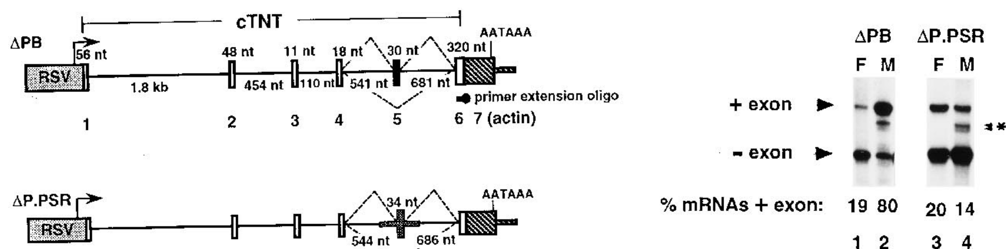

FIG. 1. Intronic elements adjacent to exon 5 regulate exon inclusion in primary skeletal muscle cultures. The cTNT genomic fragment in the ΔPB minigene contains exons 1 to 6. cTNT exon 1 is fused to the Rous sarcoma virus long terminal repeat; for 3′ end formation, cTNT exon 6 is fused to the last exon and 377 nucleotides (nt) of 3′ flanking genomic DNA from the chicken skeletal α-actin gene (62). ΔP.PSR is identical to ΔPB.SA except that segments of introns 4 and 5 were replaced by comparable segments of human β-globin intron 1 and exon 5 was replaced by a heterologous exon (see the text). Minigenes were transiently transfected into QT35 fibroblasts (F) and primary chicken embryo skeletal muscle cultures (M) and assayed by primer extension using a ^{32}P-labeled oligonucleotide complementary to exon 6 (62). The primer extension products are 169 and 199 nucleotides (ΔPB) and 169 and 203 nucleotides (ΔP.PSR). Bands were quantitated directly from the gel with a Betagen 610 imaging system. The level of exon inclusion was calculated as described in Materials and Methods. The position of the primer extension products from endogenous cTNT mRNA including exon 5 in skeletal muscle cultures is indicated by an asterisk.

model with which to study the mechanisms of regulated splicing. cTNT expression is restricted to striated (cardiac and skeletal) muscle. Alternative splicing of the cTNT pre-mRNA is developmentally regulated such that a single alternative exon (exon 5) is included in embryonic striated muscle and skipped in the adult (11). This regulatory pattern is conserved between birds and mammals (23, 33). We have established a transient transfection system to identify pre-mRNA *cis* elements required for the embryonic splicing pattern of exon inclusion in embryonic skeletal muscle (48, 62). cTNT minigenes express mRNAs that predominantly include exon 5 in primary cultures of chicken embryonic skeletal muscle, while in nonmuscle cell lines, exon 5 is spliced according to a default splicing pattern that favors exon skipping. Using this system, we previously identified a purine-rich splicing enhancer in exon 5 and demonstrated it to be a constitutive splicing element and not a muscle-specific regulatory element (48, 62). Having demonstrated that the exon enhancer activity was general, we focused on finding the elements that mediate cell-specific splicing. In this study, we describe the identification of multiple positive-acting intron elements located immediately upstream and downstream of exon 5 that act synergistically to mediate the embryonic splicing pattern of exon inclusion. Mutations in these elements prevent muscle-specific activation of exon inclusion but have no effect on the default splicing pattern in nonmuscle cells. Therefore, we have identified intronic elements that act as muscle-specific splicing enhancers (MSEs).

MATERIALS AND METHODS

Minigene constructs. Construction of ΔPB and ΔPB.SA (Fig. 1 and 2) was described previously (62). ΔPB.SA differs from ΔPB by only one nucleotide substitution in intron 4 to introduce a *Sal*I restriction site and two nucleotide substitutions in intron 5 to introduce an *Asp*718 site. ΔP.PSR is identical to ΔPB except that the last 93 nucleotides of intron 4, exon 5, and the first 70 nucleotides of intron 5 were replaced by a PCR-generated fragment containing (5′ to 3′) the last 96 nucleotides of human β-globin intron 1, the first nucleotide of globin exon 2, the 30-nucleotide skeletal troponin I (sTNI) exon 2, the last 3 nucleotides of globin exon 1, and the first 75 nucleotides of globin intron 1. Therefore, the 30-nucleotide sTNI exon 2 is flanked by the complete splice sites (exonic and intronic components) of human β-globin intron 1. sTNI exon 2 was used because this constitutive exon was demonstrated to lack elements that affect splicing in muscle or nonmuscle cells (62).

The RTB minigene (Fig. 3) was derived from the constitutively spliced sTNI gene (45). In this minigene, the Rous sarcoma virus long terminal repeat is fused to position 50 of exon 1; the last exon contains sTNI exon 4 and 15 nucleotides of exon 5 fused to nucleotide 186 of skeletal α-actin exon 7 and includes 377 nucleotides of actin 3′ flanking genomic sequence (18). To construct RTB33.51, exon 3, 111 nucleotides of intron 2, and 51 nucleotides of intron 3 from sTNI were removed by a *Pvu*II-*Cla*I deletion. The cTNT genomic fragment shown in Fig. 3 was then inserted into an *Eco*47III site located in intron 3. The sequences of unmodified and mutated cTNT genomic fragments used in this study are shown in Fig. 8. Human β-globin intron 1 was substituted for cTNT introns 4 and 5, using the megaprimer PCR cloning method (52). All constructs were confirmed by sequencing.

