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In Vivo Splicing of the β Tropomyosin Pre-mRNA: A Role for Branch Point and Donor Site Competition

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The chicken β tropomyosin gene contains two sets of alternatively spliced, mutually exclusive exons whose utilization is developmentally regulated. Exons 6A and 6B are used in nonmuscle cells (or undifferentiated muscle cells) and skeletal muscle cells, respectively. A complex arrangement of *cis*-acting sequence elements is involved in alternative splicing regulation. We have performed an extensive mutational analysis on the sequence spanning the region from exon 6A to the constitutive exon 7. A large number of mutant minigenes have been tested in transfection assays of cultured myogenic cells, and the splicing products have been analyzed by cDNA polymerase chain reaction. We demonstrate that in undifferentiated myoblasts, exon 6B is skipped as a result of a negative control on its selection, while exon 6A is spliced as a default choice. We provide evidence that the focal point of such a regulation is localized in the intron upstream of exon 6B and probably involves the blockage of its associated branch point. In differentiated myotubes, in contrast, both exons are accessible to the splicing machinery. We show that the preferential choice of exon 6B in this splicing environment depends on the existence of a competition between the two exons for the flanking constitutive splice sites. We demonstrate that both the donors and the branch points of the two exons are involved in this competition.

Split genes in eukaryotes require a precise assembly of the coding information prior to expression. The step of the maturation pathway in which the noncoding interspersed regions (introns) are excised from the full gene RNA copy (primary transcript) in order to yield the final mature message is called splicing. The definitions of coding and noncoding segments of the primary transcript may sometimes vary and lead to the stochastic or context-dependent production of different or partially different mRNAs (alternative splicing). Most of our current understanding of splicing regulation rests upon the knowledge of the *cis* elements which are involved in the selection of splice junctions. The definition of splice site strength has been a starting point in establishing the role played by splice site competition in alternative splicing. The conformity of the donor site to the consensus sequence AG:GURAGU (12, 39, 47) is considered one of the major determinants of 5′ splice site strength (1, 2, 8, 27, 58). Recognition of the donor site is mediated mainly by base pairing with the 5′ end of the RNA moiety of the U1 small nuclear ribonucleoprotein (snRNP) (60) and by the interaction of protein factors, some of which have been recently identified (10, 23, 51). A less well defined sequence (branch point sequence [BPS]), a pyrimidine-rich region (11 to 15 nucleotides [nt]), and the highly conserved AG dinucleotide define the 3′ end of introns (12, 39, 47). The BPS is usually located 18 to 40 nt upstream from the AG acceptor. Its recognition is again mediated by two orders of splicing events: first, the binding of protein factors such as U2AF (45, 56), PBP/PTB (9, 14, 40), and IBP (13, 53) to the neighboring downstream sequence; second, the recognition of its sequence by base pairing with the RNA moiety of the U2 snRNP (55, 59). Finally, an early and yet undefined role in branch site recognition is certainly played by the U1 snRNP (3, 61). As a consequence, two *cis*-acting elements are the determinants of branch point strength: (i) the presence of the downstream pyrimidine-rich region (41, 50) (which is the main binding site for the protein factors U2AF, IBP, and PBP) and of the conserved AG dinucleotide (1) and (ii) the match of the BPS to the loosely defined consensus (YNYURAY) (42, 57). It has been shown that the best BPS (or one of the best BPSs), even in higher eukaryotes, is the yeast highly conserved UACUAAC box (57). The role of splice site competition in alternative splicing has been clearly assessed for both donor (7, 26, 52, 58) and branch (37, 38) sites. Other *cis*-acting factors have been shown to be involved in the regulation of differential splicing; these factors include exon sequences (7, 17, 35), the abnormal location of the BPS with respect to the donor (49) and the acceptor (15) site, and the presence of long pyrimidine stretches (15, 19, 29) and secondary structures, which have been shown to play a role in both in vitro (6) and in vivo (33) splicing. Most of our knowledge of the *trans*-acting factors regulating alternative splicing rests on the study of *Drosophila* systems, such as sex determination (20, 21, 22), P element transposition (48), and the suppressor-of-white-apricot gene (4). However, recent studies have reported the characterization in mammalian systems of one factor, called ASF or SF2 (10, 11, 23, 24), which is part of the constitutive splicing apparatus and which can promote, in a concentration-dependent manner, the use of the proximal 5′ splice site in competition assays.

The chicken β tropomyosin gene has provided us with a model system for studies of alternative splicing. Exons 6A and 6B of the gene are mutually exclusively spliced in order to yield two classes of mRNAs. Exon 6A is shared by RNAs expressed in smooth muscle and nonmuscle tissues (the two species differ in their transcription start sites), while exon 6B is present only in the skeletal muscle-specific transcript. When muscle cells differentiate in culture, myoblasts show preeminent use of exon 6A whereas myotubes use preferentially exon 6B, but in no cases are the two exons spliced together (30, 32). We have previously shown that a minigene containing the genomic fragment spanning the region from exons 5 to 7 of the gene contains all of the necessary and sufficient *cis* information to faithfully reproduce splicing

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regulation (31). Mutagenesis of regions around the splice acceptor site of exon 6B allowed us to identify negatively acting *cis* elements which prevent splicing of the skeletal muscle-specific exon in myoblasts. The 5′ half of the exon and the upstream pyrimidine-rich region located between the branch point (position -105) and the AG acceptor contain these negative elements (15, 29). We have recently shown (33) that at least part of this negative control depends on the existence of a stem-and-loop structure which involves exon sequences. Mutation of either arm of the stem induces constitutive splicing of exon 6B in myoblasts, while a reconstituted stem formed by different (or partially different) base-pairing sequences reverts to a wild-type behavior, both in myoblasts and in myotubes. This secondary structure is necessary but not sufficient for splicing regulation in myoblasts, since modification of distinct sequence elements (29) can lead to constitutive use of this normally skipped exon in myoblasts. As a consequence, this stem (which we refer to as stem I) could be part of either a more complex secondary structure (6, 30) or a complex regulation system involving structural as well as nonstructural elements. In this report, we analyze the role played by other potential elements, such as donor and branch sites, in this regulation. We demonstrate that splice sites flanking the alternatively spliced exons are suboptimal and that competition involving both donor and branch sites is the main determinant of exon choice in myotubes but not in myoblasts. Finally, we provide evidence to show that most of the regulation of exon 6B splicing is focused around the branch point/acceptor region upstream of this exon.

MATERIALS AND METHODS

Plasmid constructions and site-directed mutagenesis. The wild-type minigene (pSVβ-alt) contains exons 5 to 7 of the chicken β tropomyosin gene inserted between the simian virus 40 (SV40) early promoter and transcription termination site (31). Mutagenesis was performed essentially by the Kunkel method (25) on the single-stranded form of pSVβ-alt, with minor modification. *Taq* polymerase (purchased from Beckman or Cetus) was used for the extension-ligation step of the reaction. Extensive sequencing was performed to confirm that unwanted changes were not introduced.

