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Cis-elements involved in alternative splicing in the rat β-tropomyosin gene: the 3′-splice site of the skeletal muscle exon 7 is the major site of blockage in nonmuscle cells

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ABSTRACT

We have been using the rat β-tropomyosin (β-TM) gene as a model system to study the mechanism of alternative splicing. The β-TM gene spans 10 kb with 11 exons and encodes two distinct isoforms, namely skeletal muscle β-TM and fibroblast TM-1. Exons 1 – 5, 8, and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as in smooth muscle cells, whereas exons 7 and 10 are used exclusively in skeletal muscle cells. Our previous studies localized the critical elements for regulated alternative splicing to sequences within exon 7 and the adjacent upstream intron. We also demonstrated that these sequences function, in part, to regulate splice-site selection *in vivo* by interacting with cellular factors that block the use of the skeletal muscle exon in nonmuscle cells (1). Here we have further characterized the critical *cis*-acting elements involved in alternative splice site selection. Our data demonstrate that exon 7 and its flanking intron sequences are sufficient to regulate the suppression of exon 7 in nonmuscle cells when flanked by heterologous exons derived from adenovirus. We have also shown by both *in vivo* and *in vitro* assays that the blockage of exon 7 in nonmuscle cells is primarily at its 3′-splice site. A model is presented for regulated alternative splicing in both skeletal muscle and nonmuscle cells.

INTRODUCTION

Alternative RNA splicing is a fundamental process in eukaryotes, that contributes to tissue-specific and developmentally regulated patterns of gene expression (2,3). The cellular factors and mechanisms that are responsible for the selection of alternative splice sites in complex transcription units are beginning to be understood (for reviews see 2–4). The most progress has been made in *Drosophila* systems such as in the genes of the sex determination pathway, suppressor of *white apricot* and *Drosophila P*-transposase. In these systems alternative splicing is subject to regulation by factors that either inhibit or activate the use of alternative 5′ or 3′ splice sites (3–5). In contrast, in vertebrate systems, much less is known about the mechanisms and cellular factors involved in regulated alternative splicing, but a number of features in the pre-mRNA have been implicated in alternative splice site selection. These include the relative strengths of 5′ and 3′ splice sites (6–8), intron size (9), the pyrimidine content of a 3′ splice site (10,8), the location of branch points (11–15), multiple alternative branch points (16,17,11,12), branch point sequences (8,18,19,20), intron sequences between a 3′ splice site and upstream branch point (14,15,20), and exon sequences (1,12,21–23,,24–26,20,27,28,29,30).

We have been using the rat β-TM gene as a model system to investigate the mechanisms responsible for developmental and tissue-specific alternative RNA splicing (1,12,14,31–33). This gene spans 10 kb of DNA with 11 exons and encodes two distinct isoforms, namely skeletal muscle β-TM and fibroblast TM-1 (Figure 1). Exons 1 through 5, 8 and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as smooth muscle, whereas exons 7 and 10 are used exclusively in skeletal muscle. Unlike most branch points that have been mapped, in which a single adenosine residue located 18–40 nucleotides from a 3′ splice site is used during lariat formation (34), *in vitro* splicing of exon 5 to exon 7 (skeletal muscle-type splice) involved the use of multiple branchpoints which are located an unusually long distance (144, 147 and 153 nucleotides) from the 3′ splice site of exon 7 (32). We subsequently investigated the functional role of the intron sequences between the distant branchpoints and the 3′ splice site of the skeletal muscle exon 7. Our results demonstrated that two distinct functional elements are present in this region (14). The first element is comprised of a polypyrimidine tract located 89–143 nucleotides upstream of the 3′ splice site, which specifies the location of the branch points used, 144–153 nucleotides

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upstream of exon 7. The second element is comprised of intron sequences located between the polypyrimidine tract and the 3′ splice site of exon 7. This region contains an important determinant in alternative splice site selection because deletions or mutations of these sequences results in the use of the skeletal muscle-specific exon in nonmuscle cells (1,14). We found that sequences contained within the 5′ end of exon 7 are also involved in blocking the use of exon 7 in nonmuscle cells (1). In addition, mutations that inactivated the 5′- and 3′-splice sites of exon 6 did not result in the use of the skeletal muscle-specific exon 7 in nonmuscle cells, demonstrating that splice site selection *in vivo* is not regulated by a simple *cis*-acting competition mechanism, but rather by a mechanism that inhibits the use of exon 7 in certain cellular environments (1).

