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RNA 1998 4: 1523-1536

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Enhancer elements activate the weak 3' splice site of α -tropomyosin exon 2

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ABSTRACT

We have identified four purine-rich sequences that act as splicing enhancer elements to activate the weak 3' splice site of α -tropomyosin exon 2. These elements also activate the splicing of heterologous substrates containing weak 3' splice sites or mutated 5' splice sites. However, they are unique in that they can activate splicing whether they are placed in an upstream or downstream exon, and the two central elements can function regardless of their position relative to one another.

The presence of excess RNAs containing these enhancers could effectively inhibit in vitro pre-mRNA splicing reactions in a substrate-dependent manner and, at lower concentrations of competitor RNA, the addition of SR proteins could relieve the inhibition. However, when extracts were depleted by incubation with biotinylated exon 2 RNAs followed by passage over streptavidin agarose, SR proteins were not sufficient to restore splicing. Instead, both SR proteins and fractions containing a 110-kD protein were necessary to rescue splicing. Using gel mobility shift assays, we show that formation of stable enhancer-specific complexes on α -tropomyosin exon 2 requires the presence of both SR proteins and the 110-kD protein. By analogy to the *doublesex* exon enhancer elements in *Drosophila*, our results suggest that assembly of mammalian exon enhancer complexes requires both SR and non-SR proteins to activate selection of weak splice sites.

Keywords: exon enhancers; pre-mRNA splicing; SR proteins

INTRODUCTION

An increasing number of genes are being identified that utilize regulated alternative splicing as a means to increase isoform diversity and control gene expression (Smith et al., 1989; Valcarcel et al., 1995; Adams et al., 1996). Typically, pre-mRNAs that are subject to regulated splicing contain weak splice sites enabling more efficient repression of splicing or requiring the presence of activating elements. Since the conserved splicing signals at the 5' splice sites, branch points, and 3' splice sites are relatively lacking in sufficient information to allow unambiguous identification (Black, 1995), the mechanism by which exons are recognized from

introns remains incompletely understood. In cases of alternative splicing, the mechanism of differential exon recognition is also unknown.

Recently, splicing enhancers have been identified that function to activate the splicing of introns containing weak splice sites. Originally these elements were described as purine-rich exon sequences, but enhancers can also reside within introns, and non-purine-rich enhancer elements have also been identified (Lavigne et al., 1993; Sun et al., 1993; Tian & Maniatis, 1993; Watakabe et al., 1993; Xu et al., 1993; Yeakley et al., 1993; Tanaka et al., 1994; Tian & Koe, 1995; Coulter et al., 1997). Purine-rich enhancers bind SR proteins, a conserved family of essential splicing factors, and apparently activate splicing by forming complexes that communicate across introns and exons (Wu & Maniatis, 1993; Kohtz et al., 1994; Staknis & Reed, 1994; Abovich & Rosbash, 1997). These bridge complexes occur early during spliceosome formation, linking initial splicing events to alternative exon selection. The fact that SR proteins have been shown to display substrate specificity and differential activity depending on their phosphorylation state suggests that SR proteins could

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conceivably regulate multiple alternative splicing events (Fu, 1995; Manley & Tacke, 1996; Xiao & Manley, 1997). However, the assembly of enhancer complexes may require the binding of both SR proteins and specific splicing regulatory proteins as in the *Drosophila doublesex* gene. Regulation of splicing of *doublesex* pre-mRNAs requires the binding of SR proteins and the splicing regulators *tra* and *tra-2* to exon-enhancer elements to activate the weak female-specific 3' splice site (Hedley & Maniatis, 1991; Ryner & Baker, 1991; Tian & Maniatis, 1992; Lynch & Maniatis, 1996).

We have been studying the alternative splicing of α -tropomyosin (α -TM) exons 2 and 3 (Lin & Patton, 1995; Perez et al. 1997; Gooding et al. 1998). Here we report the identification of four purine-rich sequences in exon 2 that function to activate the weak 3' splice site of exon 2 as well as a variety of heterologous 3' splice sites and mutated 5' splice sites. These elements can activate splicing whether they are present in an upstream or downstream exon and, for at least the two central elements, regardless of their position relative to one another. In addition to the binding of SR proteins, these enhancer elements also bind a 110 kD protein that appears to be required for stable enhancer complex formation and the activation of weak splice sites.

RESULTS

Identification of enhancer elements in α -TM exon 2

Exons 2 and 3 of α -TM are mutually exclusive exons with exon 3 included in the mRNA in most cell types (the default pathway) whereas exon 2 selection is smooth-muscle specific (Wieczorek et al., 1988; Lees-Miller et al., 1990). The regulation of splicing of these exons occurs by competition between the branch point/polypyrimidine tract (BP/PPT) combinations upstream of each exon (Mullen et al., 1991), negative regulation of exon 3 selection (Gooding et al., 1994; Lin & Patton, 1995; Singh et al., 1995; Perez et al., 1997; Gooding et al. 1998), and activation of exon 2 selection by SR proteins (Lin & Patton, 1995). The involvement of SR proteins to activate exon 2 suggests the presence of enhancer elements. Examination of the sequence of α -TM exon 2 resulted in the identification of four purine-rich elements (Fig. 1A) resembling sequences previously described as splicing-enhancer elements (Tanaka et al., 1994). To examine whether these elements are involved in splicing, we tested whether the exon 2 purine-rich elements could competitively inhibit pre-mRNA splicing by sequestering essential splicing factors. As shown in Figure 1B, increasing concentrations of competitor RNAs encoding wild-type exon 2 effectively inhibited splicing. In contrast, competitor RNAs containing mutations in each of the four purine-rich elements were

unable to block splicing. A series of competition experiments were performed using competitor RNAs containing individual and combinations of mutations in the four purine-rich elements to ascertain the role of each element (Fig. 1C). While RNAs containing all four elements were the most effective competitors (RNA 10), all RNAs that contained the third purine-rich element were also effective competitors of splicing (RNAs 4, 6, and 8). Mutations in the two central purine-rich elements severely compromised the ability to inhibit splicing (RNA 9; AX mutation), whereas mutation of just the second element did not alleviate the inhibition of splicing (RNA 8). Thus, these elements appear to form a series of target sequences that bind a necessary splicing factor(s) and titration of this factor(s) inhibits splicing.

