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RNA 1999 5: 378-394
Exonic splicing enhancers contribute to the use of both 3′ and 5′ splice site usage of rat β-tropomyosin pre-mRNA

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ABSTRACT
The rat β-tropomyosin gene encodes two tissue-specific isoforms that contain the internal, mutually exclusive exons 6 (nonmuscle/smooth muscle) and 7 (skeletal muscle). We previously demonstrated that the 3′ splice site of exon 6 can be activated by introducing a 9-nt polyuridine tract at its 3′ splice site, or by strengthening the 5′ splice site to a U1 consensus binding site, or by joining exon 6 to the downstream common exon 8. Examination of sequences within exons 6 and 8 revealed the presence of two purine-rich motifs in exon 6 and three purine-rich motifs in exon 8 that could potentially represent exonic splicing enhancers (ESEs). In this report we carried out substitution mutagenesis of these elements and show that some of them play a critical role in the splice site usage of exon 6 in vitro and in vivo. Using UV crosslinking, we have identified SF2/ASF as one of the cellular factors that binds to these motifs. Furthermore, we show that substrates that have mutated ESEs are blocked prior to A-complex formation, supporting a role for SF2/ASF binding to the ESEs during the commitment step in splicing. Using pre-mRNA substrates containing exons 5 through 8, we show that the ESEs within exon 6 also play a role in cooperation between the 3′ and 5′ splice sites flanking this exon. The splicing of exon 6 to 8 (i.e., 5′ splice site usage of exon 6) was enhanced with pre-mRNAs containing either the polyuridine tract in the 3′ splice site or consensus sequence in the 5′ splice site around exon 6. We show that the ESEs in exon 6 are required for this effect. However, the ESEs are not required when both the polyuridine and consensus splice site sequences around exon 6 were present in the same pre-mRNA. These results support and extend the exon-definition hypothesis and demonstrate that sequences at the 3′ splice site can facilitate use of a downstream 5′ splice site. In addition, the data support the hypothesis that ESEs can compensate for weak splice sites, such as those found in alternatively spliced exons, thereby providing a target for regulation.

Keywords: E complex; exon definition; purine-rich motifs; SF2/ASF

INTRODUCTION
For most eukaryotic genes, pre-mRNA splicing is an essential step in the production of functional mRNAs that can be translated into their cognate gene product. Splicing also serves as a versatile regulatory mechanism for the posttranscriptional control of gene expression. Through the differential splicing of introns, two or more alternative isoforms of a protein can be produced from the same pre-mRNA transcript. The process of pre-mRNA splicing is carried out by the spliceosome, where, following the initial recognition of splice sites at the 3′ and 5′ ends of exons by splicing factors, two

 transesterification reactions take place, joining together the exons with the concomitant excision of the intron. The cis elements on the pre-mRNA are the 5′ splice site and the 3′ splice site, which also include the branch point and the polypyrimidine tract (Kramer, 1996). The initial pairing of splice sites is a highly regulated process and the various cis and trans elements that are involved in this commitment step are still being characterized. The process of splicing must take place with a high degree of specificity and fidelity and, in spite of these stringent requirements, the cis elements that govern splice site usage have been found to be loosely conserved.

The lack of conservation of splice sites in metazoan pre-mRNAs, combined with the presence of sequences within introns that match splice site consensus equally if not better than the true splice sites, leads to the ques-
tion of how the proper splice sites are chosen and paired (reviewed in Horowitz & Krainer, 1994). A simple answer is that the splicing machinery uses sequence information other than only the splice sites themselves to make an accurate choice. These sequences can either promote or repress splice site usage. cis Elements that promote splice site usage can lie within intron as well as exon sequences in the pre-mRNA. The first observation that exon sequences and splice site proximity play a role in splicing came from studies using model pre-mRNA substrates (Reed & Maniatis, 1986). Later, it was found that activation of a weak 3' splice site of an alternatively spliced exon in β-tropomyosin (β-TM) pre-mRNA requires sequences in the downstream exon (Helfman et al., 1988). Several classes of exonic splicing enhancers (ESEs) have since been characterized in different systems (reviewed in Wang et al., 1997). The best characterized ESE is found in the Drosophila doublesex (dsx) pre-mRNA where the ESE is a 13nt repeat element (dsxRE) and is essential for exon inclusion (Tian & Maniatis, 1993). In higher eukaryotes, ESEs have been characterized in several systems, such as the immunoglobulin M system, cardiac troponin T, bovine growth hormone, and fibronectin (Lavigneur et al., 1993; Sun et al., 1993; Watakabe et al., 1993; Xu et al., 1993). In many of these systems the ESEs are enriched in purines, having a general consensus of (GAR)n. The initial characterization of purine-rich ESEs was in immunoglobulin M, where it was shown that it was necessary for upstream 3' splice site usage. Moreover, sequences containing the purine-rich ESEs could functionally replace the dsxRE in the Drosophila dsx pre-mRNA (Watakabe et al., 1993). In general, deletion or interruption of the ESEs causes skipping/aberrant splicing of the exon from which they were removed.

Evidence has been accumulating that SR proteins function via direct recognition of exon sequences. Initially, SR proteins were found to bind directly to, and be required for, the function of purine-rich elements in the exons of certain alternatively spliced pre-mRNAs (Lavigneur et al., 1993; Sun et al., 1993; Tian & Maniatis, 1993; Ramchatesingh et al., 1995; Yeakley et al., 1996). SF2/ASF can be crosslinked to the last exon of the bovine growth hormone, which contains a purine-rich ESE required for removal of the upstream intron (Sun et al., 1993). SR proteins can also be UV crosslinked to pre-mRNA in the E (early) complex (Staknis & Reed, 1994), and site-specific labeling studies indicate that the crosslinking is to exon sequences. Consistent with this result, Blencowe et al. (1994) have shown that exon-containing splicing intermediates and products are preferentially immunoprecipitated from splicing reactions by using an antibody to SR proteins. In addition, exon sequences can promote U2AF binding, an effect thought to be mediated by SR proteins (Cavaloc et al., 1994).