Cell transfections. Quail ALD satellite cells transformed with a temperature-sensitive mutant of Rous sarcoma virus were obtained as previously described (36). Ten micrograms of each minigene was used to transfect 10^6 cells by the calcium phosphate precipitation method. For differentiation, cells were incubated at the nonpermissive temperature (42°C) for at least 72 h. This was a critical parameter: no switch from myoblast-specific to myotube-specific splicing could be consistently observed for myotubes maintained in culture for less than 72 h despite the morphological and biochemical (with respect to tropomyosins) differentiation observed. This observation could be due to a reduced turnover rate of minigene transcripts compared with the endogenous tropomyosin mRNAs and to the persistence of myoblast transcripts in differentiated myotubes. Transiently expressed transcripts were harvested 24 to 48 h (myoblasts) or 72 h (myotubes) after incubation at the nonpermissive temperature. To overcome variability due to differentiation leakage, each mutant minigene was transfected at least twice, and the splicing pattern of a wild-type minigene transfected in parallel was analyzed for each set of experiments. In a number of cases, the splicing pattern of the endogenous transcript was also analyzed. When the splicing patterns of different minigenes were directly compared (for instance, Mut16 and Mut16/6A*cons*), care was taken to transfect the same cells in parallel with all of the DNAs.

cDNA PCR analysis of transcripts. cDNAs of the harvested transcripts were obtained essentially as described previously (31). Oligonucleotide SV, complementary to SV40 sequences located in the 3′ end of each minigene, was used as primer for all first-strand synthesis. Ten to 30 μg of total RNA was reverse transcribed in each 20-μl reaction, and 2 to 5 μl was subsequently used for the amplification reaction. Amplifications were performed between antisense oligonucleotide SV or 6B*ant* (complementary to exon 6B sequences) and sense oligonucleotides 6A, 6B, and 5, complementary to sequences in exons 6A, 6B, and 5, respectively. All reactions were performed in *Taq* polymerase buffer (according to Cetus specifications) containing 30 to 50 pmol of each oligonucleotide, 100 μM deoxynucleoside triphosphate, and 2.5 U of *Taq* polymerase (purchased from Cetus or Beckman) in a final volume of 50 μl. Amplifications were performed on a Crocodile Appligene polymerase chain reaction (PCR) cycler. The basic cycle (20 s at 94°C; 30 s at 55°C; 1 min at 72°C), after the first denaturation step of 3 min at 94°C, was repeated 15 to 20 times. We have verified that under our experimental conditions, amplification is exponential until at least 25 cycles. Each amplification experiment was repeated at least twice. Some of the transcripts were also analyzed by controlled primer extension (29), with essentially the same results. Amplification products were separated on nondenaturing 6% polyacrylamide gels.

The PCR products were quantitated by subsequent Southern blot hybridization (oligonucleotides SV and 5 were used as probes) or, alternatively, by performing amplifications directly with radioactive oligonucleotides (oligonucleotides SV and 5), which were added to the reaction mixture at 1/200 of the amount of the cold oligonucleotide (0.15 to 0.25 pmol, total amount). Dried gels (or hybridized blots) were scanned on a Molecular Dynamics β scanner apparatus. Some variability was observed when the amounts of amplification products of different lengths were compared in different experiments (for instance, compare bands at 163 and 239 bp for Mut16 in Fig. 4B and 5B). This variability was due mainly to the different batches of reverse transcriptase, and particularly *Taq* polymerases, used in the different experiments, since no variability was observed in repeated amplifications of the same RNA with the same enzymes. It has been proposed (62) that a number of artifacts, such as the formation during the last annealing step of heteroduplexes which migrate anomalously in nondenaturing gels, can be obtained by using PCR to analyze cDNA molecules which share common regions like those described in this report. However, such artifacts are more likely to arise when amplification reactions reach a nonexponential phase, since, as the enzyme is limiting (or the DNA too concentrated), the single strands may reanneal (eventually forming heteroduplexes) before they are copied by the *Taq* polymerase. When the enzyme is in excess (and the reaction is exponential, as we have verified for all of our experiments), the final step is not a denaturation-renaturation step but a synthesis step for nearly all of the molecules. Nevertheless, we verified that this is indeed the case by running the products of a number of amplification reactions on denaturing and nondenaturing polyacrylamide gels (data not shown). Finally, a different technique (controlled primer extension [31]) was used to analyze splicing products from a number of minigenes described in this report, while the cDNA PCR technique was used to analyze previously described (29, 33) mutants (this

report and data not shown). Again, essentially the same results were obtained.

RESULTS

A competition between exons 6A and 6B is responsible for preferential use of exon 6B in myotubes but not of exon 6A in myoblasts. In earlier reports, we and others (6, 15, 29, 33) suggested that skipping of exon 6B in myoblasts is a result of a tissue-specific inhibition of splicing which involves sequence elements located around the acceptor site of the exon. Modifications of these *cis* elements lead to the constitutive use of the skeletal muscle-specific exon (6B) in undifferentiated cells (myoblasts). Exclusive use of exon 6A is no longer a major choice in splicing of primary transcripts derived from these mutant minigenes (29), and thus mRNA containing exons 5, 6A, and 7 is virtually absent. The occurrence of such a down-regulation on exon 6A recognition as a result of sequence modifications clustered around the acceptor site of exon 6B (located some 300 bp downstream) prompted us to consider the possible existence of a competition between the two exons. Two mutant minigenes were constructed to test this hypothesis: MutΔ6A and MutK6B. MutΔ6A harbors a precise deletion of exon 6A and of the upstream intron (which has previously been shown [31] to play no role in exon 6B splicing inhibition), while in MutK6B, both splice sites of exon 6B have been destroyed. In the latter case, the exon was not deleted to account for the possibility that sequences around exon 6B participate in the regulation of exon 6A splicing through the formation of alternative secondary structures as we have previously proposed (30). The two minigenes were transfected into cultured myogenic cells, and the transcripts isolated before (myoblasts) and after differentiation (myotubes) were analyzed by cDNA PCR. Amplifications were performed between amplimers directed against exon 5 (sense) and exon 7/SV40 (antisense) sequences (3′ end region of each minigene; see Fig. 1 and Materials and Methods for details). The size and exon composition of each expected amplification product are indicated in Fig. 2. Analysis of the splicing products derived from these two minigenes is shown in Fig. 3 (lanes MutK6B and MutΔ6A Mb [myoblasts] and Mt [myotubes]) together with the products derived from the wild-type minigene (lanes wild type Mb and Mt) and summarized in Table 1.

Deletion of exon 6A did not induce the use of the skeletal muscle exon in myoblasts, since the main mRNA derived from MutΔ6A contains mainly exon 5 spliced directly to exon 7 (major amplification band at 187 bp in Fig. 3). This result confirms that the negative control existing on exon 6B splicing is independent of the presence of exon 6A and upstream sequences and demonstrates that preferential splicing of exon 6A in myoblasts is not the result of a competition between the two exons. As expected, in myotubes, this negative control is efficiently overcome and the major mRNA produced now contains exon 6B (major amplification band at 263 bp).

Upon differentiation, there is an overall reduction (by a factor varying from 2 to 5) in the amount of total transcripts which is consistently observed in all our experiments. This finding may be relevant to the existence of a small down-regulation of the SV40 promoter or to a reduction in the total plasmid copy number in myotubes (which were cultivated for 24 to 48 h longer than were myoblasts; see Materials and Methods). In any case, we have verified that splicing regulation is not affected by a change in the amount of primary transcript of at least 100-fold (data not shown). This reduction in the total amount of mature transcripts is, within the limits of experimental error, the same for all of the transfected minigenes.

Exon 6A is mainly skipped in myotubes in the presence of an unmodified exon 6B. However, when the splice sites of exon 6B were inactivated in MutK6B, exon 6A was efficiently recognized in this splicing environment. In fact, in myoblasts and in myotubes, the same abundance ratio of the transcripts containing exons 5, 6A, and 7 to the minor species containing exons 5 and 7 is observed (Fig. 3; compare amplification bands of 263 and 187 bp in lanes MutK6B Mb and Mt).

These results show that the preeminent skipping of exon 6A which is observed in myotubes (and in 6B-activating mutant minigenes in myoblasts) depends on the presence of an active exon 6B and suggest that competition between the two exons is the most probable mechanism underlying the preferential use of exon 6B in this splicing environment.

