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The SR splicing factors ASF/SF2 and SC35 have antagonistic effects on intronic enhancer-dependent splicing of the β-tropomyosin alternative exon 6A

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Exons 6A and 6B of the chicken β-tropomyosin gene are mutually exclusive and selected in a tissue-specific manner. Exon 6A is present in non-muscle and smooth muscle cells, while exon 6B is present in skeletal muscle cells. In this study we have investigated the mechanism underlying exon 6A recognition in non-muscle cells. Previous reports have identified a pyrimidine-rich intronic enhancer sequence (S4) downstream of exon 6A as essential for exon 6A 5′-splice site recognition. We show here that preincubation of HeLa cell extracts with an excess of RNA containing this sequence specifically inhibits exon 6A recognition by the splicing machinery. Splicing inhibition by an excess of this RNA can be rescued by addition of the SR protein ASF/SF2, but not by the SR proteins SC35 or 9G8. ASF/SF2 stimulates exon 6A splicing through specific interaction with the enhancer sequence. Surprisingly, SC35 behaves as an inhibitor of exon 6A splicing, since addition to HeLa nuclear extracts of increasing amounts of the SC35 protein completely abolish the stimulatory effect of ASF/SF2 on exon 6A splicing. We conclude that exon 6A recognition *in vitro* depends on the ratio of the ASF/SF2 to SC35 SR proteins. Taken together our results suggest that variations in the level or activity of these proteins could contribute to the tissue-specific choice of β-tropomyosin exon 6A. In support of this we show that SR proteins isolated from skeletal muscle tissues are less efficient for exon 6A stimulation than SR proteins isolated from HeLa cells.
Keywords: alternative splicing/ASF/β-tropomyosin/exon selection/SC35

Introduction

The removal of introns from pre-mRNA precursors is an essential and often regulated step in the expression of eukaryotic genes. Although there has been much progress in the last few years in defining the molecular mechanisms and components involved in constitutive pre-mRNA splicing (reviewed in Lamond, 1993; Moore *et al.*, 1993), the mechanisms by which splice sites are selected remain obscure (Black, 1995). Specific recognition of the correct 5′- and 3′-splice sites is a fundamental step in the regulation of alternative splicing. The splice sites of most alternatively spliced exons are close to the consensus sequences and cannot, therefore, be the sole determinants of splice site selection. Clearly other elements, *in cis* and *in trans*, are needed to define which splice site will be chosen by the splicing machinery in a given cell environment. Studies of vertebrate genes aimed at understanding the regulation of alternative splicing have led to the identification of sequences essential for the tissue-specific choice of alternative exons (Mardon *et al.*, 1987; Hampson *et al.*, 1989; Streuli and Saito, 1989; Helfman *et al.*, 1990; Libri *et al.*, 1990; Black, 1992; Lavigueur *et al.*, 1993; Sun *et al.*, 1993; Watakabe *et al.*, 1993; Gooding *et al.*, 1994; Del Gatto and Breathnach, 1995; Ryan and Cooper, 1996). Notable among these, purine-rich elements identified in several vertebrate exons, called splicing enhancers, are required for efficient splicing of the resident exons. In general, exonic splicing enhancers appear to activate splicing of the upstream intron (Lavigueur *et al.*, 1993; Watakabe *et al.*, 1993; Xu *et al.*, 1993). These enhancer sequences contain contiguous repeats of the motif GARGAR (R = purine). Specific binding of SR proteins has been shown for enhancers present in exons of several genes. A direct correlation between SR protein binding and activation of splicing has been established for ASF/SF2 in the case of the growth hormone gene (Sun *et al.*, 1993) and SRp40 and SRp55 in the case of the troponin T gene (Ramchatesingh *et al.*, 1995). The SR proteins are essential splicing factors implicated in early steps of the spliceosome formation pathway (Krainer *et al.*, 1990; Fu and Maniatis, 1992b). In fact, commitment of a pre-mRNA to splicing requires binding of a specific SR protein to the pre-mRNA (Fu, 1993; Kohtz *et al.*, 1994; Staknis and Reed, 1994). Studies of *in vitro* protein–protein interactions and using the yeast two-hybrid system show that the individual RS domains of the SR proteins interact with themselves and with each other (Wu and Maniatis, 1993; Kohtz *et al.*, 1994). In particular, SC35 and ASF/SF2 have been shown to interact with both the 70 kDa protein of the U1 snRNP and the 35 kDa subunit of the splicing factor U2AF (Wu and Maniatis, 1993). These obervations suggest a role of these proteins in early 5′- and 3′-splice site interactions. They function as both essential and alternative splicing factors *in vivo* and *in vitro* (Fu, 1995). At least for some of them, alternative splicing activity can be antagonized by increasing the concentration of the general RNA binding protein hnRNP A1 (Mayeda *et al.*, 1992, 1993). This has led to the idea that some cases of tissue-specific splicing may be regulated by simply altering the concentrations or activities of constitutive pre-mRNA splicing factors. In contrast, genetic studies in *Drosophila melanogaster* have identified several *trans*-acting proteins that specifically modulate alternative splicing. Regulatory proteins have been identified that can either activate or repress the use

of specific 3′-splice sites involved in sex determination (Hodges and Bernstein, 1994).

We have used the chicken β-tropomyosin gene as a model to understand the mechanisms underlying the tissue-specific choice of mutually exclusive exons. This gene generates a number of different protein isoforms as a result of tissue-specific alternative splicing of its pre-mRNA. It contains three pairs of mutually exclusive exons. In two of these, the exon choice is related to the use of different promoters or polyadenylation signals. The third pair of exons (6A and 6B) are mutually exclusive and selected in a tissue-specific manner independent of the promoter or polyadenylation site choice. Exon 6A is incorporated in fibroblasts and smooth muscle cells, while exon 6B is skeletal muscle specific (Libri *et al.*, 1989a,b). Several sequences involved in the choice of exons 6A and 6B have been identified both *in vivo* and *in vitro*. Sequences at the 3′-end of the intron between exons 6A and 6B and within exon 6B repress the use of the exon 6B 3′-splice site in non-muscle cells (Libri *et al.*, 1990; Gallego *et al.*, 1992). A pyrimidine-rich region present in the intron downstream of exon 6A (S4) has been shown to be essential for recognition of the 5′-splice site of exon 6A (Balvay *et al.*, 1992; Gallego *et al.*, 1992).

In this study we sought to identify possible factors that interact with the S4 pyrimidine-rich sequence to facilitate exon 6A splicing. Preincubation of HeLa nuclear extracts with an excess of RNA containing this sequence specifically inhibits exon 6A recognition by the splicing machinery, suggesting that a specific factor is being sequestered so as to inhibit splicing. The S4 sequence can be replaced by a purine-rich sequence (P3AS, similar to the exonic enhancer sequences) as well as its complementary polypyrimidine sequence (P3S) with no change in the splicing efficiency of exon 6A. The fact that the purine-rich sequence stimulates exon 6A splicing prompted us to test the possible role of SR proteins in enhancer-dependent activation of exon 6A. ASF/SF2 stimulates exon 6A splicing through specific interaction with the intronic enhancer sequence. In contrast, the protein SC35 counteracts the stimulatory effect of ASF/SF2 on exon 6A splicing. These results demonstrate that the ratio of the proteins ASF/SF2 to SC35 influences the splicing efficiency of exon 6A *in vitro*. This leads to the hypothesis that variations in the level of these proteins could contribute to exon 6A exclusion in muscle cells. Indeed, we show that SR proteins prepared from skeletal muscle cells support exon 6A splicing poorly as compared with a HeLa SR protein preparation.

Results

A nuclear factor specifically recognizes intron sequences responsible for exon 6A recognition in non-muscle cells

We have previously identified a pyrimidine-rich sequence of 33 nt in the intron starting 37 nt downstream of the 5′-splice site of exon 6A that is essential for efficient recognition of this splice site (S4; see Figure 3A). Previous studies support the idea that exon 6A activation by the S4 sequence involves specific interactions with *trans*-acting factors present in HeLa cell nuclear extracts (Balvay *et al.*, 1992; Gallego *et al.*, 1992). To test this hypothesis we have examined the ability of an RNA containing exon 6A and downstream intron sequences to specifically interact with factors present in HeLa nuclear extracts, using native gel electrophoresis and binding competition. ^{32}P-Labeled RNAs were incubated with HeLa nuclear extracts and the resulting complexes were separated on native acrylamide gels. As shown in Figure 1, RNAs containing exon 6A and 200 nt of downstream intron sequences (712 Pmac) associate with extract proteins leading to retarded mobility. The same RNA molecule lacking the S4 sequence (33 nt) does not form a complex after incubation with HeLa nuclear extracts (Δ4 Pmac, deletion of the S4 region). The same result was obtained using RNAs where the 5′- (Δ10) or 3′-half (Δ11) of the S4 sequence was deleted. We have previously shown that both of these deletions (Δ10 and Δ11) weaken the use of the exon 6A 5′-splice site. As a control we analyzed complex formation using an RNA (Δ6 Pmac) where deletion of 25 nt of intron sequences (Δ6) just upstream of the S4 sequence has no effect on exon 6A splicing efficiency (Balvay *et al.*, 1992; Gallego *et al.*, 1992). This RNA gives a band of retarded mobility similar to wild-type RNA (Figure 1, Δ6 Pmac). These results suggest that HeLa nuclear extracts contain a factor(s) that interacts with the 33 nt intronic sequence defined by S4.

To confirm the specificity of this interaction, competition experiments using wild-type and mutant RNAs were performed. ^{32}P-Labeled wild-type RNA (712 Pmac) was incubated with HeLa nuclear extracts in the presence of different competitor RNAs. Addition of unlabeled wild-type RNA 712 Pmac or Δ6 Pmac reduced complex formation, while addition of Δ4 Pmac had very little effect on complex formation (data not shown). Taken together these results show that the S4 intron sequence specifically recognizes a protein(s) present in HeLa nuclear extracts.