To examine whether the inhibition of splicing by exon 2 RNA is substrate specific, additional competition experiments using wild-type or mutant exon 2 enhancer elements were performed. As shown in Figure 1B, 400 ng of wild-type exon 2 competitor RNA effectively prevented splicing of an α -TM substrate. Inhibition of β -globin pre-mRNA splicing required approximately 100 ng of competitor RNA (Fig. 4C), whereas the splicing of an adenovirus-derived pre-mRNA substrate was not affected at the highest concentration of competitor RNA tested (5 μ g; data not shown). Thus, the factor(s) sequestered by the exon 2 enhancer elements displays substrate specificity as judged by the differing levels of competitor RNAs needed to inhibit splicing.

We next utilized various 3-exon alternatively spliced substrates to examine how these elements regulate splicing. As shown in Figure 2A, in vitro splicing of wild-type α -TM in HeLa nuclear extract leads predominately to 1–3 splicing regardless of the presence (wt) or absence (mt, AX mutation) of the two central purine-rich elements, consistent with the fact that the BP/PPT upstream of exon 3 effectively outcompetes the corresponding elements upstream of exon 2 (Mullen et al., 1991). In contrast, when the BP/PPT upstream of exon 2 was identical to that upstream of exon 3 (133, wt), exons 1 and 2 were predominately spliced together, but mutation of the exon 2 enhancer elements resulted in an increase in the amount of 1–3 splicing (133, mt). This suggests that the purine-rich elements indeed function as splicing enhancers leading to increased 1–2 splicing. To more clearly illustrate this effect, a substrate was used that contains a pyrimidine tract of intermediate strength upstream of exon 2 (pGen; Mullen et al., 1991). Splicing of this substrate in the presence of the exon 2 enhancer elements (pGen, wt) produced increased levels of 1–3 splicing relative to the 133 construct but still generated mostly 1–2 splicing. However, when the two central purine-rich elements were mutated (pGen, mt), a more dramatic increase in 1–3 splicing was observed, consistent with enhancer activity in the purine-rich elements in exon 2.

Comparison of the 1–2 to 1–3 spliced product ratio between these substrates demonstrates combinatorial action between the BP/PPT and enhancer sequences in 3'-splice-site selection (Buvoli et al., 1997). In the presence of a strong BP/PPT (133), little effect of the splicing enhancers in exon 2 can be detected. In contrast, decreasing the strength of the BP/PPT (pGen) more clearly demonstrates activation of the weak 3' splice site of exon 2 by enhancer elements.

For analysis of the effect of the α -TM exon 2 enhancer elements on in vivo splicing, model substrates were prepared that contain the first four exons of α -TM. As with the in vitro splicing constructs, wild-type α -TM was compared to substrates that have altered BP/PPTs upstream of exons 2 and 3. These constructs were transfected into smooth muscle cells (SMCs) and HeLa cells and the levels of 1–2 versus 1–3 splicing were determined with the percentage of 1–2 splicing shown in Figure 2B. With wild-type α -TM mini-genes, inclusion of exon 2 was clearly evident in cultured SMCs whereas exclusion of exon 2 predominated in HeLa cells (Fig. 2B). Mutation of the two central purine-rich elements in an otherwise wild-type α -TM mini-gene decreased the amount of 1–2 splicing in SMCs but had no effect in HeLa cells, consistent with the in vitro data shown in Figure 2A. For the BP/PPT mutants, the effects of the exon 2 enhancer elements in SMCs again demonstrated combinatorial action between enhancer elements and the strength of the BP/PPT (Fig. 2B).

FIGURE 1. Purine-rich sequences within α -tropomyosin exon 2. **A:** α -tropomyosin splicing pattern and sequence of exon 2. The first four exons of α -tropomyosin are shown with the splicing pattern observed in default and smooth muscle cells. The branchpoint and polypyrimidine tracts upstream of exons 2 and 3 are shown as circles and rectangles, respectively. The filled rectangles within exon 2 depict the purine-rich sequences (B, A, X, or M) as boxed below. The sequence of α -TM exon 2 is shown with arrows identifying the mutations that disrupt the four purine-rich regions. The letters refer to newly created restriction sites (*Bsp* E1, *Afl* II, *Xho* I, *Bam* H1) introduced in the mutated constructs. **B:** Wild type exon 2 RNA inhibits splicing of α -tropomyosin. In vitro splicing reactions utilizing an α -tropomyosin pre-mRNA substrate (DS) were performed for the indicated times in the presence of increasing amounts of competitor RNAs containing exon 2 with either wild-type (wt E2) or mutated (E2-baxm) purine-rich enhancer elements. Splicing products and intermediates are depicted to the left. **C:** Competitive inhibition of splicing by the exon 2 purine-rich sequences. Competitor RNAs containing the indicated sequences (1–10) were incubated with an α -TM pre-mRNA substrate (DS; Smith & Nadal-Ginard, 1989) under splicing conditions. In the absence of competitor (0), spliced products and intermediates accumulate as depicted to the left. The filled rectangles correspond to the wild-type purine-rich sequences as indicated in **A** and their absence refers to mutation of a given element as shown in **A**. Multiple gels were placed adjacent to one another accounting for the slight difference in mobility of the spliced product between groups.

When the BP/PPT upstream of exon 2 was identical to that upstream of exon 3 (133), proximal splice sites were exclusively joined together (1–2 splicing) and the exon 2 enhancer elements had little effect on 3'-splice-site choice. When the BP/PPT upstream of exons 2 and 3 were identically weak (122; i.e., both introns contain the weak branch point upstream of exon 2), proximal splice sites were again paired (1–2 splicing) and mutation of the enhancer elements slightly decreased exon 2 selection. In HeLa cells, splicing of the 122 and 133 constructs also resulted primarily in the pairing of proximal splice sites (1–2 splicing) and mutation of the enhancer elements had little effect.

