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Modulation of Exon Skipping and Inclusion by Heterogeneous Nuclear Ribonucleoprotein A1 and Pre-mRNA Splicing Factor SF2/ASF

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The essential splicing factor SF2/ASF and the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) modulate alternative splicing *in vitro* of pre-mRNAs that contain 5' splice sites of comparable strengths competing for a common 3' splice site. Using natural and model pre-mRNAs, we have examined whether the ratio of SF2/ASF to hnRNP A1 also regulates other modes of alternative splicing *in vitro*. We found that an excess of SF2/ASF effectively prevents inappropriate exon skipping and also influences the selection of mutually exclusive tissue-specific exons in natural β -tropomyosin pre-mRNA. In contrast, an excess of hnRNP A1 does not cause inappropriate exon skipping in natural constitutively or alternatively spliced pre-mRNAs. Although hnRNP A1 can promote alternative exon skipping, this effect is not universal and is dependent, e.g., on the size of the internal alternative exon and on the strength of the polypyrimidine tract in the preceding intron. With appropriate alternative exons, an excess of SF2/ASF promotes exon inclusion, whereas an excess of hnRNP A1 causes exon skipping. We propose that in some cases the ratio of SF2/ASF to hnRNP A1 may play a role in regulating alternative splicing by exon inclusion or skipping through the antagonistic effects of these proteins on alternative splice site selection.

Pre-mRNA splicing is an essential process in the expression of most eukaryotic protein-coding genes. The 5' and 3' splice sites and the branch site, which exhibit limited sequence conservation, are necessary but not sufficient for the accuracy of splicing (for reviews, see references 21, 35, and 55). In the case of constitutively spliced pre-mRNAs, strict fidelity in splice site selection is necessary for correct protein synthesis. On the other hand, flexibility in the recognition of alternative splice sites can result in an increase in the coding capacity of many genes and allows the possibility of regulating the process of splicing. Thus, many genes can express multiple protein isoforms by alternative splicing, often in a tissue-specific or developmentally regulated manner (for reviews, see references 41 and 59). To investigate the molecular mechanisms responsible for the specificity of constitutive and alternative splice site selection, we are characterizing several protein factors that can modulate the selection of alternative splice sites *in vitro*.

A versatile mode of regulated alternative splicing involves a choice between exon inclusion and exon skipping (for a review, see reference 59). To date, several *cis*-acting elements and *trans*-acting factors have been reported to influence alternative exon selection in a number of systems. The importance of internal exon length, and of a downstream intron element, for neuron-specific N1 exon inclusion during alternative splicing of *src* pre-mRNA has been documented (5, 6). A deletion within exon 19 of the dystrophin gene, leading to skipping of this exon, is the basis for one form of Duchenne muscular dystrophy (43). *In vitro* splicing of preprotachykinin pre-mRNA suggested that the strength of the downstream 5' splice site and its interaction with U1 small nuclear ribonucleoprotein determines whether an alternative exon is included (20, 38). In some pre-mRNAs,

specific elements in the alternative exon influence its inclusion (10, 23, 24, 40, 42, 62). Another critical factor for the selection of internal alternative exons is the relative strength of the competing splice sites, as influenced, for example, by the composition of the polypyrimidine tract upstream of the 3' splice site in the preceding intron (12, 23, 29, 40, 50). A recent exon definition model proposes that internal exons are defined by recognition of the upstream 3' splice site, followed by a search for the downstream 5' splice site (56, 61). Recently, systematic *in vitro* and *in vivo* analyses of model pre-mRNAs showed that several *cis*-acting elements, including the 5' and 3' splice sites, the branch site, the polypyrimidine tract, the length of the internal exon, and the downstream intron, cooperate to determine the selection of the internal exon (12, 13).

We have reported that the essential human splicing factor SF2/ASF (17, 37), which is a member of the SR protein family (63), and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) regulate alternative pre-mRNA splicing *in vitro* through antagonistic effects on 5' splice site selection. Proximal 5' splice sites are preferred at high concentrations of SF2/ASF, whereas distal 5' splice sites are favored at high concentrations of hnRNP A1 (16, 33, 44). SF2/ASF was also shown to cause retention of an alternative adenovirus E1A intron by a mechanism other than 5' splice site competition (26). Recently, the splicing factor SC35, which is also a member of the SR protein family (14, 63), was shown to have *in vitro* splicing activities equivalent to those of SF2/ASF (15). Furthermore, it was shown that both SF2/ASF and SC35 also favor the proximal site in a pre-mRNA containing duplicated 3' splice sites. This effect is counteracted not by hnRNP A1 but rather by an activity designated SF7 (15, 45), which is potentially related to an activity that regulates utilization of an adenovirus 3' splice site *in vitro* (64). Thus, in constitutively spliced pre-mRNAs, the combined stimulation of proximal 5' and 3' splice sites by SF2/ASF or SC35

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may be part of the cellular mechanisms that prevent inappropriate exon skipping and ensure the accuracy of splicing, whereas an excess of hnRNP A1 (or SF7) might be expected to cause inappropriate exon skipping, which should be deleterious to the cell. On the other hand, the mechanisms of alternative exon inclusion and skipping may be governed at the level of 5' and/or 3' splice site selection and could also involve SF2/ASF and hnRNP A1. To address these questions, we have examined the influence of SF2/ASF and hnRNP A1 on splice site selection *in vitro*, using several natural and model constitutively or alternatively spliced pre-mRNAs.