Among the various roles SR proteins play in splicing, such as in influencing the choice between alternate splice sites (Krainer et al., 1990a; Fu et al., 1992), their function in the commitment of a pre-mRNA to splicing is best understood (reviewed in Fu, 1995; Chabot, 1996; Manley & Tacke, 1996; Valcarcel & Green, 1996). SR proteins are detectable in the earliest pre-spliceosome complex—the E/commitment complex. It has been suggested that SR proteins act in this capacity by recruiting U1 snRNP to weak 5' splice sites. SR proteins have also been shown to be involved in protein–protein interactions. They have been shown to interact with U2AF35, a component of U2AF, and with U1-70K, a component of U1 snRNP, using the two hybrid system and gel overlays (Wu & Maniatis, 1993). Since U2AF interacts with the polypyrimidine tract at the 3' splice site and U1 snRNP interacts with the 5' splice site, a model has been proposed where the interaction of SR proteins both with U1-70K and with U2AF35 can occur across introns as well as across exons (Wu & Maniatis, 1993). Interactions bridging the exon support the exon definition hypothesis. The exon definition model is an alternate view of splice site selection where a choice is made that spans exons instead of introns. According to this model, the strength of a splice site determines not only the removal of the intron in which it is present, but also the removal of the intron flanking the other side of the exon, where splice site pairing is proposed to occur across exons. So, a weak splice site on one side of an exon has an effect on the removal of the intron on the other side leading to an exon skipping phenotype (Roberson et al., 1990; Berget, 1995).

Because of the overwhelming evidence that SR proteins bind to exon sequences, it was the interest of several groups to derive a consensus binding site for each of the SR proteins. Using SELEX with assays based on selection of binding sites, consensus sequences were derived for several SR proteins. These results confirmed that one of the high affinity binding sites of SF2/ASF, a member of the SR protein family, was purine rich and similar to the (GAR)n consensus (Tacke & Manley, 1995). More recently, the SELEX procedure was used based on splicing activity to look for functional sites of interaction of several SR proteins. A class of enhancers for SF2/ASF with the consensus sequence of SRSASGA (S represents G/C, R represents G/A) was derived using a score matrix program (Liu et al., 1988). Although degenerate, this sequence contains the flexibility to have a purine-rich consensus sequence.

The β-TM gene comprises 11 exons spanning 10 kb. The pre-mRNA encoded by this gene is alternatively spliced to yield two isoforms: a skeletal muscle-specific isoform corresponding to skeletal muscle β-TM and a non-muscle/smooth muscle specific isoform called TM-1 in fibroblasts or smooth muscle. Exons 1–5, 8, and 9 are the common exons expressed in both isoforms. Exons 7 and 10 are skeletal muscle-specific exons and
exons 6 and 11 are the non-muscle/smooth muscle-specific isoforms (Helfman et al., 1986). We have focused on the regulation of the mutually exclusive splicing event of exons 6 and 7. It has been shown that the repression of the skeletal muscle-specific exon 7 in non-muscle cells is not because of cis competition but rather via inhibitory sequences at its 3’ splice site (Helfman et al., 1988, 1990; Guo et al., 1991). Previous studies of the alternative splicing of exon 6 revealed that pre-mRNAs containing exon 5-intron 5-exon 6 (E5-i5-E6) of the rat β-TM gene cannot be spliced by HeLa nuclear extract in vitro. This inability of the 3’ splice site of exon 6 to be utilized can be overcome by three different modifications: (1) first joining exon 6 to the downstream exon 8 (Helfman et al., 1988), (2) introducing a 9-nt poly(U) tract upstream of the 3’ splice site of exon 6, and (3) strengthening the 5’ splice site of exon 6 to a U1 consensus binding site (Takahara et al., 1994; Fig. 1A). Additionally, it was seen that splicing of exon 5 to exon 6 was competed by the addition of exon 6/8 sequences and not by other sequences, indicating the importance of these exon sequences (Takahara et al., 1994). Examination of the sequences in exon 6 and exon 8 revealed the presence of several purine-rich motifs. Two are present in exon 6 and three in exon 8 of the general consensus GARGAR (Fig. 1B). The purine-rich sequences described here are reminiscent of those characterized in the cardiac troponin T gene (Xu et al., 1993), which have been shown to act as ESEs.

In this report, we show that only certain motifs act as ESEs and contribute to downstream splice site usage in vitro and in vivo and that they exert their effect before the A-complex stage of spliceosome assembly. We also show through UV crosslinking that SF2/ASF can interact with these sequences. Furthermore, we show that the sequences in exon 6 play an important role for both 3’ and 5’ splice site usage. Our data involving sequences in and around exon 6 provide an insight into the exon definition model.

**FIGURE 1.** Internal exons of β-TM and regulation of their splicing. A: Activation of exons in their respective tissue types are shown by arrows. Exons 6 and 7 are the alternatively spliced exons being present in β-TM mRNA in smooth/nonmuscle cells and skeletal muscle cells, respectively. B: Organization and localization of ESEs in exons 6 and 8. Representation of ESEs in exons 6 and 8 are shown as the striped bars within the exon. Two motifs, 6-1 and 6-2, are present in exon 6 and three motifs, 8-1, 8-2, and 8-3, are present in exon 8. The general consensus of the motifs is GARGAR and mutagenesis of the motifs was made by substituting TCT for the first, fifth, and seventh nucleotides, respectively, changing the motif to TARTARTAR. Mutagenesis was done using oligonucleotides and PCR.
RESULTS

Several purine-rich motifs are present within exons 6 and 8

Previous observations revealed a role for sequences within exons 6 and 8 with respect to 3' splice site usage of exon 6 (Helfman et al., 1988; Tsukahara et al., 1994). Inspection of sequences in exons 6 and 8 revealed the presence of five purine-rich motifs (Fig. 1B) similar to ESEs characterized in other systems (Lavigne et al., 1993; Sun et al., 1993; Watakabe et al., 1993; Xu et al., 1993). Two of the motifs are present in exon 6, termed 6-1 and 6-2, and three of the motifs are in exon 8, termed 8-1, 8-2, and 8-3. We directed our studies to these purine-rich motifs to determine if they play a functional role in splice site usage. To disrupt the purine content of the motifs, three Pu → Py substitutions were made in each repeat (see Fig. 1B for details of substitutions).

Certain purine-rich motifs in exons 6 and 8 act as exonic splicing enhancers

Splicing efficiency of the different pre-mRNAs, mutated at individual purine-rich motifs, was analyzed using an in vitro splicing assay (Fig. 2). Mutations in motifs 6-1, 6-2, and 8-2 are the most deleterious for the removal of intron 5 (Fig. 2, lanes 2, 3, and 5). Furthermore, when mutations to motifs 6-1, 6-2, and 8-2 are introduced together, they abolish 3' splice site usage completely (Fig. 3, lane 10). Motif 8-1 contributes the least to 3' splice site usage. This is not surprising, as this element contains the sequence AAAGAAGAC and is the most divergent from the canonical GARGARGAR repeats, as seen in Figure 1B. These results demonstrate that only some of the purine-rich motifs within exons 6 and 8 are required and they act in combination with each other. So, in spite of the motifs having a similar consensus, the fact that certain motifs are required for 3' splice site activation implies a dependence on context for the purine-rich motifs to have an effect on 3' splice site usage.