Transient transfection into primary skeletal muscle cultures and QT35 cells, RNA extraction, and primer extension were performed as described previously (48, 62). Primary skeletal muscle cultures were prepared from embryonic day 11 chicken breast muscle. At this developmental stage, >90% of endogenous cTNT mRNA in breast muscle tissue includes exon 5 (11), and this splicing pattern is retained in primary muscle cultures. The transition to cTNT exon 5 skipping is not reproduced in these cultures, consistent with the inability of these cells to express other markers of late developmental stages (55). The splicing patterns of the endogenous gene and transfected minigene mRNAs from ΔPB, ΔPB.SA, and ΔP.PSR were assayed by primer extension of total cellular RNA, using an oligonucleotide (E630) complementary to cTNT exon 6 (5′-GTCTCCTCTTC CTCCTCGTCTACCTGATCC-3′). For cotransfection studies, 2 μg of ΔPB.SA or ΔP.PSR and 8 μg of MyoD or myogenin expression plasmid (13, 17) were cotransfected into QT35 quail fibroblast cultures. RNA was harvested 2 and 3 days later and was assayed by primer extension using the E630 oligonucleotide. An oligonucleotide complementary to sTNI exon 4 (5′-AGGTGCTGCCGCC GGGCGGTGGCTG-3′) was used for primer extension of total cellular RNA in Fig. 3 to 7. Some transfections were assayed by low-cycle reverse transcription-PCR (RT-PCR) (data not shown). The quantitative nature of the RT-PCR assay was established on in vitro-transcribed RNAs synthesized from the cloned RT-PCR products of construct RTB5.1. The percent exon inclusion for all minigenes was calculated as [cpm exon inclusion/(cpm exon inclusion + cpm exon skipping)] × 100. Results for each construct were determined from at least three independent transfections. In Fig. 3 to 7, the significance of the difference of the mean percent exon inclusion in muscle and nonmuscle cultures was determined by Student's *t* test. For all constructs marked as "+" for regulation in muscle cultures, the probability that the mean percentages of exon inclusion in muscle and nonmuscle cells differ by chance is 1 in 100 ($P < 0.01$). Constructs marked as "−" do not express statistically significantly higher levels of exon inclusion in

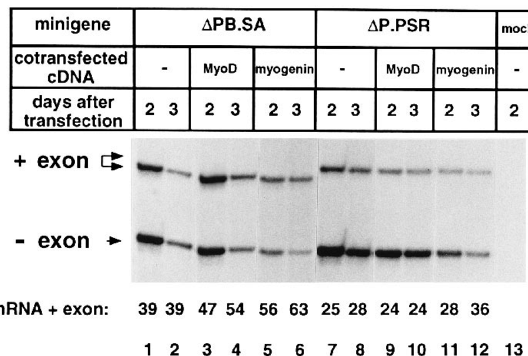

FIG. 2. Myogenin and MyoD induce the muscle-specific splicing pattern in nonmuscle cells. QT35 cultures were transiently cotransfected with 2 μg of either ΔPB.SA or ΔP.PSR and 8 μg of cDNA expression plasmid for MyoD or myogenin. RNA was harvested 2 and 3 days after transfection and assayed by primer extension. The level of exon inclusion was determined directly from the gel as described in Materials and Methods.

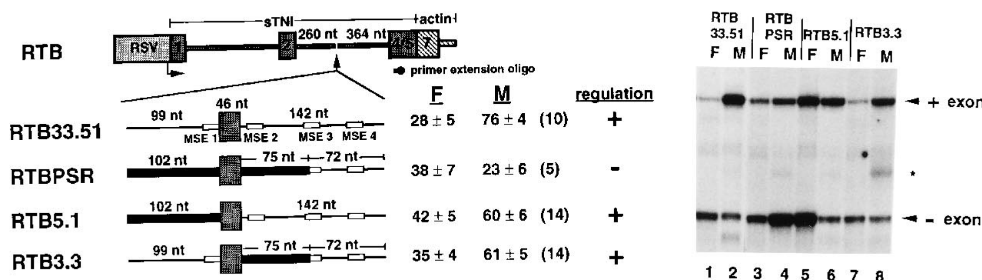

FIG. 3. Regulatory elements in cTNT introns 4 and 5 mediate muscle-specific regulation of a heterologous exon. The RTB minigene (top) was derived from the constitutively spliced sTNI gene. In RTB33.51, the last 99 nucleotides (nt) of cTNT intron 4 and the first 142 nucleotides of cTNT intron 5 (cTNT introns are represented by thin lines) flank a heterologous exon derived from sTNI exon 2 (see Materials and Methods). This is the same exon as in ΔP.PSR (Fig. 1) except that a 12-nucleotide stuffer was inserted to increase the general efficiency of exon inclusion in muscle and nonmuscle cells. Thick lines in RTBPSR, RTB5.1, and RTB3.3 represent comparable regions of human β-globin intron 1. The four MSEs defined in this study are indicated by open boxes and numbered 1 to 4 from 5′ to 3′ (see Fig. 8 for nucleotide sequences of all unmodified and mutant constructs). Minigenes were transiently transfected into QT35 (F) and primary skeletal muscle (M) cultures, and the levels of exon inclusion were assayed by primer extension using an oligonucleotide complementary to sTNI exon 4. The sizes of the primer extension products are 165 and 119 nucleotides for mRNAs including and excluding the alternative exon, respectively. Percent exon inclusion was calculated as described in Materials and Methods. Results are presented as the mean percent exon inclusion ± standard deviation. The number of independent transfections is indicated in parentheses. Statistical analysis of the results in Fig. 3 to 7 is described in Materials and Methods. The asterisk indicates the primer extension product of the endogenous sTNI mRNA in skeletal muscle cultures.

muscle than in nonmuscle cultures. Representative primer extension results are presented in the figures.

RESULTS

Activation of cTNT exon 5 inclusion requires proximal intronic elements. Our transient transfection assay for regulated splicing of cTNT minigenes has been described previously (48, 62). Briefly, cTNT minigenes such as ΔPB (Fig. 1) were transfected into chicken primary skeletal muscle cultures and the quail QT35 fibroblast line. Primary cultures from embryonic skeletal muscle retain the embryonic splicing pattern observed in muscle tissue in that the endogenous and minigene cTNT mRNAs predominantly include exon 5 (Fig. 1, lane 2, and reference 62). The embryonic splicing pattern is also reproduced in primary cultures of embryonic cardiac muscle (data not shown). In QT35 and other nonmuscle cell lines, which are expected to lack gene-specific regulatory factors for cTNT alternative splicing, the exon was predominantly skipped (Fig. 1, lane 1, and reference 62). Forced expression of cTNT cDNAs that include or exclude exon 5 has shown that the steady-state ratio of the alternatively spliced mRNAs in muscle and nonmuscle cells is not determined by differences in mRNA stability (our unpublished data). These results indicated that exon skipping is the default splicing pathway in nonmuscle cells and strongly suggested that exon inclusion in embryonic striated muscle is mediated by a positive-acting mechanism.