To dissect the individual roles played by the branch point or polypyrimidine tract, a construct was prepared in which the wild-type branch points were left intact but the polypyrimidine tract upstream of exon 2 was increased in strength and made identical to that upstream of exon 3 (1^{B2P3}23). For this construct, the

FIGURE 2. Purine-rich elements activate the weak 3' splice site of exon 2. **A:** In vitro splicing analysis. Pre-mRNA substrates containing the first three exons of α -TM were spliced in HeLa nuclear extract with either wild-type (wt) or mutated (mt) exon 2 enhancer elements (AX mutation). In 133, the branch point/polypyrimidine tract upstream of exon 3, whereas in pGen the polypyrimidine tract upstream of exon 2 was replaced with a generic pyrimidine tract. The precursor and spliced products are as indicated to the left. It should be stressed that selection of exons 2 and 3 is mutually exclusive. **B:** In vivo splicing analysis. A primer extension assay (Perez et al., 1997) was used to determine the levels of 1–2 versus 1–3 splicing of α -TM mini-genes transfected into SMCs and HeLa cells. Only the percentage of 1–2 splicing is shown. Mini-gene constructs containing altered branch point/polypyrimidine tracts upstream of exons 2 and 3 with either intact (wild-type) or mutated (AX) exon 2 enhancer elements were analyzed. The branch point/polypyrimidine tracts upstream of exon 2 (B2P2) and exon 3 (B3P3) were in their normal positions (wild-type α -TM), the polypyrimidine tract upstream of exon 2 was replaced with the polypyrimidine tract from upstream of exon 3 (1^{B2P3}23), or both introns contained B2P2 (122) or B3P3 (133).

splicing pattern was similar between the two cell types and mutation of the enhancer elements clearly decreased exon 2 selection (Fig. 2B). Interestingly, nearly the same level of 1–2 splicing was noted in HeLa cells with the 1^{B2P3}23 and 122 constructs containing intact enhancer elements. However, mutation of the enhancer elements clearly decreased the levels of 1–2 splicing for the 1^{B2P3}23 construct but not for the 122 construct. Apparently, competition between branch point/polypyrimidine tracts and activation by enhancer elements both play a role in 3' splice site selection. When the competing BP/PPT were of equal strength, proximal 3' splice sites were preferentially paired and no activation by enhancer elements was detectable. In contrast, when the strength of the BP/PPTs differed, the activation of weak branch points by the exon 2 enhancer elements was clear. Together, the in vivo and in vitro results suggest that 3' splice site selection depends on combinatorial effects between the enhancer elements and the strength of the BP/PPT.

Activation of splicing of heterologous substrates by the α -TM exon 2 enhancer elements

The two central purine-rich elements were next introduced into the 3' exon of heterologous splicing substrates that differed only in the strength of their polypyrimidine tracts (Fig. 3A). Identical branch point sequences (UACUAAC) were followed either by a weak polypyrimidine tract derived from the intron upstream of α -TM exon 2 (Dpy1) or by a polypyrimidine tract containing 15 pyrimidines interrupted by 4 adenosines (Dpy2; Roscigno et al., 1993). In addition, a GAG sequence was incorporated at the 3' splice site in the Dpy1 substrates whereas CAG was used in the Dpy2 constructs. A GAG at the 3' splice site results in a partial block to the second step of splicing, allowing visualization of the effect of the enhancer elements on both steps of splicing (Smith et al., 1989). In the absence of enhancer elements, no splicing was observed

FIGURE 3. The exon 2 purine-rich elements activate heterologous 3' splice sites independent of position. **A:** Order effects. Two-exon adenovirus-derived pre-mRNA substrates were prepared with identical branch point sequences (UACUAAC; open circles) but with two different polypyrimidine tracts. Dpy1 is a relatively weak pyrimidine tract from upstream of α -TM exon 2 and contains a GAG incorporated at the 3' splice site to partially block the second step of splicing. Dpy2 contains 15 pyrimidines interrupted by four adenosines but with a normal CAG at the 3' splice site. Splicing was performed in the presence or absence of the two central exon 2 purine-rich elements (A and X) in their normal order (E), with the two elements in the reverse order (E-r), or with one or both of these elements mutated (E-a or E-ax). **B:** Suppression of splice site mutations. Adenovirus-derived splicing substrates (as depicted) were prepared that contain a mutation of the 5' splice site (5'm; CAG/GUGAGU to CAA/GUGGUGU), deletion of the pyrimidine tract (Δ Dpy), or replacement of the pyrimidine tract with a polypurine tract (Pu) in the presence (E) or absence of the two central exon 2 enhancer elements (A and X). All substrates contained a GAG at the 3' splice site. The precursors and products of splicing are as depicted to the right. **C:** Position effects. In vitro splicing reactions utilizing adenovirus derived substrates were incubated for the indicated times (min). The Dpy1 substrate is as in **A**. Other substrates (as depicted) contained the exon 2 enhancer elements (A and X) in either their normal position (E) or with one (U-E) or two (U-E+E) copies of the elements in the upstream exon (retaining the original exon length). All substrates contained a GAG at the 3' splice site. The precursors and products of splicing are as depicted to the right.

for either substrate, even after prolonged incubation in HeLa nuclear extract (Fig. 3A, lanes 1 and 2). In contrast, inclusion of the central pair of purine-rich elements restored splicing (E). Mutation of the first of this pair of enhancer elements (E-a) only slightly reduced splicing efficiency whereas mutation of both enhancer elements (E-ax) blocked splicing entirely. As judged by the accumulation of splicing intermediates, the GAG mutation in Dpy1 shows that the enhancer elements act prior to the second step of splicing. Thus, the α -TM exon 2 purine-rich sequences function as splicing-enhancer elements in heterologous splicing substrates.

