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Exon as well as Intron Sequences Are *cis*-Regulating Elements for the Mutually Exclusive Alternative Splicing of the β Tropomyosin Gene

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The β tropomyosin gene contains two internal exons which are spliced in a mutually exclusive manner. Exon 6B is specifically included in the mature transcripts expressed in skeletal muscle or cultured myotubes, while exon 6A is a myoblast- or smooth muscle-specific exon. The intron between them, which is never spliced in normal conditions, contains two characteristic features: first, the unusual location of the branch point at position −105 from the acceptor, and second, the presence of a very long pyrimidine stretch upstream of the skeletal muscle exon. In this study we designed a number of sequence modifications to investigate the role of these two elements and of a computer-predicted secondary structure in the mutually exclusive splicing of the two exons. We found that mutations in the skeletal exon as well as in the upstream intron could change in vivo the tissue-specific pattern as well as the mutually exclusive character of the two exons. Our results suggest that the unusual position of the branch point does not prevent the utilization of exon 6B in myoblasts and that the region around the acceptor site of exon 6B and the polypyrimidine tract have an important role in this control. Last, we discuss the possible implications of secondary structures.

Splice site selection in alternative splicing is a process which leads to the production of multiple products from a single gene. Even though the mechanism of constitutive RNA splicing is rather well understood, little is known about the factors which govern the optional utilization of functional exons. *cis*-Acting elements have been proposed as determinants of splice site selection. Exon sequences have been shown to act as both negative (49) and positive (7, 28, 36) regulatory elements, being able, if mutated, to promote or prevent utilization of the flanking splice sites.

The relative strengths of donor 5′ splice sites have been shown to play a role in the competition for the same acceptor 3′ splice site; the closer the donor sequence is to the consensus AG/GURAGU, the stronger it is considered to be (1, 2, 13, 49, 53). Similarly, branch point competition has been proposed (32, 37, 51, 52, 54) to be involved in alternative splicing, although branch point strength is more loosely defined in pre-mRNA splicing of higher eucaryotes than it is in yeast splicing. A role for the pyrimidine content of the 3′ splice site has been proposed by Fu et al. (12), while secondary structures of the primary transcript have also been suggested to be implicated in determining splice site accessibility (5, 6, 8, 26, 38, 46, 50). Finally, a number of elements, such as the distance between the branch point and the donor site (32, 44) or the acceptor site (6, 14) and the intron size (13) have been correlated with a low efficiency of excision of introns; different rates of splicing of partially overlapping introns could result in alternative splice site selection. Lastly, *trans*-acting factors are believed to play a role in determining the tissue or development stage specificity of differential processing (3, 4, 25, 27, 47).

The chicken β tropomyosin gene is a model system to study alternative splicing. By the use of two promoters, alternative terminal exons and two mutually exclusive internal exons, this gene codes for three tropomyosin isoforms, two of which are expressed in adult muscle tissues (smooth or skeletal muscle), while the third is expressed in nonmuscle cells and undifferentiated myoblasts (26, 27a). The two internal mutually exclusive exons (exons 6A and 6B) have a strict tissue specificity; exon 6A is present only in mRNA of smooth muscle, nonmuscle cells, and undifferentiated myoblasts, while exon 6B is used exclusively in skeletal muscle and in differentiated myotubes in culture. Similarly, exons 9A and 9B (the 3′-terminal exons) are expressed specifically in skeletal muscle (exon 9A) or in all the other cell types (exon 9B). Two different transcription initiation sites are used according to whether the gene is expressed in adult muscle tissues (skeletal and smooth muscles) or in undifferentiated myoblasts and nonmuscle cells (see Fig. 2).

We have previously shown by minigene transfection of cultured myogenic cells (27) that all the necessary information for the tissue specificity as well as the mutually exclusive character of exons 6A and 6B is contained in a 0.9-kilobase (kb) genomic fragment containing the two alternative exons flanked by two constitutive exons. Stably transfected myoblasts show a pattern of splicing in which exon 6A is spliced between exons 5 and 7 while exon 6B is almost completely excluded from the mature transcript; after differentiation of the same cells, exon 6B is included in the mature transcript in a majority of splicing events while exon 6A is skipped. In these stable transfectants, the two exons are never spliced together.

An implication of these results is the necessary existence of at least one *trans*-acting factor which would be needed for the specific pathways of splicing. During transient transfection of the same construction, however, the developmental regulation is lost, since both myoblasts and myotubes display the same myoblast-type pattern of splicing (exons 5-6A-7), while the mutually exclusive character of the two exons is nearly completely retained (27; our unpublished results).

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We have suggested (27) that two partially independent levels of control could account for the tissue specificity and for the mutually exclusive character of the two exons.

In this report we show that at least two sequence segments act as negative controlling elements in the regulation of mutually exclusive alternative splicing of exons 6A and 6B. First, a mutation of 16 nucleotides (nt) in exon 6B transforms this exon from a muscle-specific exon into a constitutive one which is included in the mature transcript irrespective of the state of cell differentiation. At the same time, the mutually exclusive character of the two exons is partially lost, since exon 6A can now be spliced to exon 6B even though joining of the two exons is still a minor splicing pathway.

A similar mode of splicing can be obtained by making mutations in the conserved long pyrimidine stretch which precedes the acceptor site of exon 6B. These results are consistent with a model in which a secondary structure governs the regulation of mutually exclusive splicing of the two exons. Possible roles for the unusual distance between the branch point and the acceptor site and the conserved polypyrimidine stretch are also discussed.

MATERIALS AND METHODS

Vector constructions. The wild-type minigene construction pBS/SVβalt has been described previously (27). It contains exons 5 to 7 of the β tropomyosin gene (26) inserted between the simian virus 40 (SV40) early promoter and transcription termination site. Site-directed mutagenesis was performed with the commercial kit purchased from Amersham Corp. on the single-strand form of the wild-type minigene construction. The following synthetic oligonucleotides were used for the different mutant constructions.

Mut14: 5'-CTTCCCTGTGCTCCTTCCGCTCCCAGATCTGATGGCCCCTGCTTGGGATGTA-3'
Mut15: 5'-GGTGGAGCTGCTGGGGCTGGGCAGATCTGCGTCCCAACTCCCCTCCAGGGTCCTTCACTGGGGTGAAGAA-3'
Mut16: 5'-GGCCCCTGCTTGGGATGTAGAATTCCACCACTGGATGAGGAGGAGCTGAAAATTGTCACC-3'
Mut35: 5'-ACCAACAACTTGAAGCTTCTCCTCCGAGGTCCGTGACAAGGTATGACCTGGT-3'
Mut39: 5'-CCTTCATCACCCCCTCCCCTCGGACCTCGGAGGAAGCTTGGGATGTAGTAAATGT-3'

The minigene MutΔBglII was obtained from Mut14 after restriction at the *Bgl*II site added by mutagenesis, S1 nuclease treatment, and ligation. Ten additional nucleotides were accidentally deleted. All the mutants were sequenced to confirm that unwanted changes were not introduced.