Control experiments were performed to ensure that exon 6A and exon 6B sequences were absent from transcripts derived from minigenes MutΔ6A and MutK6B, respectively (data not shown).

The following experiments were performed to assess the role played by splice sites in this competition.

Exons 6B and 6A have suboptimal donor sites. Alternative splicing is often related to the existence of nonconsensus donor sites (7, 26, 52, 58) which, in turn, has been shown to be somehow related to the weakness of the sites (27). The donor sites of exons 6A and 6B match poorly the consensus AG:GUAAGU. The donor site of exon 6B (AG:GUAUGA; nonconsensus nucleotides are underlined) was converted to the consensus sequence in the mutant minigene Mut6B*cons*. Similarly, the donor site of exon 6A (AG:GUACTG), containing three deviations from the consensus, was also converted to the sequence AG:GUAAGU in the simple mutant minigene Mut6A*cons* and in the double mutant Mut16/6A*cons* (see below for description). Analyses of the splicing products are shown in Fig. 4A (amplifications between exons 6A and 7/SV40 [lanes 6A] or exons 6B and 7/SV40 [lanes 6B]) and 3B (amplifications between exons 6B and 5) and summarized in Table 1. Exon composition and amplification patterns of the splicing products are shown in Fig. 2.

When the donor site of exon 6B is converted to the consensus in mutant Mut6B*cons*, the use of this exon is increased in myoblasts, although not at the level of a constitutive exon. In fact, the abundance ratios of species 5-6A-7 to species 5-6B-7 are about 1:2 in this mutant and 5:1 to 10:1 in the wild type (compare the bands at 154 and 125 bp in lanes 6A and 6B for wild type Mb and Mut6B*cons* in Fig. 4A). This result shows that the donor site of exon 6B is not optimal, even if its conversion to the consensus is not sufficient per se to induce full activation of the exon.

We have tested the effect of strengthening the donor site of exon 6A in a context in which this exon is a minor splicing choice. First, the single mutant Mut6A*cons* was tested in myotubes, which, if transfected with the wild-type minigene, produce as a main species the 5-6B-7 mRNA. As shown in Fig. 4A, an increase of the 5-6A-7 transcript is observed in myotubes transfected with Mut6A*cons* compared with the wild type, since the two bands at 154 and 125 bp have approximately equal intensities for the mutant (lanes 6A and 6B for Mut6A*cons* Mt), whereas they are present in a ratio of 1:2 to 1:3 for the wild type (lanes 6A and 6B for wild type Mt). Since the band at 125 bp may derive from the amplification of both 5-6B-7 and 5-6A-6B-7 mRNAs (Fig. 2), we

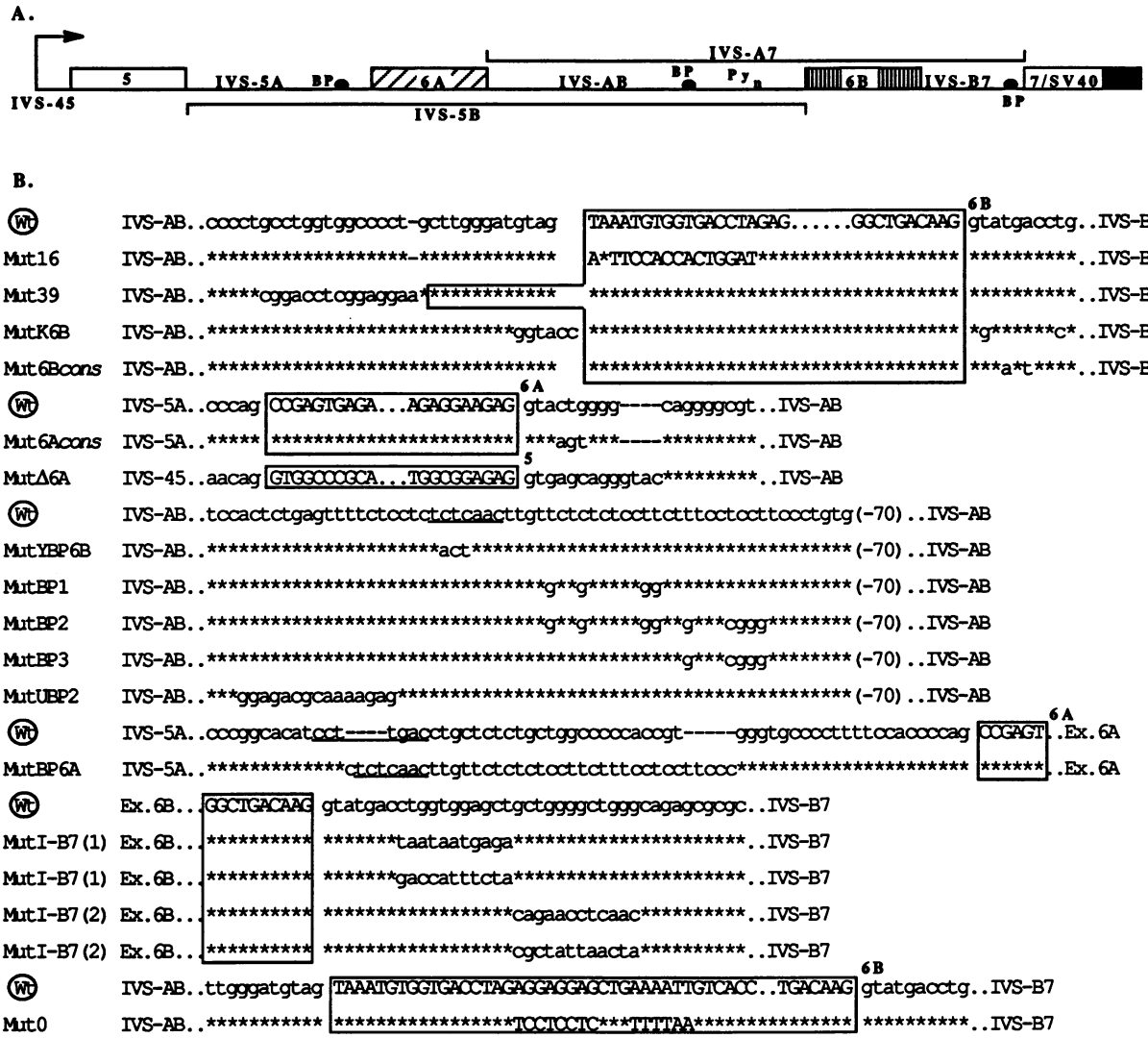

FIG. 1. (A) Schematic diagram of the minigene. All possible IVSs, according to the different splicing patterns, are indicated. BP and Py_n indicate the branch points in all introns and the long pyrimidine stretch in IVS-AB, respectively. (B) Sequences of all minigenes used for transfections. The wild-type sequence is shown for each group of mutants of the same region of the gene, and for all minigenes the sequence differences are highlighted (asterisks indicate nucleotide identity, and dashes indicate missing nucleotides). Exon sequences are boxed, and the name of the exon is indicated in the upper right corner of each box. Note that in Mut39 a longer exon 6B is present, since a 12-nt upstream AG acceptor is used instead of the wild-type one (29). The branch point sequences are underlined. For mutants which are entirely contained in IVS-AB (MutYBP6B, MutBP1, etc.) the distance from the acceptor site of exon 6B is indicated at the right of the sequence. For double mutants, the single sequence modifications are indicated separately; for instance, Mut16/BP1 contains the mutations indicated in both lines MutBP1 and Mut16.

performed amplifications between exons 6B and 5 to evaluate the relative contributions of the two species. The results (Fig. 4B, compare lanes wild type and Mut6A*cons* Mb and Mt) clearly indicate that exon 6B, whenever it is used, is systematically spliced to exon 6A in this mutant (lack of the band at 163 bp in Mut6A*cons*), both in myoblasts and in myotubes, to obtain the only mature species, 5-6A-6B-7. In other words, exon 6A is now a constitutive exon, whereas exon 6B can be skipped in about 50% of the splicing events.