Competitor S4 RNA specifically inhibits in vitro splicing of exon 6A

If the binding of a factor(s) to the S4 sequence is essential for exon 6A activation, then addition of an excess of unlabeled RNA containing the S4 sequence should specifically inhibit splicing of exon 6A. To test this hypothesis, we have determined the splicing efficiency of exons 6A–7 in the presence of increasing amounts of S4-containing RNA. The reference pre-mRNA used as substrate contains exon 6A, 194 nt of the downstream intron, 90 nt of the intron upstream of exon 7 and 38 nt of exon 7. The RNA used as competitor (S4 Pmac, 160 nt) is equivalent to 712 Pmac but lacking exon 6A and the first 37 nt of the intron downstream. Addition of an excess of S4 Pmac RNA to the splicing reaction strongly inhibited the splicing of β-tropomyosin exons 6A–7, as attested by the decrease in accumulation of mRNA and lariat intron (Figure 2, 6A–7). Similar results have been obtained when an RNA containing only the 33 nt of the S4 sequence was used as competitor (data not shown). To determine whether this inhibition is specifically associated with the presence of the enhancer sequence, competition experiments were performed on pre-mRNAs lacking the enhancer sequence (S4). The splicing efficiency of this pre-mRNA (6A–Δ4–7) is 4- to 5-fold lower than that of the wild-type pre-mRNA (6A–7). However, a measurable level of splicing independent of the presence of the

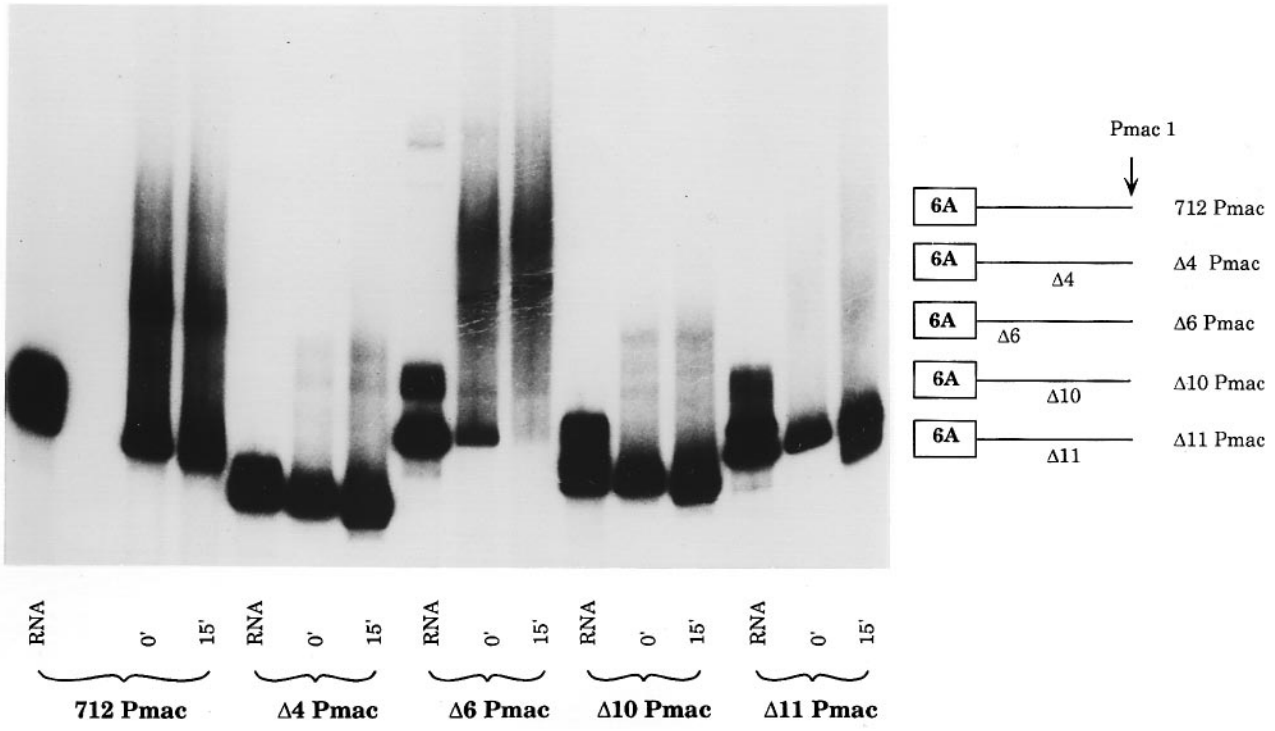

Fig. 1. HeLa cell nuclear extracts contain a factor(s) that specifically binds to the S4 intron sequence. ^{32}P-Labeled RNAs were incubated with HeLa cell nuclear extracts in splicing buffer for the indicated times and the resulting complexes were separated on a 4% non-denaturing acrylamide gel. For each transcript the first lane corresponds to RNA incubated in the absence of nuclear extract. The incubation times are indicated. A schematic diagram of the different RNAs used in the gel mobility assay are shown on the right. Δ4 is a deletion of the S4 sequence (33 nt). Δ10 and Δ11 are deletions of the 5′- and 3′-half respectively of the S4 sequence. Δ6 is a deletion of 25 nt of intron sequence just upstream of the S4 sequence.

enhancer sequence was still detected. We expect this level of splicing to be unaffected by the competition experiments described above. In fact, addition of the competitor RNA S4 Pmac had only a limited effect on the splicing efficiency of pre-mRNA lacking the intronic enhancer sequence S4 (Figure 2, 6A–Δ4–7). This result implies that the competitor RNA does not behave as a general inhibitor of splicing. We have, as well, determined the splicing activity of β-tropomyosin exons 6B–7 (this RNA does not contain the S4 enhancer sequence) in the presence of the same amounts of competitor S4 Pmac RNA. As shown in Figure 2 (right panel, 6B–7), addition of 1.5 pmol (60-fold molar excess) of S4 Pmac RNA has no effect on the splicing efficiency of β-tropomyosin exons 6B–7. Furthermore, neither human β-globin nor adenovirus pre-mRNA splicing efficiency was affected by the presence of RNA competitor S4 Pmac (data not shown). These results suggest that a titratable factor present in HeLa cell nuclear extracts is able to stimulate exon 6A recognition through interaction with the S4 sequence.

A purine-rich sequence can replace the S4 sequence for exon 6A 5′-splice site activation

We asked whether a sequence rich in the GAR elements, characteristic of exonic enhancer sequences, could replace the S4 sequence for splicing activity. The nucleotide composition of the sequences used to replace S4 are shown in Figure 3A. The results showing the effect of these substitutions on the splicing efficiency of exons 6A–7 are presented in Figure 3B. Pre-mRNA 6A–P3AS–7, in which the S4 enhancer sequence was substituted by a purine-rich sequence, gave a similar or higher level of splicing than the wild-type transcript (Figure 3B, 6A–P3AS–7). Replacement of S4 by the pyrimidine-rich sequence P3S (which is complementary to the P3AS sequence) fully restored splicing efficiency between β-tropomyosin exons 6A and 7 (Figure 3B, 6A–P3S–7). Similar results have been obtained after transfection of myoblasts with a DNA containing equivalent changes to the S4 sequence (Balvay *et al.*, personal communication). In contrast, deletion of the S4 sequence (6A–Δ4–7), or replacement of the S4 sequence by the S5 sequence (6A–S5–7), led to a decrease in splicing efficiency (Figure 3B). Splicing efficiency of 6A–Δ4–7 and 6A–S5–7 was reduced 5-fold and 3-fold respectively compared with that of 6A–7. Note that the S5 sequence, which corresponds to the intronic 32 nt sequence immediately downstream of the S4 sequence, can be deleted with no effect on exon 6A recognition *in vivo* (Balvay *et al.*, 1992). Similar results have been obtained *in vitro* (Gallego *et al.*, unpublished observations).

The fact that three different sequences could act as enhancers for activation of the β-tropomyosin exon 6A 5′-splice site raised the question of whether they are recognized by a common factor. To answer this question, we tested the effect of addition of competitor RNA S4 Pmac on the splicing efficiency of pre-mRNAs containing P3AS and P3S as enhancer sequences. The results in Figure 3C show that splicing efficiency of both pre-mRNAs (6A–P3AS–7 and 6A–P3S–7) was reduced in the presence of an excess of RNA containing the S4 sequence, as attested by the strong decrease in accumulation of the mRNA. However, higher concentrations of competitor RNA (between 20 and 40%) are needed to reach an

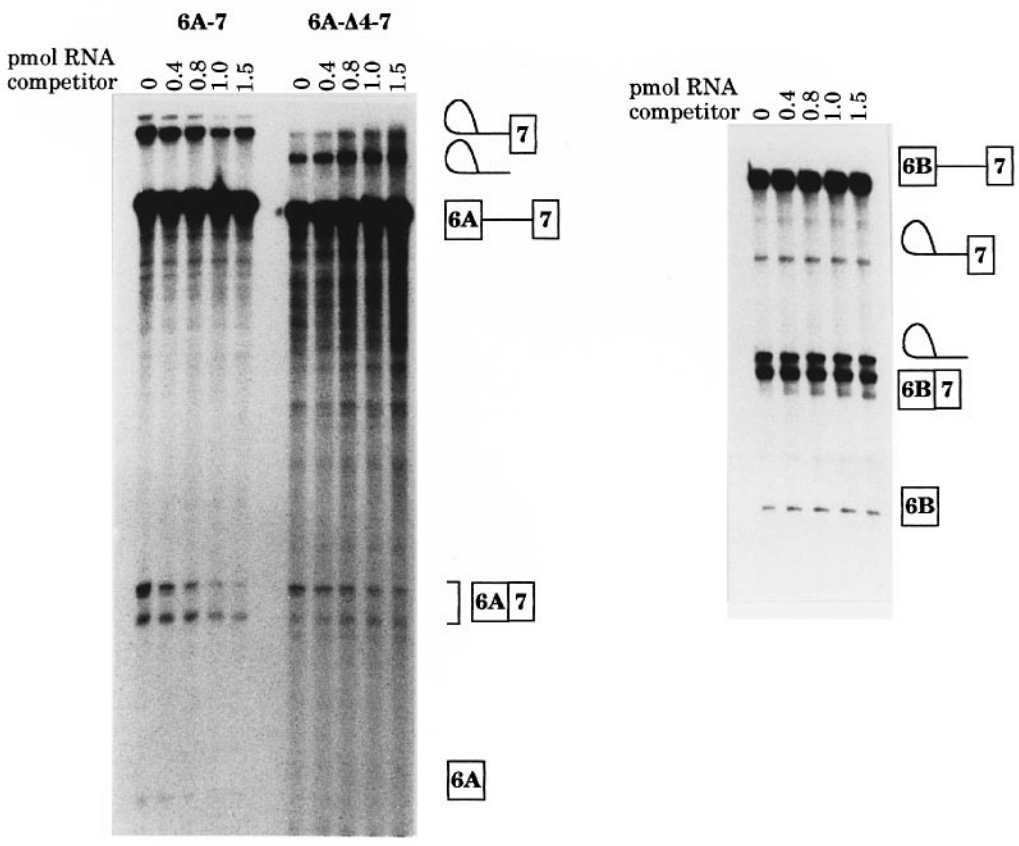

Fig. 2. Specific inhibition of exon 6A splicing by competitor RNA containing the S4 sequence. ^{32}P-Labeled pre-mRNA substrates were incubated with HeLa cell nuclear extracts under standard splicing conditions, either in the absence or presence of unlabeled competitor RNA (S4 Pmac). The substrate in the left panel (6A–7) contains β-tropomyosin exon 6A (63 nt), 194 nt of the downstream intron fused to 90 nt of the intron upstream of exon 7 and 38 nt of exon 7. The pre-mRNA 6A–Δ4–7 is equivalent to 6A–7 but lacks the 33 nt corresponding to the S4 sequence. The substrate on the right consists of β-tropomyosin exons 6B and 7 and the complete intron between them. Splicing reactions were carried out for 2 h. The products of the reaction were separated on a 7% denaturing polyacrylamide gel. Schematic representation of the splicing reaction substrate, intermediates and products are indicated on the right. Boxes represent exons and lines represent introns.

equivalent level of inhibition when compared with the wild-type pre-mRNA 6A–7 (Figure 2). Thus, we conclude that the wild-type enhancer S4 Pmac can titrate a factor(s) needed for enhancer function of the P3AS as well as P3S sequences. In a similar way, an excess of competitor RNA containing either the P3S or the P3AS sequence inhibited splicing of exons 6A–7 independent of the nature of the enhancer sequence (data not shown). Therefore, these results suggest that the three enhancer sequences tested interact with a common set of proteins present in HeLa nuclear extracts.

