RNA Secondary Structure Repression of a Muscle-Specific Exon in HeLa Cell Nuclear Extracts

BEATRICE CLOUET D'ORVAL, YVES D'AUBENTON CARAFA, PASCAL SIRAND-PUGNET, MARIA GALLEGO, EDWARD BRODY, JOELLE MARIE

The chicken β-tropomyosin pre-messenger RNA (pre-mRNA) is spliced in a tissue-specific manner to yield messenger RNA's (mRNA's) coding for different isoforms of this protein. Exons 6A and 6B are spliced in a mutually exclusive manner; exon 6B was included in skeletal muscle, whereas exon 6A was preferred in all other tissues. The distal portion of the intron upstream of exon 6B was shown to form stable double-stranded regions with part of the intron downstream of exon 6B and with sequences in exon 6B. This structure repressed splicing of exon 6B to exon 7 in a HeLa cell extract. Derepression of splicing occurred on disruption of this structure and repression followed when the structure was re-formed, even if the structure was formed between two different RNA molecules. Repression leads to inhibition of formation of spliceosomes. Disrupting either of the two double-stranded regions could lead to derepression, whereas re-forming the helices by suppressor mutations reestablished repression. These results support a simple model of tissue-specific splicing in this region of the pre-mRNA.

DURING THE GROWTH OF AN ORGANISM, PROTEINS THAT are present only in a particular tissue or stage of development are synthesized. Frequently, multiple different, yet closely related, proteins are generated by the modulation of splicing of a single pre-mRNA species. Various exons may be included or excluded from the splicing apparatus, but how such tissue-specific regulation of pre-mRNA splicing occurs is not well understood.

The chicken β-tropomyosin pre-mRNA is spliced in a tissue-specific manner to yield mRNA's coding for different isoforms of this protein (*1*). Unrelated to transcription initiation or RNA processing elsewhere in the pre-mRNA molecule, exons 6A and 6B are selected in a tissue specific, mutually exclusive fashion (*2*). Exon 6B is included in skeletal muscle, whereas exon 6A is present in all other tissues (Fig. 1). In a HeLa cell nuclear extract, a synthetic precursor containing exon 6A, intron (intervening sequence, IVS AB), exon 6B, intron (IVS B7), and exon 7 in that order gives predominantly exon 6A splicing to exon 7; exon 6B is seen as part of a large intron (Fig. 1). Deleting or substituting part of the long polypyrimidine stretch by pBR322 sequence in the 3′ portion of IVS AB leads to a stimulation of 6A-6B splicing (*3*). These mutations lead as well to a stimulation of 6B-7 splicing, even though they are in the intron upstream of exon 6B (*3*). Two different, but not mutually exclusive, explanations for this result can be proposed. First, stimulation of 6A-6B splicing, concomitant with a diminution of 6A-7 splicing, might stimulate 6B-7 splicing as a consequence of competition for splice sites. Second, the mutations in IVS AB might disrupt pre-mRNA secondary structure which sequesters exon 6B. Disrupting this structure would allow IVS B7 to be removed during splicing of exon 6B to exon 7 (Fig. 1). Although splice site competition may play a role in promoting 6B-7 splicing, we now show that in the absence of competing splice sites, RNA secondary structure can determine whether this muscle specific splice takes place.

IVS AB RNA repression of 6B-7 splicing in cis. In order to prove that RNA secondary structure can affect 6B-7 splicing, we eliminated the 5′ splice site, as well as two cryptic 5′ splice sites from the upstream region of IVS AB. The resulting pre-mRNA, transcribed from this plasmid, pPmac, includes the branchpoint of intron IVS AB as well as the pyrimidine rich segment of 96 nucleotides (nt) just downstream of this branchpoint and the final 9 nt of IVS AB; this sequence is followed by exon 6B, IVS B7, and exon 7 in that order (Figs. 1 and 2A). This pre-mRNA is almost completely inactive as a substrate for the splicing reaction that removes IVS B7 (Fig. 2B). In contrast, a pre-mRNA, transcribed from the pSma plasmid (*4*), which has at its 5′ end only the last 9 nt of IVS AB in front of exon 6B, IVS B7, and exon 7, is spliced very efficiently in vitro (Fig. 2B). In these experiments there are no

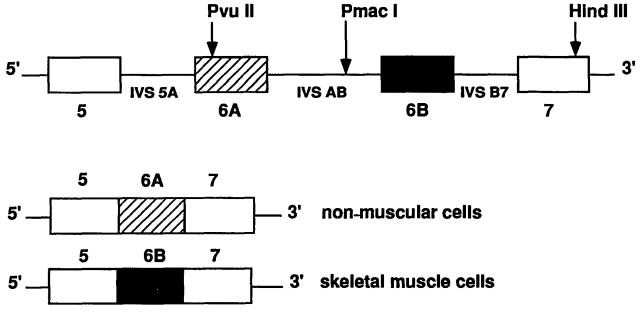

Fig. 1. Internal organization of the chicken β-tropomyosin gene showing the two mutually exclusive exons. Open boxes represent common exons. The hatched box represents the non-muscular exon 6A. The filled box represents the skeletal muscle-specific exon 6B. Exon 5, 71 nt; IVS 5A, 218 nt; exon 6A, 76 nt; IVS AB, 366 nt; exon 6B, 76 nt; IVS B7, 124 nt; exon 7, 63 nt. The Pvu II–Hind III restriction fragment was inserted into the Sma I–Hind III sites of the pSP65 vector to obtain the pSP65 700 construct (*3*). The Pmac I restriction site used to generate the pPmac transcript is indicated.