Wild-type exon 6/8 sequences can inhibit splicing of exon 5 to exon 6

ESEs have been shown to play a role in the initial commitment to splicing of a pre-mRNA in other systems. Since the purine-rich ESEs in exons 6 and 8 are essential for 3' splice site usage of exon 6, we hypothesized that they were recognized by a nuclear factor that acted at the E/commitment complex step. To address this hypothesis, a splicing-competition assay was carried out where wild-type and mutant competitor RNAs were preincubated with nuclear extract prior to the addition of ATP/creatine phosphate (CP; ATP is required for A-complex formation but not for E-complex formation) and substrate RNA. The RNA competitors used in these studies spanned exons 6 and 8 (132 nt), but lacked the splice sites at the 5' and 3' ends of the joined exons. A competitor RNA that contained mutations (see Fig. 1B for details) in the three ESE motifs, 6-1, 6-2, and 8-2, was also constructed because our previous results (Fig. 2) showed that these three motifs had the strongest enhancer activity. A range of 10- to 50-fold molar excess of RNA competitor was used. Addition of increasing amounts of the wild-type competitor inhibits the splicing of exon 5 to exon 6, as seen by the decrease of product mRNA (Fig. 3, lanes 2–5). However, addition of mutant competitor has a minimal effect on abolishing the splicing of exon 5 to exon 6 (Fig. 3, lanes 6–9). These results indicate that the purine-rich motifs in exons 6 and 8 are essential for 3' splice site activation of exon 6.

In the case of the pre-mRNA containing the mutated ESEs (p4d55 6-1/2, 8-2) the possibility exists that a silencer element was inadvertently created in the process of mutating the ESEs, which could then inhibit
ESEs in β-TM act before A-complex formation

The initial splice site recognition and commitment to splicing is thought to be mediated by a number of factors. A pre-mRNA is committed to splicing when its processing cannot be contested by the addition of excess RNA. This commitment step occurs in the E complex that forms subsequent to the H (hnRNP) complex and prior to A-complex formation (Reed & Palandjian, 1997). These complexes can be visualized in a spliceosome-assembly assay in which labeled pre-mRNAs are incubated under splicing conditions and electrophoresed through a native gel. This assay has been well characterized using the adenovirus pre-mRNA substrate (Ad-1; Konarska & Sharp, 1986), and we used it to test the hypothesis that the observed ESE-mediated effect occurred prior to A-complex formation, in the absence of ATP, as suggested by the previous experiments. Figure 4, lanes 1, 11, and 21, show the migration of the Ad-1, p4d55 mutant, and p4 wild-type (wt) pre-mRNAs alone. Upon addition of nuclear extract, ATP and CP, the three pre-mRNAs assembled into H complex at zero time point (Fig. 4, lanes 2, 12, and 22). This complex is formed by both the specific and nonspecific binding of hnRNP proteins onto pre-mRNAs. The Ad-1 pre-mRNA assembled efficiently into A and then into B and C complexes with increasing time at 30 °C (Fig. 4, lanes 3 and 4). The β-TM p4d55 wild-type pre-mRNA assembled efficiently into A complex (Fig. 4, lanes 23 and 24) but B- and C-complex assembly is inefficient compared to Ad-1 pre-mRNA-mediated complex assembly. However, the mutant p4d55 pre-mRNA did not assemble into A complex and was blocked at H-complex formation (Fig. 4, lanes 13 and 14). These results imply that the inefficiency of mutant pre-mRNA splicing lies in its inability to form a commitment complex.

To further assess the role of β-TM exon 6/8 sequences, we tested the ability of the wild-type and mutant exon competitors to contest A-complex formation in the three different pre-mRNAs. The Ad-1 pre-mRNA was not inhibited in A-complex formation by either the wild-type (Fig. 4, lanes 5, 6, and 7) or mutant pre-mRNA competitors (Fig. 4, lanes 8, 9, and 10). As expected, the addition of wild-type or mutant competitor had no effect on the mutant pre-mRNA that is blocked at H complex (Fig. 4, lanes 15–20). Addition of increasing concentrations of the wild-type competitor (Fig. 4, lanes 25–27) to the wild-type pre-mRNA inhibited A-complex formation, whereas addition of the mutant competitor to wild-type pre-mRNA blocked A-complex formation only at high concentrations. These results complement the splicing competition assay (Fig. 3) and indicate that competition by these purine-rich exon sequences is specific for the β-TM pre-mRNAs.

UV crosslinking of sequences in exons 6/8 reveals the binding of an ~30 kDa protein

Because it was clear that ESEs play a role in 3' splice site activation, presumably via the interaction with a nuclear factor, we used UV crosslinking to assay for proteins that interact with these sequences. This type of assay is used routinely to identify factor(s) that bind/interact specifically with nucleic acid probes. The probes used for UV crosslinking were the same as that used for the competition experiments, that is, wild-type or mutant sequences (mutated at 6-1, 6-2, 8-2) spanning...
exons 6 and 8. The labeled probes were incubated with nuclear extract and subjected to UV crosslinking, after which they were treated with RNase before being electrophoresed in an SDS-PAGE system. Several differences in binding ability were observed between wild-type and mutant probes. There was a significant difference in the affinities between the probes to a broad band of 33 kDa (Fig. 5A, lanes 1 and 2). Our initial studies focused on the identification of this protein(s). While the wild-type probe binds the lower portion of the band with greater intensity, the mutant probe binds the upper portion of the band with greater affinity (Fig. 5A, lane 1 vs. 2).