ASF/SF2 but not SC35 or 9G8 rescues splicing of exon 6A in the presence of an excess of S4 RNA

The SR proteins have been shown to bind to purine-rich enhancer sequences present in the exons of several alternatively spliced genes (Lavigueur *et al.*, 1993; Sun *et al.*, 1993; Heinrichs and Baker, 1995; Ramchatesingh *et al.*, 1995; Tacke and Manley, 1995; Wang *et al.*, 1995). The fact that a purine-rich sequence can replace the S4 sequence for exon 6A activation led us to ask whether SR proteins participate in enhancer-dependent splicing of β-tropomyosin exon 6A. We initially tested the ability of the different enhancer sequences to bind SR proteins. Uniformly labeled RNAs corresponding to the three different enhancer sequences (S4, P3S and P3AS) and the S5 sequence were UV crosslinked in splicing buffer with purified total SR proteins from HeLa cell extracts (purified as previously described; Zahler *et al.*, 1992). The results in Figure 4 show that the three enhancer sequences interact with the SR proteins and especially with the 30 kDa SR proteins. In contrast, the S5 sequence, which is not able to activate exon 6A 5′-splice site recognition, interacts poorly with the SR proteins. These results indicate a correlation between enhancer activity and the capacity to interact with the 30 kDa SR proteins. Of the known SR proteins, ASF/SF2, SC35 and 9G8 (Cavaloc *et al.*, 1994) possess molecular weights of 30 kDa. To determine whether this binding was functionally significant, we have analyzed the ability of the recombinant proteins ASF/SF2, SC35 and 9G8 (each isolated from baculovirus-infected insect cells) to rescue splicing activity of exons 6A–7 in the presence of the RNA competitor S4 Pmac. The activity of the individual preparations was determined as their capacity to complement a cytoplasmic S100 fraction with a human β-globin splicing substrate.

The results in Figure 5 show that addition of the protein ASF/SF2 in the presence of 0.8 pmol competitor RNA S4 Pmac strongly increased the splicing efficiency of pre-mRNAs containing any of the enhancer sequences

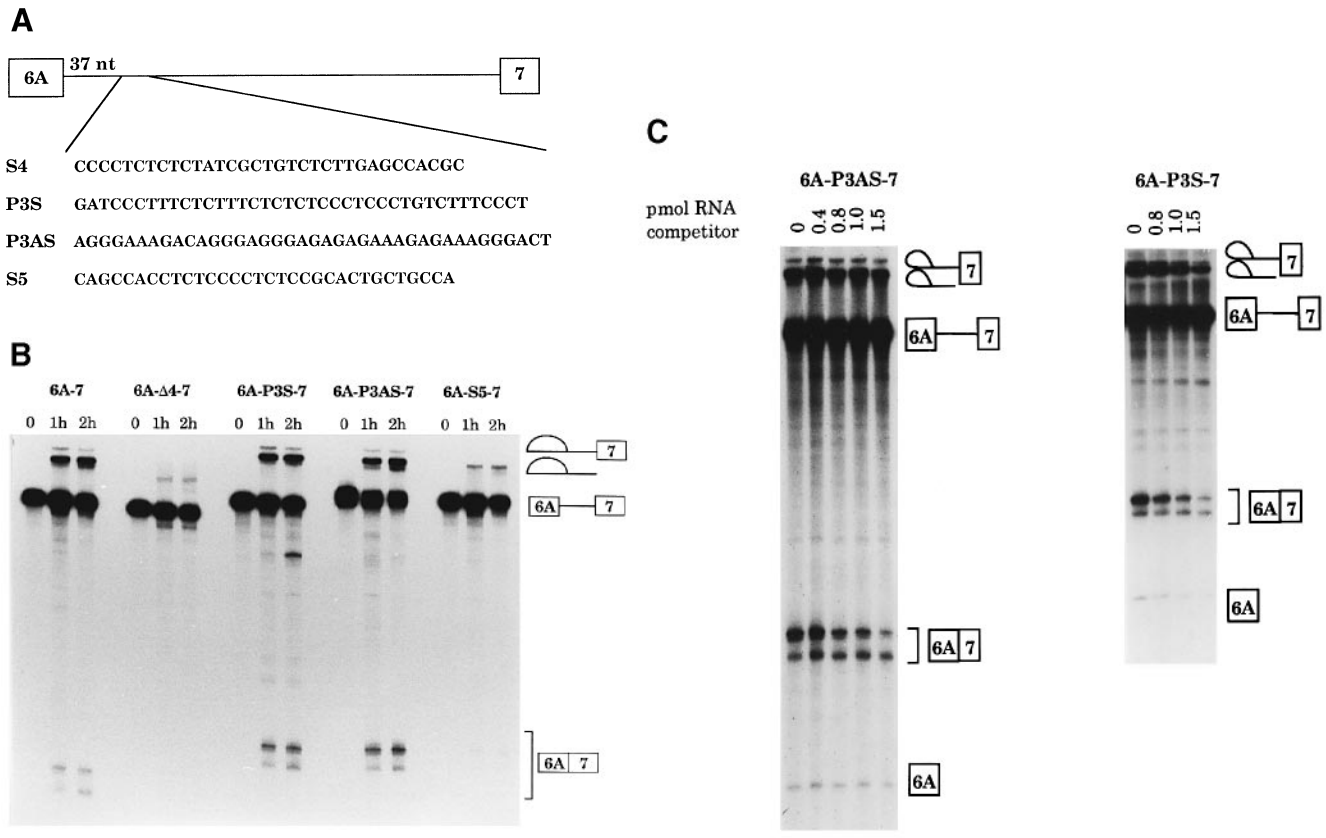

Fig. 3. Two different sequences can replace S4 for exon 6A activation. (**A**) Diagrammatic representation of the pre-mRNA containing exons 6A and 7. The intron location of the enhancer sequence S4 and its distance to the exon 6A 5′-splice site are indicated. The nucleotide composition of the sequences used to replace S4 (P3S, P3AS and S5) are presented at the bottom of the diagram. (**B**) Splicing efficiency of exons 6A–7 of the S4 mutant pre-mRNAs. The names of the different pre-mRNAs are indicated at the top. 6A–7, wild-type pre-mRNA; 6A–Δ4–7, pre-mRNA lacking the S4 sequence; for pre-mRNAs 6A–P3S–7, 6A–P3AS–7 and 6A–S5–7 sequence S4 was replaced by sequences P3S, P3AS and S5 respectively. Reaction mixtures were incubated under splicing conditions in 60% nuclear extract for the indicated times. The position of the pre-mRNA, products and intermediates of the reaction are indicated on the right. (**C**) Splicing of mutant pre-mRNAs in the presence of S4 competitor RNA. The nature of the pre-mRNA tested as well as the amount of competitor RNA added to the reaction are indicated at the top. Reaction mixtures were incubated for 2 h under standard splicing conditions with 35% nuclear extract.

(Figure 5, 6A–7, 6A–P3S–7 and 6A–P3AS–7). However, as expected, addition of ASF/SF2 protein had only a limited effect on the splicing efficiency of a pre-mRNA lacking an enhancer sequence (Figure 5, 6A–Δ4–7). Quantification data show that splicing of 6A–Δ4–7 is 27% stimulated after addition of ASF/SF2, while that of 6A–7 is 125% stimulated. Stimulation of 6A–P3S–7 and P3AS–7 pre-mRNAs by ASF/SF2 was roughly the same as that of 6A–7 pre-mRNA. From these results, we suggest that the level of activation observed on 6A–Δ4–7 is most likely associated with the role of the protein ASF/SF2 as a general splicing factor. However, these results also indicate that in addition to its general role, ASF/SF2 acts specifically through interaction with the enhancer sequences. In agreement with *in vitro* splicing experiments, UV crosslinking shows that purified ASF/SF2 binds to the three enhancer sequences, while no binding is observed on the S5 sequence (Figure 6). In contrast, neither the SC35 nor 9G8 proteins are able to rescue splicing of pre-mRNAs containing either of the enhancer sequences (data not shown). We conclude that the binding of ASF/SF2 to the enhancer intronic sequence downstream of exon 6A is functionally significant and responsible for activation of exon 6A splicing.

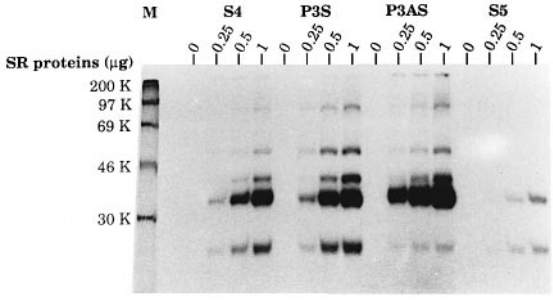

Fig. 4. UV crosslinking of SR proteins to the three enhancer sequences. The different RNAs tested (S4, P3S, P3AS and S5) are indicated at the top of the Figure. Aliquots of 50 fmol RNA were incubated with the indicated amount of HeLa SR proteins in splicing buffer with ATP and creatine phosphate for 15 min at 30°C prior to irradiation. Crosslinked proteins were resolved by electrophoresis on a 10% polyacrylamide gel. M, ^{14}C molecular weight markers.