Cells and transfections. Quail embryo myoblasts transformed with a temperature-sensitive mutant of Fujinami sarcoma virus (*ts*NY240) were obtained as previously described for Rous sarcoma virus-transformed quail myoblasts (29). A 10-μg amount of each minigene construction was used to transfect 10^6 cells by the calcium phosphate precipitation method (18). Transiently expressed transcripts were harvested after 24 or 48 h.

Oligonucleotide controlled primer extension. The method used to analyze the transcripts obtained from minigene constructions has been described previously by Erster et al. (9). In this method (Fig. 1), primer extension is performed on transcripts previously subjected to oligonucleotide-directed RNase H digestion. In a first step, an oligonucleotide directed against the sequence of interest (mostly exons 6A, 6B, and 5) is annealed to the RNA (usually 10 to 30 μg) and used to direct RNase H cleavage. Annealing of the cleavage oligonucleotide (2 to 3 pmol) was performed in 50 mM Tris hydrochloride (Tris-HCl, pH 8.3)–70 mM KCl–25 mM $MgCl_2$ for 5 min at 65°C followed by 10 min at 37°C. RNase H (1 U; Amersham) was subsequently added, and the samples were further incubated for 30 min at 37°C. After two phenol extraction, the cleaved transcripts were ethanol precipitated together with 100,000 to 400,000 cpm of a 5'-end-labeled oligonucleotide directed against SV40 sequences for primer extension.

The following oligonucleotides were used: oligonucleotide SV (26-mer) is directed against SV40 sequences at the 3' end of the minigene constructions and was used as the labeled primer in all cDNA syntheses. Oligonucleotides 5 (20-mer), 6A (21-mer), and 6B (22-mer) are directed against exons 5, 6A, and 6B, respectively. Oligonucleotide I was directed against the last 21 nt of the intron between exons 6A and 6B. For all the oligonucleotides employed, the presence of a single point of annealing was verified by RNase H-directed cleavage of uniformly labeled T3 transcripts.

When oligonucleotide 6B was used for RNase H cleavage, a reverse transcription product of 117 nt was obtained if the transcript contained exon 6B. When oligonucleotide 6A was used, two primer extension products were obtained of 124 or 200 nt, according respectively to the presence of exon 6A alone or of exon 6A spliced to exon 6B. Last, when the cleavage oligonucleotide was directed against exon 5, primer extension products of 225 nt (exons 6A or 6B alone) or 301 nt (exons 6A and 6B together) were observed (Fig. 1B).

Primer extension was performed with 10 U (0.5 μl) of reverse transcriptase (Boehringer) for each sample. The products were analyzed on a 6% polyacrylamide gel.

Secondary-structure predictions. The secondary structure around exon 6B was predicted by using the Zuker algorithm (55) as previously described (26) with the difference that the values of free-energy variations reported by Freier et al. (10) were used.

RESULTS

We have previously (26, 27) proposed a model according to which the mutually exclusive character of exons 6A and 6B would be mainly *cis* regulated by the existence of two alternative secondary structures, which would in turn allow the utilization of one or the other exon. The most stable of the two would allow the default, myoblast-type splicing pattern, while in myotubes the presence of muscle-specific factors would force the pre-mRNA into a conformation allowing the exclusive utilization of exon 6B. Such factors would be present in limiting amounts and be titrated in transient transfection assays by an overproduction of primary transcript (27).

A possible secondary structure, based on computer prediction, is shown in Fig. 2B (26). This structure would allow the default splicing pattern by hiding exon 6B from the splicing machinery in myoblasts or in transiently transfected myotubes. To test this model, we have designed a number of sequence modifications which would disrupt stable stems in such a hypothetical structure. At the same time we analyzed the role of the abnormally located branch point (position −105 from the acceptor [17]) in the intron between the two alternative exons and the effect of mutations in the conserved polypyrimidine stretch (26) upstream of the acceptor of exon 6B.

Negative *cis*-acting element is located in exon 6B. As can be seen in Fig. 2, 15 nucleotides were modified in mutant Mut16 at the 5' end of exon 6B. Splice signals were unchanged and extensive sequence analysis was performed to confirm that unwanted changes were not introduced.

This mutant was used to transfect quail myoblasts, and RNA was isolated and analyzed with the controlled primer extension method (see Materials and Methods). Essentially, mRNA was annealed to the different oligonucleotides and digested by RNase H, and primer extension was performed with a common oligonucleotide (SV) directed against SV40 sequences.