As a control, the sequence of the two central elements, as well as the nucleotides between these sequences, was reversed with the expectation that such a change would abolish enhancer function. Surprisingly, splicing was activated regardless of whether the sequence was in the reverse (noncomplementary) order (Fig. 3A). However, examination of the sequence of the two central enhancer elements revealed that the purine-rich elements are maintained even if the sequence is reversed.

normal (E): CTCAGCAA**AAGAGA**AGCTGCTGCGGG
CGT**CGGAGG**ACGAGCGG
reverse (E-r): GGCGAGCAG**GAGG**CTGCGGGCGTC
GT**CGAAGAGAA**ACGACTC

Despite this fortuitous arrangement, the results shown in Figure 3A demonstrate that splicing is activated regardless of the relative positions of the two central enhancer elements.

The interplay between the strength of the BP/PPT, enhancer elements, and splicing efficiency suggested that the purine-rich elements in exon 2 might be able to substitute entirely for the pyrimidine tract. As shown in Figure 3B, the exon 2 enhancer elements restored efficient splicing of a substrate lacking a pyrimidine tract (Δ Dpy versus Δ DpyE). However, the resulting mRNA was generated by activation of a cryptic 3' splice site within the downstream exon, accounting for the decreased size of the mRNA (Fig. 3B). Apparently, decreasing the distance between the branch point and the wild-type 3' splice site AG from 26 to 7 nt caused enhancer-dependent activation of a new, downstream 3' splice site. The newly selected 3' splice site results from the use of the first AG within the downstream exon that is apparently a suitable distance (27 nt) from the branch point. When the polypyrimidine tract was replaced by a polypurine stretch, retaining the original distance between the branch point and the 3' splice site, cryptic splice sites were not activated nor was the original 3' splice site utilized regardless of the presence of enhancer elements (Pu versus PuE; Fig. 3B). Thus, the exon 2 enhancer elements can activate selection of 3' splice sites lacking pyrimidine tracts, but there appears to be both a distance and sequence constraint between the branch point and the 3' splice site.

We next wanted to assess whether the exon 2 enhancer elements could activate the splicing of a substrate containing a 5' splice site mutation and whether they could function when placed in the upstream exon. Accordingly, a substrate was prepared containing a mutated 5' splice site (CAG/**GUGAGU** to CAA/**GUGGU**). These positions were altered simply to weaken the 5' splice site without abolishing recognition by U1 snRNA. Splicing of this substrate showed that the exon 2 enhancer elements could partially rescue the splicing of this mutation but, in addition, a cryptic 5' splice site located five nt downstream of the wild-type splice site was also activated (Fig. 3B).

When one or two sets of the two central exon 2 enhancer elements (A and X) were placed in the upstream exon, cryptic 5' splice site activation was again noted, but no use of the authentic 5' splice site was detected (Fig. 3C). Interestingly, mRNA was produced only when two sets of enhancer elements were present in the upstream exon, suggesting that these elements can act in concert. When the enhancer elements were placed over 150 nt downstream of the 3' splice site, the normal 3' splice site was activated without activation of cryptic 5' splice sites (data not shown). Together, these results suggest that the exon 2 enhancer elements can rescue multiple splicing defects and/or weak splice sites independent of position, with the caveat that cryptic splice sites may be activated. In the presence of enhancer elements, but in the absence of wild-type splice sites, it appears that the splicing machinery searches for the most suitable match to conventional splice sites within a confined range of sequence.

SR proteins interact with the α -TM exon 2 enhancer elements

In most cases, enhancer elements mediate their effects by providing a binding site for SR proteins that then promote protein–protein interaction between exon enhancers and branch points and/or across exons to bridge 5' and 3' splice sites (Fu, 1995; Reed, 1996; Abovich & Rosbash, 1997). To determine whether SR proteins were being sequestered by the enhancer elements in the α -TM exon 2 competitor RNAs, competition of α -TM splicing was performed in the presence of purified SR proteins. As shown in Figure 4A, competitively inhibited splicing reactions could be restored by the addition of total SR proteins. To determine which SR protein might be responsible for the restoration of splicing, UV crosslinking experiments were performed using radioactively labeled exon 2 RNA and either HeLa nuclear extract or purified SR proteins. The major crosslinked proteins with either protein preparation migrated at approximately 35 kD, and the binding of these proteins could be competitively inhibited by incubation of excess cold exon 2 RNA (Fig. 4B). Mutant competitor RNAs did not inhibit the crosslinking, sug-

FIGURE 4. SR proteins bind the exon 2 enhancer elements. **A:** SR proteins restore splicing to nuclear extracts competitively inhibited by exon 2 RNA. Splicing reactions utilizing an α -TM substrate with the indicated amount of exon 2 competitor RNA were supplemented with purified SR proteins and incubated for the indicated time. Splicing products and intermediates are depicted to the left. **B:** UV crosslinking of SR proteins to exon 2. RNAs encoding exon 2 were incubated in either HeLa nuclear extract (HeLa NE) or in SR proteins purified from HeLa cells (HeLa SR). Following UV crosslinking and RNase digestion, 32 P-labeled proteins were analyzed by SDS PAGE. Crosslinking was performed in the presence or absence of the indicated amount of competitor RNAs containing either wild-type exon 2 (wt) or with mutations disrupting the purine-rich elements (mt). Molecular weight markers are at the left. **C:** Recombinant SC35 and SF2/ASF relieve the inhibition of splicing by exon 2 RNA competitor. Competitive inhibition of β -globin splicing was performed by inclusion of wild-type exon 2 competitor RNA in the presence of increasing concentrations of either recombinant SC35 or SF2/ASF.

gesting a specific protein–RNA interaction. Among the SR family, SC35 and ASF/SF2 migrate at approximately 35 kD. When UV crosslinking was performed with radioactively labeled exon 2 RNAs and purified SC35 or ASF/SF2, both proteins were shown to interact with the exon 2 enhancer elements (data not shown). In addition, competitive inhibition of β -globin splicing reactions by addition of excess exon 2 RNA could be relieved by addition of either exogenous SC35 or ASF/SF2, suggesting that these two proteins are capable of interacting with the α -TM exon 2 enhancer elements (Fig. 4C). Similar concentrations of the two proteins

were used in Figure 4C, yet rSF2/ASF appeared to relieve the inhibition of splicing much more effectively than did rSC35. While functional differences between SR protein family members have been demonstrated (Fu, 1993; Screaton et al., 1995; Wang & Manley, 1995; Zahler & Roth, 1995), it is not clear whether the differences in Figure 4C are due to distinct binding-site preferences or differential activity between the two protein preparations. Nevertheless, these results suggest that the exon 2 purine-rich elements bind SR proteins and act as splicing enhancers to activate weak splice sites.