It was possible that the high levels of exon 5 inclusion in muscle cells reflected an intrinsically stronger constitutive splicing machinery as a result of higher activities of constitutive splicing factors rather than a gene-specific activation of exon inclusion. If this were the case, any alternatively spliced exon would be expected to be included at higher levels in muscle cells than in nonmuscle cells. To determine whether a high level of alternative exon inclusion was a general feature of skeletal muscle cultures, cTNT exon 5 and segments of cTNT introns 4 and 5 in ΔPB were replaced with a heterologous alternatively spliced exon to generate construct ΔP.PSR (Fig. 1). In this construct, the last 93 nucleotides of cTNT intron 4 were replaced by 96 nucleotides of intron 1 from the human β-globin gene, the first 70 nucleotides of cTNT intron 5 were replaced by the first 75 nucleotides of β-globin intron 1, and cTNT exon 5 was replaced with sTNI exon 2 (see Materials and Methods). The sizes of the introns flanking the alternative exon were essentially preserved. The chimeric ΔP.PSR construct provided an alternatively spliced exon that lacked *cis*-acting elements capable of mediating cell-specific regulated splicing. sTNI exon 2 and the globin intron are constitutively spliced in their natural contexts. sTNI exon 2 was used since it does not contain sequence that affects splicing in muscle or nonmuscle cells (62). We took advantage of the relationship between exon size and splicing efficiency (5, 15, 62) to balance the size of the ΔP.PSR exon against the intrinsic strength of the β-globin splice sites so that mRNAs including and excluding the ΔP.PSR exon were expressed in both cell types. We tested enhancer-negative exons of different sizes and identified one that gave a level of exon inclusion in QT35 cells that was similar to that of ΔPB. This allowed a direct comparison of cell-specific differences in ΔPB and ΔP.PSR alternative splicing. The minigenes were transiently transfected into QT35 and primary muscle cultures. Splicing was assayed by primer extension using an oligonucleotide that anneals to cTNT exon 6, and the levels of exon inclusion were quantitated directly from the gel as previously described (48, 62).

The two minigenes express similar levels of exon inclusion in QT35 cells (Fig. 1, lanes 1 and 3), indicating that the two alternatively spliced exons are recognized with equal efficiency in the absence of regulatory factors. The level of ΔPB exon inclusion is much higher in embryonic skeletal muscle cultures than in nonmuscle cultures, as previously described (Fig. 1, lanes 1 and 2). This is not the case for ΔP.PSR. In fact, in five independent transfections, the level of exon inclusion is lower in the muscle cultures than in nonmuscle cultures (Fig. 1, lanes 3 and 4). We conclude that preferential exon 5 inclusion in embryonic muscle cells is not due to an intrinsically high efficiency of alternative exon recognition. These results strongly suggest that enhanced inclusion of cTNT exon 5 in embryonic muscle is mediated by interactions between positive-acting regulatory factors and target sequences in the cTNT pre-mRNA. We previously demonstrated that sequence within exon 5 was not required for regulated splicing (62); therefore, target sequences essential for regulation are within the intronic regions replaced by globin intron 1 in ΔP.PSR.

The myogenic transcription factors myogenin and MyoD induce muscle-specific splicing in QT35 fibroblasts. The results presented in Fig. 1 suggested that cTNT exon inclusion is mediated by sequence-specific regulatory factors that were

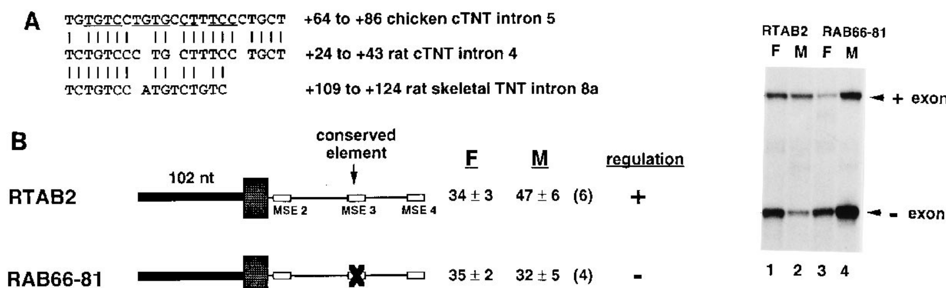

FIG. 4. A conserved element in intron 5 serves as a muscle-specific splicing enhancer. (A) Conserved sequence in the introns downstream of embryo-specific alternative exons in three muscle-specific genes. Nucleotides (nt) are numbered such that +1 is the first nucleotide of the intron downstream from the alternative exon. The GenBank accession numbers are M80829 (rat cTNT) and X03783 (rat sTNT). Nucleotide substitutions and deletions in the RAB66-81 mutation that disrupts splicing in chicken cTNT intron 5 are underlined (see Fig. 8 for nucleotide sequences). Thin lines represent cTNT intron 5; thick lines represent globin intron 1 substitutions. The RTAB2 construct contains the minimal segment of intron 5 that regulates alternative splicing in muscle (see Fig. 5). Results are presented as described in the legend to Fig. 3. (B) Primer extension assay of transfected constructs in QT35 (F) and primary muscle (M) cultures.

present in embryonic muscle but not in nonmuscle cells. To determine if factors regulating alternative splicing were induced as part of the skeletal muscle differentiation program, a cTNT minigene was transiently cotransfected with a MyoD or myogenin cDNA expression clone (provided by E. Olson, Dallas, Tex.) into QT35 fibroblasts (13, 17). MyoD and myogenin are two of four transcription factors that are capable of activating skeletal muscle-specific gene transcription when expressed in a variety of nonmuscle cells (reviewed in reference 47). MyoD and myogenin were cotransfected with cTNT minigene ΔPB.SA, which is identical to ΔPB (Fig. 1) except for three nucleotide substitutions that introduce restriction sites. These substitutions affect the basal level of exon inclusion in muscle and nonmuscle cells but do not affect the level of regulated splicing in muscle cultures. Splicing of minigene mRNA was assayed by primer extension 2 and 3 days following transfection. In four independent experiments, the level of ΔPB.SA exon inclusion was consistently increased when this construct was cotransfected with MyoD or myogenin (Fig. 2, lanes 1 to 6), while a smaller or no increase in the level of exon inclusion was observed for ΔP.PSR (lanes 7 to 12). In some experiments, cotransfection with myogenin induced 80% exon inclusion of the ΔPB.SA alternative exon (data not shown). Therefore, the muscle-specific splicing pattern was induced in QT35 cells by myogenic transcription factors. As in primary muscle cultures, the induced regulation in QT35 cells required regulatory elements that are within 99 nucleotides upstream and 70 nucleotides downstream of the exon. Myogenin and MyoD are unlikely to be directly involved in regulating splicing. It is more likely that the factors that regulate splicing are induced as part of the myogenic differentiation program. We conclude that exon inclusion in embryonic skeletal muscle requires the expression of muscle-specific splicing factors.