The authors are at the Centre de Génétique Moléculaire, C.N.R.S. associated with the Université de Paris VI, Gif-sur-Yvette, 91198 France.

competing reactions for utilization of the 5′ and 3′ splice sites of IVS B7. The 159 nt in IVS AB between the 5′ ends of pPmac RNA and pSma RNA suffice to suppress splicing of the next downstream intron, IVS B7.

To localize the repressing elements in this 159-nt segment, we constructed sequential deletions of the pPmac plasmid starting at the Pmac I site and progressing rightward into the IVS AB toward exon 6B (Fig. 2A). In vitro splicing of these deleted pre-mRNA's shows that removal of about 110 nt starting from the Pmac I site (which corresponds approximatively to the mutant d27) has only a small effect on the derepression of splicing of the IVS B7 intron. This derepression becomes substantial when deletion of the 50 nt upstream of exon 6B begins (Fig. 2, B and C). Removing the branchpoint of IVS AB does not affect the repression. This repression cannot be due to the differences in size of the expanded exon 6B among the pPmac, the deleted mutants, and pSma constructs. When different fragments of pBR322 DNA are cloned upstream of the Sma I site, the resultant synthetic pre-mRNA's contain extended exons 6B of the same length as the extended exon 6B of pPmac. In vitro splicing of these pre-mRNA's takes place at rates between 75 and 100 percent of that of pSma splicing (*5*).

Since the polypyrimidine stretch sequence between 9 and 50 nt upstream of exon 6B seems critical in the derepression of IVS B7 splicing, we sought to remove these sequences in the pPmac construct. If binding of a factor to these sequences is needed to repress splicing of the IVS B7 intron, deleting these sequences might prevent fixation of the factor, and consequently splicing of the IVS B7 intron might be derepressed. In the pPmac construct,