MATERIALS AND METHODS

Preparation of human SF2/ASF and recombinant hnRNP A1. The preparation of sonicated nuclear extract from 24 liters of HeLa cells (approximately 2.4×10^{10} cells) was described previously (32). SF2/ASF was recovered in the 60%- to 90%-saturated $(\text{NH}_4)_2\text{SO}_4$ pellet (57), leading to greater enrichment than in the 50 to 80% cut described originally (32). The pellet containing SF2/ASF was resuspended in 5 ml of dialysis buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] Na^+ [pH 8.0], 0.1 M KCl, 0.2 mM EDTA, 5% glycerol (vol/vol), 1 mM dithiothreitol) and dialyzed in 1 liter of the same buffer containing 0.5 mM phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation at $23,000 \times g_{\text{max}}$ (14,000 rpm; SS-34 rotor; Sorvall) for 30 min at 4°C. This was followed by selective precipitation in the presence of magnesium, a recently described method to purify SR-family proteins, including SF2/ASF (48, 57, 63). The supernatant described above was adjusted to 20 mM MgCl_2 , and the tube was rocked for 2.5 h at 4°C. The resulting precipitate was collected and dissolved in 0.5 ml of dialysis buffer. Two different preparations of partially purified SF2/ASF, approximately 1 mg of protein per ml, were used for the experiments described below. Although SF2/ASF polypeptide is the predominant protein in these concentrated preparations (more than 50% pure), other SR proteins known to have SF2/ASF-like activities (15, 30, 48, 63) are also present. Further fractionation by Phenyl-Superose (Pharmacia) chromatography, as described previously (32), yielded essentially pure SF2/ASF that was devoid of SC35 and other SR proteins. However, pure HeLa cell SF2/ASF, or recombinant SF2/ASF, could not be obtained in soluble form at as high a concentration and was therefore only partially active in the assays reported here. We expect that other SR proteins are also active in these assays.

The preparation of soluble recombinant hnRNP A1 (0.2 mg/ml) in *Escherichia coli* was as described previously (44).

Construction of template plasmids. Plasmid pSP64-p6(8/9), containing a rat β -tropomyosin minigene, was derived from pSP64-p2p6(8/9) (28) by restriction at a unique *Nco*I site in the middle of exon 6. The ends were filled in with DNA polymerase I Klenow fragment and ligated to *Hind*III linkers. The DNA was digested with *Hind*III and recircularized, resulting in the removal of exon 5 through the *Nco*I site of exon 6 (see Fig. 1). Pre-mRNAs beginning in exon 5 or exon 6 were respectively made by runoff transcription from pSP64-p2p6(8/9) linearized with *Bam*HI or from pSP64-p6(8/9) linearized with *Eco*RI. Template plasmids of the pSP64-DUP series, containing duplicated human β -globin first exon, first intron, and second exon units (kindly provided by R. Kole) (12), were linearized with *Bam*HI. The DUP23, DUP33, DUP51, and DUP171 constructs have

internal exons of 23, 33, 51, and 175 nucleotides (nt), respectively (31). Rabbit β -globin wild-type template plasmid, pSP64-Rchr β G(*Pvu*II-*Bgl*II) (kindly provided by M. Aebi and C. Weissmann) (1), was linearized with *Ava*I.

***In vitro* splicing assays.** ^{32}P -labeled and $^7\text{mGpppG}$ -capped pre-mRNA substrates were transcribed from each linearized template DNA with SP6 RNA polymerase essentially as described previously (34, 46). Standard *in vitro* splicing reactions were carried out at 30°C for 3.5 to 4.5 h in 25 μl with the indicated amounts of HeLa cell nuclear extract, S100 extract, SF2/ASF fraction, and recombinant hnRNP A1 as described previously (36, 47), except that 1 mM instead of 3.2 mM MgCl_2 was used for splicing the β -tropomyosin pre-mRNAs (see Fig. 1). RNA products were analyzed by electrophoresis on 4% (see Fig. 1), 5.5% (see Fig. 2 and 6), or 6.5% (see Fig. 3 and 5) polyacrylamide-7 M urea gels followed by autoradiography, as described previously (46).

RESULTS

SF2/ASF prevents inappropriate exon skipping in a natural alternatively spliced pre-mRNA. The alternative splicing pattern of rat β -tropomyosin pre-mRNA is regulated in a tissue-specific manner (27). This pre-mRNA includes two internal mutually exclusive exons: exon 6, which is used in fibroblasts and smooth muscle, and exon 7, which is uniquely used in skeletal muscle (27) (Fig. 1, diagrams).