We also tested the effects of converting the donor site of exon 6A to the consensus in a context in which exon 6B was activated by a mutation (Mut16) which modifies the secondary structure blocking the use of this exon in myoblasts (33). All transcripts derived from Mut16 contain exon 6B, either in the 5-6B-7 mRNA or in the 5-6A-6B-7 mRNA, which are present in an abundance ratio of approximately 2:3 (this ratio is the highest found in our experiments but was somewhat dependent on the batches of reverse transcriptase and *Taq* polymerase which were used; see Materials and Methods for details). The minigene containing both mutations was called Mut16/6A*cons*. As shown in Fig. 4B (lanes Mut16 and Mut16/6A*cons*), a consistent enhancement of the species containing all four exons (5-6A-6B-7) is observed for this mutant since, again, the band at 163 bp (amplification of species 5-6B-7) is lacking. Interestingly, in this double mutant, species 5-6A-7 was more abundant than in mutant Mut16 (data not shown).

Finally, similar results were obtained with a third mutant minigene in which the donor site of exon 6A was converted to the donor site of exon 5 (sequence AG:GUGAGC) (2a).

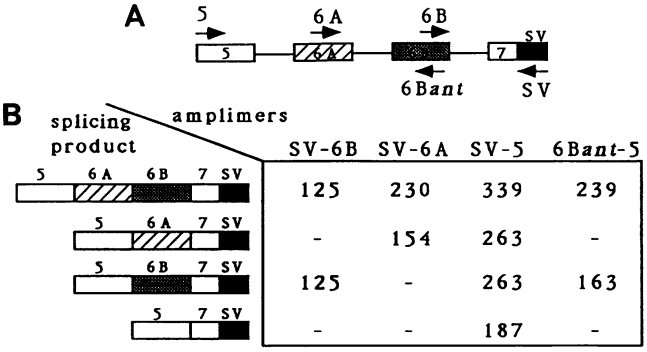

FIG. 2. Schematic representation of all possible splicing patterns and the expected cDNA PCR products. (A) The position of each oligonucleotide used is indicated on a schematic minigene. (B) Each row indicates the sizes (in base pairs) of the amplification products obtained from all possible mRNAs (indicated schematically on the left) with the amplimers indicated above the columns. For instance, transcript 5-6B-7 (third row) will yield three products of 125, 263, and 163 bp after cDNA PCR between amplimers SV-6B, SV-5, and 6B*ant*-5, respectively. Since in the minigenes containing mutation Mut39, a longer exon 6B is present (a 12-nt upstream acceptor is used), the amplification products containing exon 6B deriving from these minigenes are 12 bp longer; for instance, amplifications of transcript 5-6B-7 with amplimers SV and 5 will yield a product of 275 bp instead of 263 bp (see Fig. 5).

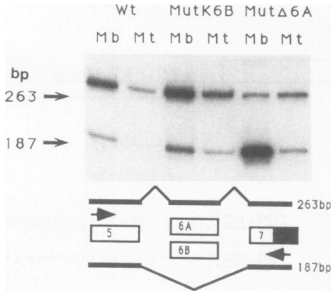

FIG. 3. cDNA PCR analysis of transcripts derived from transfected cells. The minigene used and the cell type (myoblasts [Mb] or myotubes [Mt]) are indicated for each lane. Schematic diagrams of the amplifications performed (the oligonucleotides used are indicated by arrows) are shown. Amplification was performed between amplimers SV and 5. Two main products indicating either the skipping of both exons 6 (187 bp) or the inclusion of one of them (6A or 6B; 263 bp) are obtained. Wt, wild type.

Exons 6B and 6A have competing branch sites. The two major known determinants of branch site strength are the sequence (42, 57) and the pyrimidine content (41, 50) of the region immediately downstream. In the chicken β tropomyosin gene, the branch point upstream of exon 6B has an abnormal position (−105 with respect to the AG acceptor) (15) and is followed by a highly pyrimidine rich region extending virtually to the end of the intron (30). We have previously shown (15, 29) that at least the 3′ part of the pyrimidine stretch exerts a negative control on exon 6B

TABLE 1. Amounts of splicing products in various minigenes

Minigene[a]	Amt of splicing product[b]			
	5-6A-7[c]	5-6B-7	5-6A-6B-7	5-7
Wild type				
Mb	++++++	+	++	+
Mt	++	+++++	+	+/−
MutK6B				
Mb	++++++	−	−	++
Mt	++++++	−	−	++
MutΔ6A				
Mb	−	+	−	++++++++
Mt	−	+++++	−	++
Mut6A*cons*				
Mb	++++++++++	ND	+	ND
Mt	++++	ND	++++	ND
Mut16/6A*cons*	++	ND	++++++++	ND
Mut6B*cons*	+++	+++++	+	?
MutYBP6B	+++	+++	+	?
Mut16	+/−	++++	++++++	ND
Mut16/BP1	++++	ND	+++	++
Mut16/BP2	+/−	++++++++	+/−	++
Mut16/BP6A	+/−	ND	++++++++	+
Mut39	+	+++++++	+	ND
Mut39/BP1	++++++++	ND	+/−	+
Mut39/BP2	+++	++++	+	+
Mut39/BP6A	++++	++	+++	+
MutUBP2 (Mb, Mt)	+	++++	+	?
MutBP3	++++	++++	+	?
Mut0 (Mb)	++++++	+	++	+
MutI-B7(1)	+++++	+	+	?
MutI-B7(2)	+++++	+	+	?

[a] Mb, myoblasts; Mt, myotubes.
[b] Abundance relative to the abundances of other splicing products derived from the same minigene. ND, not detected; ?, data not available.
[c] Exon composition.