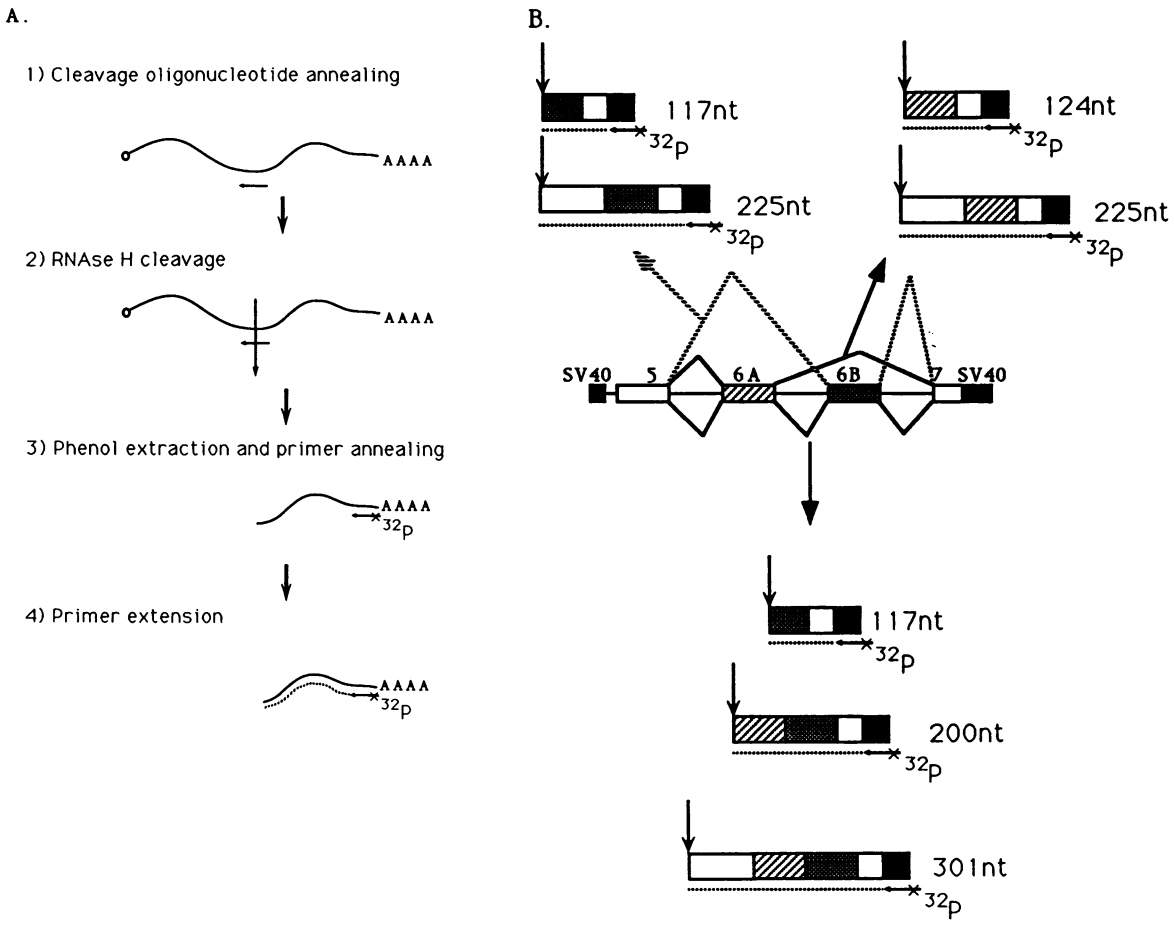


FIG. 1. (A) Schematic representation of the method used to analyze transcripts derived from the minigene constructions (controlled primer extension). (B) Splicing patterns and exon composition of most of the expected splicing products. The point of RNase cleavage for each transcript is shown by arrows; the size of the reverse transcription product of each cleaved RNA is indicated.

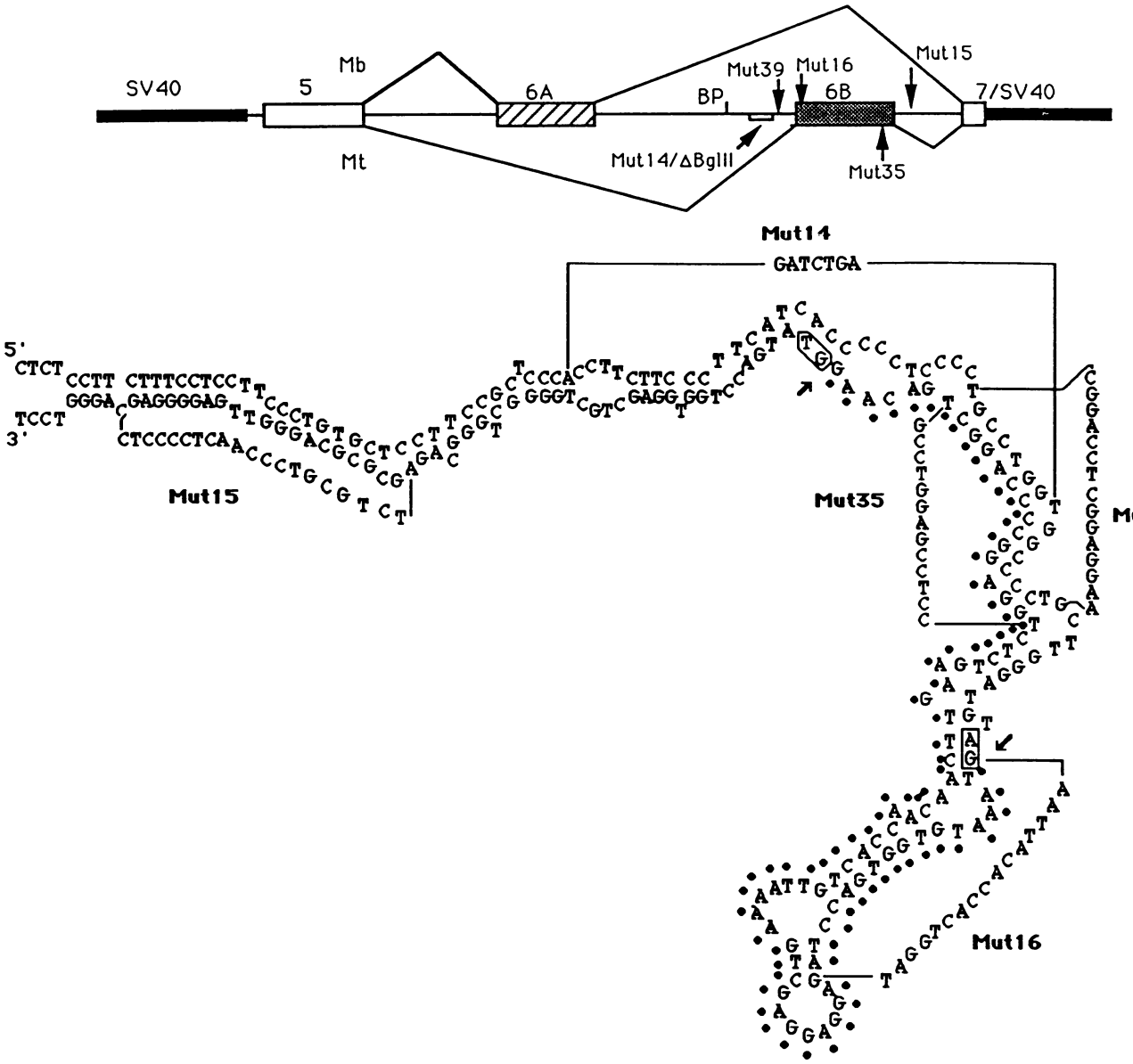
As shown in Fig. 3, a dramatic change in the pattern of splicing was observed compared with cells transfected with the wild-type construct. First, no differences were observed between the splicing products obtained in myoblasts (Fig. 3, Mut16) or in myotubes in either transient or stable transfections (data not shown). In this mutant, exon 6B now behaved as a constitutive exon and was always present, while exon 6A behaved as a "cassette" type exon which could be either included or excluded from the mature transcript.

Indeed, when extension of oligonucleotide SV was blocked by oligonucleotide 6A (Fig. 3, Mut16, lane 6A), the primer extension product which was detected had a size of 200 nt (indicative of a transcript in which exon 6A is spliced to exon 6B) instead of the 124 nt expected if exon 6B were skipped and exon 6A were joined directly to exon 7 (Fig. 3, WT, lane 6A). Furthermore, the presence of a full-length transcript in lane 6A (band at 330 nt) showed that inclusion of exon 6A was not an obligatory pathway of splicing.