To study the regulation of β -tropomyosin alternative splicing, we constructed minigenes that include the natural exons and introns from exon 5 or 6 to exon 8 and include exon 9 prespliced to exon 8. The resulting pre-mRNAs are designated 5-6-7-8/9 and 6-7-8/9 (the numbers correspond to the exons; “-” denotes an intron, and “/” denotes splicing). In agreement with a previous report (28), *in vitro* splicing in nuclear extract resulted in skipping of exons 6 and 7 to generate an inappropriately spliced 5/8/9 RNA (Fig. 1A, lane 1, products indicated by asterisks), which is not observed from the endogenous β -tropomyosin gene *in vivo*. Upon addition of SF2/ASF, this inappropriate exon skipping event was markedly prevented, thereby allowing inclusion of the fibroblast-type exon 6, the expected pattern for HeLa cells (Fig. 1A, lanes 2 and 3, products indicated by F). Since splicing of exon 5 to exon 6 does not occur efficiently *in vitro* (28), only a small amount of fully spliced fibroblast-type 5/6/8/9 mRNA was detected, and instead the corresponding 5-6/8/9 intermediate accumulated (Fig. 1A, lanes 2 and 3). An even greater stimulation of exon 6 to exon 8 splicing, at the expense of inappropriate exon 5-to-exon 8 skipping, was obtained with a small amount of SF2/ASF to complement an S100 extract that is unable to support splicing in the absence of added SF2/ASF (32) (Fig. 1A, lanes 4 to 6). Compared with nuclear extract, S100 extract plus SF2/ASF yielded reduced amounts of pre-mRNA and lariat RNAs (derived from inappropriate skipping and fibroblast-type splicing) because of the higher overall splicing efficiency.

The results of the experiment described above show that SF2/ASF prevents inappropriate exon skipping of the mutually exclusive tropomyosin exons 6 and 7, a reaction not observed with the endogenous gene *in vivo*. In the case of pre-mRNAs containing alternative 5' splice sites and a common 3' splice site, hnRNP A1 counteracts the effects of SF2/ASF on proximal 5' splice site selection, thereby causing activation of distal 5' splice sites (44). Hence, we tested whether hnRNP A1 would also cause inappropriate exon skipping with the β -tropomyosin pre-mRNA. Significantly,

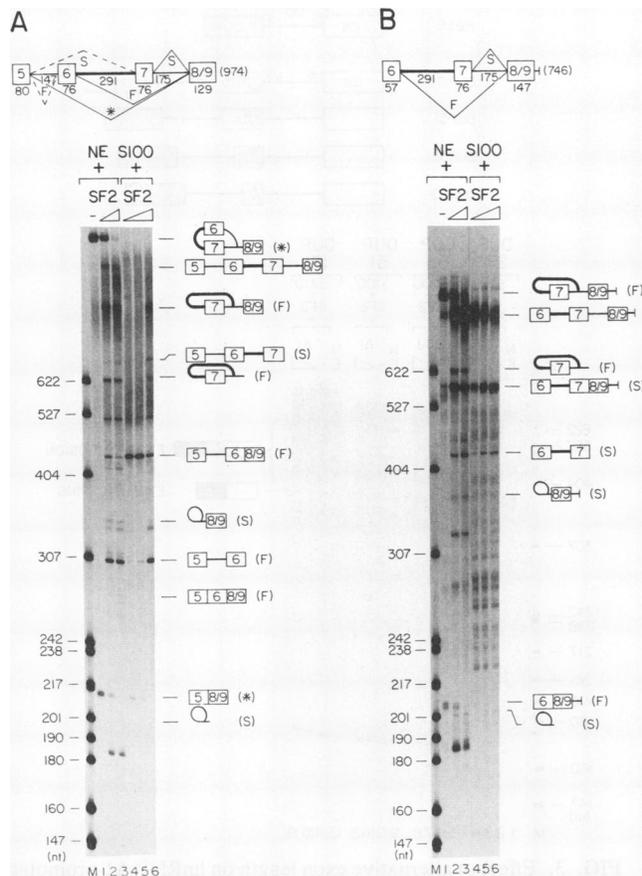


FIG. 1. Effects of SF2/ASF (SF2) on splicing of β -tropomyosin pre-mRNAs in vitro. The structures of the pre-mRNAs and splicing products are shown schematically at the top and side of each panel. The exon and intron sizes are indicated in nucleotides. Alternative splicing patterns and their products are indicated by S (skeletal muscle pattern), F (fibroblast pattern), and * (inappropriate splicing). All splicing products were characterized in detail previously (28) or otherwise identified by their electrophoretic mobilities before and after enzymatic debranching (58). The sizes of the pBR322/*Hpa*II DNA markers (lanes M) are shown in nucleotides. (A) Analysis of splicing products of β -tropomyosin 5-6-7-8/9 pre-mRNA. The splicing reactions contained nuclear extract (NE) (5 μ l) supplemented with SF2/ASF (lane 1, none; lane 2, 2.5 μ l; lane 3, 5 μ l) or S100 extract (7 μ l) complemented with SF2/ASF (lane 4, 1 μ l; lane 5, 2 μ l; lane 6, 4 μ l). (B) Analysis of splicing products of β -trypomycin 6-7-8/9 pre-mRNA. The reaction mixtures contained either nuclear extract (5 μ l) plus SF2/ASF (lane 1, none; lane 2, 2.5 μ l; lane 3, 5 μ l) or S100 extract (7 μ l) plus SF2/ASF (lane 4, 0.5 μ l; lane 5, 1 μ l; lane 6, 2 μ l).

an excess of hnRNP A1 did not promote this kind of inappropriate exon skipping event (data not shown). Thus, the in vitro modulation of alternative splicing by hnRNP A1 appears to be restricted to specific types of splice site competition (see below).