Specific association of a 110-kD protein with the exon 2 enhancer elements.

Since the absence of a band in UV crosslinking assays does not imply a lack of binding, we also performed biotin-selection experiments and Northwestern blots to identify additional proteins that associate with the exon 2 enhancer elements. Probing a Western blot of HeLa nuclear extract or S100 extract with wild-type and AX mutant RNA probes resulted in the identification of two proteins of approximately 110 kDa and 85 kDa that bound the wild-type RNA probe much more efficiently than the mutant sequence (Fig. 5A). The 85-kDa protein appears to be a breakdown product of the 110-kDa protein because freshly prepared extracts lack the 85-kDa protein but it becomes predominant with time and/or repeated cycles of freezing and thawing (data not shown). These proteins were also detected in SMCs and uterus tissue (data not shown).

To confirm that the 110-kDa protein binds specifically to the exon 2 enhancer elements, RNA transcripts containing the enhancer elements were prepared in the presence of biotinylated CTP, incubated with nuclear extract, and passed over streptavidin agarose resin to purify associated proteins. After extensive washing, the bound proteins were released by boiling and separated on SDS gels. In agreement with the hypothesis that the 110-kDa protein binds to the exon 2 enhancer elements, Northwestern blots of the affinity-purified proteins showed the presence of the 110-kDa protein (and the 85-kDa breakdown product) (Fig. 5B). Absence of biotinylated CTP and competition experiments using unlabeled RNA competitors showed that the interaction between the 110-kDa protein and the enhancer elements was specific. Probing the same blot with antibodies against the SR protein family (mAb104) showed that SR proteins were also affinity purified under these conditions (Fig. 5C). In the absence of competitor RNAs, proteins reacting with mAb104 included species migrating at approximately the positions of SRp75, SRp50, SRp40, SRp35, and Srp20 (Zahler et al., 1992). In addition, two high-molecular-weight proteins (~150 kDa) also reacted with mAb104 that were not found in purified SR protein preparations. The 110-kDa protein did not react with mAb104. Thus, the exon 2 enhancer elements stably associate with both SR proteins and a 110-kDa non-SR protein.

Stable enhancer complex formation and splicing activity requires both the 110-kDa protein and SR proteins

Since SR proteins alone were sufficient to restore splicing to extracts inhibited by the addition of exon 2 competitor RNAs (Fig. 4A,C), it was unclear what role, if any, the 110-kDa protein might play in splicing. However, splicing reactions inhibited by competitor concen-

FIGURE 5. SR proteins and a 110-kDa protein bind the α -TM exon 2 enhancer elements. **A:** Northwestern blot. Approximately 80 μ g of HeLa nuclear extract or S100 extract were loaded per lane onto a 9% polyacrylamide SDS gel, transferred to nitrocellulose membranes, and probed with either a wild-type 32 P-labeled α -TM exon 2 RNA probe (wt E2) or an AX mutant RNA probe (mt E2). Size markers are as indicated. **B:** Affinity purification. RNAs encoding the exon 2 enhancer elements were prepared in the presence or absence of biotin-CTP and incubated with HeLa nuclear extract. Bound proteins were recovered by passage over streptavidin agarose, washed, eluted by boiling, and subjected to SDS PAGE on 9% gels. After transfer to nitrocellulose, the blots were probed with a wild-type 32 P-labeled exon 2 RNA in the presence (20 pmol) or absence of wild-type or mutant cold competitor RNAs as indicated. **C:** Western blot. The same filter shown in **B** was incubated with 1 M KCl to remove the RNA probe and Western analysis was performed using an antibody against SR proteins (mAb 104). The two 150-kDa proteins are visible just near the top of the figure which represents the origin of the separating gel.

trations higher than those used in Figure 4 could not be rescued simply by the addition of proportionately larger amounts of SR proteins (data not shown). Thus, to determine whether other factors, including the 110-kDa

protein, were required for enhancer-dependent activation of splicing, HeLa nuclear extracts were depleted of enhancer binding proteins by incubation with large amounts of biotinylated exon 2 RNA followed by passage over streptavidin-agarose. The splicing activity of extracts treated in this manner was then determined. As shown in Figure 6A, treatment of nuclear extract with biotinylated exon 2 RNA (biotin-E2 depleted NE) inhibited splicing, whereas splicing in control reactions that lacked the biotinylated RNA (control NE, mock NE) was unaffected. In contrast to the competition experiments, the addition of SR proteins was unable to restore splicing to the depleted extract, suggesting that at least one additional factor had also become limiting. To test whether this factor might be the 110-kDa protein, depleted extracts were supplemented with fractions enriched in the 110-kDa protein either individually

or in combination with SR proteins. Interestingly, splicing activity was regained only upon the addition of both SR proteins and fractions enriched in the 110-kDa protein (Fig. 6A). Northwestern blots of the depleted extracts revealed that only a small amount (~10%) of the 110-kDa protein remained, consistent with effective depletion of the 110-kDa protein (data not shown). Furthermore, addition of fractions containing the 110-kDa protein was shown to stimulate splicing in partially depleted extracts in which the addition of SR proteins alone was sufficient to support low levels of splicing (data not shown).