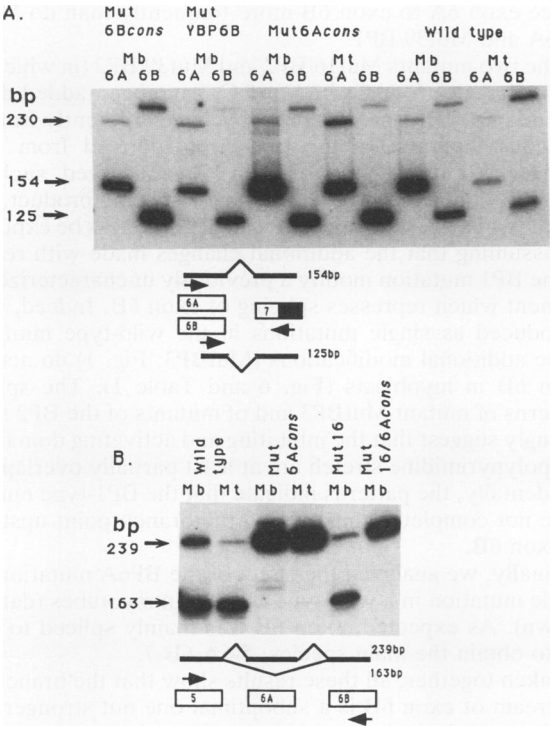

FIG. 4. cDNA PCR analysis of transcripts derived from transfected cells. (A) Amplifications were performed between amplimers SV and 6A (lanes 6A) or SV and 6B (lanes 6B). In lanes 6A, the bands at 154 and 230 bp derive from amplification of the mRNA containing exons 5-6A-7 and 5-6A-6B-7, respectively, while the bands 125 bp in lanes 6B derive from amplification of either the species containing both exons 6A and 6B (5-6A-6B-7 transcript) or the species containing exons 5, 6B, and 7 (see Fig. 2). The relative amounts of the two species can be obtained by analysis of the data shown in panel B. In the case of minigene Mut6A*cons* expressed in myoblasts, the ratio of bands at 154 and 125 bp is considered to be underestimated in this experiment, since because of the large amount of the 5-6A-7 transcript, the amplification reaction between primers SV and 6A is nearer to a linear phase (enzyme becoming limiting or enhanced reannealing of the two strands before the amplification step) than is amplification between primers SV and 6B at the same cycle number. We verified in repeated experiments with various numbers of cycles that this ratio is higher than in the wild type (data not shown). For the same reason (reaction near to a linear phase), the amount of the product of 230 bp in the same lane (5-6A-6B-7 transcript) is considered to be underestimated, since when the enzyme becomes limiting, amplification of the more abundant species 5-6A-7 outcompetes amplification of the minor species 5-6A-6B-7 (our unpublished observation). (B) Amplifications performed between oligonucleotides 6B*ant* and 5. Bands at 163 and 239 bp indicate the absence and presence, respectively, of exon 6A in spliced products containing exon 6B. Mb, myoblasts; Mt, myotubes.

splicing in myoblasts. The whole pyrimidine-rich region could play a double role in regulation: in myoblasts it would assist in masking exon 6B to the splicing apparatus, while in myotubes it would mainly specify a branch site stronger (and, as a consequence, more competitive) than the one upstream of exon 6A.

To assess the role played by the branch points of the two exons in regulation, we changed their relative strengths by modifying their sequences or the pyrimidine content of the downstream region. The BPS upstream of exon 6B (UCU CAAC) was changed to the preferred UACUAAC box (57) in mutant MutYBP6B, which should make it stronger. As expected, this modification has a moderate activating effect on splicing of the exon (Fig. 4A; compare lanes wild type Mb and MutYBP6B), since the two mRNAs containing exons 5, 6A, and 7 (amplification band at 154 bp) and 5, 6B, and 7 (amplification band at 125 bp) are present in roughly equimolar amounts. This finding indicates that the BPS upstream of exon 6B is a suboptimal one, but again, its conversion to a better BPS is not able to induce full activation of the exon.

If competition between the two branch points plays a role in regulation, the expected consequence of lessening the strength of the branch point of exon 6B would be an enhancement of exon 6A use in a context in which this exon is normally a minor choice. A similar result is expected if the strength of the branch point of exon 6A is improved. We have constructed two kind of mutants. First, four (BP1-type mutants) or eight (BP2-type mutants) Gs were introduced in the pyrimidine-rich region downstream of the branch point of exon 6B (Fig. 1B). These mutants are expected to lessen the strength of the branch point by altering the pyrimidine content of the associated sequence. Second, the branch site upstream of exon 6B together with the following 30 nt was duplicated upstream of exon 6A to obtain the BP6A-type mutants (Fig. 1B). We expected the primary transcripts derived from these minigenes to have two equivalent branch points upstream of exons 6A and 6B. To obtain a context in which exon 6A is not already a major choice, we coupled these mutations to two previously described (29) exon 6B-activating mutations, Mut16 (described above) and Mut39 (described below), to obtain the double mutants Mut16/BP1, Mut16/BP2, Mut39/BP1, and Mut39/BP2.

Mut39 is an exon 6B-activating mutation located in IVS (intervening sequence)-AB (Fig. 1) which, unlike Mut16, splices exon 6A poorly, either alone (species 5-6A-7) or together with exon 6B (species 5-6A-6B-7). The major transcript made from this minigene (species 5-6B-7) contains a modified exon 6B, since a 12-nt upstream AAG acceptor is used instead of the wild-type one. These minigenes were tested in the usual transfection assay.

The amplifications between exons 6A and 7/SV40, exons 5 and 6B, and exons 5 and 7/SV40 are shown in Fig. 5 and schematically summarized in Table 1. Sizes and exon compositions of the amplified products are shown in Fig. 2. Since the three amplification reactions have different efficiencies, the data cannot be directly compared, but the amplification band derived from species 5-6A-6B-7 (which is present among the products of each reaction) can be taken as a common reference to evaluate the relative abundances of the three mRNA species (5-6A-6B-7, 5-6A-7, and 5-6B-7) (see the legend to Fig. 5).

From inspection of the data of Fig. 5, the following conclusions can be drawn. When BP1- and BP6A-type mutants are analyzed, a general reduction in the abundance of the species 5-6B-7 is observed. The major splicing products are the two species 5-6A-7 and 5-6A-6B-7, and the distribution of the two mRNAs varies among the different mutants. Mut16/BP1 produces approximately equal amounts of the two species, while in Mut39/BP1, the major transcript is by far 5-6A-7. In Mut16/BP6A, the major transcript contains species 5-6A-6B-7, while this transcript is less abundant in Mut39/BP6A than is the 5-6A-7 mRNA (approximate ratio between the two of 3:4). In other words, in all of these mutants, the use of exon 6A has been increased. BP6A-type mutants easily splice out IVS-AB compared with BP1-type mutants. Similarly, Mut16/BP6A and Mut16/BP1