Effect of SF2/ASF on the selection of tissue-specific exons. Previous in vitro and in vivo studies of rat β -tropomyosin pre-mRNA splicing suggested that alternative splicing of exon 5 to exon 6 (fibroblast pattern) or exon 5 to exon 7 (skeletal muscle pattern) depends on prior joining of either alternative exon to exon 8 (28). Thus, alternative splicing of the mutually exclusive exon 6 or 7 to the downstream common exon 8 appears to be the critical event in this type

of regulated splicing. We therefore tested whether SF2/ASF can influence this alternative splicing event through its polar activity on 5' splice site selection.

When transcripts of the 5-6-7-8/9 minigene construct were used (Fig. 1A, diagram), the predominant modulation caused by SF2/ASF was a switch from inappropriate exon 5-to-exon 8 skipping (products indicated by asterisks in Fig. 1A) to the fibroblast splicing pattern (Fig. 1A, lanes 1 to 6, products indicated by F). However, a small amount of skeletal muscle-type splicing of exon 7 to exon 8 was also specifically stimulated by SF2/ASF addition (Fig. 1A, lanes 2, 3, and 6, products indicated by S). Using transcripts of the shorter 6-7-8/9 minigene construct (Fig. 1B, diagram), we observed strong activation of the proximal skeletal muscle-type splicing (products indicated by S) at the expense of the distal fibroblast-type splicing (products indicated by F) upon addition of SF2/ASF (Fig. 1B, lanes 2 to 6).

The quantitative difference in exon 7 inclusion between the two pre-mRNAs suggests that the factors that block exon 7 use in nonmuscle cells (see Discussion) are more effective with the longer, more natural substrate. In both cases, the SF2/ASF-induced changes are consistent with the known effect of SF2/ASF on alternative 5' splice site selection (16, 33), since the 5' splice site of the fibroblast-type exon 6 is distal and that of the skeletal muscle-specific exon 7 is proximal, relative to the downstream common exon 8. Thus, as previously reported, SF2/ASF activity does not operate in a sequence-specific manner but rather senses the relative positions of alternative 5' splice sites (16, 33). Significantly, the addition of excess hnRNP A1 did not counteract the activation of the exon 7 splice by SF2/ASF (data not shown; see Discussion).

SF2/ASF promotes alternative exon inclusion. To study the substrate requirements for splicing modulation by SF2/ASF and hnRNP A1 in detail, a series of model pre-mRNAs designed to study exon skipping and inclusion (12) were used. First, we tested the effect of SF2/ASF activity on exon skipping with these model substrates. In agreement with a previous report (12), the model pre-mRNAs DUP33 and DUP23, which include a 33- or 23-nt internal exon, were spliced predominantly via exon skipping under standard conditions in HeLa cell nuclear extract (Fig. 2, lane 1; Fig. 3, lanes 1 and 5). These pre-mRNAs are also spliced via exon skipping when transiently expressed in HeLa cells (12). Upon the addition of SF2/ASF to nuclear extract, DUP33 pre-mRNA gave rise to an exon inclusion spliced product, which was markedly promoted as the concentration of SF2/ASF increased (Fig. 2, lanes 1 to 4). Accumulation of 531-nt RNAs, in which only one of the two introns has been excised (12), was also stimulated by SF2/ASF, consistent with the suppression of exon skipping. Complementation of S100 extract with SF2/ASF resulted exclusively in exon inclusion even at low SF2/ASF concentrations (Fig. 2, lanes 4 to 6). The DUP23 substrate also showed stimulation of exon inclusion in S100 extract complemented with SF2/ASF, although with reduced efficiency (Fig. 3, lanes 1 and 2). The partial suppression of exon skipping with this substrate is also reflected in the accumulation of 521-nt RNAs, in which one of the two introns was spliced out (Fig. 3, lane 2). Thus, the ability of SF2/ASF to promote exon inclusion appears to be dependent on the length of the internal exon.

Abundance of hnRNP A1 in HeLa cell nuclear and S100 extracts. The difference in the magnitude of the SF2/ASF effect in nuclear and S100 extracts suggests that the latter extract contains less hnRNP A1, which is known to counteract the effect of SF2/ASF on alternative 5' splice site

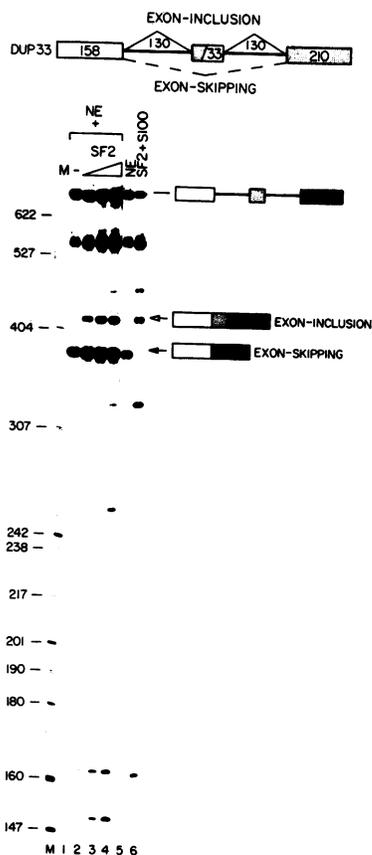


FIG. 2. Effects of SF2/ASF (SF2) on splicing of a model β -globin pre-mRNA in vitro. The structure of the DUP33 model pre-mRNA (12) is shown schematically. The two introns and their flanking exon sequences are identical. The exon and intron sizes are indicated in nucleotides. The structures of unspliced pre-mRNA and of the two mRNAs resulting from alternative exon inclusion or skipping are represented on the right. All splicing products were previously characterized in detail (12). The sizes of the pBR322/*Hpa*II DNA markers (lane M) are shown in nucleotides. The reaction mixtures contained either nuclear extract (NE) (6 μ l) plus SF2/ASF (lanes 1 and 5, none; lane 2, 2 μ l; lane 3, 4 μ l; lane 4, 8 μ l) or S100 extract (6 μ l) plus SF2/ASF (0.7 μ l) (lane 6).