It has been shown in several systems that SR proteins can bind to ESEs (Sun et al., 1993; Ramchatesingh et al., 1995; Yeakley et al., 1996). We were interested in seeing if SR proteins or a subset of them bind to the ESEs in exons 6 and 8. SR proteins can be purified in a two-step purification, namely an ammonium sulfate fractionation (60–90%) followed by magnesium chloride precipitation (20 mm; Zahler et al., 1992). In an effort to separate the broad crosslinking band into more discrete bands, we tested the crosslinking abilities of these probes using different ammonium sulfate fractions. Nuclear extract was subjected to several ammonium sulfate precipitations, that is, 0–30%, 30–60%, and 60–90%. The 60–90% cut was further subjected to a 20 mm MgCl₂ precipitation. When these different fractions were crosslinked to the wild-type and mutant probes, we observed that the two 35-kDa crosslinking products could now be separated. The wild-type probe binds the 35-kDa band present in the SR preparation (Fig. 5A, compare lanes 9 and 10), whereas the mutant probe binds an activity that is precipitated by a 30–60% ammonium sulfate cut (Fig. 5A, compare lanes 5 and 6). Since the only differences between these two probes is the 3-nt substitution in three motifs, we think that a binding site for a nuclear factor may have been created. Since pre-mRNA substrates containing this mutation cannot be spliced, this
activity was not further investigated. We do not think that this is an inhibitory factor for reasons discussed above (Fig. 3). SF2/ASF purified from HeLa cells was tested for its ability to crosslink wild-type and mutant probes, and it was seen that there was binding to the wild-type probe by SF2/ASF as compared to the mutant probe (Fig. 5B, compare lanes 3–5 with 8–10). We propose that because the 3’ splice site of exon 6 is poor, SF2/ASF binds via the ESEs, thereby contributing to recognition of the splice site.

**Competition by wild-type exon sequences can be rescued by SF2/ASF**

Wild-type sequences inhibit splicing of substrate p4d55 (Fig. 3) and were able to interact with SF2/ASF (Fig. 5B). To determine if SF2/ASF was sufficient to rescue the inhibitory effects of competition by the wild-type sequences, competition assays were performed as described in Figure 3. Additional protein was added to some of the reactions (Fig. 6, lanes 10–18). Since nuclear extract contains all the protein components required for splicing, it could rescue splicing of p4d55 pre-mRNA (Fig. 6, lanes 10–11). An SR preparation also had the ability to rescue splicing of the p4d55 substrate in this competition assay (Fig. 6, lanes 14–15). We tested a HeLa cell-purified SF2/ASF preparation to complement the UV crosslinking studies described earlier, and we observed that SF2/ASF alone could rescue the splicing of the p4d55 substrate (Fig. 6, lanes 16–17), although after UV crosslinking, other proteins were also bound to the exon sequences. On the other hand, splicing could not be rescued by the addition of S100, a cytoplasmic fraction that lacks only SR proteins in order to be splicing competent (Fig. 6, lane 18). Despite the fact that U2AF and U1 snRNP act at the E complex, addition of a 0–60% ammonium sulfate fraction containing these two factors could not rescue splicing (Fig. 6, lanes 12–13).

Taken together, the spliceosome assembly, UV crosslinking, and competition experiments demonstrate that the ESEs bind SR proteins, most notably SF2/ASF, at the E complex stage of splicing. This interaction presumably occurs so that the splicing machinery is recruited to an otherwise inefficiently used splice site. These results demonstrate the specificity of the RNA–protein interaction in the context of ESEs and SF2/ASF.

In an effort to promote the splicing of the mutant pre-mRNA, additional SR proteins were added to the splicing reactions of mutant substrates. As shown in Figure 7, lanes 2–5, addition of total SR proteins, HeLa purified SF2, recombinant SF2, or SC35 did not activate splicing of the mutant pre-mRNA. The same procedure was carried out using wild-type pre-mRNA to show that increasing the concentration of protein is, in itself, not inhibitory to splicing (Fig. 7, lanes 7–10). This indicates that merely increasing the local concentration of SF2/ASF is not sufficient to activate splicing, but actual interaction with exon sequences is crucial in its ability to promote splicing. Lanes 11–16 show splicing
of wild-type pre-mRNA in the presence of competitor. Similar to results shown in Figure 6, SR proteins, HeLa-purified SF2, and recombinant SF2 rescue splicing. However, addition of SC35, an SR protein of similar mobility to SF2, lacks the ability to rescue splicing. This result demonstrates a substrate specificity between SR proteins and ESEs.

**Predicted SF2/ASF binding sites correlate with ESEs in exons 6 and 8**

Specific exonic target sequences recognized by several SR proteins under splicing conditions have been identified using a functional SELEX approach (Liu et al., 1988). The sequences identified for SF2/ASF were then analyzed to determine a consensus sequence, using the program GIBBS sampler, which was found to be SRSASGA (S represents G or C, R represents purine). The defined motifs were used to generate a score matrix, according to the frequency of each nucleotide at each position. This score matrix was then used to analyze the distribution of high score motifs in exons 6 and 8 of β-TM (see Fig. 8). The top panel shows the distribution of ESE motifs detected by this method. The y-axis represents the strength of the ESE motif and the x-axis represents exons 6 and 8 joined together with exon 6 spanning nt 1–76 and exon 8 spanning nt 77–132. The bar below this graph represents exons 6 and 8, with the purine-rich motifs analyzed in this study indicated by shaded blocks. There is a correlation between the GARGARGAR motifs and the potential SF2/ASF interaction sites picked up using the score matrix program. Next, the sequence with mutations in the purine-rich motifs (see Fig. 1B for details) was analyzed in the same manner, and it was seen that the three point mutations to each motif eliminated most of the predicted SF2/ASF interacting sites.

The fact that two completely independent approaches gave rise to strikingly similar results indicates the rel-
evancy of these sequences to 3’ splice site usage and that SF2/ASF is involved in this process via functional interaction.

p2p6(8/9) as a model for exon definition

The β-TM substrate p2p6(8/9) which spans exons 5 through 9 (E5-i5-E6-i6-E7-i7-E8-E9) exhibits an exon-skipping phenotype in vitro for reasons that are unclear. This substrate skips the nonmuscle exon 6 in an in vitro splicing assay using nuclear extract prepared from HeLa cells and is processed to an E5-E8-E9 product. However, when the 3’ splice site of exon 6 is improved to include a strong polypyrimidine tract containing a stretch of nine uridines, p2p6(8/9)pU, the exon inclusion product is obtained (E5-E6-E8-E9). Moreover, a splicing intermediate that utilizes the 5’ splice site of exon 6 is also seen (E5-i5-E6-E8-E9; Tsukahara et al., 1994). Exon 7, being skeletal muscle-specific, is not utilized in either substrate. These observations are in agreement with the exon definition hypothesis (Berget, 1995) in which a strong splice site on one side of an exon is communicated across that exon and improves the use of the splice site on the other side. Because exon 6 contains two purine-rich motifs that have been demonstrated to act as ESEs, we were interested in seeing if they also play a role in defining and bridging exon 6, thereby promoting its recognition.