SC35 inhibits splicing of exon 6A in an enhancer-specific manner

We have tested the capacity of a SR protein preparation to rescue splicing activity between exons 6A and 7 in the presence of the competitor RNA S4 Pmac. Activity of the

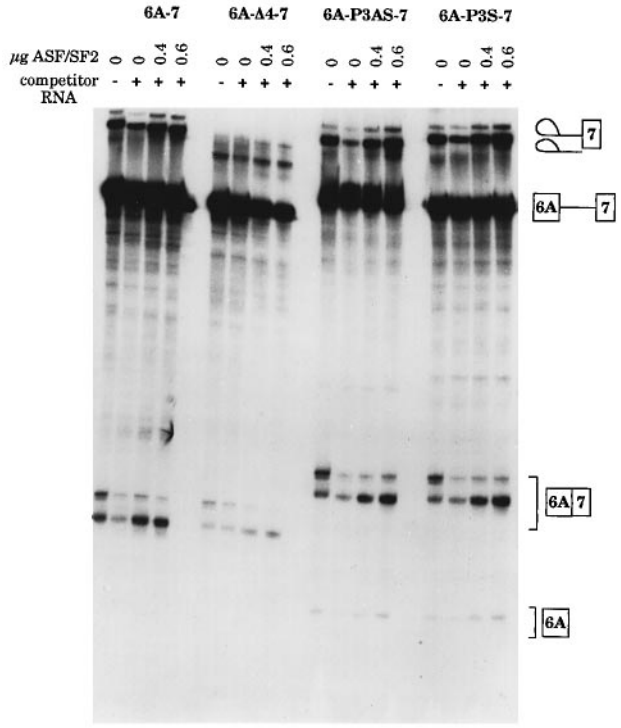

Fig. 5. ASF/SF2 stimulates splicing of enhancer-containing pre-mRNAs in the presence of competitor RNA. The names of the different pre-mRNAs used as substrates for the splicing reaction are indicated at the top. The presence or absence of 0.8 pmol competitor RNA (S4 Pmac) on the splicing reaction is indicated by the signs + or –. The amounts of recombinant ASF/SF2 added to each reaction are indicated. Reaction mixtures were incubated under splicing conditions for 2 h. The splicing products and intermediates are represented on the right.

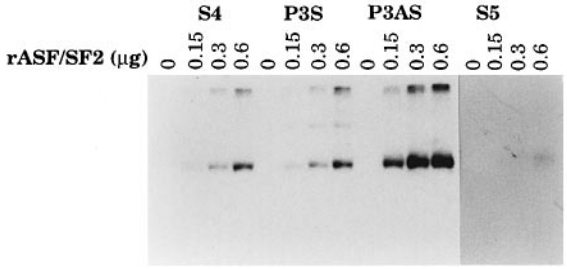

Fig. 6. Recombinant ASF/SF2 binds specifically to the three enhancer sequences. The different RNAs tested (S4, P3S, P3AS and S5) are indicated at the top of the Figure. Aliquots of 50 fmol RNA were incubated with the indicated amount of proteins in splicing buffer with ATP and creatine phosphate for 15 min at 30°C prior to irradiation. Crosslinked proteins were resolved by electrophoresis on a 10% polyacrylamide gel. The negative control lanes (S5) are overexposed relative to the others in order to emphasize the absence of protein interaction.

SR proteins was controlled with a β-globin or adenovirus splicing substrate after addition to S100 extract.

Results presented in Figure 7 indicate that addition of SR proteins can rescue splicing of exons 6A–7 for pre-mRNAs containing either the wild-type enhancer sequence S4 (Figure 7, 6A–7) or the purine-rich enhancer P3AS (Figure 7, 6A–P3AS–7). As expected, no stimulation of splicing activity for a precursor RNA lacking an enhancer sequence (Figure 7, 6A–Δ4–7) was detected after addition of SR proteins. Surprisingly, splicing of the pre-mRNA containing the pyrimidine-rich enhancer sequence P3S was not activated after addition of the SR proteins (Figure 7, 6A–P3S–7). This result is in apparent contradiction to those described above showing that the SR protein ASF/SF2 is able to rescue splicing of exons 6A–7 independent of the nature of the enhancer sequence (Figure 5).

The simplest hypothesis to explain this discrepancy would be the presence in the total SR preparation of a protein able to counteract the effect of ASF/SF2 on exon 6A activation. As mentioned above, the three enhancer sequences UV crosslinked a protein(s) of 30 kDa in a total SR protein preparation. We decided to determine whether the 30 kDa SR proteins SC35 and 9G8 could counteract ASF/SF2 for exons 6A–7 splicing. In fact, addition of recombinant SC35 protein to the *in vitro* splicing reaction strongly inhibited splicing of exons 6A–7 for pre-mRNAs containing the P3S enhancer sequence (Figure 8, 6A–P3S–7) and to a lesser extent that containing the wild-type enhancer sequence S4 (Figure 8, 6A–7). Addition of 0.6 μg SC35 reduced the splicing efficiency of 6A–7 and 6A–P3S–7 pre-mRNA by 55 and 75% respectively. This result may explain the inability of a total SR protein preparation to rescue splicing of exons 6A–7 in the presence of the P3S enhancer sequence. In contrast, only a limited effect was observed on the splicing efficiency of the pre-mRNA containing the enhancer sequence P3AS (15%), indicating that SC35 does not have a general inhibitory effect on exon 6A recognition (Figure 8, 6A–P3AS–7). We conclude that exon 6A splicing inhibition by SC35 is correlated with the nature of the enhancer sequence.

To further confirm that the inhibitory effect of SC35 was specific, we tested the effect on exons 6A–7 splicing of addition of the proteins 9G8 and ASF/SF2 to a HeLa nuclear extract. Addition of equivalent or higher amounts of the SR protein 9G8 had no effect on the splicing efficiency of exons 6A–7 (data not shown). In agreement with the results in Figure 5, addition of ASF/SF2 stimulated splicing of pre-mRNAs containing any of three enhancer sequences (data not shown). We conclude that the inhibitory effect of SC35 on exons 6A–7 splicing is specific to this SR protein and is not a general effect induced by addition to the HeLa nuclear extract of an excess of any SR protein.

SC35 antagonizes the positive effect of ASF/SF2 in exon 6A splicing

The simplest hypothesis to explain the inhibitory effect of SC35 on the splicing efficiency of exons 6A–7 is competition with ASF/SF2 on the enhancer sequence. In order to obtain a functional test for the ability of SC35 to directly counteract the effect of ASF/SF2 on exon 6A activation we prepared HeLa nuclear extracts depleted of SR proteins. We used as a SR-depleted HeLa nuclear extract the fraction that precipitates at 65% ammonium sulfate. This SR-depleted HeLa nuclear extract showed very little activity in supporting splicing of exons 6A–7 (Figure 9, lane 1 for each pre-mRNA). No splicing activity was detected when a human β-globin pre-mRNA was used as substrate (data not shown). Addition of SC35 was not able to stimulate splicing of exons 6A–7, whatever the nature of the enhancer sequence (Figure 9, lanes 2 and 3 for each pre-mRNA). However, as expected, splicing

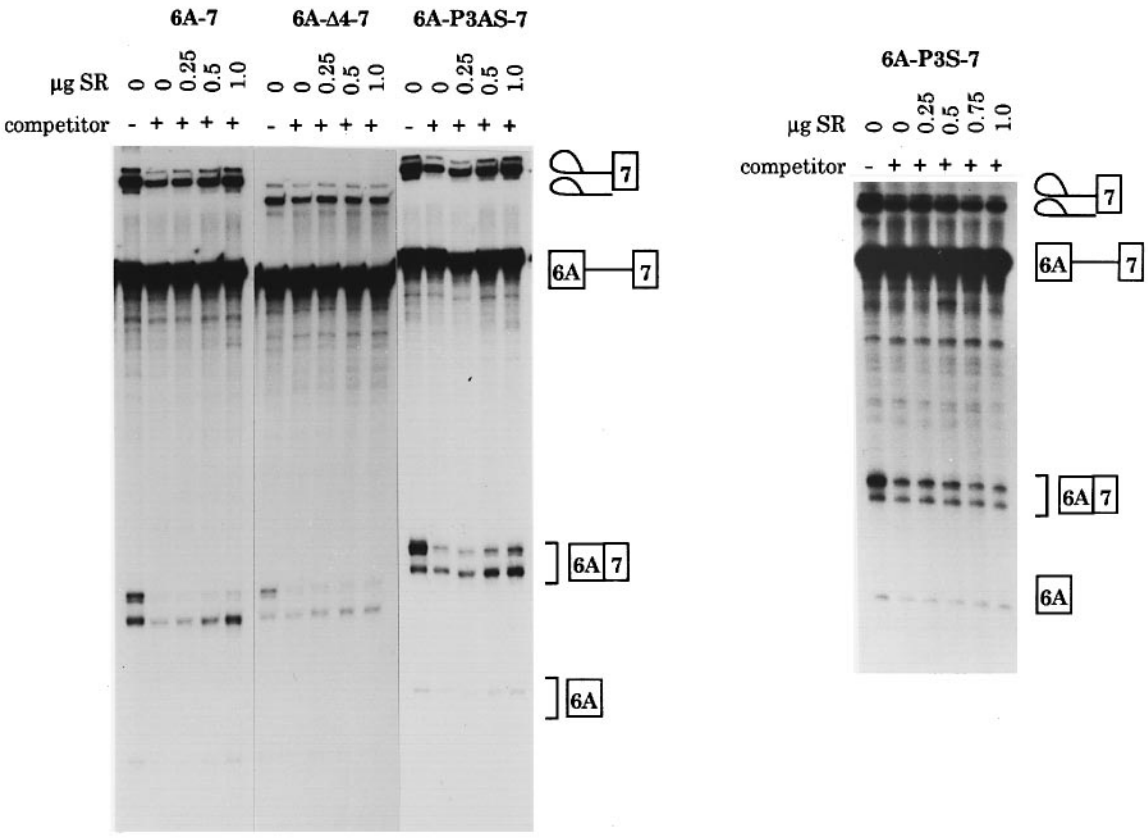

Fig. 7. The SR proteins stimulate exon 6A splicing in an enhancer-specific manner. The pre-mRNA substrates containing an enhancer sequence (6A–7, 6A–P3S–7 and 6A–P3AS–7) or lacking the S4 sequence (6A–Δ4–7) were incubated under standard splicing conditions. The absence or presence of 0.8 pmol competitor RNA (S4 Pmac) is indicated (– or +). The amount of SR proteins from HeLa cells added to each reaction is indicated in μg. After 2 h incubation, splicing products were separated in a 7% denaturing acrylamide gel. The position of the precursor, products and intermediates are shown on the right.

of the three pre-mRNAs used as subtrate was stimulated after addition of ASF/SF2 (lane 4 for each pre-mRNA). To answer whether SC35 could directly counteract the splicing stimulatory effect of ASF/SF2, we added increasing amounts of SC35 to the ASF/SF2-containing extract. The results of this experiment indicate that addition of SC35 has an inhibitory effect on the splicing efficiency of pre-mRNAs containing either the wild-type enhancer sequence S4 or the P3S enhancer sequence (Figure 9, lanes 5–7, 6A–7 and 6A–P3S–7). In contrast, very little effect on the splicing of exons 6A–7 was detected after addition of equivalent amounts of SC35 for the pre-mRNA containing the P3AS enhancer sequence (Figure 9, lanes 5–7, 6A–P3AS–7), which confirmed the results presented in Figure 8. Addition of 0.4 μg SC35 reduced splicing of 6A–P3S–7 and 6A–7 by 90 and 75% respectively, while splicing of 6A–P3AS–7 was only reduced by 20%. Taken together these results strongly suggest that recognition of β-tropomyosin exon 6A depends upon the ratio of ASF/SF2 to SC35. These two proteins have an antagonistic effect on intronic enhancer-dependent activation of β-tropomyosin exon 6A.