Muscle-specific regulation requires intronic elements located upstream and downstream of exon 5. Results from our initial attempts to define regulatory elements in the ΔPB minigene suggested that functionally redundant elements were located throughout introns 4 and 5 (data not shown). To avoid the complications of redundant elements, we sought to define minimal cTNT genomic fragments that were necessary and sufficient to regulate exon inclusion in embryonic muscle cells. To accomplish this, we designed a minigene containing minimal cassettes from cTNT introns 4 and 5 flanking a heterologous exon whose inclusion was enhanced by intronic sequences. To produce this construct, cTNT genomic fragments containing 99 nucleotides of intron 4 and 142 nucleotides of intron 5 were cloned into a constitutively spliced sTNI gene to generate construct RTB33.51 (Fig. 3). In RTB33.51, cTNT exon 5 was replaced by the sTNI heterologous exon used in ΔP.PSR (Fig. 1) to eliminate a known splicing enhancer and simplify interpretation of the data. We demonstrated previously that the enhancer in exon 5 is not required for regulated splicing in muscle cells (62). As in ΔP.PSR, we sought to balance exon size with splice site strength such that a detectable level of default exon inclusion was expressed in nonmuscle cells. This allowed us to determine the effects of mutations on the default splicing pattern in nonmuscle cells as well as on the regulated splicing pattern in muscle cells. To increase the level of exon inclusion in both cell types, it was necessary to increase the size of this exon by insertion of a 12-nucleotide stuffer. Because this exon does not contain sequences required for regulated splicing, exon modifications can be used to manipulate the general efficiency of exon recognition without interfering with the signals for muscle-specific regulation. Using this

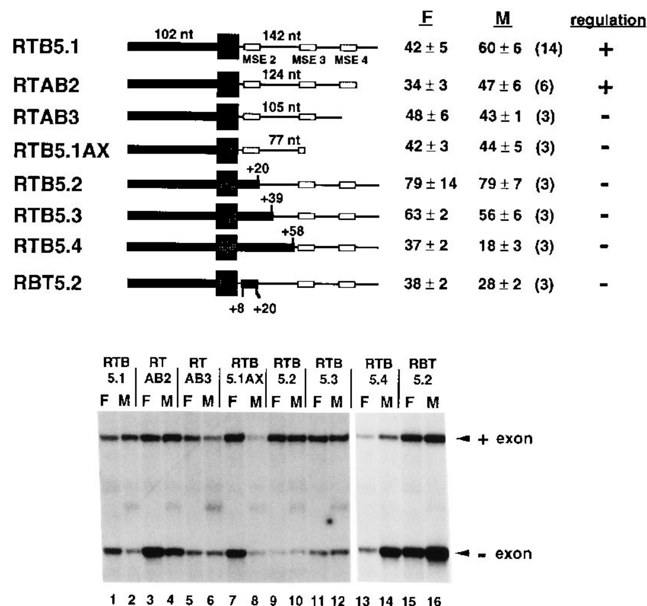

FIG. 5. Deletion and substitution analysis to localize regulatory elements in intron 5. Thin lines represent cTNT intron 5; thick lines represent globin intron 1 substitutions (see Fig. 8 for nucleotide sequences). Results are presented as described in the legend to Fig. 3. nt, nucleotides; F, QT35 fibroblasts; M, muscle cells.

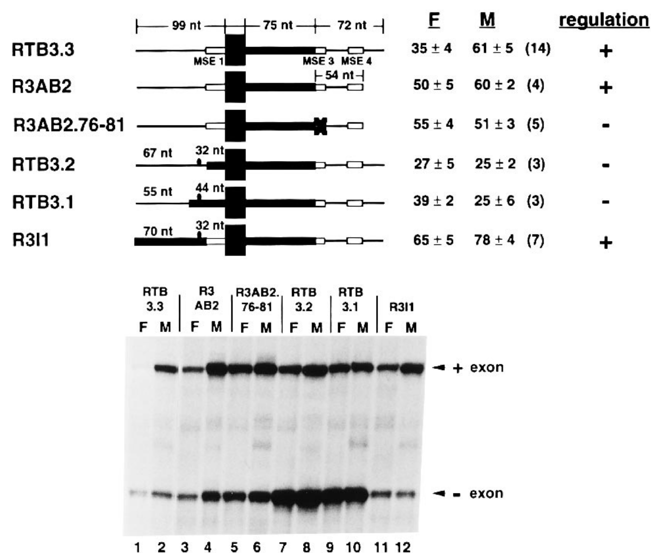

FIG. 6. Deletion and substitution analysis to localize regulatory elements in introns 4 and 5 that mediate synergistic interactions across the exon. Thin lines represent cTNT introns 4 and 5; thick lines represent globin intron 1 substitutions. The positions of the cTNT and globin branch sites are indicated by closed ovals in RTB3.1, RTB3.2, and R3I1. Results are presented as described in the legend to Fig. 3. nt, nucleotides; F, QT35 fibroblasts; M, muscle cells.

approach, we were able to distinguish intronic elements that mediate muscle-specific effects from those that affect splicing in both muscle and nonmuscle cells.

RTB33.51 was transfected into muscle and nonmuscle cultures, and splicing was assayed by primer extension using an oligonucleotide complementary to sTNI exon 4. The level of exon inclusion was quantitated directly from the gel. The results showed that the cTNT intron segments in RTB33.51 regulated splicing of the heterologous exon (Fig. 3, lanes 1 and 2). Additional upstream and downstream intron segments did not increase the level of exon inclusion in muscle cells (data not shown), indicating that RTB33.51 contains the critical cTNT intronic elements required for regulation.

To identify the pre-mRNA sequences that are required for enhanced exon inclusion in embryonic muscle, cTNT introns flanking the heterologous exon in RTB33.51 were systematically replaced by comparable regions of human β-globin intron 1. The overall results of this analysis, presented in Fig. 3 to 7, demonstrate that at least four regions within the cTNT intron segments in RTB33.51 mediate exon inclusion in embryonic skeletal muscle. We refer to these regions as MSEs because mutations prevent activation of exon inclusion in muscle cells but do not affect the default splicing pattern in nonmuscle cells. Each MSE was defined by a single mutation that eliminated preferential exon inclusion in embryonic skeletal muscle. The precise boundaries of these elements remain to be identified. The sequences of unmodified and mutated intron segments used in this study are presented in Fig. 8.