Inclusion of exon 6 is dependent on exon sequences

To determine if exon sequences, in particular the purine-rich elements in exon 6, are required for its inclusion, we introduced the same type of mutations as described above that disrupt the motifs. Three purine → pyrimidine substitution mutations were made in the wild-type, p2p6(8/9) → p2p6(8/9)6-1/2, as well as in the construct containing the strong polypyrimidine tract, p2p6(8/9)pU → p2p6(8/9)pU6-1/2, (see Fig. 9 for details of mutations in and around exon 6). Figure 10 shows a time course experiment of splicing of these four substrate RNAs. Consistent with the previous study, the wild-type pre-mRNA skips exon 6 (Fig. 10, lanes 1–5) and the pre-mRNA substrate with the poly U
trc

FIGURE 9. Mutations to the sequences in and around exon 6. Only the sequences targeted for substitutions are shown. Arrows pointing down are down-regulating substitutions and arrows pointing up are splice site strengthening substitutions. pu: mutations to the polypyrimidine tract at the 3' splice site of exon 6, substituting to a stretch of nine uridine residues. Cs: mutations to the 5' splice site of exon 6, converting it to an exact complement of the U1 snRNA. 6-1 and 6-2 are the mutations within exon 6 disrupting the purine stretch with pyrimidine residues.

ESEs and splice site usage

elements within exon 6 act as ESEs and may play a role in promoting interactions across the exon between a strong and weak splice site.

3' and 5' splice sites' use of exon 6 requires ESEs 6-1 and 6-2

To make analysis of 3' and 5' splice site usage easier, pre-mRNAs derived from substrate p2p6(8/9) were constructed and used in in vitro splicing assays (Fig 11A). The first part focuses on exon 5 to exon 6 and the second, exon 6 to exon 8. Both constructs include the splice sites around exon 6. First we analyzed the splicing of exon 5 to exon 6 in the substrate p5, which comprises exon 5 through exon 6 plus 19 nt of intron 6, including the 5' splice site of exon 6 (E5-i5-E6-Di6; Fig 11A). As shown previously (Helfman et al., 1988; Tsukahara et al., 1994), the wild-type substrate cannot be spliced (Fig. 11A, lane 1) and mutations in ESEs 6-1 and/or 6-2 show no effect (Fig. 11A, lanes 2-4). A polyU mutation strengthening the polypyrimidine tract at the 3' splice site allows exon 6 to be spliced to exon 5 (p5pU; Fig. 11A, lane 5; Tsukahara et al., 1994). When we mutated ESEs 6-1 and 6-2 in this context, however, the ability to use the 3' splice site of exon 6 was lost, suggesting a cooperation between the polypyrimidine tract and the exon sequences (Fig. 11A, lanes 6-8). It was shown previously that the 3' splice site of exon 6 could also be utilized when the 5' splice site upstream of this exon was changed to a U1 snRNA consensus binding site (p5pCs; Fig. 11A, lane 9; Tsukahara et al., 1994). It was proposed that this occurs through an exon-bridging mechanism. To determine if exon sequences are required for this interaction, mutations to ESEs 6-1 and 6-2 were made in this context. A loss of 3' splice site use was then observed in this substrate, indicating the necessity of the ESEs (Fig. 11A, lanes 10-12). Because we observed that ESEs 6-1 and 6-2 are required if even one of the splice sites is weak, we tested the necessity for these exon sequences when both splice sites were strong, that is, polyU at the 3' splice site and U1 snRNA consensus at the 5' splice site (p5pUCs). This substrate can be spliced very efficiently and even in the context of exon mutations retains this ability (Fig. 11A, compare lane 13 with lanes 14-16), suggesting an alternative mechanism that bypasses ESEs 6-1 and 6-2.

A similar set of experiments was performed to analyze the use of the 5' splice site of exon 6 (Fig. 11B). Substrates used spanned a part of intron 5, including the 3' splice site of exon 6, through exon 9 (Δi5-E6-i6-E7-i7-E8-E9) and are called Δp2p6(8/9) or its derivatives pU and Cs. The 5' splice site of exon 6, although suboptimal, is still functional, as seen by the splicing of exon 6 to exon 8 (Fig. 11B, lanes 1,2). When the purine motifs were mutated in this context, Δp2p6(8/9)6-1/
6-2, there was no usage of the 5’ splice site (Fig. 11B, lanes 3, 4). The presence of a strong 3’ splice site upstream of this splice site, \( \Delta p2p6(8/9)pU \), shows some improvement of 5’ splice site usage (Fig. 11B, lanes 5, 6). However, this splicing event is also dependent on the presence of purine-rich motifs 6-1 and 6-2 (Fig. 11B, lanes 7, 8). When the 5’ splice site is improved to a consensus sequence, \( \Delta p2p6(8/9)Cs \), it markedly improves 5’ splice site usage but it is also sensitive to the nature of the exon sequences (Fig. 11B, compare lanes 9, 10 vs. 11, 12). When both the splice sites are strong, \( \Delta p2p6(8/9)pUCs \), however, there is no longer a dependence on purine-rich motifs 6-1 and 6-2 (Fig. 11B, compare lanes 13, 14 vs. 15, 16).

This result complements the data obtained on the 3’ splice site usage of exon 6, suggesting that when one or both splice sites around an exon are weak, then the presence of ESEs within the exon promote splicing. However, when both the splice sites are strong, then the ESEs are redundant and a different mechanism is utilized for splice site usage.

**ESEs are required for exon 6 inclusion in vivo**

To correlate ESEs’ requirement in vitro with an in vivo situation, we employed a transient transfection assay. Minigene \( \beta\)-TM constructs had been used previously for in vivo transfection experiments (Helfman et al., 1988, 1990; Guo et al., 1991; Guo & Helfman, 1993; Caceres et al., 1994). These constructs have an SV40 promoter and a 5’ UTR followed by genomic sequences spanning exons 5–9. We had shown previously using RT-PCR that the SV40 p2 constructs undergo two types of processing events in vivo, as exon 6 is only partially included in nonmuscle cells while exon 7 is always suppressed.