In order to study in greater detail how sequences in and around exon 7 prevent the use of this exon in nonmuscle cells, we have further analyzed the *cis*-elements involved in splice site selection. These studies extend our previous observations and demonstrate that the critical *cis*-elements for regulated alternative splicing are confined to sequences within exon 7 and the adjacent upstream intron. We also demonstrate that the blockage of exon 7 in nonmuscle cells is primarily at its 3′-splice site.

MATERIALS AND METHODS

Plasmid construction and transfection

For *in vivo* studies designed to analyze the internal pair of alternative exons of the rat β-TM gene, plasmids pSV40-p2 and pSV40-p2(7/8) were used (12). For *in vitro* splicing assay, pSP64-p2(7/8) was used (12). The chimeric adenovirus-β-tropomyosin minigene was derived from a clone kindly provided by Douglas Black (28), by replacing sequences from HindIII site in intron 1 to SalI site in intron 2 of adenovirus major transcription unit with β-tropomyosin sequences, spanning 163 nucleotides upstream of exon 7 to 25 nucleotides downstream of exon 7. Mutations were introduced into these clones from previously described mutants (1) by standard subcloning methods (35). Transfections and RNA harvesting were performed as described previously (1).

PCR assays

For analyzing the splice products from adenovirus chimeric gene transcripts, the primers previously described by Black were used (28). They are (1) CTCAAGGGGCTTCATGATGTCC, complementary to the β-globin trailer sequences in pEVRF1; (2) TCCGATCCGGTCGATGCGGACTCG, complementary to the third exon of the adenovirus major late transcription unit; (3) CAGCGCCTTGTAGAAGCGCGTATG, homologous to the leader sequence of pEVRF1. To visualize the PCR products by autoradiography, primer 3 was 5′-end labeled with ^{32}P-γATP (35). To make cDNA of the mRNA, 3 μg of total cellular RNA were annealed to 3 ng primer 1 and reverse transcribed in a 30-μl reaction according to the protocol of Smale and Baltimore (1989). After reverse transcription, the sample was diluted to 60 μl, and 5 μl of which was used for PCR.

For analyzing the splice products from β-TM minigenes, four primers were used. They are: (1) TTTTGGAGGCCTAGGCTTTT, homologous to the SV40 leader sequences; (2) TTTGCCACAGATCTTTCAGC, complementary to exon 9 sequences of β-TM; (3) CACCTCGGCTCTCTC, complementary to exon 5 sequences of β-TM; (4) GTCCGCTTGGGCTTCCAGAG, complementary to exon 7 sequences. Primer 1 was 5′ end labeled with ^{32}PγATP. cDNAs were made with same method as above, except that 200 pmol of random hexamers were used in the reverse transcription.

The PCR reactions included 20 cycles of 94° for 1 min, 55° for 1 min, and 72° for 1 min 45 sec., followed by 72° for 10 min. The PCR products were extracted with pheneol/chloroform, ethanol precipitated, and analyzed on a 6% polyacrymide/8M urea gel. The gel was dried and analyzed by autoradiography.

Synthesis of RNA and *in vitro* splicing reaction

The ^{32}P-labeled pSP64-p2(7/8) were synthesized *in vitro*, primed with CAP analog, as described (36). HeLa cell nuclear extracts were prepared as described (37). *In vitro* splicing reactions, as described previously (12), were carried out at 30° for 120 min. 15 μl nuclear extract were used in a reaction volume of 25 μl that consisted of 1 mM $MgCl_2$, 500 μM ATP, 20 mM creatine phosphate, 2.6% (wt/vol) polyvinyl alcohol, 60 mM KCl, 0.12 mM EDTA, and 0.6 mM dithiothreitol (DTT).

RESULTS

Intron sequences upstream and downstream of exon 7 are sufficient to regulate the suppression of exon 7 flanked by heterologous exons

Our previous studies have identified *cis*-acting elements that inhibit the use of exon 7 (skeletal muscle exon) in nonmuscle cells (14,1). The elements are located in the intron immediately of exon 7 and at the 5′-end of exon 7, since mutations (deletions or base substitutions) at these regions result in the activation of exon 7 splicing in nonmuscle cells. To determine if these elements are sufficient to suppress exon 7 splicing in a nonmuscle cell environment, we have made a chimeric minigene shown in