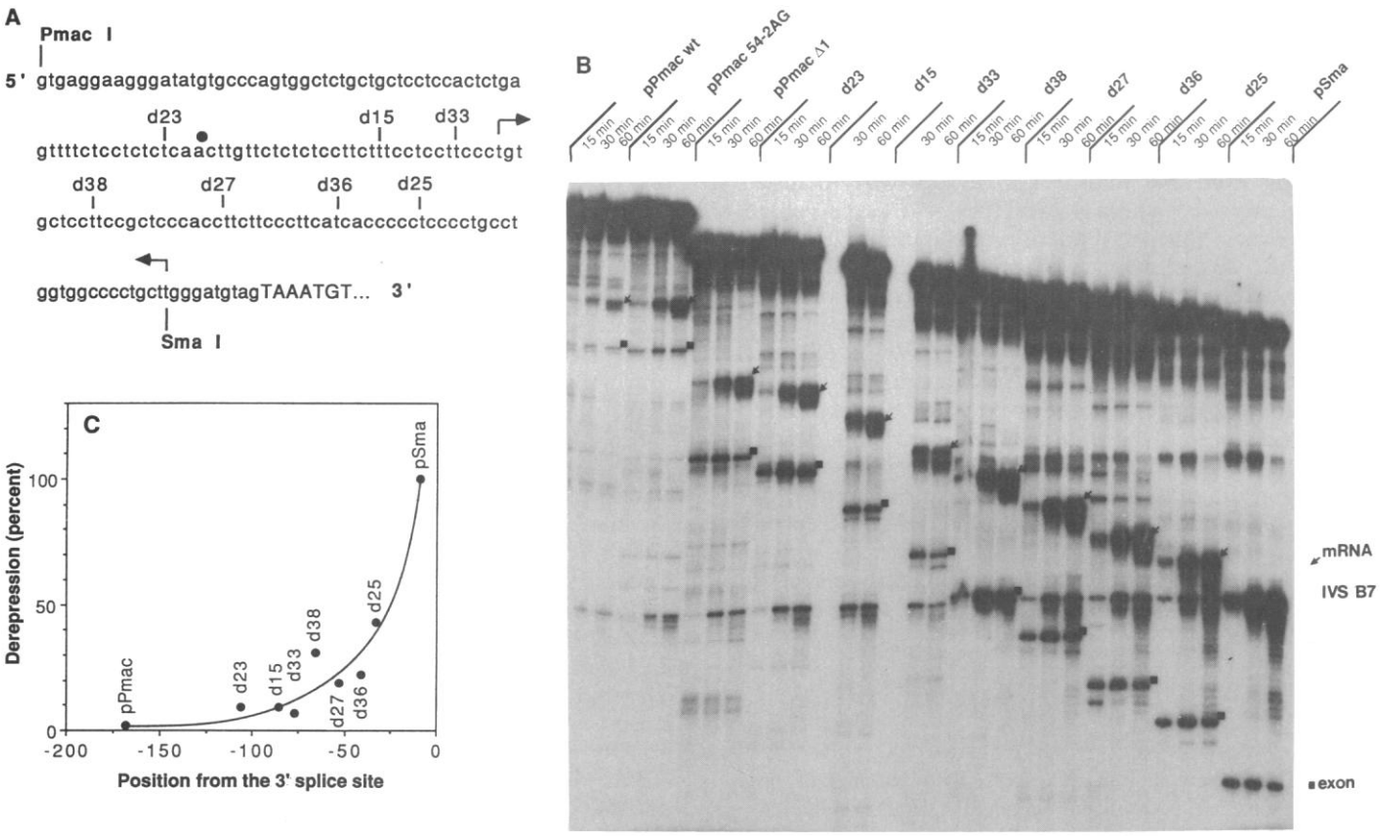

Fig. 2. Deletion of IVS AB sequences derepresses the splicing of exon 6B to 7. (**A**) Nucleotide sequence of IVS AB in the pPmac pre-mRNA. The pPmac plasmid was derived from the pSP65 700 by subcloning the Pmac I–Hind III restriction fragment into the Sma I–Hind III sites of the pSP65 vector. The Pmac I site starts 168 nt upstream of exon 6B. Plasmids containing deletions from the Pmac I site toward exon 6B were obtained from the pSP65 700 vector by digestion with Pmac I and Sac I restriction enzymes. The DNA was treated with exonuclease III and then digested by nuclease S1. Klenow fragment of DNA polymerase I was then added to generate blunt ends. The DNA was then ligated with T4 DNA ligase. Sequences of all deletion mutants were determined by double-stranded DNA sequencing (*33*). The arrows indicate the polypyrimidine sequence which has either been deleted in the pPmac Δ1 construct or replaced by a pBR322 sequence in pPmac 54-2AG. For these two mutants, previous constructs were used in which deletion of 65 nt of the pyrimidine rich sequences or replacement by 63 nt of pBR322 had already been done (*3*). From these plasmids, p700 Δ1 and p700 54-2AG, the Pmac I–Hind III restriction fragment was subcloned into the Sma I–Hind III sites of the pSP65 vector. The pSma I precursor was generated from the p700 Δ1 by subcloning the Sma I–Hind III restriction fragment into the Sma I–Hind III sites of the pSP65 vector. Lower case letters, intron nucleotides; upper case, exon 6B. The Sma I restriction site indicates the 5′ end of the pSma precursor. The dot indicates the position of the branchpoint in IVS AB. Positions of deletion mutants obtained by exonuclease III digestion are indicated. The lengths of the various transcripts from pPmac to pSma were 436, 375, 353, 345, 333, 320, 308, 300, and 276 nt. (**B**) In vitro splicing reactions of the different precursors. ^{32}P-labeled precursors were made by in vitro transcription, and spliced in 60 percent (v/v) HeLa cell nuclear extract with 1 mM $MgCl_2$ (*3*). The splicing products were analyzed on a 6 percent denaturing polyacrylamide gel. In this figure, the mRNA and IVS B7 intron of the pSma precursor migrate at the same position. For quantification of these data, these products were analyzed on different percentage sequencing gels in order to separate the mRNA and IVS B7 intron. This allowed us to know what portion of the total radioactivity was contributed by each product. (**C**) Graphic representation of derepression of splicing of the IVS B7 intron. The Y axis represents the percent of derepression observed for the deletion mutants relative to the pSma precursor. This value was arbitrarily set at 100 percent for the pSma precursor. Since the final lariat is of constant length, whereas the various mRNA's are not, the quantification of final lariat production was presented here. The quantification of mRNA (length corrected) gives a similar pattern. Derepression was calculated as the amount of spliced IVS B7 intron product divided by the amount of input precursor RNA. Scanning of the autoradiographs was done with a micro densitometer (Vernon Phi 5) from a lighter exposure than the one shown in (B). The *x*-axis represents the position of the deletion mutants in the IVS AB intron starting at the Pmac I site and progressing toward exon 6B.

internal deletion of part of the pyrimidine-rich region that leads to the pPmac Δ1 precursor has only a small effect on the derepression of IVS B7 splicing (Fig. 2B). In the same way, the substitution of part of the polypyrimidine stretch by a pBR322 sequence in the pPmac 54-2AG precursor also has a minimum effect (Fig. 2B). In these two mutated precursors, the branchpoint and the 29-nt polypyrimidine stretch just downstream of the branchpoint have been maintained. The reason why these two precursors are repressed is discussed below. As expected from the results with the exonuclease generated deletions, deleting the IVS AB branchpoint hexanucleotide from pPmac pre-mRNA did not affect the repression of IVS B7 removal (*5*).