selection (44). In agreement with this prediction, immunoblot analysis with an anti-hnRNP A1 monoclonal antibody (a gift from G. Dreyfuss) demonstrated much lower levels of hnRNP A1 in S100 extract than in nuclear extract (Fig. 4). Comparison with known amounts of purified recombinant hnRNP A1 indicates that our preparation of nuclear extract contains approximately 350 ng (10 pmol) of hnRNP A1 per μ l of extract. The concentration of hnRNP A1 in S100 extract is less than 10-fold lower (30 ng or 0.9 pmol/ μ l of extract) (Fig. 4 and data not shown).

In the experiments reported here (e.g., in Fig. 3), the range of added hnRNP A1 was 200 to 800 ng (6 to 23 pmol), compared with 2.1 μ g (61 pmol) in 6 μ l of nuclear extract, or approximately 10 to 40% of the endogenous hnRNP A1. For SF2/ASF polypeptide, we calculated a concentration of 150 ng (5.4 pmol)/ μ l of nuclear extract and a concentration about 30-fold lower in S100 extract (data not shown). In Fig. 2, e.g., the endogenous SF2/ASF in 6 μ l of nuclear extract is therefore 0.9 μ g (32 pmol); we estimate the range of added SF2/ASF as 0.6 to 2.4 μ g (22 to 87 pmol), or approximately

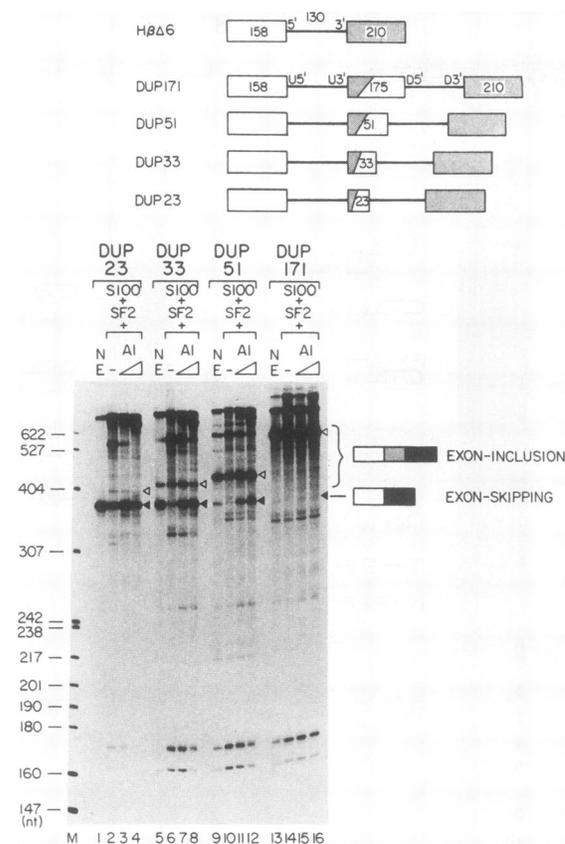


FIG. 3. Effect of alternative exon length on hnRNP A1-promoted exon skipping. The structures of the β -globin pre-mRNA (36) and of the model DUP23-171 derivatives (12) are shown schematically. The exon and intron sizes are indicated in nucleotides. The upstream (U) and downstream (D) introns and their adjacent exon sequences are identical. The alternative spliced products are indicated by triangles (\blacktriangleleft , exon inclusion products; \blacktriangleleft , exon skipping products). All splicing products were previously characterized in detail (12). The sizes of the pBR322/*Hpa*II DNA markers (lane M) are shown in nucleotides. The reaction mixtures contained either nuclear extract alone (NE) (6 μ l) or S100 extract (6 μ l) plus SF2/ASF (SF2) (0.7 μ l) plus recombinant hnRNP A1 (lanes 2, 6, 10, and 14, none; lanes 3, 7, 11, and 15, 1 μ l; lanes 4, 8, 12, and 16, 4 μ l).

70 to 270% of the endogenous SF2/ASF. This latter estimate does not take into account the likely contribution by other SR polypeptides to the overall SF2/ASF activity. In addition, the fraction of active SF2 and hnRNP A1 molecules in the extracts and purified preparations is unknown.

hnRNP A1 promotes skipping of short internal exons. We next analyzed the substrate requirements for regulation of exon skipping by hnRNP A1. It was shown previously, with the model pre-mRNAs used here, that exon inclusion or skipping is dependent on the length of the internal alternative exon in vitro and in vivo (12). In agreement with that study, the spliced products obtained under standard splicing conditions in HeLa cell nuclear extract gradually switched from exon skipping to exon inclusion as the length of the internal exon increased (Fig. 3, lanes 1, 5, 9, and 13). In S100 extract complemented with SF2/ASF, the ratio of exon inclusion to exon skipping significantly increased, relative to the ratio in nuclear extract, even with the shortest internal exon (Fig. 3, lanes 1, 2, 5, 6, 9, and 10). The addition of hnRNP A1, under