Mobility shift experiments were then performed to determine whether both SR proteins and the 110-kDa protein are necessary for stable, enhancer-specific complex formation. Purified SR proteins and fractions containing partially purified 110-kDa protein were incubated

FIGURE 6. SR proteins and fractions containing the 110-kDa protein are required for stable enhancer complex formation and splicing activity. **A:** Splicing activity. HeLa nuclear extract was incubated with biotinylated exon 2 RNA and streptavidin-agarose. The agarose beads were then pelleted, and the supernatant (biotin-E2 depleted NE) was assayed for splicing activity. Control extract was carried through the above incubations but buffer was substituted for the RNA and streptavidin agarose. Similarly, mock-depleted extract was treated with streptavidin-agarose, but not biotinylated RNA. SR proteins alone could not restore splicing to the depleted extracts but the combination of SR proteins and fractions containing the 110-kDa protein was sufficient to rescue splicing. **B:** Enhancer complex formation. A 25–45% ammonium sulfate fraction of HeLa nuclear extract containing the 110-kDa protein was subjected to further purification in which the presence of the 110-kDa protein was assayed by Northwestern blots (see Materials and Methods). Enriched fractions were incubated with wild-type exon 2 RNA probes in the presence or absence of wild-type (wt) or mutant (mt) competitor RNAs and purified SR proteins and subjected to native gel electrophoresis. Only the combination of SR proteins and the 10-kDa protein could form a stable, enhancer-specific complex.

with radioactively labeled exon 2 RNAs in the presence of wild-type and mutant competitor RNAs and subjected to nondenaturing gel electrophoresis (Fig. 6B). Stable, enhancer-specific complexes were observed only when both purified SR proteins and fractions containing the 110-kDa protein were combined. Together, these data support an important role for the 110-kDa protein in enhancer specific complex formation and the activation of weak splice sites.

DISCUSSION

We have identified four purine-rich sequences in exon 2 of α -TM that act as splicing enhancers. Exons 2 and 3 of α -TM are in competition with one another, with exon 2 included only in smooth muscle mRNA. Previous work has shown that in SMCs, exon 3 is subject to negative regulation mediated by *cis*-acting sequences flanking exon 3 (Gooding et al., 1994; Lin & Patton, 1995; Singh et al., 1995; Perez et al. 1997; Gooding et al. 1998). However, SR proteins have also been shown to activate exon 2 inclusion (Lin & Patton, 1995). This article shows that activation of exon 2 splicing is due to the presence of enhancer elements mediating the binding of SR proteins, including at least SC35 and ASF/SF2, in combination with a 110-kDa protein. The activation of the weak 3' splice site of exon 2 has been studied primarily in HeLa nuclear extracts, whereas intact HeLa cells incorporate exon 2 at very low levels. Apparently, the BP/PPT upstream of exon 2 is so weak that enhancer elements are needed to activate its splicing, but such activation occurs only when exon 3 selection is repressed. Thus, it seems that the factors that bind to the exon 2 enhancer elements are constitutively active in all cells but are not sufficient to activate exon 2 unless additional factors repressing exon 3 are present. In this way, competition between these two exons is because of the strength of competing 3' splice sites derived from combinatorial contributions by the branch point, polypyrimidine tract, and enhancer elements (Buvoli et al., 1997). As for the regulation of α -TM in SMCs, it is likely that a combination of negative factors coupled with the formation of enhancer-specific activating complexes allows SMCs to incorporate exon 2 rather than exon 3. It should be stressed that unless SMCs possess enhancer-complex-specific proteins, such as a unique complement of SR proteins or specifically modified SR proteins (Xiao & Manley, 1997), the exon 2 enhancer elements are essentially constitutive components and may be only indirectly involved in smooth-muscle-cell regulation of α -TM splicing. As for the 110-kDa protein, Northwestern blots using partially purified protein and a variety of enhancer sequences suggest that it binds multiple enhancer elements (data not shown). Coupled with its widespread expression pattern, it appears that unless the

110-kDa protein is specifically modified in SMCs, it should also be viewed primarily as a constitutive splicing component.

Position effects of the enhancer elements in α -TM exon 2

The two central enhancer elements can function in heterologous substrates within either the upstream or downstream exon and in reverse order. In addition, these enhancer elements can partially rescue a 5' splice site mutation. To our knowledge, these are the first enhancer elements that activate both 5' and 3' splice sites in a position-independent manner. It remains possible that these unique properties are because of individual effects from each of the four purine-rich elements in exon 2, with the caveat that activation of splicing was most pronounced in the presence of the third element. The recent demonstration that enhancer elements in the caldesmon and troponin T genes are multipartite (Elrick et al., 1998) suggests that the combination of multiple enhancer elements in exon 2 and the sequence of each of these elements confers unique enhancer properties enabling position-independent activation of both 5' and 3' splice sites.

Activation of weak 3' splice sites by the exon 2 enhancer elements revealed a distance constraint between the position of the branch point and the 3' splice site AG in the heterologous substrates that we have used. With suboptimal positioning, cryptic splice sites became activated. In particular, when the branch point was moved too close to the wild-type 3' splice site AG, the next AG within the downstream exon was chosen. Remarkably, the distance between the newly activated 3' splice site AG and the branch point was nearly identical to the wild-type distance (27 versus 26 nt). However, replacing the pyrimidine tracts with purines to recreate the exact wild-type branch point to AG distance did not restore splicing even in the presence of enhancer elements. Previous reports have demonstrated a distance constraint regarding activation of splicing by enhancer elements (Lavigne et al. 1993; Tian & Maniatis, 1994) and the inhibitory effects of inserting purines between the branch point and 3' splice site on 3' splice site selection (Reed, 1989; Roscigno et al., 1993; Coolidge et al., 1997). For the exon 2 enhancer elements, there is both a distance and a sequence constraint restricting 3' splice site activation.

Regarding α -TM splicing, the fact that the exon 2 enhancer elements are position independent results in a potentially unique situation. Conceivably, these elements could work to activate selection of either exon 2 or exon 3. However, based on our results showing that selection of competing 3' splice sites results from combinatorial effects (Buvoli et al., 1997), it appears that the strong splicing signals driving selection of exon 3

mask or obviate enhancer function during default exon selection.