IVS AB RNA repression of 6B-7 splicing in trans. Since the size and the functional sequence elements of this 159-nt region of RNA cannot account for its ability to repress splicing of the downstream IVS B7 intron, we investigated its role in forming the secondary structure of the repressed pre-mRNA. This was of interest because this sequence, which contained the polypyrimidine stretch, has been predicted to form a stable RNA-RNA duplex with a G rich region in the middle of IVS B7 (*1*). Therefore, RNA from the Pmac I site to the 3′ splice site of IVS AB (pRep RNA) and pSma pre-mRNA were transcribed from separate plasmids. When unlabeled pRep RNA was hybridized to labeled pSma pre-mRNA, splicing of exon 6B to exon 7 was completely repressed even at a molar ration of pRep RNA to pSma RNA of 2:1 (Fig. 3A) (*6, 7*). As a control, we used an RNA complementary to pRep, called pARep. Incubation of this RNA with the pSma precursor only slightly affected splicing of the IVS B7 intron, except at very high ratios of pARep to pSma RNA where some nonspecific inhibition of splicing was seen (*8*).

The transrepression depended on incubation of the two RNA's together before the addition of the nuclear extract. If nuclear extract was added to the pSma pre-mRNA before the unlabeled pRep RNA, no inhibition was seen. The repression of splicing of the IVS B7 intron was due to a hybrid formed between the pRep RNA and the pSma precursor (Fig. 3B). For this experiment, splicing reactions were performed after hybridization of ^{32}P-labeled pRep or pARep RNA's to unlabeled pSma precursor. When we analyzed the reactions on native gels, only hybridization of the pRep RNA to the pSma RNA led to an RNA-RNA duplex, which remained stable even during incubation with nuclear extract (Fig. 3B). Incubation of pARep RNA with pSma pre-mRNA did not lead to formation of an RNA-RNA duplex. The transrepression was specific for sequences that were in the distal portion of IVS AB (pRep RNA). A 2′OCH$_3$ oligonucleotide, whose sequence was identical to the last 15 bases at the 3′ end of pRep RNA, inhibited pSma pre-mRNA splicing to