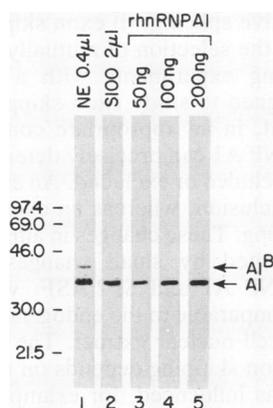


FIG. 4. Abundance of hnRNP A1 in HeLa cell nuclear and S100 extracts. The indicated amounts of the S100 and nuclear extract (NE) preparations used throughout this study were analyzed by immunoblotting. Purified recombinant hnRNP A1 (rhnRNP A1) (50 to 200 ng) was used as a concentration standard. Samples separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were electroblotted onto nitrocellulose, incubated with an anti-hnRNP A1 monoclonal antibody (9H10; a gift from G. Dreyfuss) at a 1/500 dilution, and then subjected to alkaline-phosphatase-based detection, as described previously (44). The relative mobilities of hnRNP A1 (34 kDa) and its alternatively spliced isoform A1^B (38 kDa), which contains a unique internal exon (7), are indicated. The relative mobilities of prestained protein size markers (Amersham) are shown in kilodaltons.

otherwise identical conditions, promoted exon skipping with the DUP23, DUP33, and DUP51 pre-mRNAs in a concentration-dependent manner (Fig. 3, lanes 2 to 4, 6 to 8, and 10 to 12). This was also reflected at the level of 521-, 531-, and 549-nt RNAs, in which only one of the two introns is spliced out. An increase in the ratio of exon skipping to exon inclusion was also seen upon the addition of hnRNP A1 to nuclear extract (data not shown). An excess of hnRNP A1 failed to promote significant exon skipping with the DUP171 pre-mRNA, which contains the longest internal exon (Fig. 3, lanes 14 to 16). Therefore, there appears to be an upper limit on the length of the internal alternative exon in order for hnRNP A1 to promote exon skipping, although these particular substrates do not address the possible contributions of sequence context and higher-order structure.

A strong upstream polypyrimidine tract prevents hnRNP A1-induced exon skipping. Another critical parameter that influences the selection of internal alternative exons in these model pre-mRNAs is the pyrimidine content of the upstream 3' splice site (12, 13). Thus, the major spliced product of DUP33 pre-mRNA in nuclear extract is generated by exon skipping (Fig. 5, lane 1), whereas the mutant transcript DUP33-Y5, which has an uninterrupted polypyrimidine tract in the upstream intron, is spliced exclusively by exon inclusion *in vivo* (12) and in nuclear extract (lane 5). Splicing of wild-type DUP33 in S100 extract complemented with SF2/ASF resulted in partial exon inclusion (Fig. 5, lane 2), which reverted to exon skipping, as expected, upon hnRNP A1 addition (lanes 3 and 4). In contrast, hnRNP A1 addition failed to promote exon skipping in the case of the mutant DUP33-Y5 pre-mRNA (Fig. 5, lanes 6 to 8). Splicing of the DUP33-Y1, -Y2, and -Y3 substrates (12), which have pyrimidine contents intermediate between those of DUP33 and DUP33-Y5, showed that hnRNP A1-mediated exon skipping decreased gradually as the pyrimidine content near the 3'

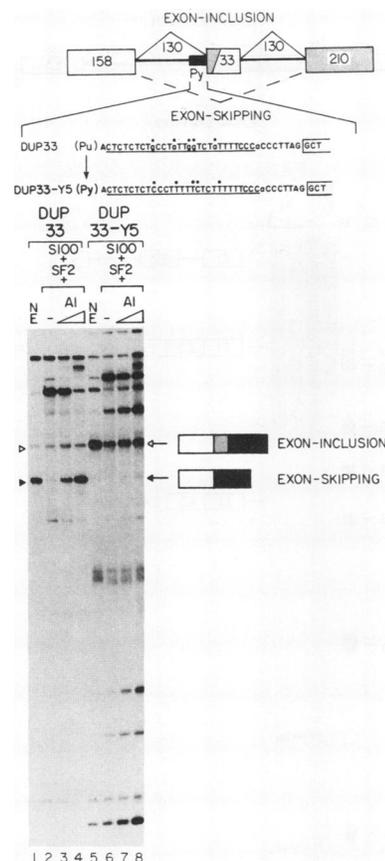


FIG. 5. Effect of upstream polypyrimidine tract composition on hnRNP A1-promoted exon skipping. The structures of the model pre-mRNAs (12) and the sequences of the polypyrimidine tracts and 3' splice sites in each upstream intron are shown. The dots indicate the five nucleotide differences (transversions) between the two templates. The exon and intron sizes are indicated in nucleotides. The structures of the two mRNAs resulting from alternative exon inclusion or skipping are represented on the right. All splicing products were previously characterized in detail (12). The reaction mixtures contained either nuclear extract alone (NE) (6 μl) or S100 extract (6 μl) plus SF2/ASF (SF2) (0.7 μl) plus recombinant hnRNP A1 (lanes 2 and 6, none; lanes 3 and 7, 1 μl; lanes 4 and 8, 4 μl). Pu, purine; Py, pyrimidine.