When extension was blocked by oligonucleotide 5, two products were obtained, one with a length of 225 nt and the second with a length of 301 nt (Fig. 3, Mut16, lane 5). The former major product had the expected size for exon 5 spliced to exons 6B and 7, while the latter corresponded to exon 5 spliced to exons 6A, 6B, and 7. Splicing of exon 6A to exon 6B was directly confirmed by the observation that extension of a labeled oligonucleotide against exon 6B could be partially blocked by oligonucleotide 6A (data not shown).

The existence of two mRNAs (one containing and the other lacking exon 6A) was also confirmed by the presence of two major full-length transcripts in the free extension reaction with lengths of 330 nt and 406 nt (Fig. 3, Mut16, lane F).

FIG. 2. (A) Schematic representation of the minigene constructions. Position of sequence modifications in mutants is shown by arrows. The splicing patterns of the wild-type minigene in myoblasts (Mb) and myotubes (Mt) after stable transfection are indicated. Open boxes, Common exons; shaded box, skeletal muscle/myotube-specific exon; hatched box, smooth muscle/myoblast-specific exon; solid boxes, SV40 sequences. (B) Computer-predicted secondary structure around exon 6B. Exon sequences are indicated by asterisks; donor and acceptor sites are boxed. Positions of the different mutations as well as the sequence modifications are indicated. (C) Sequence of the minigene constructions around exon 6B. Exon 6B sequences (5′ and 3′ ends) are in capital letters; in lowercase letters are intron sequences. The mutated regions are underlined; the position and extension of deletions are indicated. Branch point locations are relative to the acceptor used (indicated by arrows in Mut39 and Mut14).


SV40
5
Mb
Mt
6A
BP
Mut39
Mut16
6B
Mut15
7/SV40
Mut14/ΔBglII
Mut35
Mut14
GATCTGA
Mut15
Mut35
Mut39
Mut16


-105
-60
-50
-40
-30
-20
-10
WT tcaActtgttctc..ccgctcccaccttcttccttcatcacccccteccctgcctggtggcccctgcttgggatgtagTAAATGTGGTGACCTAGAG...
Mut16 tcaActtgttctc..ccgctcccaccttcttccttcatcacccccteccctgcctggtggcccctgcttgggatgtagAATTCCACCACTGGATGAG...
-51
34nt
Mut14 tcaActtgttctc...ccgctcccagatctgatggcccctgcttgggatgtagTAAATGTGGTGACCTAGAG...
-63
42nt
ΔBglII tcaActtgttctc.....ccggcccctgcttgggatgtagTAAATGTGGTGACCTAGAG...
-94
Mut39 tcaActtgttctc..ccgctcccaccttcttccttcatcacccccteccctcggacctcggaggaagcttgggatgtagTAAATGTGGTGACCTAGAG...
+30
WT Ex6B ....TCTCTGGAGGCCCAGGCTGACAAGgtatgac.....cagagcgcgcagggttgaggggagcaggt...
Mut35 Ex6B ....TCTCTCCTCCGAGGTCCGTGACAAGgtatgac.....cagagcgcgcagggttgaggggagcaggt...
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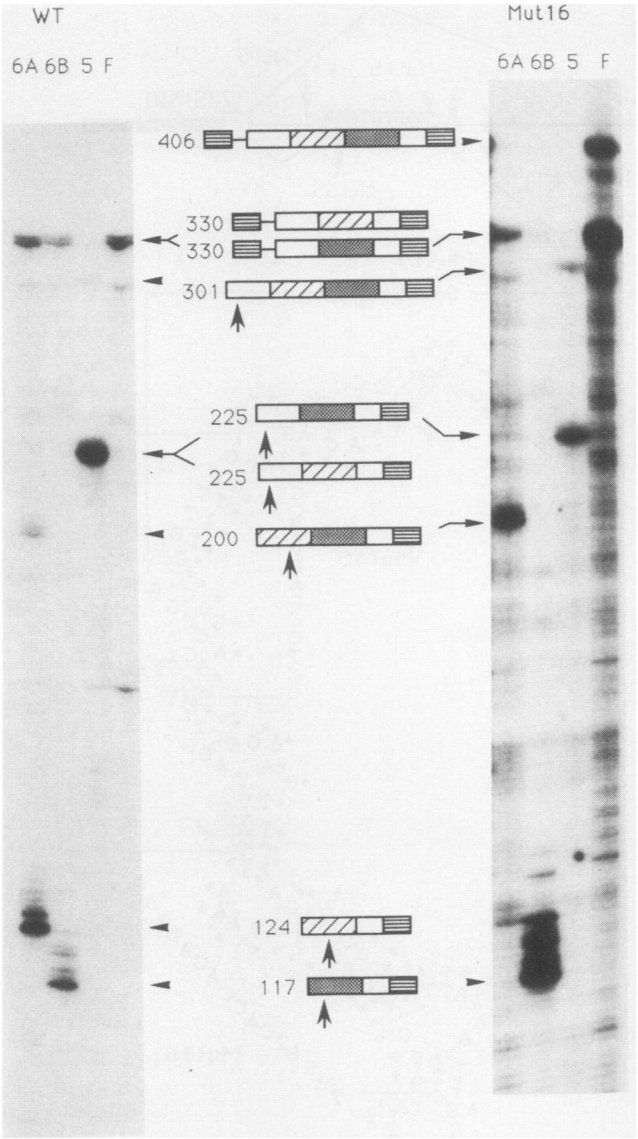

FIG. 3. Controlled primer extension analysis of transcripts after transient expression of the wild-type (WT) and mutant Mut16 (Mut16) minigene constructions in myoblasts. The sizes and the exon composition of each reverse-transcribed splicing product are indicated. Additional bands are probably due to premature reverse transcription termination or to RNase H nibbling. Large arrows indicate the points of RNase H cleavage. Lanes 6A, 6B, and 5, mRNAs cleaved with RNase H after annealing with oligonucleotides against exons 6A, 6B, and 5, respectively. Lanes F, Free reverse transcriptase extensions. Open boxes, Common exons; shaded boxes, skeletal muscle/myotube-specific exon; hatched boxes, smooth muscle/myoblast-specific exon; striped boxes, SV40 sequences; thin lines, intron sequences.

Lastly, when oligonucleotide 6B was used to block the extension of oligonucleotide SV, a 117-nt product was obtained, which indicates that exon 6B was spliced to exon 7. It has to be noted that there was no full-length extension product, which suggests that all the molecules of transcript contained exon 6B (Fig. 3, Mut16, lane 6B).