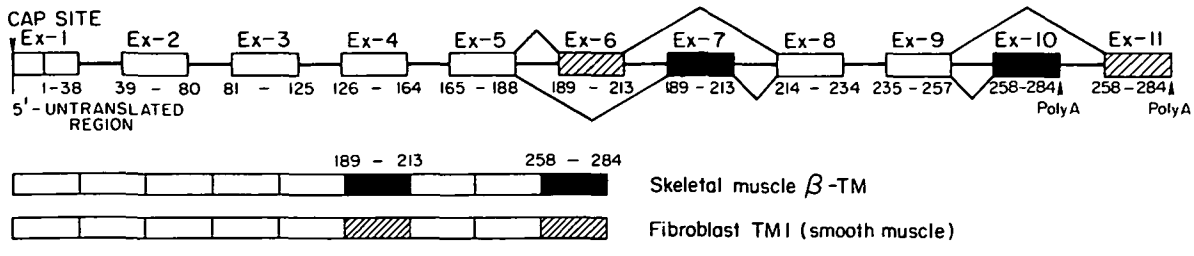

Figure 1. Exon-intron organization of the rat β-tropomyosin gene. Open boxes represent common exons, hatched and closed boxes represent tissue-specific exons as indicated. Horizontal lines represent introns; they are not drawn to scale. The amino acids encoded by each exon are indicated. The CAP site and polyadenylation sites are also indicated.

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Figure 2. The chimeric minigene was composed of adenovirus major late transcription unit driven by cytomegalovirus (CMV) promoter/enhancer, the tropomyosin exon 7 and its immediate flanking region was inserted replacing the adenovirus exon 2. The inserted TM gene segment contained sequences immediately upstream of the branch point of exon 7 through 25 nucleotides downstream of exon 7. We also prepared chimeric minigenes with β-TM segments containing three individual mutations. We previously characterized these three mutations, termed int-3, int-5 and d3-5, in the context of β-TM gene sequences and found that they are capable of activating exon 7 splicing in nonmuscle cells (1). The int-3 and int-5 mutants contain clustered point mutations of GGATCC instead of wild-type sequence TGTCTC and ACGCCC, respectively. The d3-5 mutant contains a 22 nucleotide deletion from int-3 to int-5 position. The wild type and mutant heterologous minigenes were transfected into HeLa cells. After 48 hours incubation, cytoplasmic RNA was isolated and analyzed by reverse transcription-PCR analysis. As shown in Figure 2, the splice product for the wild-type heterologous minigene is adenovirus exon 1 spliced to adenovirus exon 3, and the use of exon 7 of the TM gene is not detected. On the other hand, mutants int-3 and int-5 can partially activate the use of exon 7 flanked by adenovirus exons, as shown by the splice product of adenovirus exon 1 to TM exon 7 to adenovirus exon 3 (Figure 2). Furthermore, mutant d3-5, which completely activated exon 7 usage in the context of the natural TM gene flanking exons (1), almost completely activated exon 7 splicing in the heterologous genes. These results demonstrate that exon 7 and its immediate flanking intron regions are sufficient for the regulation of exon 7 splicing in a nonmuscle cellular environment.

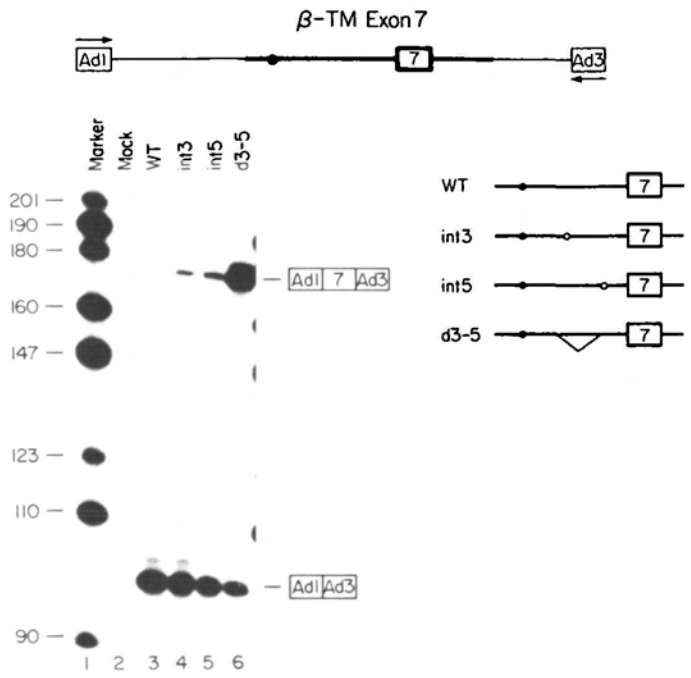

Figure 2. Exon 7 and its flanking intron sequences are sufficient to regulate the suppression of exon 7 when flanked by heterologous exons derived from adenovirus. (*Top*) Schematic diagram of the chimeric minigene used in HeLa cell transfection. The position of the branchpoint sequences (BPS) (black dot) and adjacent polypyrimidine tract (black box) in intron 6 are also indicated. The wild type and mutant chimeric minigenes (*right*) were transfected into HeLa cells and the cytoplasmic RNA harvested 48 hours later. The RNAs were analyzed by RT-PCR assay using a primer complementary to adenovirus exon 3 and a primer homologous to adenovirus exon 1 that is 5′ end labeled with ^{32}P-γATP. Arrows (*Top*) represent the primers used in the PCR assay. The top bands in the autoradiograph correspond to splice products of adenovirus exon 1 to exon 7 of TM gene to adenovirus exon 3. The bottom bands correspond to splice products adenovirus exon 1 to adenovirus exon 3. Marker lane are markers using pBR322 digested with Msp I.