splice site increased (data not shown). These data show that a strong polypyrimidine tract element, made up of uninterrupted pyrimidines, overcomes the modulatory activity of hnRNP A1 that leads to internal exon skipping.

hnRNP A1 does not promote inappropriate exon skipping of a natural constitutively spliced pre-mRNA. The preceding experiments described above with natural and model alternative splicing substrates showed that the stimulation of exon skipping by hnRNP A1 depends on structural features of the substrates. In the case of the model pre-mRNAs, a long internal exon or a strong upstream 3' splice site abrogated the exon skipping activity of hnRNP A1. We hypothesized that the exon and intron sequences of constitutively spliced pre-mRNAs have evolved to minimize inappropriate exon skipping. If this is the case, an excess of hnRNP A1 should not affect the splicing of natural constitutively spliced pre-mRNAs containing multiple exons.

We chose to analyze the splicing of wild-type rabbit β-globin pre-mRNA, which contains three exons and two

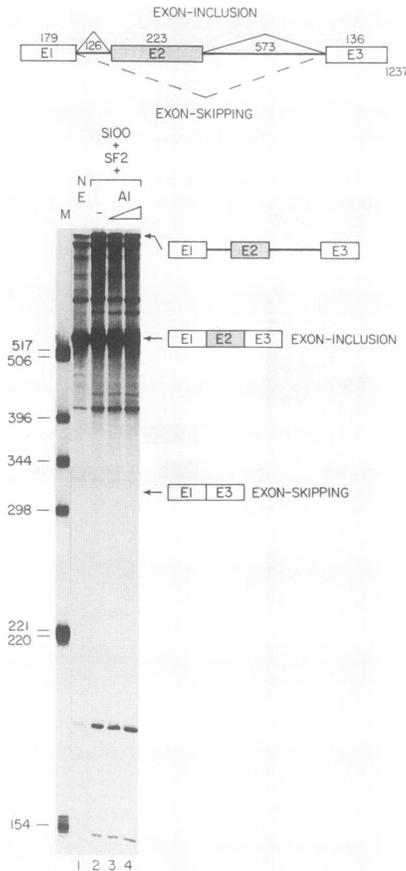


FIG. 6. Effect of hnRNP A1 on constitutive splicing of rabbit β -globin pre-mRNA in vitro. The structure of the pre-mRNA is shown schematically with the exon and intron sizes in nucleotides (1). The pre-mRNA and final spliced product are represented on the right. The expected position of the inappropriate exon skipping product is also indicated on the right. The sizes of the pBR322/*Hinf*I DNA markers (lane M) are shown in nucleotides. The characterization of the splicing products was described previously (1). The reaction mixtures contained either nuclear extract alone (NE) (lane 1, 15 μ l) or S100 extract (6 μ l) plus SF2/ASF (SF2) (0.7 μ l) plus recombinant hnRNP A1 (lane 2, none; lane 3, 2 μ l; lane 4, 5 μ l).

introns (E1-E2-E3), because trace amounts of inappropriate E2 skipping were previously detected with this substrate in some preparations of nuclear extract (1). Fully spliced E1/E2/E3 mRNA was efficiently generated in nuclear extract and in S100 extract complemented with SF2/ASF, while an RNA of the expected size for E1/E3, reflecting inappropriate skipping of E2, could not be detected with our extracts (Fig. 6, lanes 1 and 2). Significantly, the addition of hnRNP A1 did not generate detectable levels of E1/E3 mRNA or inhibit accurate E1/E2/E3 splicing (Fig. 6, lanes 2 to 4).

DISCUSSION

Influence of the ratio of SF2/ASF to hnRNP A1 on alternative splicing. Previous work showed that the ratio of SF2/ASF to hnRNP A1 specifies the selection of alternative 5' splice sites in vitro in the case of pre-mRNAs that contain alternative 5' splice sites that compete for a common 3' splice site (44). We have examined whether this system of two antagonistic protein factors can influence two other

modes of alternative splicing: (i) exon skipping versus exon inclusion and (ii) the selection of mutually exclusive exons.

In vitro splicing experiments with a series of model pre-mRNAs designed to study exon skipping and inclusion demonstrated that, in an appropriate context, the ratio of SF2/ASF to hnRNP A1 can precisely determine whether the internal exon is included or excluded. An excess of SF2/ASF promotes exon inclusion, whereas an excess of hnRNP A1 favors exon skipping. These changes in alternative splice site selection are elicited by small changes in the relative amounts of hnRNP A1 and SF2/ASF, within a range of concentrations comparable to the endogenous levels of these factors in HeLa cell nuclear extract. The ability of hnRNP A1 to promote exon skipping depends on the context of the alternative exon as influenced, for example, by the size of this exon and by the relative strength of the polypyrimidine tract in the preceding intron.