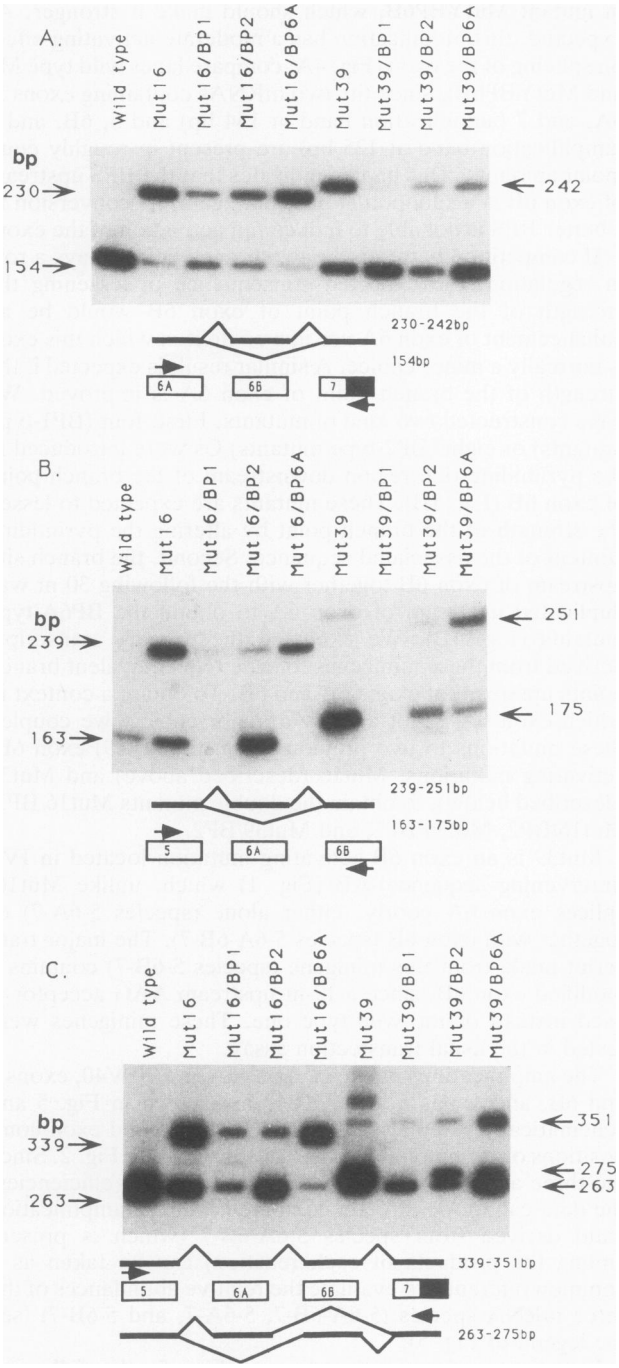

FIG. 5. (A) Amplifications performed between oligonucleotides SV and 6A. All transcripts were derived from myoblasts. Bands at 230 and 154 bp are as in Fig. 4A. Since in mutants of the Mut39 series a 12-nt-longer exon 6B is present, amplification bands derived from mRNAs containing exons 5, 6A, 6B, and 7 migrate slightly slower (242 instead of 230 bp). (B) Amplifications performed with amplimers 6B*ant* (antisense) and 5. Bands at 163 and 175 bp (for minigenes of the Mut16 and Mut39 series, respectively; see above) indicate the presence of mRNAs containing exons 5, 6B, and 7, while bands at 239 and 251 bp (for minigenes of the Mut16 and Mut39 series, respectively) result from amplification of species 5-6A-6B-7. (C) Amplifications between oligonucleotides SV and 5. In mutants of the Mut16 series, bands at 263 bp derive from amplification of mRNA species containing one of the two mutually exclusive exons, while the band at 339 bp indicates splicing of both exons. In mutants of the Mut39 series, bands at 263, 275, and 351 bp indicate the use of only exon 6A (species 5-6A-7), only exon 6B (species 5-6B-7), and both exons (5-6A-6B-7), respectively. The higher-molecular-weight band in Mut39 is of unknown origin (29). These data derive from Southern blot hybridization of amplification products. Oligonucleotides SV (A and C) and 5 (B) were used as probes. The data from different amplifications cannot be directly compared, since the three reactions have different efficiencies (data not shown), but the amount of species 5-6A-6B-7 (which is present in each reaction) can be taken as a common reference. For instance, for Mut16/BP2 in panel A, it can be inferred that species 5-6A-6B-7 is roughly twice as abundant as species 5-6A-7, while in panel B it can be observed that species 5-6B-7 is present in about 30-fold molar excess compared with species 5-6A-6B-7. Thus, the ratio between the molar amounts of species 5-6A-7 and 5-6B-7 is about 60.

splice exon 6A to exon 6B more frequently than do Mut39/BP6A and Mut39/BP1.

The two mutants Mut16/BP2 and Mut39/BP2 (in which five extra changes, four Gs and one C, have been added downstream of the BP1 modifications) behave differently from the previous mutants. In the transcripts derived from these minigenes, a reactivation of exon 6B is observed, such that the 5-6B-7 mRNA is by far the major splicing product. This result (which was unexpected) can nevertheless be explained by assuming that the additional changes made with respect to the BP1 mutation modify a previously uncharacterized *cis* element which represses splicing of exon 6B. Indeed, when introduced as single mutations in the wild-type minigene, these additional modifications (MutBP3; Fig. 1) do activate exon 6B in myoblasts (Fig. 6 and Table 1). The splicing patterns of mutant MutBP3 and of mutants of the BP2 series strongly suggest that the inhibiting and activating domains of the polypyrimidine stretch are at least partially overlapping. Incidentally, the patterns indicate that the BP1-type mutants have not completely inactivated the branch point upstream of exon 6B.

Finally, we analyzed the effect of the BP6A mutation as a single mutation in a wild-type context in myotubes (data not shown). As expected, exon 6B was mainly spliced to exon 6A to obtain the main species, 5-6A-6B-7.

Taken together, all these results show that the branch site upstream of exon 6B is a suboptimal one but stronger than the branch site upstream of exon 6A and that competition between the two sites is at least one of the elements responsible for the preferential use of exon 6B in myotubes.

Mutations activating exon 6B splicing in myoblasts are clustered in IVS-AB and in the 5′ half of the exon. We have previously shown (33) that a stem-and-loop structure (stem I) formed by 6B exon sequences is one of the elements implicated in splicing regulation. Nevertheless, the presence of this stem is necessary but not sufficient for inhibition of splicing of the skeletal muscle-specific exon, since other mutations located in the upstream intron are able to induce the full activation of the exon in the presence of an intact stem I (29). Stem I must be part of a more complex regulatory system which is responsible for exon skipping in myoblasts. We have tested the hypothesis that stem I could be part of a more complex secondary structure, as we (29, 30) and others (5, 6) have previously proposed. Figure 6A shows the secondary structure around exon 6B as proposed by Clouet-d'Orval et al. (5) on the basis of chemical and mutational analysis. This structure has been shown to be involved in splicing inhibition of the intron downstream of exon 6B (IVS-B7) in vitro, and some of the exon 6B-activating mutations that we have previously described (29)