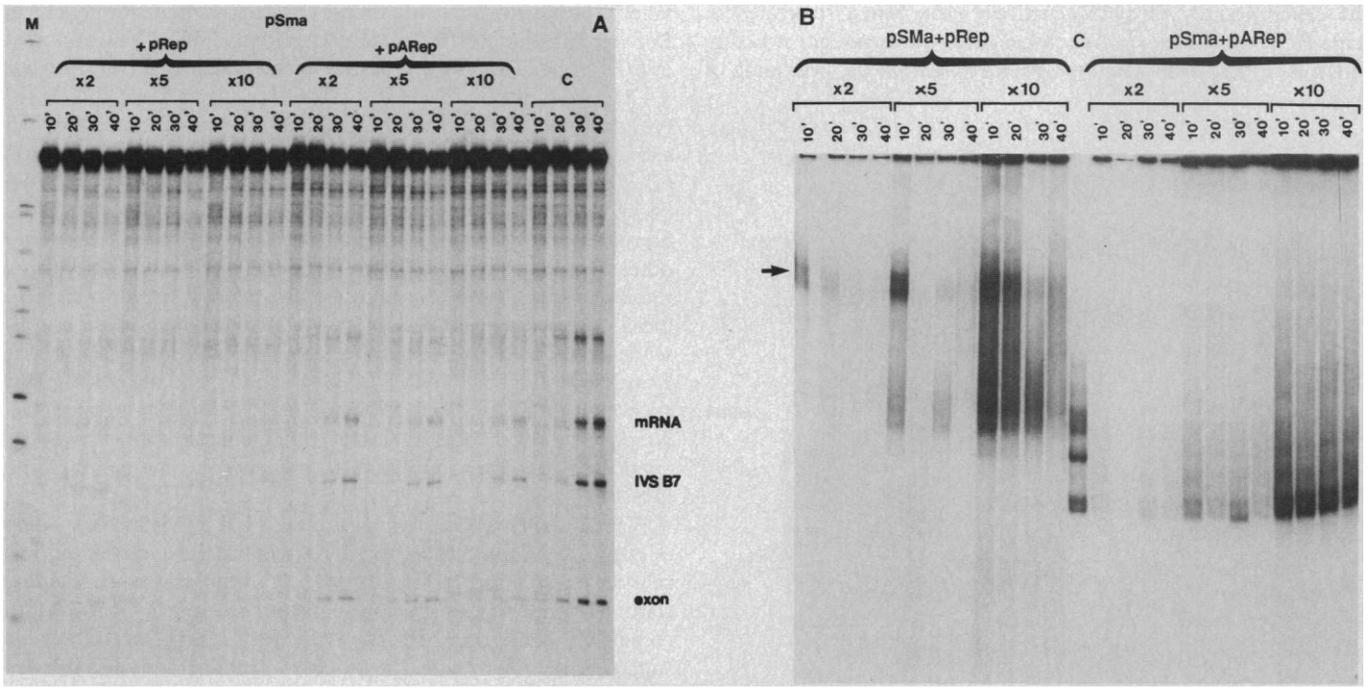

Fig. 3. Repression of IVS B7 splicing in trans by an RNA from the upstream IVS AB intron. (**A**) Analysis of the splicing products. The pRep and pARep fragments were derived from the mutant pPmac Mut16 (*12*) (Fig. 4) by subcloning the Pmac I–Eco RI restriction fragment into a pGEM3Z vector. Changing the first 16 nt of exon 6B in the construction of the mutant Mut16 introduces an Eco RI restriction site at the junction of IVS AB–exon 6B. Unlabeled capped pRep and pARep precursors were obtained by in vitro transcription with, respectively, SP6 or T7 RNA polymerase. The RNA's were purified on a 4.5 percent denaturing polyacrylamide gel and quantified by ethidium bromide staining. In vitro transcribed unlabeled RNA's were incubated with ^{32}P-labeled pSma substrate (10^5 cpm; 0.02 pmole) in molar ratios of 2:1, 5:1, and 10:1, in 10 μl of 20 mM Hepes (pH 7.6), 100 mM KCl, 5 mM $MgCl_2$ at 56°C for 5 minutes and slowly cooled to 30°C. The RNA's were then diluted up to 50 μl in splicing conditions with HeLa cell nuclear extract (to 40 percent v/v). C indicates the splicing experiment with pSma in the absence of pRep or pARep; the ^{32}P-labeled markers (M) of Hpa II–digested pBR322 DNA are at the left. Splicing reactions were carried out for 10, 20, 30 and 40 minutes. The splicing products were then fractionated on a 5 percent denaturing polyacrylamide gel. (**B**) Formation of a hybrid between pRep RNA and pSma precursor RNA is involved in the repression of IVS B7 splicing. The experimental conditions are the same as in Fig. 3A except that pRep or pARep RNA's were ^{32}P-labeled and the pSma precursor RNA was unlabeled. For analysis of the behavior of pRep RNA and for avoiding confusion of hybridization with entry into splicing complexes, the splicing reactions were treated with proteinase K and extracted with phenol (*3*). Labeled pRep or pARep RNA's were then analyzed on a 6 percent native polyacrylamide gel (acrylamide:bis = 38:2) at 4°C. The arrow indicates the hybrid formed between pRep and pSma RNA. Lane C represents pRep and pARep mixed together. The level of hybridization correlates with the repression of splicing of the IVS B7 intron up to 20-minute incubation time. Longer incubation times induce a disruption of the RNA-RNA duplexes with a concomitant small activation of the splicing reaction. This disruption could be due to an unwinding activity present in HeLa cell nuclear extracts (*34*).

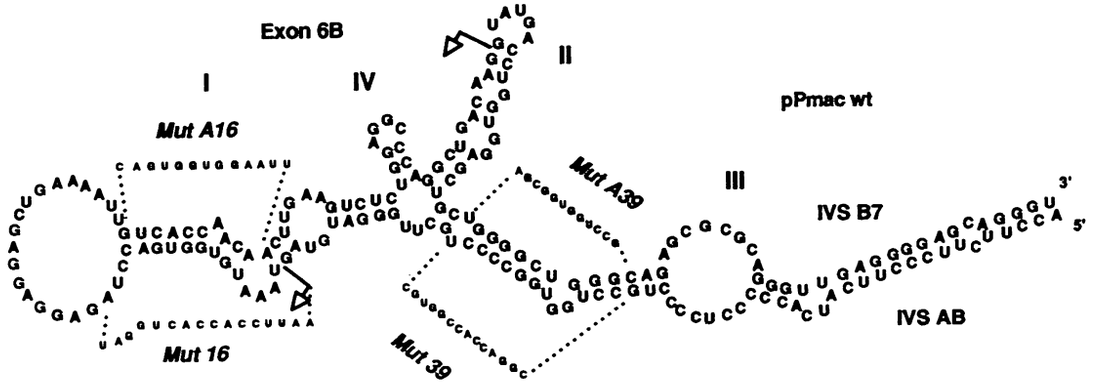

Fig. 4. Experimentally determined secondary structure of the region around exon 6B in the pPmac construct. The structure was determined with the use of enzymatic and chemical probes. The structure shows the last 70 nt of IVS AB, exon 6B, and the first 60 nt of IVS B7. The arrows indicate the junctions intron-exon. The nucleotides that have been changed in various mutant precursors are indicated. I, II, III, and IV refer to the stem loop structures.

about 50 percent of that of the control (*5*); this was what would be expected from the exonuclease digestion data shown in Fig. 2C. In contrast, a 2′OCH_3 19-nt fragment that hybridized to exon 6B (forming a duplex with the large loop of stem-loop I shown in Fig. 4) had no effect on pSma pre-mRNA splicing (*5*).

Effect of secondary structure in and around exon 6B on IVS B7 splicing. Although calculated secondary structures are useful when experiments are planned, it is not always possible to predict the experimentally or phylogenetically determined structures of large RNA molecules (*9*). We investigated the secondary structure of the β-tropomyosin wild-type pre-mRNA from the beginning of exon 6A to the end of exon 7, as well as the structure of various mutant pre-mRNA's. This was done by enzymatic and chemical probing of these RNA's (*10*). We record (Fig. 4), on the basis of such probing (*11*), the structure of a segment of pre-mRNA starting 70 nt upstream of exon 6B (thus, in the long pyrimidine stretch in the distal portion of IVS AB) and finishing in the purine rich region of IVS B7.