Consistent with the results for ΔP.PSR (Fig. 1), regulated splicing in muscle cultures is lost when both the upstream intron segment and the first 70 nucleotides of the downstream intron are replaced (RTBPSR) (Fig. 3, lanes 3 and 4). Reintroducing either cTNT intron 4 or the first half of intron 5 in RTBPSR restored regulated splicing in muscle cultures (RTB3.3 and RTB5.1, respectively) (Fig. 3, lanes 5 to 8). While the level of regulation in these two constructs is reduced compared with that of RTB33.51, it is reproduced in multiple transfections and is sufficient to permit analysis of the *cis* elements that mediate regulation. These results demonstrate that the first 142 nucleotides of intron 5 are sufficient to regulate splicing of a heterologous exon (RTB5.1). In addition, the downstream 72-nucleotide region of intron 5 is not sufficient to regulate splicing (RTBPSR); however, this segment plus the last 99 nucleotides of intron 4 are sufficient to regulate splicing of a heterologous exon (RTB3.3). We conclude that regulatory elements are located both upstream and downstream of the alternative exon.

A conserved sequence in the downstream intron is required for regulated splicing. To identify conserved regulatory elements, we compared the nucleotide sequences of chicken cTNT introns 4 and 5 with introns flanking alternative exons that are similarly regulated (embryo-specific alternative exons of muscle-specific genes). The genomic sequences of two additional genes that fit this criterion were available in the database: rat cTNT exon 4 (which is homologous to exon 5 in the chicken) and rat sTNT exon 8a (6, 32). This comparison revealed similar sequence motifs located within the first 124 nucleotides of the downstream intron in all three genes. In the chicken gene, this motif is located at positions +64 to +86 in intron 5 (MSE 3) (Fig. 4 and 8). To determine whether this motif was required for regulated splicing, we constructed RAB66-81 (Fig. 4 and 8), which contains a 13-nucleotide mutation in the conserved motif and the first 124 nucleotides of intron 5, the minimal amount of intron 5 required for regulation (Fig. 5). The default level of exon inclusion in QT35 cells was not affected by the RAB66-81 mutation; however, this mutation eliminated regulated exon inclusion in muscle cultures (Fig. 4, lanes 3 and 4). In four independent transfections, the level of exon inclusion in muscle cultures was less than that in QT35 cells, which is characteristic of an unregulated alternative exon (ΔP.PSR and RTBPSR [Fig. 1 and 3, respectively]). We conclude that the region from positions +64 to +86 of intron 5 contains a conserved element required for activation of exon inclusion in muscle cells. The fact that the RAB66-81 mutation had no effect in QT35 cells indicates that this element is likely to be a target for muscle-specific regulatory factors rather than ubiquitously expressed constitutive splicing factors.

Multiple elements in intron 5 contribute to regulated exon inclusion in muscle. To define the 5′ and 3′ boundaries of the

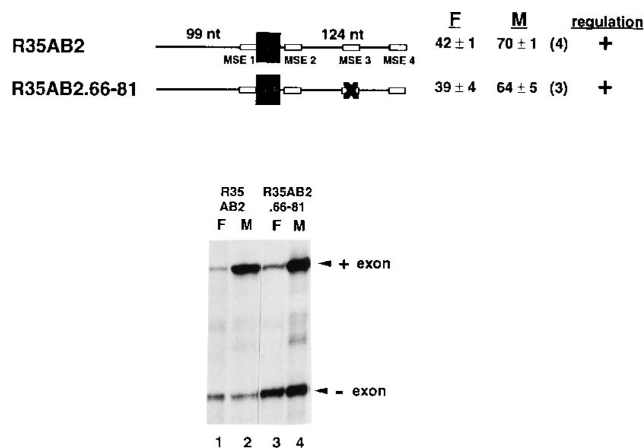

FIG. 7. The last 99 nucleotides of cTNT intron 4 compensate for loss of the conserved element in intron 5. The mutation that completely eliminates regulation in construct RAB66-81 (Fig. 4) has little effect when cTNT intron 4 is present (R35AB2.66-81). Results are presented as described in the legend to Fig. 3. nt, nucleotides; F, QT35 fibroblasts; M, muscle cells.