In conclusion, exon 7 was always spliced to exon 6B, which could in turn be spliced either to exon 6A (which is joined to exon 5) or directly to exon 5. Owing to the different sizes of the extension products, it is difficult to quantify exactly the relative abundance of the two species. We estimate that about 60% of the transcripts contained only exon 6B and 40% contained both exons 6A and 6B (Fig. 3, Mut16, compare bands in lanes 6A and 5). An enhancement of exon 6B utilization was obtained after in vitro splicing of the same mutant construction (M. Goux-Pelletan, unpublished observations).

A second mutant (Mut35) was constructed in which 13 nt were modified near the 3′ end of exon 6B (Fig. 2). Though this region was expected to play a role in the constitution of the predicted secondary structure, the splicing pathway of the mutated minigene did not show an enhancement of exon 6B utilization (Fig. 4, Mut35, lane 6B). Interestingly, this mutation seemed to have a negative effect on splicing of exon 6B, since no detectable level of this exon was observed in the spliced transcripts in two independent experiments (Fig. 4 and data not shown). However, because of the low level of exon 6B utilization in wild-type constructs (Fig. 3, WT), further experiments are needed to ensure that skipping of the skeletal muscle exon in Mut35 occurs at a significant level.

Mutational analysis of the intronic sequences around exon 6B. The intron which separates the two mutually exclusive exons has two characteristic features. First, the branch point is located far from its canonical position (i.e., at position −105 from the acceptor site of exon 6B) (17). Second, the sequence between the branch point and the AG is composed almost exclusively of pyrimidines (26). These two elements may be related; the pyrimidine stretch may play a passive role in pushing away the branch point, whose distance from the acceptor site would be the only determinant of splice site selection. On the other hand, the polypyrimidine tract may be important per se, for instance by binding to *trans*-acting factors or by base-pairing with other sequences in secondary structures of the primary transcript, as we have recently proposed (26) (Fig. 2).

In order to investigate the role of this region, we designed a first mutant (Mut14) which contains two modifications: a deletion of 34 nt was made in the polypyrimidine tract, and a sequence with a good match to the branch point consensus was added at position −22 (Fig. 2). The positions of the deleted sequences in the theoretical secondary structure are shown in Fig. 2B.

This construction was transfected into muscle cells. RNA was isolated and analyzed as usual. The results are shown in Fig. 5 (section Mut14) and are very similar to the results obtained with the mutant Mut16. No difference was observed whether the transfected cells remained myoblasts (Fig. 5) or were allowed to differentiate (data not shown). Extension of the oligonucleotide SV was primarily blocked by oligonucleotide 6B and only to a minor extent by oligonucleotide 6A (Fig. 5, Mut14, lanes 6B and 6A). In the latter case (lane 6A), a product with a larger size (226 nt, see below) than the expected 124 nt was observed, which indicates that exon 6A was not spliced directly to exon 7 but through exon 6B. This conclusion was confirmed by the presence of two extension products in lane 5 (the band at 327 nt is barely visible in this exposure), which again indicates splicing of exon 6A to exon 6B in a few cases. Interestingly, the relative abundance of the two species (one in which exon 6A was spliced between exon 6B and exon 5 and the other lacking exon 6A) was different from that seen with mutant Mut16, since the species containing exon 6A was less abundant in mutant Mut14 than in mutant Mut16. All these results indicate that inclusion of exon 6B has become an

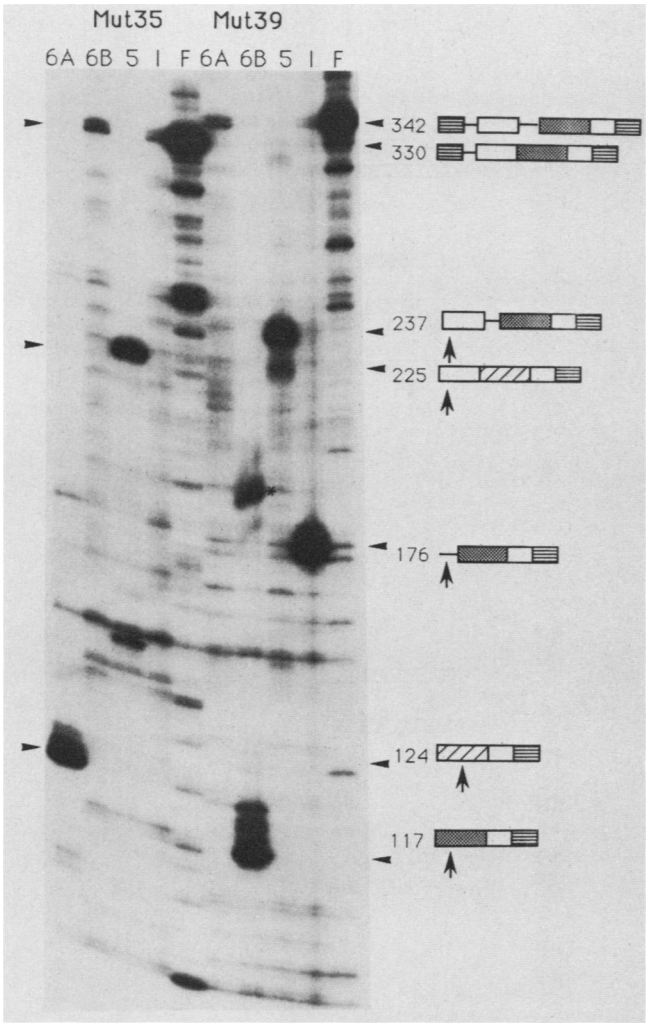

FIG. 4. Controlled primer extension analysis of transcripts after transient expression of mutants Mut35 and Mut39 minigene constructions in myoblasts. See legends to Fig. 2 and 3 for explanation of symbols and letters. Lanes I, Transcripts cleaved with RNase H after annealing with an oligonucleotide against the last 21 nt of the wild-type intron upstream of exon 6B. Note that in the case of transcripts derived from the Mut39 minigene, the annealing of this oligonucleotide (I) was limited to the 12 nt of intron downstream from the AG acceptor used in this particular construction (see text and Fig. 2C). The band marked with an asterisk in lane 6B of section Mut39 is of unknown origin.

obligatory pathway of splicing while exon 6A still remains a facultative exon. Splicing of exon 6A to exon 7 was completely abolished, and excision of the intron between the two mutually exclusive exons could now occur, though with a lower frequency than in mutant Mut16.