Intron sequences in the 5′ half of intron 6 do not affect alternative splice site selection

Recent work on the chicken β-TM gene demonstrated that the intron region 37 nucleotides downstream of the nonmuscle exon 6 was involved in the negative regulation of splicing of the skeletal muscle-specific exon 7 in nonmuscle cells (38,39). To examine if this *cis*-element is also present in rat β-TM gene, we have introduced into plasmid pSV40-p2 a series of deletion mutations ranging from 17 nucleotides to 197 nucleotides downstream of

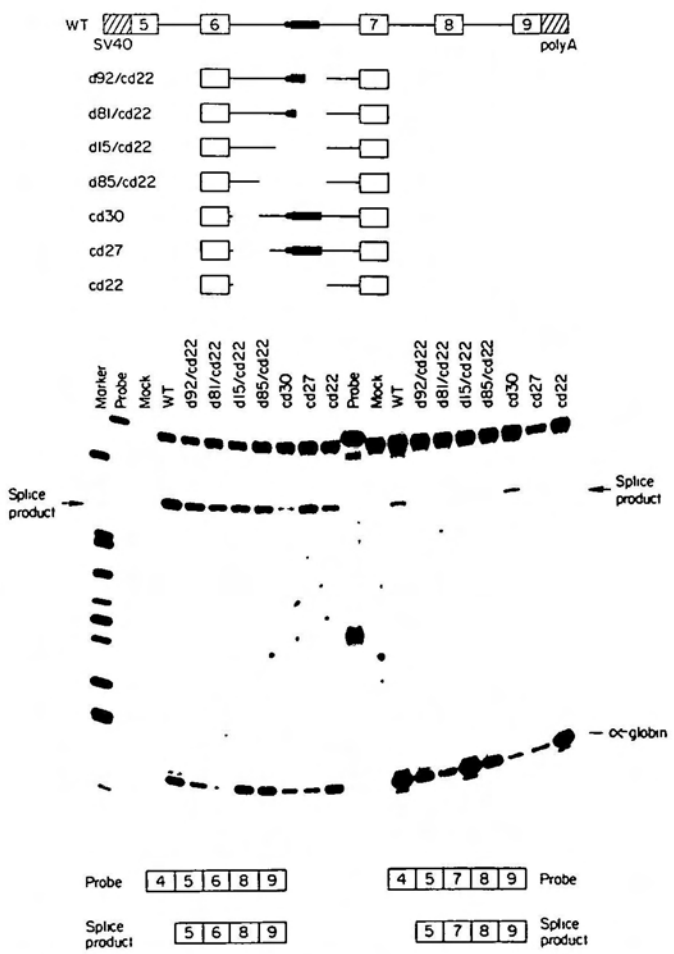

Figure 3. Deletions of sequences downstream of exon 6 does not affect alternative splice site selection in nonmuscle cells. The position of the deletions were shown previously (14). (*Top*) Schematic diagram of the tropomyosin minigene used in HeLa cell transfection. The wild type and deletion mutants were transfected into HeLa cells and the cytoplasmic RNA harvested 48 hours later. (*Middle*) Autographs of RNase pretection analysis using cDNA probes derived from cDNA clones encoding rat fibroblast TM-1 (*left*) and skeletal muscle β-tropomyosin (*right*). The band corresponding to the reference α-globin RNAs is labeled (α-globin). (*Bottom*) Schematic diagram of the structure of the RNA probe and protected fragments (splice products).