SR proteins prepared from skeletal muscle tissue do not support exon 6A splicing

Previous results have shown that exon 6A exclusion in myotubes depends upon the presence of an active exon 6B, suggesting that competition between these two exons is the mechanism allowing exclusive use of exon 6B (Libri *et al.*, 1992). It has been shown that the SR proteins are differentially expressed in a variety of cells and tissues (Fu and Maniatis, 1992a; Vellard *et al.*, 1992; Zahler *et al.*, 1993). In particular, skeletal muscle tissue SR preparations contain a proportionally lower amount of the 30 kDa proteins compared with the thymus SR proteins (Zahler *et al.*, 1993).

We have considered the hypothesis that non-recognition of the intronic enhancer sequence downstream of exon 6A in skeletal muscle cells may be related to the SR factor content of this tissue. To test this assumption, we prepared SR proteins from chicken embryo pectoral muscle tissue and compared them with HeLa SR proteins by SDS–PAGE analysis followed by immunoblotting with mAb 104 or specific antibodies directed against SC35 (αSC35; see Materials and methods). Figure 10A (left panel) shows an mAb 104 immunoblot with an equal amount of SR proteins extracted from HeLa cells and skeletal muscle (180 ng). HeLa cells and skeletal muscle exhibit a different profile of SR proteins. SRp55 and SRp20 are more abundant in skeletal muscle tissues than in HeLa cells, in contrast to SRp40 and SRp75, which are more abundant in HeLa cells than in muscle tissues. However, for the same amount of SR proteins prepared from HeLa cells or skeletal muscle, the monoclonal antibody mAb 104 recognizes roughly equivalent amounts of SRp30 proteins. In contrast, the αSC35 immunoblot

(Figure 10A, right pannel) reveals that 90 ng of skeletal SR preparation gives a stronger signal than 180 ng of the SR preparation from HeLa cells. Quantitative analysis shows that skeletal SR proteins contain 2.3 times more SC35 protein than HeLa SR proteins, referenced to an equivalent amount of total SRp30 proteins. To test the activity of the two SR protein preparations, substrate pre-mRNA was incubated in a SR-depleted HeLa nuclear extract. To facilitate the analysis, we used a pre-mRNA containing exons 5, 6A and 7 in which the 3′-splice site of exon 6A had been deleted, allowing only splicing of exon 5 or exons 6A to 7. The results in Figure 10B show that addition of 0.5 μg SR proteins from HeLa cells activated splicing of exons 5–7, as attested by accumulation of the slower migrating lariat (Figure 10B, lane 2). Increasing the amount of SR proteins to 0.75 μg allowed activation of exons 6A–7 splicing (Figure 10B, lanes 3–5). As shown in the right panel, addition of SR proteins from skeletal muscle cells supported splicing of exons 5–7 to a level comparable with that of the HeLa cell SR protein preparation. However, no significant stimulation of exons 6A–7 splicing was detected, even after addition of 1.5 μg SR proteins (Figure 10B, lane 9). Thus, these results indicate that the SR proteins from muscle cells recognize the intronic enhancer sequence downstream of exon 6A poorly *in vitro*. This also supports the idea that in muscle cells down-regulation of exon 6A may contribute to its exclusion in this type of cell.

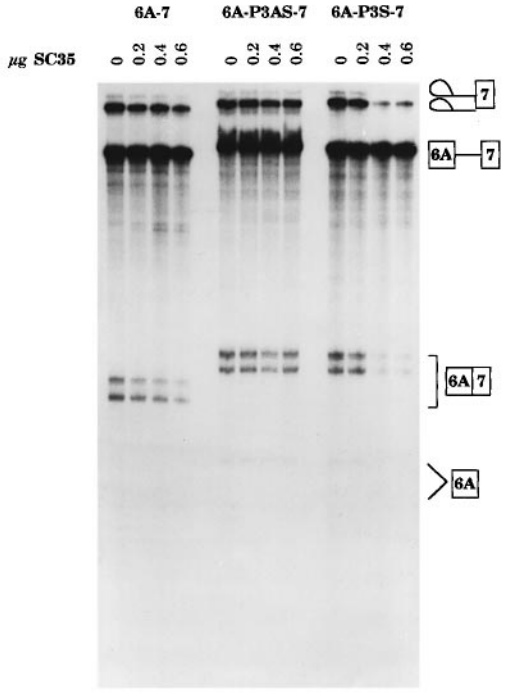

Fig. 8. Addition of SC35 to HeLa nuclear extracts inhibits splicing of exons 6A–7. Pre-mRNA subtrates containing each of the three enhancer sequences were incubated under splicing conditions in the presence of the indicated amounts of baculovirus recombinant SC35. Splicing of human β-globin was not inhibited after addition of equivalent or higher amounts of SC35 (data not shown).

Discussion

Role of ASF/SF2 as a positive regulator of β-tropomyosin exon 6A splicing

We have previously identified a splicing enhancer element (S4) in the intron downstream of chicken β-tropomyosin exon 6A as essential for its recognition (Balvay *et al.*, 1992; Gallego *et al.*, 1992). The purpose of this study was the identification of the factor(s) able to activate exon 6A recognition through binding to the S4 sequence. We show here that the SR protein ASF/SF2 is able to activate the use of exon 6A through specific recognition of the S4 enhancer sequence. This conclusion is supported by the results showing that: (i) addition of ASF/SF2 to the splicing reaction relieved the splicing inhibition generated by the presence of competitor RNA S4; (ii) ASF/SF2 binds specifically to the three enhancer sequences. This is the first demonstration that a positive acting intronic sequence can be activated through interaction with a SR protein (ASF/SF2).

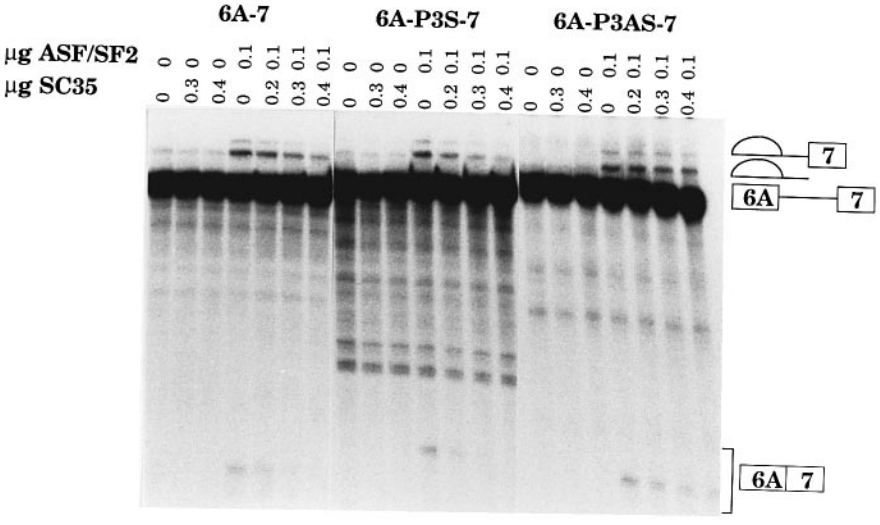

Fig. 9. SC35 antagonizes enhancer-mediated splicing activation of exon 6A by ASF/SF2. The pre-mRNA subtrates were incubated under splicing conditions with an SR-depleted nuclear extract (fraction precipitated at 65% ammonium sulfate). The indicated amounts of SC35 and/or ASF/SF2 were added to each reaction mixture. Under these conditions, addition of the SC35 protein stimulated splicing of a human β-globin substrate (data not shown).