Enhancer complex formation

Several lines of evidence suggest that SR proteins promote the functional association of factors across both introns and exons resulting in the interaction between U2AF, U1 snRNP, and SR proteins (Wu & Maniatis, 1993; Kohtz et al., 1994; Staknis & Reed, 1994; Zuo & Manley, 1994; Lynch & Maniatis, 1996; Abovich & Rosbash, 1997). RNase protection-immunoprecipitation experiments first suggested that SC35 was required for a stable interaction between U1 snRNP and U2 snRNP at the 3' splice site (Fu & Maniatis, 1992). The molecular interactions were more clearly defined when it was shown that SC35 and SF2/ASF were capable of interacting directly with the 70K protein of U1 snRNP (Wu & Maniatis, 1993; Kohtz et al., 1994) and that SC35 could interact with the 35-kDa subunit of U2AF, forming a molecular bridge across the intron between the 5' and 3' splice sites. More recently, branch point binding proteins have been identified in yeast and mammalian systems that are apparently necessary for bridge complex formation (Abovich & Rosbash, 1997; Berglund et al. 1997, 1998; Rain et al. 1998). In mammalian systems, enhancer sequences may facilitate bridge complex formation activating the splicing of suboptimal splice sites by recruiting SR proteins to facilitate the binding of U1 snRNP and U2AF.

This report demonstrates that additional proteins may be required to stabilize enhancer complex formation. We found that an activity that copurifies with a 110-kDa protein was needed to promote stable complex formation between the exon 2 enhancer elements and SR proteins. Furthermore, the depletion of enhancer binding proteins from nuclear extract using biotinylated RNA inhibited splicing, and rescue of this inhibition was achieved only upon the addition of both SR proteins and a fraction containing the 110-kDa protein. Extensive purification and identification of the 110-kDa protein is now underway to verify and refine its role in enhancer complex stabilization.

Previously, Yeakley et al. (1996) had shown that a 37-kDa protein and Srp40 are required to recruit additional SR proteins to GAA-rich enhancer elements. Combined with our results, this suggests that the activity of splicing enhancer elements could be modulated by the binding and assembly of additional nuclear factors to enhancer elements prior to the recruitment of specific SR proteins. Such a model is similar to the role played by the sex determination genes, *tra* and *tra-2*, in *Drosophila melanogaster*. The proteins encoded by these genes activate the splicing of the female-specific exon of the *doublesex* gene by forming a stable enhancer dependent complex with SR proteins (Chabot, 1996).

MATERIALS AND METHODS

Construct preparation

The α -TM single intron substrate (DS), the β -globin substrate (RR108), and the adenovirus substrate were as described (Smith & Nadal-Ginard, 1989; Smith et al., 1989). The alternative splicing constructs B2P2-E2mut, B3P3-E2mut and pGEN-E2mut were prepared by inverse PCR on wild-type templates (Mullen et al., 1991). Dpy1 and Dpy2 were derived from pPIP85 (Moore & Sharp, 1992) and contain a 30-nt deletion upstream of the branch point sequence (TACTAAC). Dpy1 contains a 21-nt polypyrimidine tract (CGCCCGTTTG TTGTGTGTCTC) derived from the intron upstream of α -TM exon 2, and Dpy2 contains a 15-nt polypyrimidine tract (GCC CATTATTATCCA) derived from PIP4 (Roscinio et al., 1993). Exon 2 mutants used as splicing competitors were prepared by inverse PCR (Perez et al., 1997) on the DS template with appropriate oligonucleotide pairs. Δ Dpy, Δ DpyE, 5'm, 5'mE, Pu, PuE, E, U-E, and U-E+E were constructed by PCR amplifying the adenovirus substrate (Moore & Sharp, 1992) with oligonucleotide primers containing the T7 transcription initiation sequence in the 5' oligonucleotides in addition to exon sequences. All oligonucleotide sequences and cloning strategies are available upon request.

In vitro transcription and splicing reactions

Capped pre-mRNA transcripts were prepared as described (Smith & Nadal-Ginard, 1989; Mullen et al., 1991). Unlabeled, uncapped competitor RNAs were prepared either by decreasing the amount of 32 P-CTP 200-fold and adding unlabeled CTP to a concentration equal to the other three rNTPs or by using the SP6 MEGAscript™ In vitro Transcription Kit (Ambion) without 32 P-CTP as per the manufacturer's instructions. Biotinylated RNA was prepared as for competitor RNA except that 100- μ M Biotin-14-CTP (Gibco-BRL) was added to the transcription reaction.

HeLa cell nuclear extracts were prepared as described (Abmayr et al., 1988) except that the final dialysis was stopped when equilibration with buffer E was reached as measured with a conductivity meter (Corning). Standard splicing reactions contained 20–40 fmol of 32 P-RNA transcript, 2.2 mM MgCl_2 , 0.5 mM ATP, 20 mM creatine phosphate, 1,000 U/mL of either RNasin (Promega) or Prime RNase Inhibitor (5'→3', Inc.), and 30–40% nuclear extract, as indicated. Competitive inhibition experiments were performed by preincubating splicing reactions with the indicated amount of competitor RNA for 10 min before adding substrate RNA. Splicing reactions were fractionated by electrophoresis through either 5, 8, 15, or 16% 19:1 polyacrylamide:bis-acrylamide gels in the presence of 8 M urea, followed by autoradiography.

In vivo splicing analysis

Smooth muscle cells (PAC1 cells; Rothman et al., 1992) and HeLa cells were grown as described (Lin & Patton, 1995). Transient transfections were done using TransIT™ polyamine transfection reagent (PanVera). Essentially, 10 μ L of the reagent were added to 0.2 mL serum-free medium and

incubated at room temperature for 5 min. Plasmid DNA (5 μ g) was then added to the diluted TransITTM reagent followed by another 5 min incubation at room temperature. Cultured cells were washed once with serum-free medium followed by a 5 h incubation with the DNA/TransITTM reagent complex at 37 °C. The medium was then replaced with complete growth medium containing serum. RNA was isolated from cultured cells 24 h after transfection using Tri-reagent (Molecular Research Center, Inc.).

For analysis of splicing patterns, an RT/PCR assay followed by primer extension of cDNA templates was performed as described (Perez et al., 1997). Quantitation was performed by determining the ratio of 1–2 to 1–3 extension products within the same lane using a Molecular Dynamics Phosphorimager.