When the transcripts from Mut14 and Mut16 were analyzed in parallel on the same gel (data not shown), an important difference arose. In the case of mutant Mut14, all the extension products obtained when extension was blocked by oligonucleotides 6A and 5 were 26 nucleotides larger than the corresponding splicing products of Mut16 (226 nt instead of 200 nt in lane 6A; 327 nt instead of 301 nt and 251 nt versus 225 nt in lane 5). The same difference was seen in the full-length reverse transcriptase products. This 26-nt difference arose from the use of an additional AG acceptor site accidentally introduced during the mutagenesis reaction (Fig. 2C). This conclusion was confirmed by the fact that the extension reaction could be blocked by an oligonucleotide covering the last 22 nt of the intron (Fig. 5, Mut14, lane I). It is interesting that in this mutant, both the newly added branch point consensus sequence and the natural exon 6B acceptor site were completely ignored by the splicing apparatus.

In mutant Mut14, both the shortened distance from the natural branch point (51 nt) and a different environment for the new acceptor site could account for the acquired constitutive pattern of splicing. To further investigate these aspects, we constructed two more mutations in this region. The first mutant (MutΔBglII; Fig. 5, ΔBglII) was derived from mutant Mut14 by deletion of the *Bgl*II site which contained the new acceptor site (Fig. 2C). In MutΔBglII, the only acceptor site present was the natural one, which was located 63 nt downstream from the branch point. The difference between this construction and the wild type is a deletion of 42 nt in the pyrimidine region.

As can be seen in Fig. 5, the primary transcript derived from this mutant minigene construction was spliced in a similar way to Mut16 or Mut14, though some important differences must be pointed out. As in the other mutants, exon 6B joined to exon 7 was still the major splicing pathway (lane 6B), but in this case the skeletal muscle-specific exon (6B) could be skipped by the splicing apparatus. In fact, as can be seen in lane 6A, a minor product existed in which exon 6A was spliced directly to exon 7 (Fig. 5, lane 6A, 124-nt product). Exon 6B was joined directly to exon 5 in the majority of splicing events, while exon 6A could be included between the two (product 5-6A-6B-7) with about the same efficiency as in mutant Mut14.

In the case of mutant MutΔBglII, two elements can still account for the change in the splicing pathway: first, the branch point is 42 nt closer to the acceptor site than in the wild-type construct; second, the sequence environment of the acceptor site has been changed, since 42 nt of the pyrimidine tract has been deleted. We therefore designed another mutant minigene construction (Mut39, Fig. 2) in which the sequence environment of the acceptor site has been modified without significantly altering the distance from the branch point. In this construction, two AG (GAG and AAG) are present within the mutated 15 nt, and the distance between them and the branch point is respectively 90 and 94 nt, i.e., only about 10 nt less than in the wild-type sequence. The position of this mutation in the predicted secondary structure is shown in Fig. 2B (note that this region is predicted to base-pair with sequences mutated in Mut35). As can be seen in Fig. 4, section Mut39, the changes in the splicing pattern were clearly more dramatic than with mutant MutΔBglII. Exon 6B was now constitutive, and exon 6A was completely ignored, as can be seen in lane 6A, where neither the band at 124 nt (splicing of exon 6A directly to exon 7) nor the band at 200 nt (sequential splicing of exons 6A, 6B, and 7) was observed. As expected from the results obtained with mutant Mut14, one of the two AG's introduced by mutagenesis was used. Interestingly, only the one farther downstream from the branch point (AAG) was utilized, while the 4-nt upstream GAG was ignored, as confirmed by partial sequencing of the transcript (data not shown). Elimination of the two AG's introduced by mutagenesis led to no significant changes in the splicing pattern of mutant Mut39, and the wild-type acceptor upstream of exon 6B was used constitutively (data not shown).

Finally, a 20-nt change in the intron downstream of exon 6B (Mut15; Fig. 2A and C), also theoretically implicated in

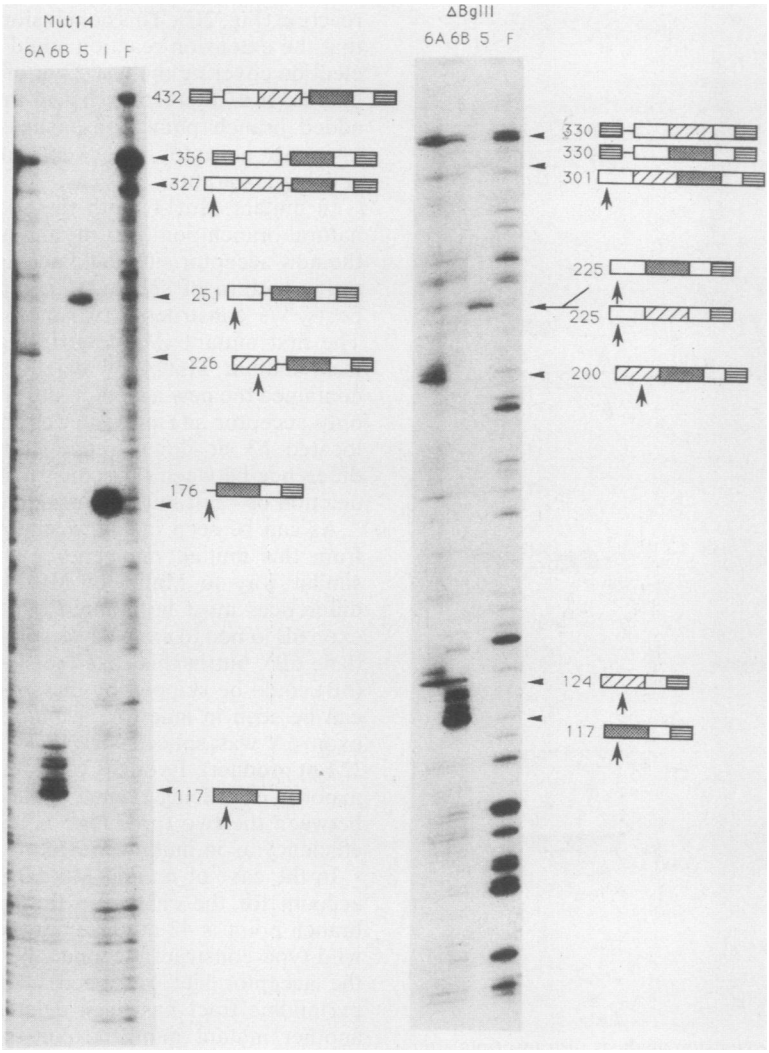

FIG. 5. Controlled primer extension analysis of transcripts after transient expression of mutants Mut14 and MutΔBglII (ΔBglII) minigene constructions in myoblasts. See legends to Fig. 2 and 3 for explanation of symbols and letters. Lane I, Transcripts cleaved with RNase H and an oligonucleotide against the last 21 nt of the intron upstream of exon 6B.

stable base-pairing (Fig. 2B), resulted in a wild-type splicing pattern (data not shown).