With respect to the experiments presented, we point out some salient features of this structure: (i) the pyrimidine rich region of IVS AB forms an RNA-RNA duplex with the purine rich region of IVS B7 (stem III). It's overall ΔG of formation is predicted to be −47.6 kcal/mol). (ii) There is a stem loop structure (stem I, ΔG = −11.1 kcal/mol) which puts the junction region of intron IVS AB and exon 6B into a double helix with a region in the middle of exon 6B. The first 16 nt of exon 6B (which include the distal most 13-bp of stem I) have been implicated in the exclusion of exon 6B in nonskeletal muscle tissue in transfection experiments (*12*). (iii) Two other stem loop structures (stems II and IV) of marginal calculated stability, include exon 6B sequences. Stem II has at its distal point the 5′ splice site of IVS B7.(AG:GU*AUG*A). The AUG of this sequence forms a single-stranded loop. Although not shown, the branchpoint sequence of IVS B7 is in a loop, with the branchpoint adenosine completely susceptible to attack by dimethyl sulfate (*11*). A single-stranded loop structure containing the branchpoint sequence has been found in the constitutively spliced human β-globin IVS 1 (*13*).

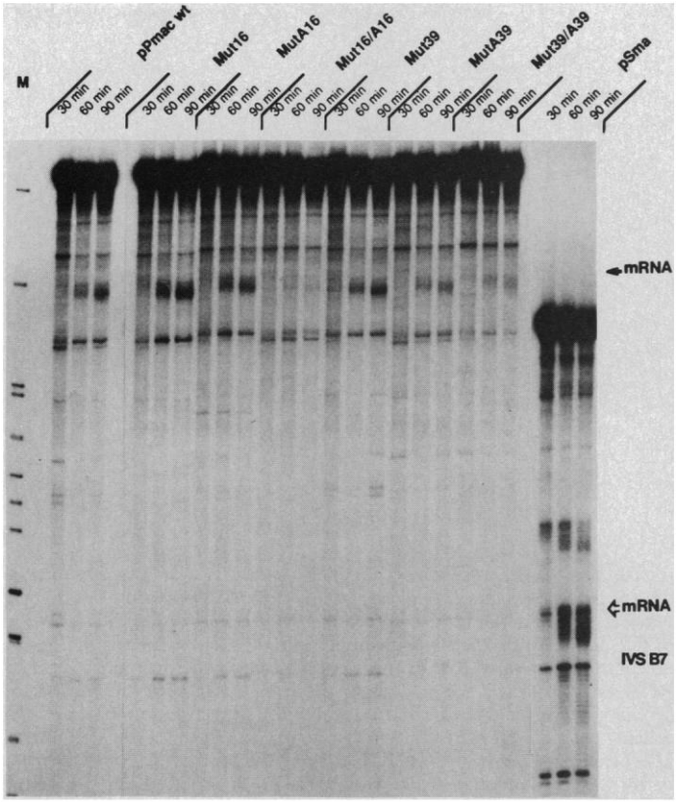

Fig. 5. Mutations introduced into stem I and stem III affect splicing of the IVS B7 intron. Splicing conditions are the same as in Fig. 2B, except that the products of the in vitro splicing reactions were analyzed on a 5 percent denaturing polyacrylamide gel. The black arrow indicates the position of mRNA for the pPmac precursor. The unfilled arrow indicates the position of the pSma mRNA.

We tested the roles of stem III and stem I in repression of splicing of exon 6B to exon 7. The stem I mutation that we introduced was pPmac Mut16 (Fig. 4). It mutates 16 nt of the wild-type structure, starting at the first nucleotide of exon 6B, and is designed to completely disrupt the structure of stem I. The experimentally supported secondary structure of this mutant (*11*) indicates that the exonic structure is changed, but that the interaction of the pyrimidine stretch and purine stretch (stem III) is maintained. Stem I is replaced by a less stable stem; the IVS AB–exon 6B junction is now in the loop at the end of this stem. The exon 6B–IVS B7 junction is now in the middle of a structure resembling stem II, but in a different conformation. Splicing of the pPmac Mut16 pre-mRNA is derepressed for exon 6B–exon 7 splicing (Fig. 5). We disrupted stem I by changing the opposite strand as well; this construction, called pPmac MutA16 (Fig. 4), is also derepressed for removal of IVS B7 (Fig. 5) (*12*). The double mutant pPmac Mut16-A16 was made to restore stem I. The calculated stability of stem I in the double mutant is somewhat greater than that of the wild-type stem I and it is completely repressed for exon 6B–exon 7 splicing, even more so than is the wild-type construct (Fig. 5). Hence, the secondary structure of stem I RNA is capable of regulating splicing

of the downstream IVS B7 intron despite the fact that stem I is far upstream of all the sequence elements thought to be required for constitutive splicing.