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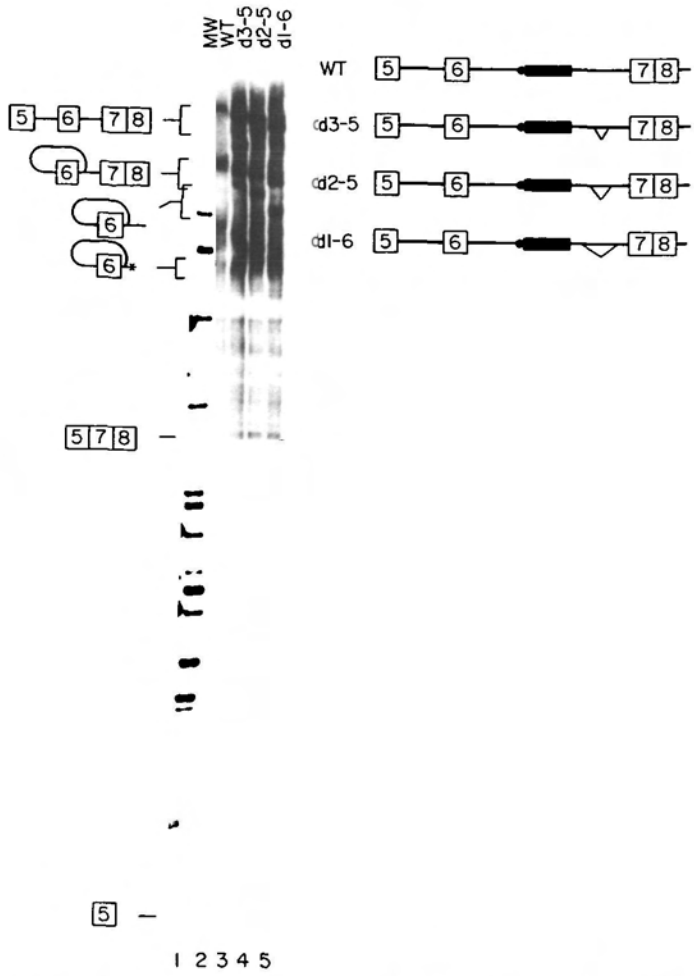

Figure 4. *In vitro* splicing of pre-mRNAs containing deletions of intron sequences upstream of exon 7. The diagram at the right represents wild type and mutant DNA templates derived from pSP64-p2(7/8) linearized with *Eco*RI. The positions of branch point sequences (black dot) and polypyrimidine tract (black box) in intron 6 are also indicated. In vitro splicing reactions were carried out for 2 hours, and the ^{32}P-labeled RNA products were seperated in a 4% denaturing polyacrylamide gel. Schematic representation of the products and intermediates are indicated to the left of autoradiographs and are (from top to bottom) pre-mRNA, lariat/exon intermediate, free lariat, free lariat but 3′ end is degraded up until the lariat branchpoints by 3′ exonucleolytic activity, splice product, and free exon 5. Marker lane are markers using pBR322 digested with *Msp*I.

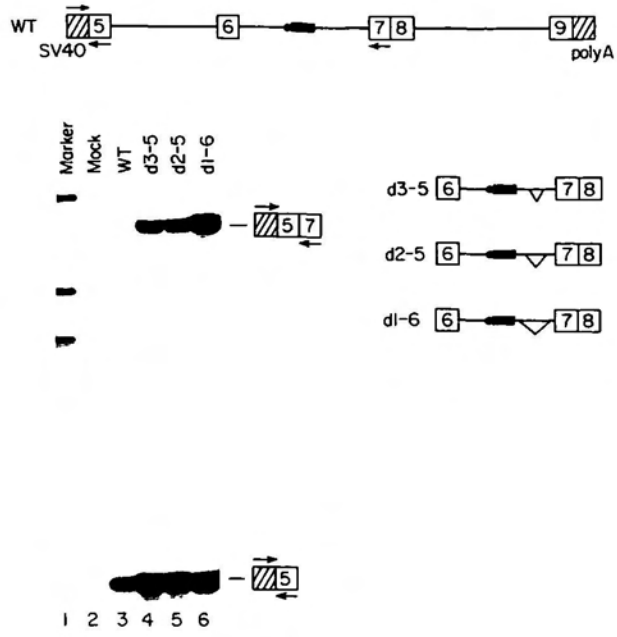

Figure 5. RNA analysis of HeLa cells transfected with tropomyosin minigenes. (*Top*) Schematic diagram of the tropomyosin minigenes used in the HeLa cell transfections. The positions of branchpoint sequences (black dot) and adjacent polypyrimidine tract (black box) are also indicated. The wild type and mutant minigenes (*right*) were transfected into HeLa cells and the cytoplasmic RNA harvested 48 hours later. The RNAs were analyzed by RT-PCR assay using primers complementary to exon 7 and exon 5 sequences, and a primer homologous to SV40 leader sequences that is 5′ end labeled with ^{32}P-γATP. Arrows (*Top*) represent primers used in the PCR assay. The top bands in the autoradiograph correspond to splice products exon 5 to exon 7. The bottom bands correspond to the reference.