DISCUSSION

The branch point location between −18 and −40 nt from the acceptor site has been shown previously to be one of the major determinants of its selection (20, 31, 35, 40, 41, 52). Other important factors are the presence of a downstream polypyrimidine stretch and of an AG dinucleotide as the acceptor site. It has been shown that mutations or deletions of the conserved AG can lower the efficiency of factor binding (16, 42, 48), prespliceosome formation (24), and 5′ splice site cleavage (35, 39) while generally inhibiting the second step of the splicing reaction (35, 39). Deletions of the pyrimidine stretch block prespliceosome formation (11) and all subsequent steps of splicing (35, 39).

Very recently a number of cases (14, 17, 21, 45) have been described in which an abnormal location of the branch point is associated with alternative splicing. Chebli et al. (6) have shown that in adenovirus E1A, excision of the 216-nt intron depends on the formation of a stable stem-and-loop hairpin which would bring back the abnormally located branch point (−59 to −51 nt from the AG site) to an operational, permitted distance of −31 to −39 nt from the acceptor. These authors (6, 14) have proposed that mainly the second step of splicing (3′ splice site cleavage and exon ligation) would be affected by the abnormal location of the branch point. Smith et al. (45) and Reed (34) have very recently shown that an important prerequisite for the utilization of branch points located upstream of their canonical position is the presence of an associated polypyrimidine tract which could compensate for the negative effect of the absence of a nearby AG acceptor. Interestingly, in three of the four known cases, the abnormally located branch point is associated with very long pyrimidine stretches.

Consistent with this hypothesis, we found that the potential branch point added at its canonical location (position −22) in mutant Mut14 was ineffective for the utilization of the 6B acceptor, as if the wild-type branch point could efficiently compete with the newly added one in spite of the fact that the latter has a better match to the consensus and is nearer to the acceptor of the former (22 nt versus 51 nt for

the former). Interestingly, only the wild-type branch point in this construction is followed by a pyrimidine-rich region. We note that the whole pyrimidine stretch is not necessary to "fix" the branch point position; in construction Mut14, only about half of the pyrimidine-rich region (50 nt) follows the branch point, while in vitro splicing studies of similar constructions (17) have shown that only 24 nt of this region are sufficient to allow branch point utilization. Similar results have been recently described by the group of D. Helfman for the corresponding region of the rat β tropomyosin gene (23).

In constructions Mut14 and Mut39, two AG's (respectively CAG and AAG) added by mutagenesis between the branch point and exon 6B were utilized instead of the wild-type acceptor. This is not caused by intrinsic inability of the wild-type acceptor to be selected in these mutants, since in a construction very similar to Mut14 (MutΔBglII) but lacking the additional AG, the wild-type acceptor was used efficiently. The utilization of such additional AG's is probably more relevant to the existence of a scanning mechanism which would select the first AG (non-GAG) downstream from the branch point, as proposed on the basis of statistical (15, 30, 33, 43), in vivo (12), and in vitro (17, 45) splicing studies.

Our results allow us to exclude a simple relationship between the abnormal location of the branch point and the skipping of exon 6B in myoblasts. In fact, in mutant Mut39, an acceptor site located in a different context but at about the same distance from the branch point (94 nt versus 105 nt) was utilized in a constitutive manner. The same result was obtained with mutant Mut16, in which only the first 14 nt of the exon have been changed while the branch point location is the same as in the wild type. Similarly, in mutant MutΔBglII, in which the distance from the branch point to the acceptor is still different from the canonical one (63 nt), the utilization of exon 6B was again a major splicing pathway. These experiments clearly show that the selection of the branch point upstream of the exon 6B acceptor is relatively independent of its position but dependent on sequence elements located in the skeletal muscle-specific exon and in the upstream intron.

Similarly, the mutually exclusive character of the two alternative exons is not directly (or solely) related to branch point location. Our mutants Mut16 and Mut39 did not differ significantly as far as the branch point location was concerned (respectively at positions −105 and −94 from the AG of exon 6B) but differed in the mutually exclusive behavior of the two alternative exons. In Mut16, exon 6A was spliced to exon 6B in about 40% of the mature transcripts, while in Mut39 no detectable joining of the two exons was observed. In mutant MutΔBglII, in spite of the reduced distance between the branch point and the acceptor (63 nt), 6A to 6B splicing was less efficient than in Mut16.

Though we have reported previously (17) that the abnormal location of the branch point can lower in vitro the efficiency of the second splicing step for the joining of exon 6A to exon 6B, it is possible either that this reduced efficiency is not relevant in vivo or that additional factors exist in vivo which are effective in overcoming such a constraint. Moreover, it should be noted that in the mutant construction tested in vitro (p54) (17), the sequence between the branch point and the acceptor is different from that of Mut39 and Mut16 and that the only possible splicing pathway is splicing of exon 6A to exon 6B, since exon 5 is absent. Therefore, it is possible that in this particular construction, spliceosome formation upstream of exon 6B is possible but splicing of exon 6A to exon 6B is still forbidden, as in Mut39.

We show that a number of *cis*-acting elements are responsible for the skipping of exon 6B in myoblasts or in transiently transfected myotubes. Mutations or deletions in at least two regions around the acceptor site of exon 6B caused the constitutive utilization of the exon. First, a negative controlling element is located at the beginning of exon 6B and was eliminated by the mutation of 15 nt in Mut16. The second element can be located in the intron between position −13 and position −62 upstream of the acceptor and has been deleted in mutant MutΔBglII or mutated in Mut39. A negative sequence element located in a similar position has been very recently described by D. Helfman et al. (23) for the rat gene. Note that the two regions are not overlapping and that sequence modifications in only one permit selection of the wild-type branch point and splicing to the first downstream AG acceptor.

It is possible that such sequence modifications interfere with the binding of a myoblast-specific factor whose presence would be necessary for skipping of exon 6B. A number of arguments can be raised against this hypothesis. First, the putative target for this factor would be considerably larger (at least 75 nt) than the binding sites of known transcription or splicing factors. Second, it would be difficult to explain the myoblast-specific splicing pattern associated with an overproduction of the primary transcript in transiently transfected myotubes. We have previously proposed (27) that a muscle-specific factor is titrated in these conditions, thus permitting by default the myoblast-specific pathway of splicing (skipping of exon 6B). Third, it would not explain the distance effects which we (17; this report) and others (22) observed after mutations in this region of the gene. As already discussed, our mutations around exon 6B had a variable effect on the utilization of exon 6A, while in vitro splicing studies of similar constructions (17) have shown that the excision of the intron between exons 6B and 7 is strongly enhanced in mutated constructions. In addition, studies from Helfman's laboratory on the splicing of the similar rat β tropomyosin gene (22) have shown that joining by mutagenesis exons 6B and 7 (in our terminology) causes constitutive utilization of the acceptor site of the joined exons 6B-7 versus the utilization of exon 6A.