We disrupted the proximal portion of stem III (proximal with respect to exon 6B) in like manner. We changed 14 nt in the ascending arm of stem III in the construction pPmac Mut39 (*12*) and 13 nt of the descending arm in the construction pPmac MutA39, and reestablished the RNA-RNA double helix in the double mutant pPmac Mut39-A39 (Figs. 4 and 5). Disrupting stem III in the construction pPmac Mut39 derepresses exon 6B–exon 7 splicing. The pPmac MutA39 has little or no effect and, in fact, this is not entirely unexpected because the calculated structure of the pPmac MutA39 pre-mRNA allows formation of an alternative structure that is almost as stable as the wild-type stem III (*14*). This alternative structure keeps the 5′ splice site of IVS B7 in a wild-type configuration. The double mutant, which reestablishes the wild-type geometry of stem III (it too is stronger than its wild-type counterpart), completely represses splicing. Again, we emphasize that these mutations do not concern any nucleotides of the 5′ splice site, the branchpoint region, or the 3′ splice site of the IVS B7, whose splicing is affected.

That derepression for all the single mutant pre-mRNA's is 10 to 20 percent of the fully derepressed pSma precursor is not so surprising; a complex secondary structure is maintained around exon 6B to prevent its use in a nonmuscular context. Changing one element in this structure may not be enough to completely relieve the inhibitory process.

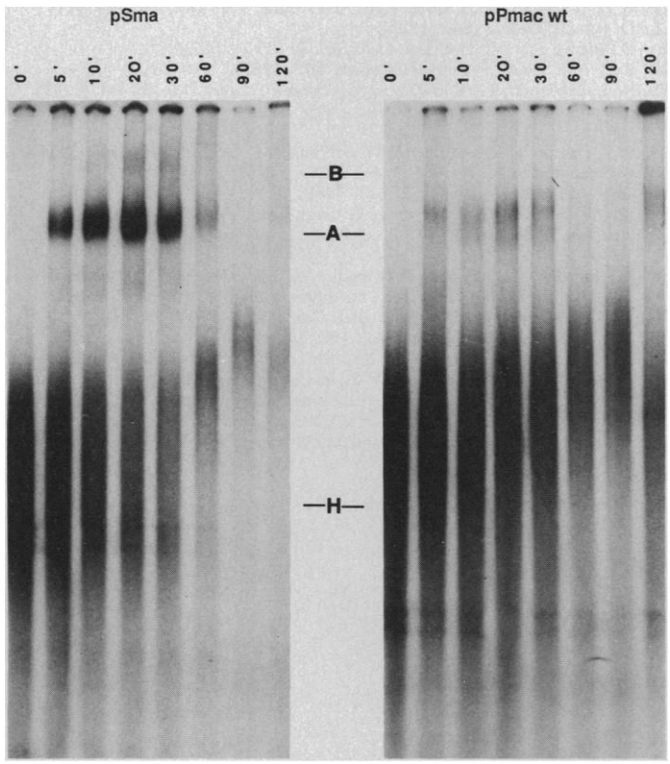

Fig. 6. Spliceosome assembly of the pPmac wild type and pSma splicing reactions analyzed by native gel electrophoresis. For kinetics of spliceosome assembly, 10^6 cpm of ^{32}P-labeled precursors were spliced in 50-μl reaction mixtures under the same conditions given in Fig. 2B. At the indicated times 4-μl portions of each splicing reaction were mixed with 2 μl of heparin (at 15 mg/ml). Samples were loaded onto a 4 percent native polyacrylamide-bisacrylamide (80:1, v/v), 1-mm-thick gel (*35*). Electrophoresis was conducted at room temperature at 4 watts for 4 hours. A, the pre-splicing complex; B, the spliceosome; H, the nonspecific complex.

The two mutants pPmac Δ1 and pPmac 54-2AG, which have been described previously and which affect the stem III can be regarded as pPmac MutA39-like mutants because they retain strong stem III–like structures involving the 29 pyrimidines just downstream of the IVS AB branchpoint (*5*).

In vitro splicing of pre-mRNA takes place on a large complex, the spliceosome, which assembles around the pre-mRNA to promote catalysis in the two splicing reactions (*15*). Mixed polyacrylamide-agarose nondenaturing gel electrophoresis distinguishes an adenosine triphosphate (ATP)–hydrolysis–independent nonspecific complex from at least two ATP-hydrolysis–dependent complexes, A and B (*16*). The A complex, which is a pre-spliceosome, depends on U1 and U2 (small nuclear ribonucleoprotein) snRNP function, ATP hydrolysis, and a number of non-snRNP proteins (*16*). The B complex (and subsequent complexes that form after splicing) represents the full spliceosome (*16*). The pSma splicing is paralleled by the rapid appearance of all three of these complexes (Fig. 6). pPmac pre-mRNA which is repressed for splicing forms the nonspecific ATP-hydrolysis–independent complex. Formation of complex A is as reduced as was the production of splicing intermediates when reaction products were analyzed on a sequencing polyacrylamide gel (Figs. 2B and 6). Thus, repression acts at or before this early step in spliceosome assembly.