Proteins

Whole cell extracts were prepared from 10⁹ HeLa cells (5 $\times$ 10⁴ cells/mL) as described (Zahler et al., 1992). Solid ammonium sulfate was added to the percentage saturation indicated in the text and precipitations were carried out as described (Englund & Seifter, 1990). SR proteins were prepared as described (Zahler et al., 1992). Fractions containing the 110-kDa protein (as judged by Northwestern analysis, see below) were prepared by FPLC as follows. The 25–45% ammonium sulfate fraction from 10⁹ HeLa cells was dialyzed against Buffer A (20 mM HEPES, pH 7.6, 0.1 M NaCl, 5 mM EDTA, 5% (w/v) glycerol, 1 mM AEBSF (Calbiochem), 1 mM leupeptin, and 0.1 mM aprotinin), applied to a Hi-Load Q Sepharose column and eluted with a 0.1–1.0 M NaCl linear gradient in Buffer A. Peak fractions eluted at 0.3–0.4 M NaCl, were combined, adjusted to 2 M NaCl and applied to a Hi-Load Phenyl Sepharose column and eluted with a 2–0.1 M NaCl linear gradient. Peak fractions eluted at 0.5–0.4 M NaCl, were pooled, and dialyzed against Buffer B (Buffer A with 20 mM HEPES, pH 6.5). Further fractionation was performed on a Resource S (Pharmacia) cation exchange column eluted with a 0.2–1.0 M NaCl linear gradient. Peak fractions were diluted to 0.2 M NaCl and applied to a polyguanosine agarose column and eluted with Buffer B containing 0.4 M NaCl. Final dialysis of all fractions was carried out against Dignam D buffer containing 5% glycerol (Dignam et al., 1983). Gel filtration fractions containing the 110-kDa protein used in splicing assays were obtained by passing 4 mL of HeLa nuclear extract over a HiPrep 26/60 Sephacryl S-300 column (Pharmacia) equilibrated in Dignam D with 5% glycerol at a flow rate of 1.0 mL/min while collecting 2 mL fractions. The presence of the protein was assayed by TCA precipitation of 0.5–0.75 mL of the fractions followed by Northwestern blot as described below.

RNA binding assays

UV crosslinking was performed as described (Patton et al., 1991) except that 50 fmol of ³²P-CTP labeled α -TM exon 2 RNA and the indicated amount of unlabeled competitor RNA was used. Substrate and competitor RNAs were premixed and added simultaneously to the binding reaction. Northwestern assays were performed essentially as described (St. Johnston et al., 1992) except that no difference in binding

was observed if the urea denaturation–renaturation steps were omitted. Briefly, proteins were electrophoresed on 9% polyacrylamide gels and transferred to nitrocellulose membranes which were then blocked for 1 h at room temperature in 25 mM NaCl, 10 mM HEPES, pH 8, 0.1 mM EDTA, 1 mM DTT, and 5% nonfat dry milk. RNA binding was performed at room temperature for 5 min in 50 mM NaCl, 10 mM MgCl₂, 10 mM HEPES, pH 8, 0.1 mM EDTA, 1 mM DTT, 2.5% nonfat dry milk, and the indicated ³²P-labeled probe (2–5 $\times$ 10⁵ cpm/mL). Membranes were then washed several times for 5 min each at room temperature in binding buffer.

Affinity selection with biotinylated RNA substrates was carried out in 0.5 mL reactions containing 40% HeLa nuclear extract, 250 mM KCl, 2.2 mM MgCl₂, 0.5 mM DTT, 1,000 U/mL RNasin (Promega), 100 pmol biotinylated RNA and the indicated amount of cold competitor RNA. Substrate and competitor RNAs were premixed and added simultaneously to the binding reaction. Mixtures were incubated at 30 °C for 10 min and put on ice. Fifty microliters of undiluted magnetic streptavidin beads (Dynal) were added and incubated at 4 °C for 1 h while nutating. The beads were collected by magnetizing the tube and the supernatant removed. Beads were washed four times in 1 mL of a buffer containing 20 mM Tris-HCl (pH 7.5), 250 mM KCl, 2.2 mM MgCl₂, and 0.1% Triton X-100. Proteins were eluted in 50 μ L sample buffer (Laemmli, 1970) and fractionated as described in the figure legends. Western blots (Harlow & Lane, 1988) were performed with undiluted culture supernatant from a hybridoma expressing mAb104 (ATCC).

Binding reactions for the gel-shift assays contained 4 μ L of protein, 12.5 fmol RNA, 5 mg/mL heparin, 20 mM HEPES, pH 8.0, 5% glycerol, 70 mM KCl and 3 mM MgCl₂ and were incubated 10 min at 30 °C. Reactions were fractionated by electrophoresis at room temperature in 5% polyacrylamide–5% glycerol–0.5 $\times$ TBE gels. Gels were prerun for 30 min at 25 V, samples were loaded, and electrophoresis was performed at 100 V (12.5 V/cm) for 2 h.

Depletion of nuclear extract with biotinylated RNA

HeLa nuclear extract was incubated for 15 min at 30 °C with 500 ng/ μ L biotinylated exon 2 RNA and 45 min at 4 °C with 20% (v/v) streptavidin–agarose beads equilibrated in Dignam D with 5% glycerol. The agarose beads were then pelleted by brief centrifugation, and the supernatant was used as depleted nuclear extract in splicing assays.

ACKNOWLEDGMENTS

We would like to thank Kristen Lynch and Tom Maniatis for the generous gift of recombinant SC35 and ASF/SF2. This work was supported by a grant from the NIH (GM50418) to J.G.P., a Sydney Blackmer Neuromuscular Disease post-doctoral fellowship from the Muscular Dystrophy Association to S.A.M., and an NIH training grant (GM08554) to B.T.D. Phosphorimager analysis was made possible by a grant from the NSF (BIR-9419667).

Received March 3, 1998; returned for revision March 26, 1998; revised manuscript received August 17, 1998

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