Our results are best explained by the existence of a secondary structure of the primary transcript around exon 6B which prevents its utilization in the absence of skeletal muscle-specific factors. Part of this structure (or an additional *cis* mechanism) would be responsible for the intrinsic low efficiency of excision for the intron between exon 6A and 6B. Short mutations or deletions would change the folding of the region and permit constitutive utilization of exon 6B while partially disrupting the mutually exclusive character of the two exons.

Such a secondary structure would act by preventing splicing factors from interacting with the branch point upstream of the skeletal exon and/or by weakening its efficiency in the competition with the branch point upstream of exon 6A for the donor site of exon 5. In a muscle environment, the structure would be disrupted by factor binding and spliceosome formation between the donor site of exon 5, and the branch point upstream of exon 6B would be the preferred choice. The location of the branch point could be important to avoid interference between the binding of muscle factors and spliceosome assembly or binding of splicing factors and disruption of the secondary structure.

We have previously proposed a model of secondary structure based on computer predictions (26). It is worth noting that this secondary structure is conserved for the

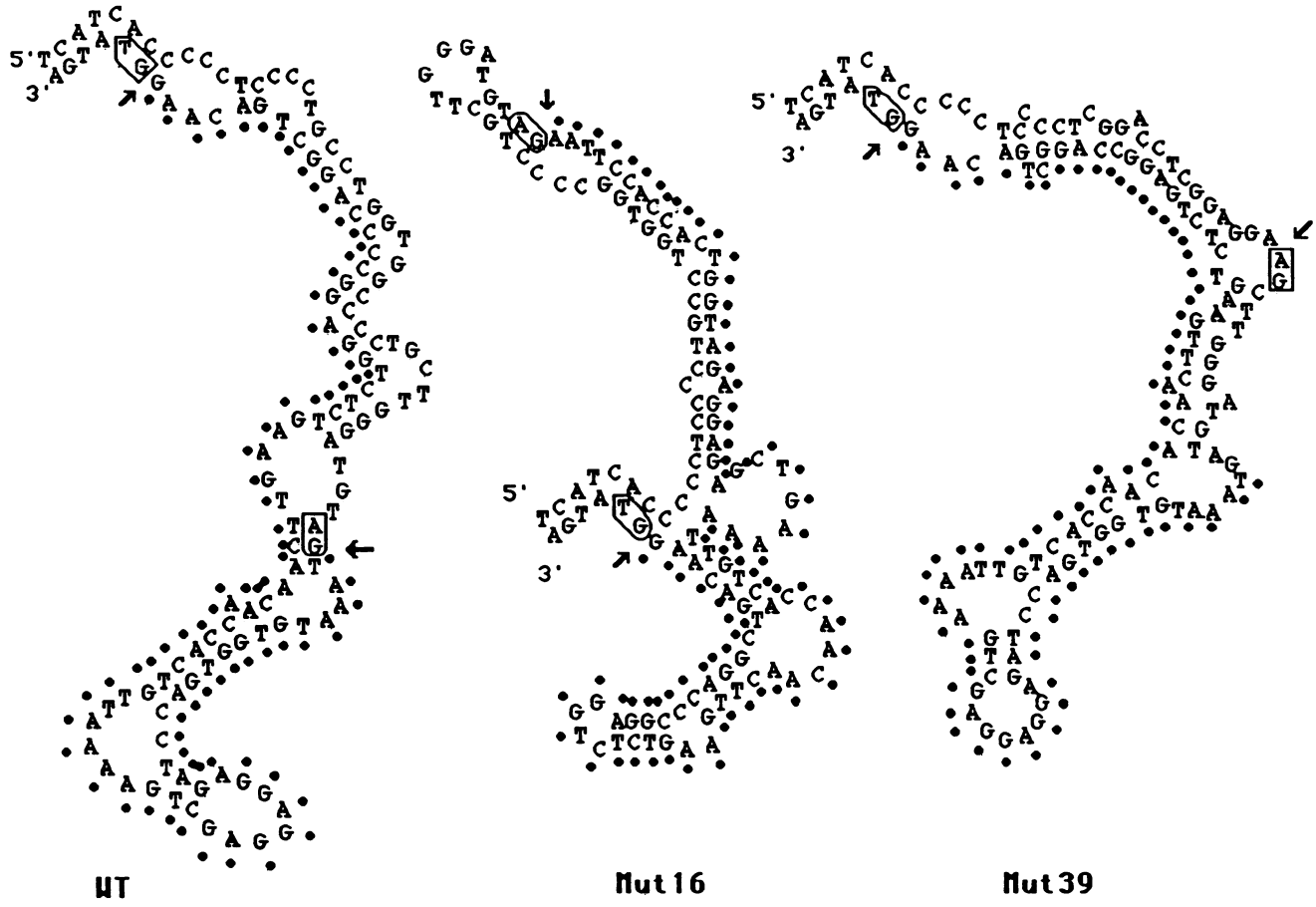

FIG. 6. Comparison of the computer-predicted secondary structures around exon 6B in the wild type, in mutant Mut16, and in mutant Mut39. Only the regions of the three transcripts with a different folding are shown. The rest of the structure is identical in the three cases. Exon sequences are indicated by asterisks; donor and acceptor sites are boxed.

corresponding region of the rat gene, as pointed out by Helfman and co-workers (23). As shown in Fig. 6, the modifications introduced in the primary sequence of Mut16 and Mut39 would change the theoretical folding of the region. Even if we cannot assess that such theoretical folding modifications are the real molecular basis for the change in the splicing pattern, it is possible that they would enhance the accessibility of exon 6B acceptor and/or the binding of splicing factors to the branch point/acceptor region.

The experiments described in this report are not fully in accordance with the folding shown (Fig. 2B) as a whole, since the sequence modifications introduced in mutants Mut15 and Mut35 were ineffective in unmasking the skeletal muscle-specific exon, despite the expected implication of these regions in stable base-pairing in the model. This result is not surprising since, as we have previously pointed out (26), a number of potentially important factors are not taken into account in computer prediction. However, it should be noted that if the sequence mutated in Mut35 is a positive element necessary to the constitutive (or alternative) splicing machinery for expression of exon 6B, it is possible that the negative effect introduced by the mutation is dominant and masks the positive effect due to the disruption of the secondary structure. Such positive elements have been described for both constitutive (36) and alternative (7, 19, 28) splicing.

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