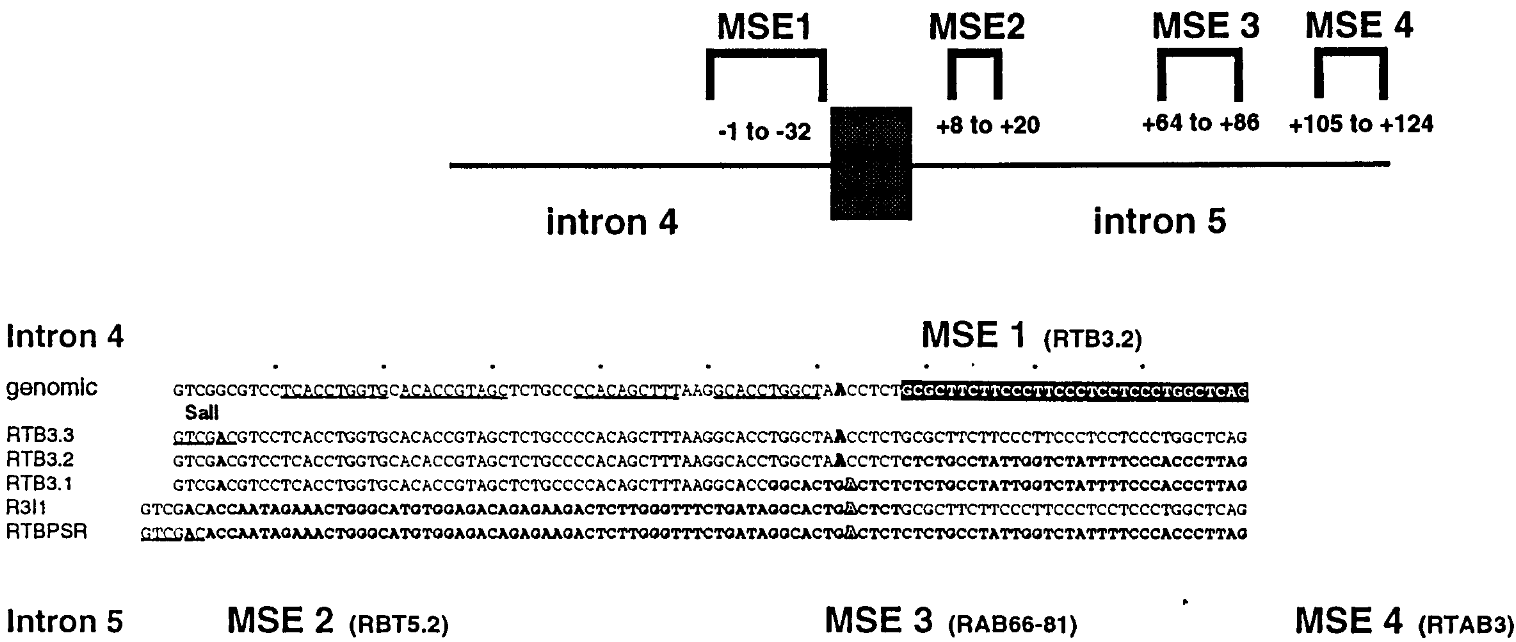

FIG. 8. The diagram at the top shows the locations of four regions required for enhanced exon inclusion in embryonic skeletal muscle. Below, the nucleotide sequences of cTNT introns 4 and 5 in all minigene constr used in this study are compared with the sequences in cTNT genomic DNA. In the genomic sequences of cTNT introns 4 and 5, the CCAYCYCT(G/C)Y repeats are underlined (or overlined where two motifs overl the conserved sequence in intron 5 is both overlined and underlined, MSEs 1 to 4 are in black background, and the name of the minigene which defined each MSE is in parentheses. The CCAYCYCT(G/C)Y conse sequence was derived from all repeats in introns 4 and 5, not just those shown. In the sequence for each construct, substitutions in genomic sequence are indicated in boldface and deletions are indicated by dashed li All constructs contain a single nucleotide substitution at the 5′ end of the intron 4 segment to generate a *Sal*I site (underlined in RTB3.3) and two nucleotide substitutions in intron 5 to generate an *Asp*718 site (underli in RTB5.1 and RTBPSR). The predominant branch sites for cTNT intron 4 and human β-globin intron 1 are indicated in outline. Note that in RTBPSR intron 5, the distance from the 5′ splice site to the *Asp*718 is 5 nucleotides longer than in the natural cTNT intron as a result of substitution of the first 70 nucleotides of cTNT intron 5 by the first 75 nucleotides of β-globin intron 1. All of the constructs that test intron 4 mutat (diagrammed in Fig. 6) contain the globin-for-cTNT intron 5 substitution as in RTBPSR.

region in intron 5 that is required for regulated splicing, we performed the deletion and substitution analysis presented in Fig. 5. The 3′ boundary was determined by deletions that sequentially removed sequence from the 3′ end of the cTNT genomic fragment. Deletion of 18 nucleotides from the 3′ end (RTAB2) (Fig. 5, lanes 3 and 4) had a slight effect on the level of exon inclusion in muscle cells. It is likely that this region contains a sequence that contributes to regulation but is not essential. The remaining 124 nucleotides of intron 5 are sufficient for regulated splicing in muscle cultures. Deletion of an additional 19 or more nucleotides (RTAB3 and RTB5.1AX) (lanes 5 to 8) eliminated regulated splicing because the level of exon inclusion observed in muscle cells decreased to or below the level of exon inclusion observed in nonmuscle cells. We conclude that the conserved element (MSE 3) is not sufficient for regulated exon inclusion; additional elements located between +105 and +124 of intron 5 are also required. The region between +105 and +124 defines a second region required for regulation, MSE 4.

To define the 5′ boundary of the regulatory elements in intron 5, the 5′ end of the intron was replaced with progressively larger segments of the identical positions from β-globin intron 1. Substitution of the first 20, 39, or 58 nucleotides resulted in the loss of regulated splicing in muscle cells (constructs RTB5.2, RTB5.3, and RTB5.4, respectively) (Fig. 5). Since the natural 5′ splice site of intron 5 is not required for regulation (RTB3.3) (Fig. 3), we expected that the regulatory element disrupted in RTB5.2 was not the 5′ splice site but an element located in the downstream portion of this substitution. This was confirmed in construct RBT5.2, in which the first seven nucleotides of cTNT intron 5 containing the natural 5′ splice site were not sufficient to restore regulated splicing. The RBT5.2 construct demonstrates that an element within positions +8 to +20 of intron 5 is required for regulation. This region is designated MSE 2.

Taken together, the results presented in Fig. 5 define a region of intron 5 between positions +8 and +124 that is sufficient for regulation. This segment contains at least three regions (MSEs 2 to 4), all of which are required for regulation in the absence of intron 4. The mutations that defined MSEs 2 to 4 (RTAB3 [Fig. 5], RAB66-81 [Fig. 4], and RBT5.2 [Fig. 5]) eliminated regulation in muscle cells without affecting the default level of exon inclusion in nonmuscle cells, indicating that recognition of elements within these regions is muscle specific.

Regulation of exon inclusion in muscle involves synergistic interactions across the exon. The last 99 nucleotides of cTNT intron 4 restored regulated splicing to RTBPSR (RTB3.3) (Fig. 3). To define the relevant regulatory elements, we first tested whether the 72-nucleotide downstream segment of intron 5 contributed to this regulation by introducing two mutations into this region. One was a deletion of 18 nucleotides from the 3′ end of the cTNT genomic segment to the 3′ end of MSE 4 (construct R3AB2) (Fig. 6, lanes 3 and 4). The 3′ end of the cTNT intron 5 fragment in R3AB2 is the same as in RTAB2, which defined the minimal amount of intron 5 required for regulation (Fig. 5). In multiple independent transfections, this deletion increased the level of exon inclusion in nonmuscle cells, but overall, exon inclusion in muscle cells remained regulated (compare RTB3.3 [Fig. 6, lanes 1 and 2] and R3AB2 [lanes 3 and 4]). The second mutation was in MSE 3, the conserved element shown in Fig. 4. While the 5′-most one-third of the conserved element is removed in RTB3.3, 14 of 23 nucleotides remain intact (Fig. 8). A mutation within these remaining nucleotides completely eliminated regulation (R3AB2.76-81) (Fig. 6, lanes 5 and 6). Therefore, the result for R3AB2.76-81 indicated that the 99-nucleotide intron 4 segment is not sufficient to regulate alternative splicing of a heterologous exon. Similarly, the result for RTBPSR indicated that the downstream 72-nucleotide segment from intron 5 was not sufficient for regulated splicing (Fig. 3). We conclude that regulated splicing in RTB3.3 requires synergistic interactions across the exon between elements located in these two regions. One of the required elements in intron 5 is the conserved region MSE 3.