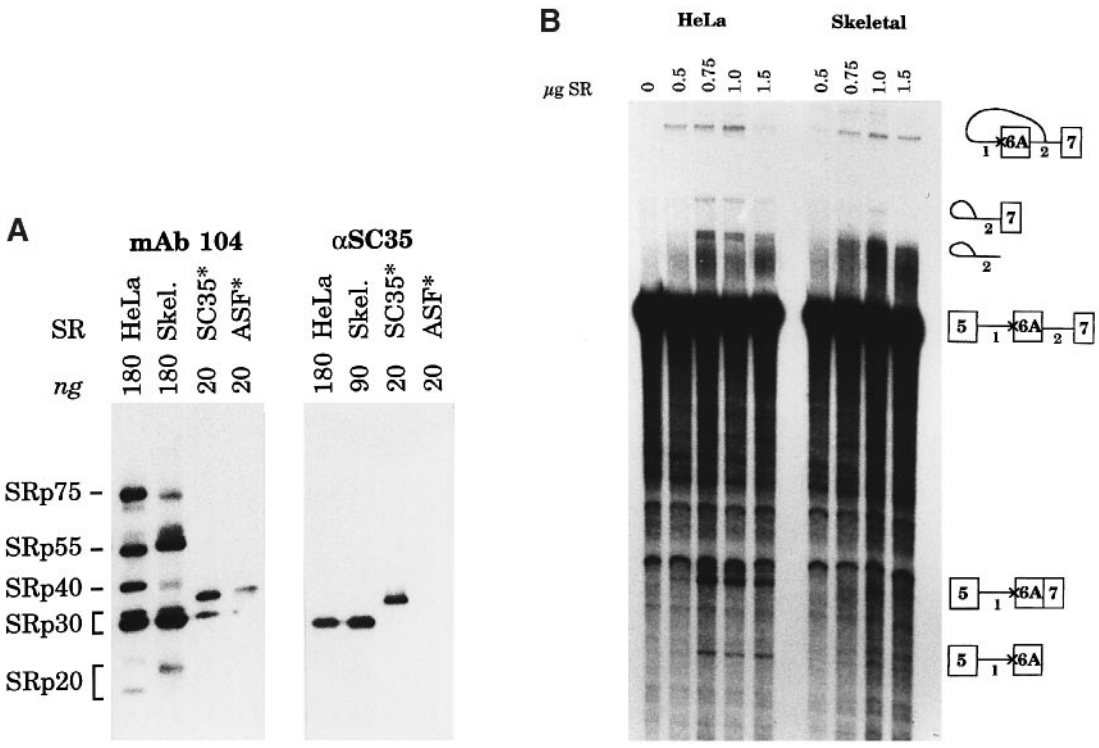

Fig. 10. SR proteins from skeletal muscle are unable to activate exon 6A splicing. (**A**) Immunoblot of SR proteins extracted from HeLa cells and skeletal tissues with mAb 104 and αSC35. SR proteins were subjected to SDS–PAGE. After transfer to nitrocellulose, blots were incubated with either mAb 104 (left pannel) or αSC35, which specifically recognizes SC35 (right pannel). The amount of SR proteins as well as the nature of the antibodies used for immunoblots are indicated at the top of the figure. SC35* and ASF*, presented as controls, refer to the recombinant proteins. Due to their histidine tag, the recombinant proteins exhibit a higher molecular weight than SR proteins isolated from tissues. Note that 90 ng skeletal muscle SR proteins versus 180 ng HeLa SR proteins were loaded on the gel (right panel). (**B**) Splicing of 5–6A–7 pre-mRNA in an SR-depleted nuclear extract supplemented with SR from HeLa cells or skeletal tissues. The substrate RNA used contains β-tropomyosin exon 5, 70 nt of the downstream intron, 63 nt of exon 6A, 194 nt of the downstream intron, 90 nt of the intron upstream of exon 7 and 38 nt of exon 7. This substrate does not contain the 3′-splice site of exon 6A. The indicated amounts of SR proteins prepared from HeLa cells or skeletal tissue were added to the SR-depleted HeLa nuclear extract (fraction precipitated at 65% ammonium sulfate). The structures of the observed bands are indicated to the right of the figure.

Several intronic splicing enhancer sequences have been described. Splicing of exon IIIB of the rat fibronectin gene depends on repeats in the downstream intron of the motif UGCAUG (Huh and Hynes, 1994). Mutation of the six motifs (AU)GGG present in the chicken β-tropomyosin intron downstream of exon 6B strongly inhibits splicing of this intron (Sirand-Pugnet *et al.*, 1995). Interestingly, as for the tropomyosin S4 enhancer sequence, the other three intronic enhancers described to date are enriched in pyrimidines and close to the 5′-splice site. One is present in the intron downstream of the N1 exon in the c-*src* gene and is essential for its inclusion (Black, 1992). The second lies downstream of the K-*Sam* exon of the fibroblast growth factor receptor 2 (Del Gatto and Breathnach, 1995). The third consists of three intronic elements located downstream of the 5′-splice site of exon 5 of cardiac troponin T, mutations of which prevent exon 5 inclusion in muscle cells (Ryan and Cooper, 1996). Thus, all these pyrimidine-rich enhancer sequences might constitute a new family of intronic enhancers. Only in the case of c-*src* were the proteins interacting with the enhancer sequence investigated. In that case, ASF/SF2 does not seem to be essential for exon N1 recognition (Min *et al.*, 1995). However, this does not exclude the possibility that other SR proteins might be involved in the activation process, as has been observed for recognition of exon 5 of cardiac troponin T (Ramchatesingh *et al.*, 1995). It will be interesting to determine whether SR proteins play a general role in the activation of non-purine-rich intronic enhancer sequences.

The involvement of ASF/SF2 in the activation of a pyrimidine-rich enhancer sequence is unexpected. Indeed, to date ASF/SF2 has been implicated in the recognition of exonic enhancer sequences rich in purines (Sun *et al.*, 1993; Ramchatesingh *et al.*, 1995). In fact, *in vitro* selection of high affinity target sequences for ASF/SF2 identified purine-rich sequences (Tacke and Manley, 1995). One possibility is that ASF/SF2 does not interact with very high affinity with the pyrimidine-rich S4 sequence. In agreement with this, we have indications that recombinant ASF/SF2 binds to a S4-containing transcript with a lower affinity than to a transcript containing GAA repeats (data not shown). This opens the possibility that the interaction of ASF/SF2 with the target sequence could be mediated or facilitated by another sequence-specific binding protein. Although ASF/SF2 is needed for enhancer-dependent stimulation of exon 6A, we cannot conclude that it is sufficient in itself. Among the factors needed for enhancer recognition, ASF/SF2 could be the only one present in limiting amounts in HeLa nuclear extracts. Supporting this hypothesis we have found that ASF/SF2 and the SR proteins fail to activate exon 6A splicing when added to

an S100 fraction, while human β-globin pre-mRNA is efficiently spliced (our unpublished observation). Recently, Tacke and Manley (1995) have shown that an element containing three copies of a high affinity ASF/SF2 binding site behaves as an exonic splicing enhancer. Consistent with our observation, they also showed that ASF/SF2 is needed, though not sufficient, to activate the enhancer in a splicing-deficient S100 extract. Therefore, these results indicate that the S100 fraction lacks some activities present in an SR-depleted nuclear extract (our studies) or in a 20–40% ammonium sulfate fraction of nuclear extract (Tacke and Manley, 1995). Conceivably, these activities are dispensable for basal splicing, but could be absolutely required for the splicing of introns with inherently weak splicing signals. That several factors may be needed to promote splicing activation has been shown by analyses of the female-specific splicing of *Drosophila doublesex* (*dsx*) pre-mRNA. The products of the *transformer* (*tra*) and *transformer*-2 (*tra*-2) genes activate *dsx* female-specific splicing by promoting the formation of a splicing enhancer complex which includes the SR proteins and probably other unidentified proteins (Tian and Maniatis, 1993; Amrein *et al.*, 1994; Lynch and Maniatis, 1996). It is possible that formation of an enhancer splicing complex constitutes a general mechanism for enhancer-mediated activation of splicing. Further experiments will be required to establish whether the mechanism of β-tropomyosin exon 6A 5′-splice site activation involves the formation of a splicing enhancer complex.

SC35 as an inhibitor of splicing

The results in Figure 8 indicate that the general splicing factor SC35 behaves as an inhibitor of β-tropomyosin exon 6A splicing and that its effect is specific, since it depends on the nature of the intronic enhancer sequence. Furthermore, we show that SC35 directly antagonizes ASF/SF2 for exon 6A splicing activation (Figure 9). These results indicate that SC35, although able to interact with the enhancer sequence, is not able to induce exon 6A splicing activation. In agreement with our observations, it has been shown that three copies of a SC35 high affinity binding site failed to function as an exonic enhancer sequence (Tacke and Manley, 1995). Since SC35 binds to the target sequence, it has been suggested that SC35 may be unable to stimulate splicing of a weak 3′-splice site by binding to exon sequences. These authors selected target sequences *in vitro* for the proteins ASF/SF2 and SC35. They found that some of these sequences can be recognized by both proteins. It would be interesting to test whether these types of sequences will work as splicing enhancers through specific interaction with ASF/SF2. Since they were selected as sequences also recognized by SC35, it is tempting to predict splicing inhibition by an excess of this protein. At present, no example of natural enhancer activation by SC35 has been reported. Indeed, it has been shown that SC35 is unable to activate enhancer-dependent splicing for either the last exon of bovine growth hormone or exon 5 of cardiac troponin T pre-mRNAs (Sun *et al.*, 1993; Ramchatesingh *et al.*, 1995). However, in both cases the lack of activity was associated with an inability of SC35 to bind the enhancer element. SC35 has been identified as a constituent of two enhancer splicing complexes; however, a direct correlation between binding of SC35 and splicing activation has not been established (Tian and Maniatis, 1993; Wang *et al.*, 1995).

Recently, Kanopka *et al.* (1996) reported the inhibition of adenovirus IIIa pre-mRNA splicing by SR proteins. The repressor element located upstream of the IIIa branch site is purine rich. They have shown that ASF/SF2 interacts with the repressor element and inhibits splicing of IIIa pre-mRNA. Thus, in contrast to what has been previously thought, this work and our work demonstrate that SR proteins can also be repressors of splicing. Other examples of proteins inhibiting splicing by counteracting the role of general splicing factors have been described. Enhancer-dependent stimulation of bovine growth hormone intron D splicing by ASF/SF2 is antagonized by hnRNP A1 (Sun *et al.*, 1993). This protein seems to bind with low specificity to the target sequence. Whether hnRNP A1 competes with ASF/SF2 for overlapping binding sites or hnRNP A1 blocks protein–protein interaction between ASF/SF2 and other factors remains to be shown. The second example is the work of two independent groups suggesting that binding of PTB to the pyrimidine tract of some introns could repress 3′-splice site use by preventing binding of the splicing factor U2AF (Lin and Patton, 1995; Singh *et al.*, 1995). Lastly, the protein Sex-lethal (Sxl) inhibits the use of the non-sex-specific 3′-splice site in the first intron of *tra* pre-mRNA. It has been shown that Sxl has a higher affinity for the non-sex-specific polypyrimidine tract than the essential splicing factor U2AF. Furthermore, it was concluded that the inability of Sxl to activate splicing is due to the lack of the splicing effector domain present in U2AF. Thus, addition of the U2AF RS domain to Sxl converts this protein from a splicing repressor to an activator (Valcarcel *et al.*, 1993).

In the example presented here, where SC35 antagonizes the enhancer-dependent splicing effect of ASF/SF2, both proteins contain an RS domain needed for protein–protein interactions. ASF/SF2 contains an RNA recognition motif (RRM) and also a divergent repeat of the RRM called the RRMH (Zahler *et al.*, 1992), which is absent in SC35. It will be interesting to determine whether the presence of this domain could explain the different activities of the two proteins.