The same type of point mutations as previously described (i.e., to motifs 6-1 and 6-2) were introduced in exon 6 as described in the in vitro system, and the effect of these mutations was tested by transfecting plasmids containing the minigenes (Fig. 12) in HeLa and 293 cells. Cytoplasmic mRNA was harvested followed by RT-PCR using reverse primers specific for exons 5 and 9. To prevent amplification of endogenous \( \beta\)-TM mRNA, the forward primer was complementary to the 5’ UTR of SV40. We observe that the ESE mutations in exon 6, individually or in combination, abolish exon inclusion completely (Fig. 12, right panel, lanes 4, 7, 8, and 9) as compared to the wild-type minigene (Fig. 12, right panel, lanes 3 and 6), which showed both the exon-inclusion as well as exon-skipping products. Since exon 7 is the same size as exon 6, we needed to rule out aberrant exon usage. A unique \( NcoI \) site is present within exon 6. Digestion of the RT-PCR products with \( NcoI \) (right panel) confirms exon 6 usage. The above results confirm the results obtained from the in vitro splicing assay system described above and demonstrate a role for the ESEs in vitro as well as in vivo.

**DISCUSSION**

Previous studies revealed that splicing of \( \beta\)-TM pre-mRNA requires an ordered pathway of splicing in which exon 6 must first be joined to exon 8 for activation of the 3’ splice site of exon 6 (Helfman et al., 1988). Those
studies suggest that sequences within exon 8 contain an important determinant for splice site usage. Inspection of the sequences within exon 8, as well as exon 6, reveals the presence of several purine-rich motifs. Motifs similar to these have been shown in other systems, including immunoglobulin, troponin T, and growth hormone to act as ESEs (Lavigneur et al., 1993; Sun et al., 1993; Watakabe et al., 1993; Xu et al., 1993). To determine if these elements play a functional role in splice site use, each element was individually mutated and analyzed for its effect. The present studies demonstrate that in addition to sequences within exon 8, sequences in the alternatively spliced exon 6 also play a critical role in the use of this exon. Although both purine-rich elements in exon 6 were essential for splice site usage, only one of the three purine-rich elements in exon 8 was required for activation of the 3' splice site of exon 6 using a substrate that contained all of exon 8 joined to exon 6 (Fig. 2).

Conservation and cooperation among ESEs

From work done in the Drosophila dsx system, it has been shown that ESEs act not in a synergistic manner, but rather in an additive manner (Lynch & Maniatis, 1995, 1996; Hertel & Maniatis, 1998). That is, the presence of multiple motifs acts to increase the possibilities of SR protein interaction promoting splice site usage and that these motifs do not interact with each other. In the case of β-TM pre-mRNAs, mutation of any one of the three motifs (6-1, 6-2, or 8-2), reduces 3' splice site usage, and mutating all three of them abolishes 3' splice site usage, suggesting that these motifs may interact with each other to build a higher order complex that then allows 3' splice site usage. Also, when a third purine-rich element was added in exon 6 in the E5-i5-E6 context, it did not activate 3' splice site usage (data not shown), suggesting that the context in which the purine-rich motif is present is critical and not merely its presence within the exon.

Interestingly, it was previously found that the splicing of exon 6 to exon 8 required at least the first 26 nt of exon 8 (Helfman et al., 1988). Those studies show that this is not a length requirement, as addition of up to 200 nt of intronic sequences downstream of exon 6 does not activate its 3' splice site. Exon sequences, specifically sequences in exons 6 and 8, were shown to compensate for the weak 3' splice site. In the studies presented here, we show that mutations in the purine-rich motif at the 5' end of exon 8, that is, 8-1 (which lies within the first 26 nt), did not abolish splice site usage, suggesting that an additional cis-acting element might be present in this region. A sequence alignment of β-TM genes from rat, human, and chicken...
shows that in addition to the purine-rich motifs, the pyrimidine-rich region at the 5′ end of exon 8 is highly conserved. This pyrimidine-rich sequence at the 5′ end of exon 8 might represent a novel element and the situation may be reminiscent of the Drosophila dsx gene, where six 13-nt repeat pyrimidine-rich ESEs interspersed by a purine-rich ESE (PRE) are present. In chicken β-TM the regulation of exon 6 is via a downstream pyrimidine-rich intronic enhancer, and activation is mediated through the interaction with SF2/ASF (Gallego et al., 1996, 1997). Such an intronic element is absent in rat β-TM. Moreover, as discussed above, downstream exon sequences and not downstream intron sequences are required for 3′ splice site activation of exon 6 (Helfman et al., 1988). Thus, although there is almost full conservation of exonic sequences, the mechanism of splicing regulation may not be fully conserved between species.

SR proteins, ESEs, and the commitment to splicing

Since SR proteins have been proposed to play various roles in pre-mRNA splicing, including the sequence specific interaction with the substrate, it has been of special interest to predict these sequences using SELEX (Tuerk & Gold, 1990). With this method, and using RNA–protein binding assays, consensus sequences have been proposed to be the binding site for each SR protein. SF2/ASF has been shown to bind to a consensus sequence that is enriched in purine residues and to have a general sequence similar to that of the purine-rich motifs of exons 6 and 8 (Tacke & Manley, 1995; Tacke et al., 1997). Using SELEX combined with a functional assay, Liu et al. (1998) carried out a search for ESE motifs specific for different SR proteins. This method is different from previous SELEX approaches, as the mode of interaction is based on function rather than binding. The consensus motif for SF2/ASF was found to be SRSASGA and prediction of these motifs in exons 6 and 7 coincides with functional sites characterized in this study (Fig. 7). Moreover exon sequences found with mutated purine-rich motifs when analyzed by the program developed by Liu et al. showed an almost complete loss of predicted SF2/ASF high-score motifs, which correlates with the UV-crosslinking data presented in this report (see Fig. 5B). Although the program predicts several sites for SF2/ASF interaction, only some of them are effective in 3′ splice site usage of exon 6 as shown by the data presented here, suggesting that the sequence by itself is not sufficient, but that context and/or positional information may also play a role in splice site selection.