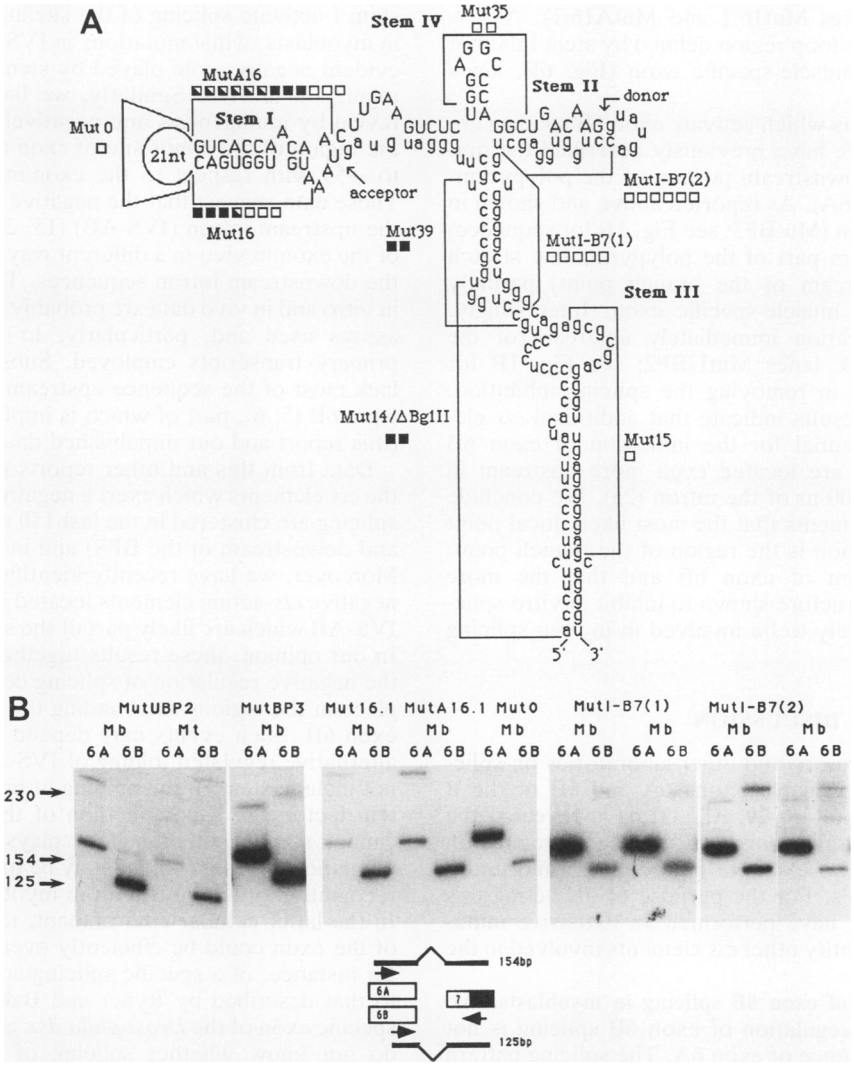

FIG. 6. (A) Folding of the primary transcript around exon 6B as described by Clouet-D'Orval et al. (5, 6). Exon and intron sequences are in uppercase and lowercase letters, respectively. The donor and acceptor sites of exon 6B are indicated. Stems I, II, III, and IV are indicated as described previously (5). The positions of mutations described in the text or in previous reports (29, 33) are indicated by brackets. For most of the mutants, different sequences have been introduced at the same location (see text). Each box represents a single mutant for which every nucleotide of the region defined by the brackets has been changed [for instance, seven different sequences have been tested for Mut16 (15 nt) and five have been tested for MutI-B7(1) (11 nt), two of which are shown in Fig. 1B]. With respect to the use of exon 6B in myoblasts, ■ indicates a strongly activating mutation (the ratio of the molar amounts of the 5-6A-7 transcript to the 5-6B-7 and 5-6A-6B-7 transcripts has to be less than 1/3), ◪ indicates a weakly activating mutation (above-mentioned ratio of around 1), and □ indicates a neutral mutation (compared with the parallel-transfected wild-type minigene). (B) cDNA PCR analysis of transcripts derived from transfected cells. The minigene names, the cell differentiation stage (myoblasts [Mb] or myotubes [Mt]), and the amplification performed (amplimers SV and 6A [lanes 6A] or SV and 6B [lanes 6B]) are indicated. Exon compositions of the amplified splicing products are as in Fig. 4A. In the case of MutI-B7(1) and MutI-B7(2), analysis of two clones of different sequence is shown.

located in the upstream intron (IVS-AB) are expected to disrupt some of the stems. We have modified the sequences of the downstream intron involved in base pairing in stems II and III (Fig. 6A). To ensure that the observed splicing patterns would not depend on the sequence introduced in a particular assay, mutagenesis was performed with a mixture of oligonucleotides, and a number of mutant minigenes bearing different sequences in the same region were isolated and tested in the usual transfection assay. Six and five mutants with different sequences were tested for stems II and III, respectively [MutI-B7(1) and MutI-B7(2); Fig. 6A]. A similar experiment has been performed on the region of stem I (7 and 12 mutants for the two sides of the stem). As shown schematically in Fig. 6A and by the data in Fig. 6B [lanes 6A and 6B for MutI-B7(1) and MutI-B7(2); see also Fig. 1B and 2 for sequences and exon compositions of the amplification products], none of the mutations introduced in the downstream intron had any activating effect on splicing of exon 6B. This is also true for previously described mutations located in the regions surrounding the donor site of exon 6B such as Mut35 (exon 6B) and Mut15 (intron IVS-B7) (29) (Fig. 6A). On the other hand, most of the sequences introduced in the regions of Mut16 and MutA16 (stem I) activated (to different degrees) splicing of the exon

(Fig. 6A; Fig. 6B, lanes Mut16.1 and MutA16.1). As expected, mutation of the loop region defined by stem I did not activate the skeletal muscle-specific exon (Fig. 6B, lanes Mut0).

Most of the mutations which activate exon 6B splicing are located in IVS-AB. We have previously described the mutations of the more downstream portion of the polypyrimidine stretch (29) (Fig. 6A). As reported above and shown in Fig. 6B, a 5-nt mutation (MutBP3; see Fig. 1B for sequence) located in the upstream part of the polypyrimidine stretch (about 20 nt downstream of the branch point) partially activates the skeletal muscle-specific exon. Interestingly, even a 15-nt modification immediately upstream of the branch point (Fig. 6B, lanes MutUBP2; see Fig. 1B for sequence) is effective in removing the splicing inhibition. Finally, preliminary results indicate that additional *cis* elements which are essential for the inhibition of exon 6B splicing in myoblasts are located even more upstream in IVS-AB, in the first 100 nt of the intron (2a). We conclude from this set of experiments that the most likely focal point of this splicing regulation is the region of the branch point/acceptor site upstream of exon 6B and that the more complex secondary structure shown to inhibit in vitro splicing of IVS-B7 is unlikely to be involved in in vivo splicing regulation.

DISCUSSION

Data from our laboratory and other laboratories on splicing of the mutually exclusive exons 6A and 6B of the β tropomyosin gene (5, 6, 15, 29, 31, 33) have revealed the existence of a complex mechanism underlying the regulation of expression of the two exons in differentiated and undifferentiated muscle cells. For the purpose of dissecting this complex problem, we have performed an extensive mutagenesis analysis to identify other *cis* elements involved in the regulation.

Negative regulation of exon 6B splicing in myoblasts. We demonstrate that the regulation of exon 6B splicing is not dependent on the presence of exon 6A. The splicing pattern of a deleted minigene lacking exon 6A and the upstream intron (MutΔ6A) shows that the skeletal muscle-specific exon is used mainly in differentiated myotubes, while the major product obtained in myoblasts contains exon 5 spliced directly to exon 7. Thus, preferential use of exon 6A in myoblasts is due not to a splicing competition between the two exons but to the existence of a specific block on splicing of exon 6B and to the default utilization of exon 6A, which is in agreement with our previous data (29, 33). We have previously shown (33) that this negative control is related to the existence of one stem-and-loop structure (referred to as stem I) located at the very beginning of exon 6B and suggested (29, 30) that this stem could be part of a more complex secondary structure. Recently, Clouet-D'Orval et al. (5, 6) have characterized the folding of the primary transcript around exon 6B on the basis of chemical probing and in vitro splicing of shortened constructions (Fig. 6A). Modifications of two stems of this structure (stems I and III) have been shown to affect in vitro splicing of the downstream exon (IVS-B7) (6).

Though there is a basic accordance between in vivo and in vitro results concerning the role played by stem I in the regulation, our mutagenesis data for intron sequences around the donor site of exon 6B (region of stems II and III) do not support this model for the in vivo regulation of splicing. In fact, only mutations introduced in the region of stem I activate splicing of the skeletal muscle-specific exon in myoblasts, while mutations in IVS-B7 failed to reveal any evident negative role played by stems II and III in splicing regulation in vivo. Similarly, we have never been able to reveal by mutagenesis any negatively acting *cis* element in the region of the donor site of exon 6B, from positions −20 to +54 with respect to the exon/intron border (Fig. 6A). These data suggest that the negative *cis* elements present in the upstream intron (IVS-AB) (15, 29) must inhibit splicing of the exon in vivo in a different way than by base pairing to the downstream intron sequences. The differences between in vitro and in vivo data are probably relevant to the different assays used and, particularly, to the differences in the primary transcripts employed. Substrates spliced in vitro lack most of the sequence upstream of the branch point of exon 6B (5, 6), part of which is implicated in the regulation (this report and our unpublished data).

Data from this and other reports (29) show that many of the *cis* elements which exert a negative control over exon 6B splicing are clustered in the last 150 nt of IVS-AB (upstream and downstream of the BPS) and in the 5′ half of exon 6B. Moreover, we have recently identified (2b) some additional negative *cis*-acting elements located in the very beginning of IVS-AB which are likely part of the same regulatory system. In our opinion, these results together suggest that much of the negative regulation of splicing depends on events taking place in the regions surrounding the acceptor/branch site of exon 6B. Such events may depend on the existence of an alternative regulated folding of IVS-AB (which may or may not include stem I), the binding of protein or ribonucleoprotein factors, or a combination of the two. At present, we cannot state whether stem I plays an active role in the regulation; its role could simply be to lessen the efficiency of recognition of exon 6B both in myoblasts and in myotubes. In the latter cellular environment, this poor recognizability of the exon could be efficiently overcome by the presence, for instance, of a specific splicing activation system similar to that described by Ryner and Baker (46) for the female-specific exon of the *Drosophila dsx* gene. In other words, we do not know whether splicing of exon 6B in myotubes depends on early disruption of stem I or whether it takes place despite its presence in this more favorable processing environment. It is interesting that at least part of the regulation (main skipping of the exon in myoblasts and better recognition in myotubes) is retained in a minigene which lacks exon 6A and IVS-AB (i.e., which contains exon 5, IVS-5A, exon 6B, and IVS-B7) but in which stem I has not been modified (our unpublished data).