To localize the components in the intron 4 segment that contribute to regulated splicing in RTB3.3, regions of intron 4 were replaced by comparable regions of β-globin intron 1. The structures of the cTNT and globin 3′ splice sites are analogous in that the positions of the branch sites are similar (−38 for cTNT intron 4 and −37 for β-globin intron 1) (Fig. 8) (9, 50). Constructs RTB3.2 and RTB3.1 (Fig. 6, lanes 7 to 10) contain replacements of the polypyrimidine tract alone (RTB3.2) and the polypyrimidine tract and the branch site of cTNT intron 4 (RTB3.1) with that of β-globin intron 1. The loss of regulation in construct RTB3.2 indicated that the polypyrimidine tract alone is required for regulation. This was confirmed by the reciprocal substitution in construct R3I1, in which the last 32 nucleotides of cTNT intron 4 were shown to be sufficient to act synergistically with intron 5 to mediate regulated splicing (Fig. 6, lanes 11 and 12). This region of intron 4 is designated MSE 1. Therefore, the regulatory component of intron 4 that cooperates with the downstream intron is within the polypyrimidine tract and is separate from the branch site. These results demonstrate that regulated splicing in embryonic muscle is mediated in part by synergistic interactions between a component in the polypyrimidine tract in intron 4 and the conserved element in intron 5.

We sought to determine the extent of functional redundancy between elements located upstream and downstream of the alternative exon. Results presented in Fig. 4 demonstrated that mutation of a conserved element in intron 5 (MSE 3) eliminated regulated splicing in muscle. To determine whether the regulatory element in intron 4 could compensate for mutations in MSE 3 in intron 5, we introduced the unmodified and mutated intron 5 segments from RTAB2 and RAB66-81 (Fig. 4) into constructs containing the 99-nucleotide cTNT intron 4 segment to generate R35AB2 and R35AB2.66-81, respectively (Fig. 7). The results for these minigenes demonstrated that mutation of the conserved element had a small effect on the level of exon inclusion in muscle cells and no effect in nonmuscle cells in the presence of intron 4. The reduced level of exon inclusion in muscle is reproducible and probably reflects a reduced regulatory capability. However, the main point of this experiment was to demonstrate that the regulatory element in intron 4 (presumably MSE 1) compensates for the loss of a conserved regulatory element in intron 5 (MSE 3), despite the fact that intron 4 does not contain a sequence that resembles the conserved element. We conclude that modulation of exon inclusion in embryonic muscle is mediated by multiple regulatory elements located upstream and downstream of the alternative exon and that these elements are diverse in sequence and redundant in function.

DISCUSSION

Alternative splicing of cTNT exon 5 is regulated during striated muscle development such that the exon is included in the embryo and skipped in the adult. The embryonic splicing pattern is maintained in primary cultures of embryonic skeletal and cardiac muscle, as demonstrated by high levels of exon inclusion for both the endogenous gene and transiently transfected minigenes. This provides an experimental system with

which to investigate the mechanism of exon inclusion in embryonic striated muscle (Fig. 1, references 48 and 62, and unpublished results).

The results presented in this study demonstrate that activation of cTNT exon 5 inclusion in embryonic skeletal muscle requires two components: (i) intronic elements located upstream and downstream of the exon and (ii) muscle-specific positive-acting factors. Our results support a model for positive regulation. First, we demonstrated previously that exon skipping is the default splicing pattern in nonmuscle cells that results from a balance of several features that make exon 5 an intrinsically poor splicing substrate (10, 12, 62). A positive-acting mechanism is required to account for enhanced inclusion of an exon that is predominantly skipped in the absence of regulatory factors. Second, muscle-specific exon inclusion requires intronic elements located upstream and downstream of the exon. Mutations in these elements decrease the level of exon inclusion in muscle cells to or below the default level observed in nonmuscle cells, suggesting that these elements serve as targets for factors that mediate muscle-specific exon inclusion. These results are consistent with a model in which positive-acting regulators in muscle cells enhance exon inclusion and not the alternative model in which a constitutively expressed repressor blocks exon inclusion in nonmuscle cells. It should be noted that this study addresses only the mechanism of exon inclusion in embryonic muscle and not the developmental transition to exon skipping in adult tissue. While the developmental switch to exon skipping may simply be due to loss of the activity mediating exon inclusion, more complex alternative models are also possible.

A number of intron (1, 5, 8, 14, 20, 21, 24, 30, 35, 40, 54, 56) and exon (4) elements have been shown to influence alternative splice site usage in vertebrates. While many of these are novel splicing elements, most are not cell specific in their activities. Only a few elements have been demonstrated to be targets for factors that mediate cell-specific splicing events. For this investigation, minigenes were constructed such that some level of exon inclusion was expressed in both muscle and nonmuscle cells. This allowed us to determine the effects of mutations on the default splicing pattern in nonmuscle cells as well as on the regulated splicing pattern in muscle. Using this approach, we have identified the first muscle-specific positive-acting splicing elements to be described. Mutations in these elements prevent activation of exon inclusion in muscle cells but have no effect on the default level of exon inclusion in nonmuscle cells. These elements are likely to be targets for muscle-specific splicing factors rather than ubiquitously expressed constitutive splicing factors. We refer to these elements as MSEs.

In some constructs, the default level of exon inclusion was affected in both muscle and nonmuscle cells. In cases in which the substituted regions included the 5′ and 3′ splice sites (RTB5.2 and R3I1), a change in the default level of exon inclusion is likely to be due to differences in the intrinsic strengths of these constitutive splicing signals. In our analysis of muscle-specific regulation, the critical question is whether the level of exon inclusion is significantly higher in muscle than in nonmuscle cells. In other constructs, the default level of exon inclusion was altered by mutations that did not involve known splicing signals. For example, deletion from the 3′ end of the intron 5 segment in RTAB3 (Fig. 5) and R3AB2 (Fig. 6) tended to increase the level of exon inclusion in nonmuscle cells. We are currently testing whether this effect was due to nonspecific changes in the spatial context of the exon or to loss of repressor sequences. Since deletions in this region did not affect the default levels of exon inclusion for RTBPSR (data not shown), this region is not likely to contain a general splicing repressor.