We observe that SR proteins, in particular SF2/ASF, interacts with the purine-rich elements in exon 6 during the commitment complex. Thus, the binding of SR proteins to sequences contained within exons 6 and 8 contributes to alternative splice site selection. It has been demonstrated that when the β-TM SV40 p2 plasmid, which shows partial skipping of exon 6 in vivo, is cotransfected with an SF2/ASF expression plasmid, the equilibrium is shifted toward an exon-inclusion phenotype (Caceres et al., 1994). hnRNP proteins coat a newly synthesized transcript in both a specific and non-specific manner, and some of these interactions play a role in alternative splice site selection. For example, some hnRNP proteins, such as hnRNP A1, can antagonize the actions of SR proteins, such as SF2/ASF, on alternative 5′ splice sites. In this regard it is worth noting that hnRNP A1 had no effect on alternative 5′ splice site usage of β-TM pre-mRNAs in vitro (Mayeda et al., 1993). In cardiac troponin T, it has recently been shown that the conserved hnRNP protein, CUG-binding protein, was found to bind to the CUG repeats within the muscle-specific splicing enhancers and regulate its alternative splicing (Philips et al., 1998). hnRNP A1 has been shown to selectively interact with certain SR proteins through its gly-rich domain (Cartegni at el., 1996). It is not known if exon 6 of β-TM is regulated in skeletal muscle via factors that affect the binding of SF2/ASF to the purine-rich motifs in exon 6. Analysis of the tissue distribution of several SR proteins showed that SF2/ASF levels are lower in skeletal muscle cells (Zahler et al., 1993; Hanamura et al., 1998). This may serve as a potential mode of regulation for exon 6 repression in this cell type. Other modes of regulation include the local concentration, as SR proteins are known to localize to speckles within the nucleus (Spector et al., 1991), or the state of phosphorylation of these proteins (Mermoud et al., 1994).

Regulation through cell type-specific splicing factors

Using the Drosophila dsx pre-mRNA, it was shown that SR proteins act in the commitment complex along with Tra/Tra2 (Tian & Maniatis, 1993). Recently, it was shown that two human homologs of Tra2, hTra2α and hTra2β, bind to purine-rich ESEs such as the GAR elements, and although they are not necessary for constitutive splicing, they are required for enhancer-dependent splicing (Tacke et al., 1998). It is thought that high concentrations of SF2/ASF can compensate for physiological concentrations of the hTra2 proteins, and that hTra2 may act by recruiting SR proteins to the enhancer. It was previously seen that the splicing of pre-mRNAs containing E5-i5-E6-E8 (p4d55) was not supported by S100 + SR proteins alone, but also required the addition of 40–60% ammonium sulfate to activate the 3′ splice site of exon 6 (Tsukahara et al., 1994). The identity of this additional component in the 40–60% ammonium sulfate fraction is not known, but those results suggest that another factor plays a critical role in the commitment of this pre-mRNA to spliceosome assem-
**ESEs and splice site usage**

bly. This system may be reminiscent of the *Drosophila* dsx system whereby Tra/Tra2 and SR proteins interact with ESEs and thereby promote upstream 3’ splice site usage (Tian & Maniatis, 1993). There is the possibility that the hTra2 proteins may be the additional component, along with S100 + SR, to activate p4d55 splicing. It is also interesting to note that unlike the 3’ splice site, the 5’ splice site of exon 6 can be spliced in the presence of S100 + SR proteins (Tsukahara et al., 1994). Thus, different subsets of factors participate in the use of the 3’ and 5’ splice sites of this exon.

**ESEs and their role in exon definition**

Most higher eukaryotic pre-mRNAs are characterized by short exons and very long introns. In these cases recognition of splice sites in an intronic polarity becomes implausible and models proposing recognition in an exonic polarity combined with experimental evidence that promotes such models have become established (Robberson et al., 1990; Berget, 1995). In such a situation, a weak splice site on one side of an exon can have an adverse effect on the removal of the intron on the other side or, alternatively, a strong splice site on one side of an exon can promote the removal of the intron on the other side. It was shown experimentally that a strong 5’ splice site improves upstream 3’ splice site usage correlating U1 snRNP and U2AF binding to these sites (Hoffman & Grabowski, 1992). Furthermore, exonic sequences were shown to play a role in similar situations (Staknis & Reed, 1994). We show here, using the sequences around exon 6 as a model substrate, that ESEs play a role in both upstream and downstream intron removal when either splice site flanking exon 6 is suboptimal. When both the 3’ and 5’ splice site around exon 6 are strong, however, ESEs seem to be dispensable towards splice site usage. These results have implications about regulation of usage of alternatively spliced exons. It is a common feature of constitutively spliced exons to have strong splice sites, thereby eliminating the requirement for other cis elements. In the case of alternatively spliced exons, splice sites are often weak, leading to exon exclusion. We show that cis elements that lie within the exon promote communication of the splice sites flanking the exon within which they reside, thus allowing their inclusion.

Further work will be required to fully characterize the role of the ESEs identified in this study. Which sequences in exon 8 are involved in the splicing of exon 6 to 8 (fibroblast-type splice) or exon 7 to 8 (skeletal muscle-type splice) are not known. It will be important to determine whether the same cis-acting elements and SR proteins mediate these splicing events in non-muscle and muscle environments. Such differences might play a critical role in regulation of tissue-specific alternative RNA splicing, and experiments are currently in progress to address these questions.

**MATERIALS AND METHODS**

**Plasmid construction: SP6-p4d55 series of mutants**

Substrates used are the same as described in Tsukahara et al. (1994). Mutations to the purine-rich motifs in these substrates were made by using oligonucleotides and PCR. Oligonucleotides used to create p4d55-1, p4d55-2, p4d55-3, p4d55-4, and p4d55-5, respectively, are 5’-CGAGCCAGGCAGCTGAGGGCTAGTTCAACATGAGAC-3’, 5’-TCGCTAGACCTACTAGGTAGCTTCGAACCATGGAC-3’, 5’-GAGGAGTATTCCACCTAAGCGTACAAATAAGAAGAG-3’, 5’-AAAGAGGAAAAATCTAACTGAGCATACTAAAATTACTTCTGAG-3’, and 5’-GAAGATCAACTTCGTTGAGCCGTAGCTGAGGAGCTCAC-3’. The mutated fragments were then subcloned into the original SP6-p4d55 parental vector.

To create the p2p6(8/9) series of mutants, oligonucleotides to 6-1 and 6-2 (see above) were used in the parental constructs described in Tsukahara et al. (1994). The SV40p2 minigene used for transfection was the same as described in Guo & Helfman (1993). To create the substrates SV40p2(6-1), SV40p2(6-2), SV40p2(6-1/2), SV40p2(6-1), and SV40p2(6-1) were subcloned a Bsu361 fragment from the p2p6(8/9) templates used for in vitro transcription were linearized at the BamHI site to provide templates for in vitro transcription.

**In vitro transcription**

For the in vitro splicing assays, 32P-labeled SP6-tropomyosin pre-mRNAs (linearized at Clal) were synthesized in vitro (primed with m7CAP analog) as described in Tsukahara et al. (1994). RNA used for spliceosome assembly was synthesized in the presence of all four radiolabeled nucleotides and gel purified. RNA used as competitor for competition experiments was synthesized similarly, but without the addition of radiolabeled nucleotides, following which it was gel purified.