Tissue-specific choice of β-tropomyosin exon 6A

A complex mechanism is responsible for the tissue-specific selection of the mutually exclusive exons 6A and 6B in the chicken β-tropomyosin gene. Both *in vivo* and *in vitro* analyses have shown that sequences in the 3′-half of the intron between these two exons and in exon 6B are involved in exon 6B repression in non-muscle cells (Goux-Pelletan *et al.*, 1990; Libri *et al.*, 1990; Gallego *et al.*, 1992). However, mutations activating exon 6B in non-muscle cells are not sufficient to induce total exclusion of exon 6A in transfected myoblasts and do not lead to exon 6A exclusion in HeLa cell extracts, suggesting that exon 6A is also regulated. We have shown here that an SR protein preparation from chicken skeletal muscle tissue does not support exon 6A splicing, while this exon is efficiently recognized by a HeLa cell SR protein preparation (Figure 10B). In contrast, both preparations stimulate splicing of exons 5–7 to an equivalent level. Differences in the relative amounts of individual SR proteins have been found between different cells and tissues (Fu and

Maniatis, 1992a; Vellard *et al.*, 1992; Zahler *et al.*, 1993). In agreement with these observations, immunoblots with mAb 104 show that HeLa cells and skeletal muscle exhibit differences in the abundance of particular SR proteins (Figure 10A). Furthermore, we have shown using specific antibodies against SC35 that the ratio of SC35 to ASF/SF2 is at least 2-fold higher in the skeletal muscle SR protein preparation than in that of HeLa cells. Changes in the relative levels or activities of these two proteins or of other SR factors exhibiting equivalent properties could have a dramatic effect on exon 6A recognition. Thus, these results strongly suggest that a shift in the SC35 versus ASF/SF2 balance in skeletal muscle cells contributes to exon 6A exclusion in these types of cells.

In summary, we have shown that ASF/SF2 and SC35 have antagonistic activities for the inclusion of exon 6A of β tropomyosin pre-mRNA. Remarkably, our present and previous studies illustrate the various roles of different subsets of the SR proteins in the regulation of alternative splicing. The mechanisms by which SR proteins stimulate 3′-splice sites through interaction with exonic enhancers are beginning to become apparent (Lavigueur *et al.*, 1993; Sun *et al.*, 1993; Tian and Maniatis, 1993; Ramchatesingh *et al.*, 1995; Tacke and Manley, 1995; Wang *et al.*, 1995). These studies indicate that the SR proteins could stabilize complex assembly on the 3′-splice site. In contrast, the mechanism of splicing activation by intronic enhancers remains largely obscure. Our study should help in the understanding of this process.

Materials and methods

Plasmid constructs

All β-tropomyosin clones were derived from a 1.7 kb chicken genomic clone spanning exons 4–7 (Libri *et al.*, 1989b). Construct 6A–7 contains fragment *Pvu*II–*Pma*cI, which includes exon 6A and 194 bp of the downstream intron, fused to fragment *Bss*HII–*Hin*dIII, which includes 37 bp of exon 7 and 90 bp of the upstream intron. Constructs 6A–P3S–7 and 6A–P3AS–7 were derived from plasmid 6A–Δ4–7, which is equivalent to 6A–7 but lacks the 33 bp of the S4 sequence. Deletion of the S4 sequence introduced a *Pst*I site which was used to insert a synthetically prepared hybrid oligonucleotide 5′-C TTT CTC TTT CTC TCT CCC TCC CTG TCT TTC CCT-3′. Insertion in this orientation generated the 6A–P3S–7 construct and in the opposite orientation 6A–P3AS–7. Construct 6A–S5–7 was derived from plasmid 6A–P3AS–7, in which the P3AS sequence was replaced by a synthetic hybrid oligonucleotide 5′-CAG CCA CCT CTC CCC TCT CCG CAC TGC TGC CA-3′. Construct 5–6A–7 was made by fusing fragment *Bam*HI–*Pma*cI (containing exon 5, the whole downstream intron, exon 6A and 194 bp of the downstream intron) to fragment *Bss*HII–*Hin*dIII (including 90 bp of the intron upstream of exon 7 and 37 bp of exon 7). The 3′-splice site of exon 6A was eliminated by deletion of the fragment *Pst*I–*Pvu*II, which includes 150 bp of the intron upstream of exon 6A and the first 13 bp of exon 6A. Deletion of the sequences S6, S10 and S11 has been described previously (Gallego *et al.*, 1992).

For UV crosslinking experiments, the synthetic hybrid oligonucleotides described above were cloned into vector pSP72, to give either RNA P3S or RNA P3AS, or into pGEM 3Z, to give RNA S5 or RNA S4 (sequences shown in Figure 3A).

RNA synthesis and splicing reactions

Capped RNAs were synthesized *in vitro* as described previously using SP6 polymerase and [α-^{32}P] UTP (Ruskin *et al.*, 1984). All transcripts were further purified by electrophoresis on polyacrylamide/urea gels.

Capped RNAs used as competitors were prepared in a scaled up transcription reaction (400 μl) and purified on a Qiagen column (with no carrier). 5′-Cap and 3′-terminal residues were protected by addition of biotinamidocaproyl hydrazide (BACH) as follows. RNA (up to 50 μg in 150 μl 3.3 mM sodium acetate, pH 5) was oxidized with 1.5 mM sodium metaperiodate for 30 min at 0°C. The hydrazones were formed by successive addition of 1 vol. 27 mM BACH in dimethylformamide/water (1:3) and 1 vol. 100 mM sodium cyanoborohydride in 0.2 M sodium phosphate, pH 8, and incubated for 30 min at 25°C. Modified RNA was then purified on a Qiagen column.

HeLa cells nuclear extracts were prepared as described previously (Dignam *et al.*, 1983) with the modifications of Abmayr *et al.* (1988).

Splicing reactions were performed as described (Gallego *et al.*, 1992) with 30 fmol ^{32}P-labeled pre-mRNA transcript and 35% nuclear extract (except when indicated). Competitor RNAs (0.8 pmol, except when indicated) and SR proteins were added prior to addition of the labeled substrate RNA. All reactions were prepared on ice and then incubated at 30°C for 2 h. Reaction products were analyzed on 7% denaturing polyacrylamide gels. Splicing intermediates and products were identified by the kinetics of their appearance. The lariat forms were characterized by their change in mobility in different percentages of acrylamide (Ruskin *et al.*, 1984) and after enzymatic debranching in HeLa S100 fractions (Ruskin and Green, 1985). All the splicing reactions were reproductible with different preparations of HeLa nuclear extracts. After drying, gels were autoradiographed and individual RNA bands were quantified using a PhosphorImager (Molecular Dynamics). Splicing efficiency was calculated either as the ratio between the final lariat value and the sum of the pre-mRNA value and the final lariat value or as the ratio between the mRNA value and the sum of the pre-mRNA value plus the mRNA value. For each precursor the number of uracil molecules were taken into account.

Recombinant proteins

The recombinant 9G8 protein, expressed in baculovirus-infected Sf9 cells, was immunopurified as described for the human 9G8 protein (Cavaloc *et al.*, 1994). Plasmid pAc-HisSC35 was obtained by insertion of an *Eag*I–*Pst*I partial digest of plasmid pB1-HPR5 (a kind gift from J.Soret) into the same restriction sites of the pAcSGhisNT-B vector (Pharmigen). pVL-HisASF was prepared by the insertion of an *Eco*RI–*Kpn*I fragment from plasmid pDS56-ASF (a kind gift from J.Manley) into the pVL1392 vector (InVitrogen). After extraction of the total proteins of Sf9-infected cells, histidine-tagged ASF/SF2 and SC35 were purified on a 1 ml Hitrap chelating column (Pharmacia). Due to their histidine tag, recombinant SC35 and ASF/SF2 proteins exhibited a higher molecular weight than SR proteins prepared from HeLa cells and skeletal muscle (Figure 10A). Protein concentrations were determined by the dye binding assay (BioRad) with bovine serum albumin as standard. Activity of the protein preparations was tested by complementation of a S100 extract using the E1A unit of adenovirus 2 and human β-globin pre-mRNAs as splicing substrates.

SR proteins and immunoblotting

Total SR proteins from HeLa cells and chicken embryo pectoral tissue were prepared as previously described (Zahler *et al.*, 1992). SR proteins were resolved by 11.5% SDS–PAGE. Protein gels were electroblotted and probed with two antibodies as previously described (Cavaloc *et al.*, 1994): mAb 104 (Zahler *et al.*, 1992) was obtained from the supernatant of a hybridoma culture (ATCC CRL-2067); αSC35 is a monoclonal antibody directed against the 15 C-terminal residues of SC35. This antibody detects only SC35 and not ASF (Figure 10A) or 9G8.

RNA mobility shift assays

The RNA mobility shift assays were performed essentially as described previously (Konarska and Sharp, 1986). Capped ^{32}P-labeled RNAs were synthesized *in vitro* and purified on acrylamide/urea gels. Binding reactions were performed in splicing buffer with ATP and creatine phosphate, 5 μg tRNA and 1% nuclear extract (25 μl final volume). The reaction mixtures were preincubated for 10 min at 30°C before addition of the ^{32}P-labeled RNA (15 fmol/40 000 c.p.m.). At the indicated times the samples were adjusted to 2 mg/ml heparin, 1.5% glycerol, 0.01% bromophenol blue and 0.01% xylene cyanol. The protein–RNA complexes were separated by native gel electrophoresis in 4% acrylamide gels (acrylamide/bis-acrylamide 30:1) using Tris–glycine as the running buffer. The gel was electrophoresed at 4°C at 15 W until the xylene cyanol had migrated 13–15 cm and then dried and visualized by autoradiography.

UV crosslinking experiments

Short RNAs were uniformly labeled with [α-^{32}P]UTP, [α-^{32}P]CTP and [α-^{32}P]GTP and purified by polyacrylamide gel electrophoresis. Aliquots of 50 fmol RNA were incubated with 500 ng tRNA, 1 μg bovine serum albumin and the indicated amount of total SR proteins or recombinant

ASF/SF2 in splicing buffer with ATP and creatine phosphate (12.5 µl final volume). After 15 min incubation at 30°C, the mixtures were transferred to a microtiter plate on ice and irradiated with UV light (wavelength 254 nm) in a Stratalinker (Stratagene) for 15 min. The irradiated samples were treated with 1 mg/ml RNase A and 10 U RNase T1 at 30°C for 30 min and analyzed by electrophoresis on a standard 10% SDS–polyacrylamide gel. Individual radioactive bands were quantified using a PhosphorImager (Molecular Dynamics).

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References

Abmayr,S.M., Reed,R. and Maniatis,T. (1988) Identification of a functional mammalian spliceosome containing unspliced pre-mRNA. *Proc. Natl Acad. Sci. USA*, **85**, 7216–7220.

Amrein,H., Hedley,M.L. and Maniatis,T. (1994) The role of specific protein–RNA and protein–protein interactions in positive and negative control of pre-mRNA splicing by transformer 2. *Cell*, **76**, 735–746.

Balvay,L., Libri,D., Gallego,M. and Fiszman,M. (1992) Intronic sequence with both negative and positive effects on the regulation of the β-tropomyosin transcript. *Nucleic Acids Res*., **20**, 3987–3992.

Black,D.L. (1992) Activation of c-src neuron-specific splicing by an unusual RNA element *in vivo* and *in vitro*. *Cell*, **69**, 795–807.