exon 6 (Figure 3). HeLa cells were transfected with minigenes containing wild type or seven deletion mutants, and incubated for 48 hrs. Cytoplasmic RNA was then isolated and analyzed by RNase protection assay. The RNase protection analysis of these RNA samples and RNA from mock-infected cells were carried out with RNA probes derived from cDNA clones encoding rat fibroblast TM-1 and skeletal muscle β-TM (Figure 3). Transient expression of wild-type minigene resulted in spliced RNA that contained exons 5+6+8+9. A low level of the skeletal muscle-type splice product, exons 5+7+8+9, was detected in cells transfected with the wild-type construct, which is similar to the observations with the chick β-TM gene in myoblast cells (20). Mutants d92/cd22, d81/cd22, d15/cd22, d85/cd22 and cd22, which deleted part or all of the polyprymidine tract in intron 6, did not affect splicing of the upstream exon, as shown by the normal levels of splicing of exons 5+6+8+9. They abolished the use of exon 7, which is consistent with our *in vitro* and *in vivo* data that this polypyrimidine tract is important for proper splicing of exon 5 to exon 7 (14). None of the mutations that deleted intron sequences from 17 to 197 nucleotides downstream of exon 6 led to significant activation of exon 7 usage, as shown by the lower levels of the spliced RNA product containing exons 5+7+8+9, relative to the level derived from the wild-type construct. These mutants also do not seem to affect the use of exon 6 (fibroblast-type splice) in HeLa cells. These results indicate that in the rat β-TM gene the intron sequences between the 5′ splice site of exon 6 and the branch sites upstream of exon 7 are not involved in the negative regulation of exon 7 splicing in nonmuscle cells.

The blockage of exon 7 splicing in nonmuscle cells occurs at its 3-splice site

As described above, we previously demonstrated that the *cis*-elements responsible for the blockage of exon 7 splicing are located in exon 7 and the adjacent upstream intron. However, the mechanism of the blockage is not clear, although we have shown that the blockage is mediated by cellular factors (1). To determine whether the blockage of exon 7 splicing occurs at its 5′-splice site, 3′-splice site, or both, we have prepared wild-type as well as three intron mutants of plasmid P2(7/8) that have exon 7 and exon 8 ligated together, and used them for both *in vitro* and *in vivo* assays (Figures 4 and 5). *In vitro* splicing reactions were carried out for 120 min. Following the reaction, the RNAs were fractionated on a 4% polyacrylamide/urea gel (Figure 4). As shown in Figure 4, wild-type P2(7/8) produced a relatively

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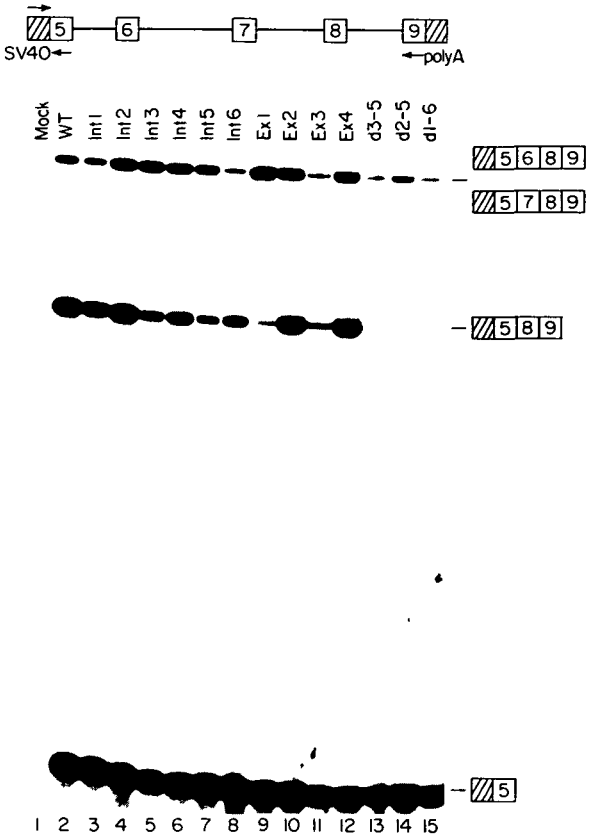

Figure 6. Mutants that have strong activation of exon 7 splicing also reduce the level of exon skipping *in vivo*. The position of base substitution mutants and deletion mutants were shown previously (1). (*Top*) Schematic diagram of the tropomyosin minigene used in HeLa cell transfection. The wild type and mutants were transfected into HeLa cells and the cytoplasmic RNA harvested 48 hours later. The RNAs were analyzed by RT-PCR assay using primers complementary to exon 9 and exon 5, and a primer homologous to SV40 leader sequences that is 5′ end labeled with ^{32}P-γATP. Arrows (*Top*) represent primers used in the PCR assay. The top bands in the autoradiograph correspond to splice products of exon 5 to 6 to 8 to 9 and/or exon 5 to 7 to 8 to 9. The middle bands correspond to skipping products of exon 5 to 8 to 9. The bottom band was produced by a primer antisense to exon 5 as a control to normalize initial starting amount of cellular RNAs being used.