RNA used for UV crosslinking was synthesized similarly, but in the presence of all four radiolabeled nucleotides and in addition they were gel purified. RNA used for competition experiments was synthesized similarly, but without the addition of radiolabeled nucleotides, following which it was gel purified.

p2p6(8/9) templates used for in vitro transcription were linearized at the BamHI site at the end of exon 9. The same series of plasmids was also used to obtain templates for the p5 series (exon 5-intron 5-exon 6) but were linearized with Sstl, which recognizes a site 19 nt downstream of exon 6. The plasmids in the Δp2p6(8/9) series were linearized at the BamHI site at the end of exon 9 to provide templates for in vitro transcription.

**In vitro splicing**

HeLa cell nuclear extracts were prepared as described (Dignam et al., 1983). In vitro splicing reactions were carried out at 30 °C for 120 min or as indicated. Standard reactions contained 60% nuclear extract, 2 mM MgCl2, 500 μM ATP, 20 mM creatine phosphate, 2.6% (w/v) polyvinyl alcohol, 15–30 ng 32P-labeled pre-mRNA, 13 mM HEPES (pH 8.0),
12% (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA, and 0.3 mM DTT in a reaction volume of 25 μL. After incubation for the indicated time, the reactions were stopped by the addition of SDS and proteinase K. The RNA was recovered as described (Krainer et al., 1984). The products of the reaction were analyzed on 4% acrylamide/7 M urea gels. For competition experiments, unlabeled RNA competitor was preincubated with the nuclear extract for 15 min prior to the addition of 32P-labeled pre-mRNA and ATP/CP containing mix.

**Spliceosome assembly**

We use the method of Konarska and Sharp (1986) but PVA was added at a concentration of 0.5% (final concentration) per reaction. Briefly, substrate pre-mRNA (radiolabeled with all 4 nt) was incubated with nuclear extract and ATP/CP mix. After an incubation at the indicated time points at 30°C, the reaction was loaded directly onto a 8:1 non-denaturing Tris-Gly gel system.

**Protein preparations**

Nuclear extract was prepared as described above. SR proteins were prepared as described by Zahler et al. (1992). SF2/ASF was a gift of Dr. Mike Murray. SC35 was a gift of Dr. Hong-Xiang Liu. Several SR preparations were a gift of Dr. Akila Mayeda (Krainer et al., 1990b; Screaton et al., 1995). S100 was a gift from Charlie Chen (Mayeda & Krainer, 1992).

**UV crosslinking**

32P-labeled RNA probe was incubated with the indicated amounts of protein in a final volume of 12.5 μL containing 2 mM MgCl₂, 500 μM ATP, 20 mM creatine phosphate, 1.3 μg tRNA and 400 ng BSA. The reactions were incubated at 30°C for 10 min and irradiated with UV light (254 nm at a distance of 7 cm) for 5 min after which RNase A and RNase T1 were added to a final concentration of 1 μg/μL and 2 U/μL, respectively, and incubated at 37°C for 15 min. Laemmli sample buffer was then added, and the samples were boiled before loading on a 12% SDS-PAGE gel.

**Derivation of score matrix**

The derivation of the score matrix is as described in Liu et al. (1998).

**Transfections and preparation of mRNA**

HeLa cells were transfected with the different SV40-p2 constructs using the calcium phosphate precipitation protocol (see Helfman et al., 1990, for transfection details).

To harvest total cytoplasmic RNA, cells were washed in cold PBS and scraped off in 1 mL of PBS/plate. Cells were pelleted and resuspended in 350 μL cold lysis buffer (150 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-Cl [pH 8.0], 200 U/mL RNasin) and NP40 was added to a final concentration of 0.5%. After an incubation on ice for 5 min, the samples were spun at 4°C. The supernatant was transferred to a fresh tube containing 4 μL of 20% SDS, mixed, and 5 μL of proteinase K (10 mg/mL) were added, and the entire reaction was incubated at 37°C for 15 min. The samples were extracted with equal volumes of phenol-chloroform (2 ×) and chloroform (1 ×). Samples were precipitated with 40 μL 3 M NaOAc and 1 mL EIOH. Pellets were washed with 75% EIOH, 25% 0.1 M NaOAc and resuspended in 50 μL of 2 × DNase buffer (20 mM MgCl₂, 2 mM DTT), 1 μL RNasin, and 3 μL DNase, and incubated at 37°C for 20 min. Twenty-five microliters of DNase stop solution (2.5 M NaOAc, 0.1 M EDTA) were added and the samples were once again extracted and precipitated as described above. Dried pellets were resuspended in DEPC water and ODs were taken.

**RT-PCR**

cDNA was made in a reverse transcription reaction as follows: 400 ng of total RNA were boiled for 3 min with 250 pmol of oligo dT and then incubated at 42°C for 120 min in a reaction volume of 30 μL containing 10 mM DTT, 500 μM dNTPs, RT buffer, and RT superscript. Six microliters of this cDNA mixture were then used as template in a 50 μL PCR reaction containing 40 μM dNTPs, 1.5 mM MgCl₂, Taq buffer, Taq polymerase, and 20 pmol each of forward and reverse primer. Primers used to analyze the spliced products of the β-TM minigene are: (1) 5’ TTTTGAGGCTAGGCTTTTT 3’, homologous to the SV40 leader sequences; (2) 5’ CACCTC GGCTCTCTC 3’, complementary to exon 5 sequences of β-TM; and (3) 5’ TTTGCCCAGATCTTTCAGC 3’, complementary to exon 9 sequences of β-TM. The PCR reactions underwent 25 cycles at 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min followed by an extension at 72°C for 10 min. An aliquot of each sample was digested with Nco1, which allows for clear distinction between exon 6 versus exon 7 usage, and analyzed by agarose gel electrophoresis and stained with ethidium bromide, following which they were photographed.

**ACKNOWLEDGMENTS**

We thank David Horowitz and Mike Murray for useful discussions and Luca Cartegni, David Horowitz, Hong-Xiang Liu, and Mike Murray for critical reading of the manuscript. We thank Hong-Xiang Liu for assistance in analyzing exon 6/8 sequences for predicted SF2 binding sites. We would like to thank Charlie Chen, Akila Mayeda, Mike Murray, and Hong-Xiang Liu for SR protein preparations and Dr. Magda Konarska for the Ad-1 plasmid as well as technical advice. The work done was supported by National Institutes of Health grant no. GM43049.

Received June 19, 1998; returned for revision July 6, 1998; revised manuscript received November 24, 1998

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