Black,D.L. (1995) Finding splice sites within a wilderness of RNA. *RNA*, **1**, 763–771.

Cavaloc,Y., Popierlarz,M., Fuchs,J.P., Gattoni,R. and Stévenin,J. (1994) Characterization and cloning of the human splicing factor 9G8: a nobel 35 KDa factor of the serine/arginine protein family. *EMBO J*., **13**, 2639–2649.

Del Gatto,F. and Breathnach,R. (1995) Exon and intron sequences, respectively, repress and activate splicing of a fibroblast growth factor receptor 2 alternative exon. *Mol. Cell. Biol*., **15**, 4825–4834.

Dignam,J.D., Lebovitz,R.R. and Roeder,R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammmalian nuclei. *Nucleic Acids Res*., **11**, 1475–1489.

Fu,X.D. (1993) Specific commitment of different pre-messenger RNAs to splicing by single SR proteins. *Nature*, **365**, 82–85.

Fu,X.D. (1995) The superfamily of arginine/serine-rich splicing factors. *RNA*, **1**, 663–680.

Fu,X.D. and Maniatis,T. (1992a) Isolation of a complementary DNA that encodes the mammalian factor SC35. *Science*, **256**, 535–538.

Fu,X.D. and Maniatis,T. (1992b) The 35-kDa mammalian splicing factor SC35 mediates specific interactions between U1 and U2 small nuclear ribonucleoprotein particles at the 3′ splice site. *Proc. Natl Acad. Sci. USA*, **89**, 1725–1729.

Gallego,M.E., Balvay,L. and Brody,E. (1992) *Cis*-acting sequences involved in exon selection in the chicken β-tropomyosin gene. *Mol. Cell. Biol*., **12**, 5415–5425.

Gooding,C., Roberts,G.C., Moreau,G., Nadal-Ginard,B. and Smith, C.W.J. (1994) Smooth muscle-specific switching of alpha-tropomyosin mutually exclusive exon selection by specific inhibition of the strong default exon. *EMBO J*., **13**, 3861–3872.

Goux-Pelletan,M., Libri,D., d'Aubenton-Carafa,Y., Fiszman,M., Brody,E. and Marie,J. (1990) *In vitro* splicing of mutually exclusive exons from the chicken β-tropomyosin gene: role of the branch point location and very long pyrimidine stretch. *EMBO J*., **9**, 241–249.

Hampson,R.K., La Follette,L. and Rottman,F.M. (1989) Alternative processing of bovine growth hormone mRNA is influenced by downstream exon sequences. *Mol. Cell. Biol*., **9**, 1604–1610.

Heinrichs,V. and Baker,B.S. (1995) The *Drosophila* SR protein RBP1 contributes to the regulation of doublesex alternative splicing by recognizing RBP1 RNA target sequences. *EMBO J*., **14**, 3987–4000.

Helfman,D.M., Roscigno,R.F., Mulligan,G.J., Finn,L.A. and Weber,K.S. (1990) Identification of two distinct intron elements involved in alternative splicing of β-tropomyosin pre-mRNA. *Genes Dev.*, **4**, 98–110.

Hodges,D. and Bernstein,I.S. (1994) Genetic and biochemical analysis of aternative RNA splicing. *Adv. Genet*., **31**, 207–281.

Huh,G.S. and Hynes,R.O. (1994) Regulation of alternative pre-mRNA splicing by a novel repeated hexanucleotide element. *Genes Dev.*, **8**, 1561–1574.

Kanopka,A., Mühlemann,O. and Akusjärvi,G. (1996) Inhibition by SR proteins of splicing of a regulated adenovirus pre-mRNA. *Nature*, **381**, 535–538.

Kohtz,J.D., Jamison,S.F., Will,C.L., Zuo,P., Luhrmann,R., Garciablanco, M.A. and Manley,J.L. (1994) Protein–protein interactions and 5′-splice-site recognition in mammalian mRNA precursors. *Nature*, **368**, 119–124.

Konarska,M.M. and Sharp,P.A. (1986) Electrophoretic separation of complexes involved in the splicing of precursors to mRNA. *Cell*, **46**, 845–855.

Krainer,A.R., Conway,G.C. and Kozak,D. (1990) Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells. *Genes Dev.*, **4**, 1158–1171.

Lamond,A.I. (1993) The spliceosome. *BioEssays*, **15**, 595–603.

Lavigueur,A., Labranche,H., Kornblihtt,A.R. and Chabot,B. (1993) A splicing enhancer in the human fibronectin alternate ED1-exon interacts with SR-proteins and stimulates U2-snRNP binding. *Genes Dev.*, **7**, 2405–2417.

Libri,D., Lemonnier,M., Meinnel,T. and Fiszman,M.Y. (1989a) A single gene codes for the β subunit of smooth and skeletal muscle tropomyosin in the chicken. *J. Biol. Chem*., **264**, 2935–2944.

Libri,D., Marie,J., Brody,E. and Fiszman,M.Y. (1989b) A subfragment of the β-tropomyosin gene is alternatively spliced when transfected into differentiating muscle cells. *Nucleic Acids Res*., **17**, 6449–6462.

Libri,D., Goux-Pelletan,M., Brody,E. and Fiszman,M. (1990) Exon as well as intron sequences are *cis* regulating elements for the mutually exclusive alternative splicing of the β-tropomyosin gene. *Mol. Cell. Biol*., **10**, 5036–5046.

Libri,D., Balvay,L. and Fiszman,M.Y. (1992) *In vivo* splicing of the β-tropomyosin pre-mRNA: a role for branch point and donor site competition. *Mol. Cell. Biol*., **12**, 3204–3215.

Lin,C. and Patton,G.J. (1995) Regulation of alternative 3′ splice site selection by constitutive splicing factors. *RNA*, **1**, 234–245.

Lynch,K.W. and Maniatis,T. (1996) Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila doublesex* splicing enhencer. *Genes Dev.*, **10**, 2089–2101.

Mardon,H.J., Sebastio,G. and Baralle,F.E. (1987) A role for exon sequences in alternative splicing of the human fibronectin gene. *Nucleic Acids Res*., **15**, 7725–7733.

Mayeda,A., Zahler,A.M., Krainer,A.R. and Roth,M.B. (1992) Two members of a conserved family of nuclear phosphoproteins are involved in pre-messenger-RNA splicing. *Proc. Natl Acad. Sci. USA*, **89**, 1301–1304.

Mayeda,A., Helfman,D. and Krainer,A.R. (1993) Modulation of exon skipping and inclusion by heterogenous nuclear ribonucleoprotein A1 and pre-mRNA splicing factor SF2/ASF. *Mol. Cell. Biol*., **13**, 2993–3001.

Min,H., Chan,R.C. and Black,L.D. (1995) The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event. *Genes Dev.*, **9**, 2659–2671.

Moore,M.J., Query,C.C. and Sharp,P.A. (1993) Splicing of precursors to mRNA by the spliceosome. In Gesteland,R.F. and Atkins,J.F. (eds), *Splicing of Precursors to mRNA by the Spliceosome*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 303–357.

Ramchatesingh,J., Zahler,M.A., Neugebauer,M.K., Roth,B.M. and Cooper,A.T. (1995) A subset of SR proteins activates splicing of the cardiac troponin T alternative exon by direct interactions with an exonic enhancer. *Mol. Cell. Biol*., **15**, 4898–4907.

Ruskin,B. and Green,M.R. (1985) An RNA processing activity that debranches RNA lariats. *Science*, **229**, 135–140.

Ruskin,B., Krainer,A.R., Maniatis,T. and Green,M.R. (1984) Excision of an intact intron as a novel lariat structure during pre-mRNA splicing *in vitro*. *Cell*, **38**, 317–331.

Ryan,J.K. and Cooper,A.T. (1996) Muscle-specific splicing enhancers regulate inclusion of the cardiac troponin T alternative exon in embryonic skeletal muscle. *Mol. Cell. Biol*., **16**, 4014–4023.

Singh,R., Valcarcel,J. and Green,R.M. (1995) Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. *Science*, **268**, 1173–1176.

Sirand-Pugnet,P., Durosay,P., Brody,E. and Marie,J. (1995) An intronic (A/U)GGG repeat enhances the splicing of an alternative intron of the chicken β-tropomyosin pre-mRNA. *Nucleic Acids Res.*, **23**, 3501–3507.

Staknis,D. and Reed,R. (1994) Direct interactions between pre-mRNA and six U2 small nuclear ribonucleoproteins during spliceosome assembly. *Mol. Cell. Biol.*, **14**, 2994–3005.

Streuli,M. and Saito,H. (1989) Regulation of tissue specific alternative splicing: exon-specific *cis*-elements govern the splicing of leukocyte common antigen pre-mRNA. *EMBO J.*, **8**, 787–796.

Sun,Q., Mayeda,A., Hampson,R.K., Krainer,A.R. and Rottman,F.M. (1993) General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. *Genes Dev.*, **7**, 2598–2608.

Tacke,R. and Manley,J.L. (1995) The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. *EMBO J.*, **14**, 3540–3551.

Tian,M. and Maniatis,T. (1993) A splicing enhancer complex controls alternative splicing of doublesex pre-messenger RNA. *Cell*, **74**, 105–114.

Valcarcel,J., Singh,R., Zamore,P.D. and Green,M.R. (1993) The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-messenger RNA. *Nature*, **362**, 171–175.

Vellard,M., Sureau,A., Soret,J., Martinerie,C. and Perbal,B. (1992) A potential splicing factor is encoded by the opposite strand of the *trans*-spliced c-*myb* exon. *Proc. Natl Acad. Sci. USA*, **89**, 2511–2515.

Wang,Z., Hoffmann,H.M. and Grabowski,P.J. (1995) Intrinsic U2AF binding is modulated by exon enhancer signals in parallel with changes in splicing activity. *RNA*, **1**, 21–35.

Watakabe,A., Tanaka,K. and Shimura,Y. (1993) The role of exon sequences in splice site selection. *Genes Dev.*, **7**, 407–418.

Wu,J.Y. and Maniatis,T. (1993) Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell*, **75**, 1061–1070.

Xu,R.S., Teng,J. and Cooper,T.A. (1993) The cardiac troponin-T alternative exon contains a novel purine-rich positive splicing element. *Mol. Cell. Biol.*, **13**, 3660–3674.

Zahler,A.M., Lane,W.S., Stolk,J.A. and Roth,M.B. (1992) SR proteins—a conserved family of pre-messenger-RNA splicing factors. *Genes Dev.*, **6**, 837–847.

Zahler,A.M., Neugebauer,K.M., Lane,W.S. and Roth,M.B. (1993) Distinct functions of SR proteins in alternative pre-messenger RNA splicing. *Science*, **260**, 219–222.

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