low level of spliced RNA of exons 5+7+8. By contrast, the three mutants that have deleted the intron regulatory element produced significantly higher amount of spliced products of exons 5+7+8 (Figure 4). For the *in vivo* assay, we have transfected SV40P2(7/8) wild type and mutant plasmids into HeLa cells and cytoplasmic RNAs were isolated 48 hrs after transfection and analyzed by reverse transcription-PCR assay. In agreement with the *in vitro* data, the wild-type construct produced the splice product with exons 5+7 at a very low level, and the mutants exhibited significantly higher amounts of exon 5 to exon 7 splice product in the *in vivo* transient expression assay (Figure 5). Although our previous data showed that splicing of exon 5 to exon 7 required precursors in which exon 7 was first joined to exon 8 (12), yet compared with the mutants that have deleted the intron regulatory sequences the wild-type still spliced very inefficiently in this construct. In addition, our previous results have shown that precursors containing exon 7, intron 7 and exon 8 exhibit efficient splicing of exon 7 to exon 8 *in vitro* (31,14).

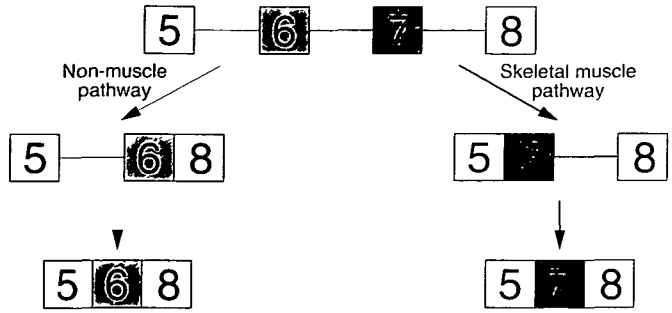

Figure 7. Model for alternative splicing of β-tropomyosin pre-mRNA in nonmuscle and skeletal muscle cells. For a discussion see text.

Collectively these results demonstrte that the blockage of exon 7 in nonmuscle cells is primarily at its 3′-splice site.

Mutants that have strong effect in activation of the exon 7 splicing also reduce exon skipping *in vivo*

By using reverse transcription-PCR method, we can detect proper as well as aberrant splice products generated in transient expression systems that could not be detected by the RNase protection assay. We have examined a series of constructs containing wild-type as well as mutants that have been previously using the RNase protection assay (1). As shown in Figure 6, using a 5′ primer containing the SV40 leader sequence and a 3′ primer complementary to exon 9 for PCR amplification, we detected a substantial amount of exon skipping product, i.e, splice product containing exons 5+8+9, when cells were transfected with the wild-type construct. However, mutants Ex1, d3-5, d2-5 and d1-6 that almost fully activate skeletal muscle exon 7 splicing in Hela cells (1), significantly reduced the amount of exon skipping.

DISCUSSION

The data presented in this paper demonstrates that the suppression of exon 7 splicing is regulated by sequences around exon 7 when put into a heterologous minigene (Figure 2). This is consistent to our previous results showing that critical elements for regulated alternative splicing of exon 7 are located at exon 7 and its upstream intron sequences (1). Although the mechanism of exon 7 blockage in nonmuscle cells is unclear, we proposed according to our previous studies, that cellular blocking factors are involved (1). Here we have shown that the blockage of exon 7 splicing is primarily at its 3′-splice site. Although it was previously shown that splicing of exon 5 to exon 7 *in vitro* and *in vivo* can occur in a nonmuscle cell system if exon 7 is first joined to exon 8 (12), our present results demonstrate that this splicing is relatively inefficient with the wild type sequences. However, mutations in the intron regulatory region upstream of exon 7 resulted in a increased splicing efficiency both *in vivo* and *in vitro* (Figures 4 and 5). These results indicate that the suppression of exon 7 splicing by blocking factors in nonmuscle cells is primarily at its 3′-splice site. Similar results have been reported for the human α-tropomyosin gene (40).

The mechanism by which the 3′-splice site of exon 7 is blocked in nonmuscle cells remains to determined. We have found that the polypyrimidine tract binding protein (PTB) can interact with the intron regulatory elements upstream of exon 7 (33). It is

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unclear at present how the binding of PTB to these sequences contributes to splice site regulation. In addition, we cannot exclude the possibility that other factors will bind to these sequences and thereby block the use of this 3'-splice site. Work is currently underway to study the interaction of factors that are known to act on 3'-splice sites, e.g., U2 snRNP, U2AF, and to determine if the interaction of such factors are regulated in nonmuscle cells.