Multiple protein factors are expected to bind to the polypyrimidine-rich region downstream of the branch point of exon 6B. Splicing factors like U2AF (45, 56) or proteins whose role in splicing has yet to be definitively demonstrated, such as IBP (13, 53) or PTB/PBP (9, 14, 40), are all known to bind pyrimidine-rich sequences well. A factor that binds the polypyrimidine region upstream of the skeletal muscle-specific exon of the β tropomyosin gene in the rat (exon 7, corresponding to exon 6B in our terminology) was recently identified by Guo et al. and was proposed to be involved in blocking the use of the exon in nonmuscle cells (16). Finally, preliminary results indicate that at least three proteins bind to a region located between exons 6A and 6B, even though the precise binding sites (and the nature of the proteins) have not yet been identified (our unpublished data).

It is possible that at least part of the regulation in myoblasts relies on the presence of regulatory proteins which would compete with splicing factors for the binding to the

polypyrimidine-rich region. However, we do not believe that the interaction of any protein with the polypyrimidine stretch may induce exon 6B skipping per se, since mutations located outside this region (i.e., located upstream of the branch point or in exon 6B) are able to disrupt the negative control. If *trans*-acting factors play a role in negative regulation in myoblasts, a complex pattern of interactions should be envisaged.

Finally, we show that both the donor and the branch sites related to exon 6B are suboptimal and that this weakness is necessary for splicing inhibition in myoblasts. In fact, as they are modified to match the respective consensus, some activation of the exon is observed. Thus, the conversion to the consensus of the donor site of exon 6B is able to relieve, if only partially, the negative control existing on the upstream acceptor site. This result can be interpreted in the light of the exon definition model (43, 52): a stronger donor site may induce a better recognition of the upstream acceptor site and partially balance the effects of the negative control.

The occurrence of weak splice sites in cases of alternative splicing has already been reported (7, 26, 52, 58) and is widespread in tropomyosin genes (18, 28, 30, 34, 44, 54). Their role is most probably a permissive one for the setup of a superimposing fine-tuning control mechanism.

Exons 6A and 6B compete for splicing in myotubes. We show that the skipping of exon 6A in myotubes depends on the presence of an active exon 6B on the primary transcript. A minigene bearing mutations on both splice sites of exon 6B produces transcripts containing mainly exons 5, 6A, and 7 in both differentiated and undifferentiated cells. We suggest that the donor and branch sites of the two exons play important (and probably distinct) roles in this competition. We show that converting the donor site of exon 6A to the consensus (Mut6A*cons*) induces two major effects in myotubes: first, it becomes a better competitor for the acceptor site of exon 7 (leading to an enhanced production of species 5-6A-7); second, it enhances splicing of the intron between exons 6A and 6B (species 5-6A-6B-7), which leads to a partial loss of the mutually exclusive character of the two exons.

Similarly, we have proposed that the branch point upstream of exon 6B could be stronger than the branch point of exon 6A and that a competition between the two may play a role in splicing regulation. In this report, we show that all of the mutants which bear a weaker branch point upstream of exon 6B or a stronger one upstream of exon 6A exhibit the common feature of using exon 6A as a main choice, which clearly indicates that branch point competition plays an important role in splicing regulation, as recently shown for exons 2 and 3 of the α tropomyosin gene in the rat (37).

The branch point mutants differ mainly in their ability to splice out IVS-AB to obtain the four-exon product 5-6A-6B-7. These differences are probably linked to the existence of an undefined constraint which lessens the spliceosome assembly rate for splicing of IVS-AB. A steric constraint has been implicated (49) in the regulation of mutually exclusive splicing in the rat α tropomyosin gene (exons 2 and 3); in this case, the branch point is too close to the donor site to permit fast and efficient spliceosome assembly. The factor which induces a very low rate of excision of IVS-AB is unknown. In our experiments, two main sequence changes affect the splicing efficiency of IVS-AB and relieve, at least partially, the constraint. First, with respect to the introduction of better splice sites flanking the IVS, we have already discussed the effect of the modification of the donor site of exon 6A; similarly, coupling mutation MutYBP6B (UCUCAAC to the preferred UACUAAC for the BPS of IVS-AB) to mutation Mut39 (exclusive use of exon 6B) enhances splicing of IVS-AB (data not shown). Second, the mutation introduced in Mut16 (and in some other exon mutants; data not shown) induces 60% splicing of exon 6A to exon 6B (compared with the exclusive use of exon 6B). It is possible that the hairpin loop structure defined by stem I is somewhat related to this constraint, which seems at least partially independent of the activation of exon 6B (in Mut39, exon 6B is activated but spliced in the unique species 5-6B-7).

The current model to accommodate all of our data is the following. In myoblasts, exon 6B is not accessible; the secondary structure of which stem I is a part, undefined elements in IVS-AB, the existence of suboptimal splice sites flanking the exon, and possibly *trans*-acting factors all contribute to the negative control of its selection.

In myotubes, a qualitatively (or quantitatively) modified splicing environment makes both exons accessible to the splicing apparatus. In an early step of branch point selection, spliceosomes begin to assemble (for instance, by binding of U1 snRNP, U2AF, and other factors) around the BPS of exon 6B (and possibly of exon 7) more rapidly than around the BPS of exon 6A. The selection of the correct donor sites for mature spliceosome assembly depends subsequently on donor site competition and on the constraints existing on IVS-AB excision. Selection of the donor site of exon 6A for splicing to exon 7 is prevented by the presence of the stronger and preferred donor site of exon 6B. Similarly, use of the donor of exon 6A in the spliceosome initiated at the BPS upstream of exon 6B is prevented by the combined effect of the constraint on IVS-AB splicing and the competition with the stronger donor site of exon 5. Mutations of the BP6A kind, providing a stronger branch site upstream of exon 6A, mainly affect the first step and allow rapid splicing of IVS-5A. The subsequent events depend on the spliceability of IVS-AB (which is linked to the type of activating mutation, Mut16 or Mut39); in Mut16/BP6A, IVS-AB is spliced faster than IVS-A7 (to obtain the main species, 5-6A-6B-7), while in Mut39/BP6A, the excision rates of the two alternative introns (IVS-AB and IVS-A7) are similar (because of the poorer spliceability of IVS-AB in this mutant) and lead to a mixture of the two species 5-6A-7 and 5-6A-6B-7. In mutants of the BP1 type (Mut16/BP1 and Mut39/BP1), the branch point upstream of exon 6B is weaker than in the wild type, and early steps of spliceosome assembly are faster in IVS-5A than in IVS-AB, leading to splicing of exon 5 to exon 6A. Subsequent joining of exon 6A to exon 6B is prevented by the existence of a stronger constraint on IVS-AB splicing due to the weaker branch point. As a consequence, the main mature species is 5-6A-7 for both Mut16/BP1 and Mut39/BP1. Finally, in Mut6A*cons* (in myotubes), the sequence modification affects mainly the donor selection events. In this mutant, assembly of spliceosomal factors around the BPS of IVS-5A is still slow, but the strong donor site of exon 6A can assemble in mature spliceosomes with both the BPS upstream of exon 6B and the BPS upstream of exon 7. IVS-5A is the last to be spliced in this minigene to obtain a mixture of species 5-6A-7 and 5-6A-6B-7.

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