Mutation analysis defined four regions in cTNT introns 4 and 5 that act synergistically as MSEs in embryonic skeletal muscle cultures (MSEs 1 to 4) (Fig. 8). Intron 5 contains at least three regions between +8 and +124 that are required for muscle-specific exon inclusion. In addition, the region from +125 to +142 contributes to regulation but is not essential. A fourth element (MSE 1) is located in the polypyrimidine tract of intron 4. Of particular interest is a 23-nucleotide sequence located in intron 5 that is conserved between the chicken and rat cTNT genes (MSE 3). This sequence is also found downstream of a third embryo-specific alternative exon in rat sTNT, suggesting that it may be involved in developmentally regulated splicing of a number of muscle-specific genes.

Each MSE was defined by a single mutation that eliminated enhanced exon inclusion in embryonic skeletal muscle cultures (MSE 1, 2, or 4) or by conservation between rat and chicken genes (MSE 3). The precise boundaries of the MSEs remain to be defined. Further experiments are necessary to identify the specific targets for regulatory factors within the regions defined as MSEs and to determine whether additional regulatory elements are located between MSEs 2 and 4 in intron 5. All four MSEs are pyrimidine rich (Fig. 8). The three elements in intron 5 (MSEs 2 to 4) have sequence features in common, suggesting the possibility that these regions contain variable repeats of a single element. MSEs 2 and 4 have a common, albeit highly variable, sequence motif, CCAYCYCT(G/C)Y (underlined in Fig. 8). Additional repeats of this motif are found throughout cTNT introns 4 and 5, including copies upstream and downstream of the genomic fragment tested in this study (data not shown). Copies of this sequence are also found upstream and downstream of rat cTNT exon 4 and sTNT exon 8a, suggesting that this motif is conserved. Interestingly, several repeats of this motif are found in the intron downstream of the c-*src* neuron-specific N1 exon within regions that are conserved between birds and mammals (5). It is doubtful that this motif plays a role in c-*src* splicing in skeletal muscle since the N1 exon is skipped in c-*src* mRNAs expressed in embryonic and young adult chicken skeletal muscle (16). This sequence is not within the intronic element from +38 to +70 that is required for neuron-specific splicing of the N1 exon, but three copies are found in the intron fragment from +70 to +142 that restores partial neuron-specific splicing (5). Therefore, a role for this motif in neuron-specific splicing cannot been ruled out. A second motif, CTTTC, is repeated five times in the first 124 nucleotides of cTNT intron 5 and is present in all three MSEs in intron 5 (MSEs 2 to 4). We are currently testing whether the specific sequences or merely the pyrimidine-rich nature of these motifs is required for regulated splicing of cTNT exon 5 in embryonic muscle.

The element within the 3′ splice site of intron 4, MSE 1, appears to be distinct from the three elements in intron 5. Despite the lack of shared sequence, MSE 1 is functionally redundant with at least two elements in intron 5: MSE 2 (compare R3I1 [Fig. 6] with RTB5.1 [Fig. 3]) and the conserved element MSE 3 (compare RAB66-81 [Fig. 4] with R35AB 2.66-81 [Fig. 7]). Therefore, the mechanism of cell-specific regulation of cTNT exon 5 involves diverse elements that exhibit redundant functions. While these elements may bind to different factors, their activities probably feed into a common pathway that most likely recruits or stabilizes binding of constitutive splicing factors to the weak splicing signals that flank exon 5. It is possible that multiple elements with overlapping functions exist to enhance the stability and specificity of this process.

Regulated splicing requires at least three of the four MSEs, indicating that regulation is mediated by synergistic interactions between multiple intronic elements. The MSEs may act as distinct binding sites for regulatory factors or as components of a single large binding site. It should be noted that this region does not contain detectable conserved secondary structures that are consistent with the mutation studies. Given that the four MSEs act synergistically, we cannot determine from the results presented in this study whether all or only a subset of the MSEs bind directly to muscle-specific regulatory factors. For example, regulatory activity could require binding of muscle-specific factors to only one MSE and constitutive splicing factors to the others. Muscle-specific regulation would be disrupted by MSE mutations that block binding of either constitutive or muscle-specific factors to the pre-mRNA.

Synergistic interactions between multiple and repeated elements appear to be a general mechanism of cell-specific regulation of pre-mRNA alternative splicing in vertebrate and invertebrate systems (5, 7, 20, 24, 28, 30, 31, 51, 56, 60). Positive regulation of the *Drosophila doublesex* (*dsx*) female-specific exon requires synergistic interactions between the 13-nucleotide *dsx* repeat elements (25, 29, 44), a purine-rich splicing enhancer (36), and a binding site for RBP1 in the 3′ splice site (26). These elements are required for cooperative interactions between gene-specific regulatory factors (Tra and Tra-2) and constitutive splicing factors (SR proteins) to assemble a multicomponent regulatory complex (57, 58). Similarly, the neuron-specific N1 exon of c-*src* has been shown to require multiple elements to regulate cell-specific splicing (5, 8). The intron downstream from the N1 exon contains positive-acting RNA elements which bind to both constitutive and neuron-specific factors in vitro (41). Both introns upstream and introns downstream of the N1 exon also contain a repeated element that specifically represses exon inclusion in vitro in HeLa nuclear extracts (8). Together, these results suggest an unexpected complexity in the regulation of cell-specific alternative splicing. The modulatory nature of regulated splicing is reminiscent of transcriptional regulation in which different *cis*-acting elements, often present in multiple copies, mediate assembly of a complex composed of ubiquitously expressed constitutive factors and cell-specific regulatory factors (59).

This study establishes the general mechanism for regulated inclusion of cTNT exon 5 in embryonic striated muscle. Models involving specific activation of either the 3′ splice site or the 5′ splice site are feasible given that regulatory elements are within the polypyrimidine tract of intron 4 and immediately downstream of the 5′ splice site in intron 5. The fact that regulatory elements located upstream and downstream of the exon act synergistically across the exon to mediate muscle-specific activation of exon inclusion suggests an alternative possibility in which these regulatory elements promote muscle-specific inclusion by recruiting or stabilizing factors involved in exon definition (49). Given that exon-spanning interactions are involved in the recognition of at least some constitutive exons (2, 4), it is likely that the modulation of these interactions is utilized to regulate cell-specific inclusion of alternative exons.

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