Using reverse transcription PCR analysis we found that transient expression of the wild type tropomyosin minigene results in both authentic splice product, i.e., exons 5+6+8+9, as well as the exon skipping product, exons 5+8+9 (Figure 6). Why exon-skipping occurred in the cells is not clear but it could be due to several possible mechanisms. One is that there may be limiting factors that promote exon 6 splicing in HeLa cells. Thus when cells were transfected with the minigene, the amount of pre-mRNA transcribed from the minigene exceeded the amount of the factors required for proper splicing. The nature of these factors are currently not known, and could be general splicing factors like SR proteins or other unknown proteins. For example, recent studies have shown that SF2/ASF and hnRNP A1 can regulate exon inclusion or skipping *in vitro* (41). *In vitro* splicing of β-TM minigene that include sequences from exon 5 to 9, resulted in exon skipping, such that the product was 5+8+9. Upon addition of SF2/ASF, this inappropriate exon skipping event was markedly prevented, thereby allowing inclusion of the nonmuscle-type exon 6, which is the expected splicing pattern in HeLa cells (41). It is possible that exon skipping observed in the transient expression assay (Figure 6) is due to insufficient amount of SF2/ASF in the cell relative to the amount of RNA expressed from the minigene. In the future, it will be interesting to co-transfect SF2/ASF cDNA with pSV40-p2 to see if the exon skipping event will be prevented by increasing the amount of SF2/ASF in the cell. Another possible mechanism for the exon skipping event generated by transient expression of the minigene is chromosome positioning. It has been shown that snRNPs and splicing factor SC35 co-localized within the speckled nuclear domains that may correspond to the sites of spliceosome assembly and splicing itself (42,43). The position of the natural β-TM gene in the chromosome relative to the spatial localization of certain splicing factors might be important for proper splicing.

Interestingly, mutations that activate the use of the skeletal muscle exon 7 in nonmuscle cells suppressed exon-skipping (Figure 6). Transient expression of the exon 7 mutant Ex1, and intron mutants d3-5, d2-5 and d1-6 in HeLa cells resulted in almost 100% splice product of exon 5+7+8+9 (Figure 6), and very little or no skipping products were detected (Figure 6). These mutations derepressed exon 7 splicing presumably by interfering with the interaction of blocking factors. Therefore, in the absence of blocking factors there could be sufficient general splicing factors binding to this region and promoting exon 7 usage. The nature of these factors is unknown. They could be the same factors required for proper exon 6 splicing or different factors specifically required for exon 7 splicing. Another feature of exon 7 is that it has a long polypyrimidine tract upstream of its 3'-splice site (14). Studies of the rat α-TM gene have shown that a long polypyrimidine tract adjacent to the branch point, as well as a strong consensus branchpoint, determine a strong 3'-splice site, and presumably provide strong binding sites for *trans*-acting factors that act early in the spliceosome formation (8). It is possible that in the absence of blocking factors, the long polypyrimidine tract upstream of exon 7 enables more *trans*-acting factors to bind, resulting in efficient exon 7 splicing and little or no skipping, whereas exon 6 does not have a strong polypyrimidine tract, and less factors bind there and therefore more skipping products will be generated.

Our previous studies indicated that the critical step in the use of the nonmuscle exon 6 occurs during the joining of exon 6 to the downstream common exon 8 (12). Although we previously demonstrated that joining exon 7 to exon 8 resulted in splicing of exon 7 in nonmuscle cells both *in vitro* and *in vivo*, it is clear from the present studies that these precursors are spliced relatively inefficiently. The data presented in this paper are consistent with a model in which the critical step in the use of the skeletal muscle-specific exon 7 is activation of it's 3'-splice site (Figure 7). In the absence of the effects of blocking factors, exon 7 becomes the default exon of choice because of its stronger 3'-splice site. In addition, although this and previous studies from our laboratory indicate that the use of the skeletal muscle exon in nonmuscle cells is subject to negative regulation via blocking factors (1,33), it remains to be determined how regulation is achieved in skeletal muscle. Whether or not regulation in skeletal muscle is via repression of blocking factors or expression of a splice site activator remains to be determined. Our results do not rule out the possibility that positive-acting factors will be required in skeletal muscle cells to promote the use of exon 7. Work is currently underway to study the factors that block the use of exon 7 in nonmuscle cells and to develop a cell-free assay system from myogenic cells.

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