RNA structure can repress a muscle-specific splicing event. Variations of splicing of single pre-mRNA can display many patterns. Such variations are an important component of differentiating vertebrate tissues, especially muscle and nerve (*17*); they also contribute to differentiation in nonvertebrates (*18*). In fact, tissue specific splicing may be the direct evolutionary result of primitive, ambiguous splicing systems (*19*). Splice site choice in some other systems can be influenced by the relative strengths and positions of the 5′ splice site (*20*), 3′ splice site (*21*), and branchpoint sequence (*22*), as well as by protein factors (*23*). Berget and co-workers were the first to emphasize that pre-mRNA's with multiple introns and exons are not spliced as linear combinations of the exon 1–IVS-exon 2 motif (*24*). Their experiments suggested positive interactions between the 5′ splice site and the 3′ splice site surrounding an exon; our data extend "exon definition" to include negative interactions.

The role of RNA secondary structure in pre-mRNA splicing is not entirely clear. Exon "skipping" could be induced in vitro in three exon-two intron constructions; artificially engineered strong RNA stems have been made between intronic sequences, thereby putting the middle exon into a loop (*25*). When such constructs were tested in vivo, essentially all exon "skipping" disappeared (*26*). Because eukaryotic nuclei contain many proteins with known (*27*) or purported (*28*) RNA helicase activity, the idea arose that perhaps RNA secondary structure of pre-mRNA was not relevant to splicing, since helicases would in any case undo pre-existing structure. Since then, Eperon and co-workers (*29*) have shown that RNA-RNA duplexes, when they include a 5′ splice site, can repress in vivo splicing. They suggested that the relative kinetics of RNA folding and splice site commitment, perhaps by U1 snRNP interaction (*30*) could determine whether or not a splicing reaction takes place.

Our experiments bear on this controversy. RNA secondary structure generated during the evolution of the chicken β-tropomyosin gene represses splicing of exon 6B to exon 7 in a HeLa cell extract. The two structures, stem I and stem III, whose importance we have shown, did not directly involve either the 5′ splice site, the 3′ splice site, or the branchpoint sequence of the IVS B7 intron. In fact one of the stem structures involves the beginning of exon 6B and the intron IVS AB, far away from the elements of IVS B7 splicing. We should like to emphasize that the repression in trans seen after mixing pRep RNA and pSma pre-mRNA involves only the wild-type sequences of the β-tropomyosin gene. Long, artificial

antisense RNA's that hybridize to exons are capable of inhibiting in vitro splicing of β-globin pre-mRNA (*31*). In one way, such experiments only reinforce the idea that a naturally evolved RNA duplex structure could contribute to a modulated splicing event.

We also know that pPmac and pSma pre-mRNA's have different tertiary structures [determined by probing with Pb(II)]. Hybridizing pRep RNA to pSma pre-mRNA not only reestablishes repression of splicing, it also reestablishes a pPmac-like tertiary structure (*11*). Although we do not understand the mechanism by which the RNA structure of the pre-mRNA leads to repression of IVS B7 splicing, we found that it does this before the formation of the A pre-spliceosome complex, thus very early in spliceosome assembly. Whether these RNA structures involving stems I and III indirectly block access of the 5′ splice site of IVS B7 to U1 snRNP binding remains to be determined. Whether or not a protein is needed to stabilize this secondary structure is an open question. Although our studies are limited to the chicken β-tropomyosin pre-mRNA, other studies (*32*) are compatible with the idea that similar secondary structure masking of the skeletal muscle specific exon takes place in the rat β-tropomyosin pre-mRNA.

Do these pre-mRNA structures really play an in vivo role in the skeletal muscle specific splicing of exon 6B to exon 7? Libri and Fiszman (*12*) have shown that precisely those secondary structures necessary for repressing exon 6B–exon 7 splicing in a HeLa cell extract are necessary for excluding exon 6B from being spliced into mRNA in myoblasts. If RNA secondary structure alone can allow exon 6B to be skipped in nonskeletal muscle cells, potentially simple models of tissue specific splicing arise. If only skeletal muscle possessed a helicase that modified or melted the secondary structure around exon 6B, its presence plus cis competition for splice sites could explain the mutually exclusive tissue-specific splicing in this region. Alternatively, skeletal muscle may specifically lack a factor that stabilizes the secondary structure around exon 6B. This work suggests that searching for such factors could contribute to our understanding of β-tropomyosin tissue specificity.

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3. M. Goux-Pelletan *et al.*, *EMBO J.* **9**, 241 (1990). The intron IVS AB was characterized by a far upstream branchpoint (−105 nt from the 3′ splice site) and a long polypyrimidine stretch. We showed that deletion of 63 nt of the polypyrimidine stretch or its substitution by pBR322 sequences increased not only the splicing of IVS AB, but also the splicing of IVS B7.
4. The pSma plasmid was derived from plasmid pSP65 700 Δ1 (*3*) by subcloning the Sma I–Hind III fragment into the pSP65 vector.
5. J. Marie and E. Brody, unpublished observations.
6. In order to demonstrate stable repression in trans, it is necessary that both pSma RNA and pRep be synthesized with a 5′-7MeGpppG-5′ cap structure. When the pRep RNA is not capped, repression is seen early in the incubation, but is reversed later during the course of incubation. Moreover, higher molar ratios of repressor RNA to pre-mRNA are needed to see even this transient repression. This may be due to a 5′ → 3′ exonuclease activity in HeLa cell nuclear extracts whose activity is inhibited by capping of the 5′ ends of the primary transcript.
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