A different mode of alternative splicing was studied with pre-mRNAs derived from a rat β -tropomyosin gene. The natural transcripts from this gene are processed in a tissue-specific manner, in part by incorporation of the mutually exclusive exons 6 and 7 (Fig. 1A, diagram). Previous mutational analyses identified critical *cis*-acting elements, within exon 7 and in the adjacent upstream intron, that specifically block the use of the skeletal muscle-specific exon 7 in nonmuscle cells in vivo (23, 29). It was shown that the lack of exon 7 utilization in nonmuscle cells is due not to a simple *cis* competition mechanism but rather to the action of *trans*-acting factors that block the use of this exon in certain cellular environments (23). A protein factor that binds specifically to the regulatory region upstream of exon 7 was recently purified from HeLa cells by using a gel mobility shift assay (23, 51) and shown to be identical to PTB, a previously identified polypyrimidine tract-binding protein (19, 52), one isoform of which is also known as hnRNP I (4, 18, 53). This was an unexpected finding, since the regulatory element is not composed of consecutive pyrimidines (23, 51). Whereas the binding of PTB may be involved in the negative regulation of exon 7 inclusion, no *trans*-acting factors that positively promote inclusion of this exon in skeletal muscle have been documented. In the present study, we showed that SF2/ASF influences alternative splicing of β -tropomyosin in vitro, allowing inclusion of exon 7 in a nonmuscle cell extract. In this particular instance, hnRNP A1 did not antagonize SF2/ASF to inhibit use of the proximal exon 7 and promote use of the distal exon 6.

The mechanism by which SF2/ASF promotes the β -tropomyosin skeletal muscle-specific splicing pattern in HeLa cell extracts remains unknown. One possibility is that activation of the proximal 5' splice site downstream of exon 7 by excess SF2/ASF overcomes the negative regulation exerted by PTB and/or other factors at the regulatory elements located upstream of, and within, exon 7. Alternatively, the exon 7 blocking factor(s) could be a specific target for SF2/ASF, or the two factors may compete for binding to the same sequence, e.g., the regulatory element in the intron preceding exon 7. In this regard, we note that hnRNP A1, a known antagonist of SF2/ASF (44), was also reported to bind at 3' splice sites under splicing conditions in nuclear extract (60). Perhaps the blocking factor(s) stoichiometrically antagonizes SF2/ASF during splicing of β -tropomyosin pre-mRNA, in a manner analogous to that of hnRNP A1 with many other pre-mRNAs. The concentration of SF2/ASF protein (relative to the concentration of hnRNP A1) varies considerably in different cell lines and tissues (25). Hence, it is possible that regulation of the ratio of SF2/ASF to PTB and/or to

other factors plays an important role in the tissue-specific control of β -tropomyosin alternative splicing.

Although SF2/ASF is able to promote inclusion of exon 7 *in vitro*, it is unclear how this relates to the regulated inclusion of this exon in muscle cells *in vivo*. Transient transfection experiments with wild-type and mutant rat β -tropomyosin minigenes revealed that blockage of the skeletal muscle-specific exon 7 in nonmuscle cells is primarily the result of inhibition of the 3' splice site of exon 7 (22). However, any changes in activities that alter the relative use of the 5' splice site of exon 7, and hence the relative balance of splice site competition, could lead to alterations in normal splicing regulation. The observed effects of SF2/ASF on β -tropomyosin *in vitro* splicing may reflect the additional influence of this factor on alternative 3' splice site selection (15). This activity of SF2/ASF is not counteracted by hnRNP A1 but rather by an activity designated SF7 (45). Further purification and characterization of SF7 will be necessary before we can assess whether the ratio of SF2/ASF to SF7 can also influence exon skipping or exon inclusion with the substrates described here.

We note that highly purified SF2/ASF polypeptide showed somewhat reduced activity in some of the assays described here compared with concentrated preparations that contain additional SR polypeptides with SF2/ASF activity, as originally defined (15, 16, 30, 32, 33, 48, 63). Therefore, it is possible that individual SR polypeptides make different qualitative or quantitative contributions to the exon inclusion properties reported here.

Influence of the ratio of SF2/ASF to hnRNP A1 on constitutive splicing. Our data obtained with natural and model pre-mRNAs support the idea that the activity of the general splicing factor SF2/ASF on splice site selection might be part of the normal cellular mechanisms that prevent exon skipping and ensure the fidelity of constitutive splicing (33). We found that appropriate amounts of SF2/ASF effectively prevented inappropriate exon skipping in rat β -tropomyosin pre-mRNA. In addition, we found that the ability of hnRNP A1 to promote exon skipping by counteracting SF2/ASF is substrate dependent. Some of the important structural parameters of the substrate appear to be the length of the alternative exon and the relative strength of the preceding 3' splice site. We interpret these results as indicating that the ratio of SF2/ASF to hnRNP A1 affects splice site selection when there is an appropriate balance of *cis* competition strengths between the relevant splice sites. The same structural parameters should influence presumptive interactions between factors bound at the splice sites of adjacent introns, which result in exon definition (20, 38, 56, 61). Hence, SF2/ASF and hnRNP A1 could be involved in some of the molecular interactions responsible for exon definition. In addition, the ability of hnRNP A1 to influence splice site selection may be restricted by the presence of specific sequences that render the substrate susceptible to regulation by other factors, as in the case of β -tropomyosin.

hnRNP A1 did not cause inappropriate exon skipping *in vitro* either with a constitutively spliced rabbit β -globin pre-mRNA or with an alternatively spliced rat β -tropomyosin pre-mRNA. Since hnRNP A1 is an abundant nuclear protein (2, 53), the fact that it is unable to promote exon skipping indiscriminately *in vitro* is significant. Thus, even high levels of hnRNP A1, as are found in proliferating cells (8, 11, 39, 49, 54), should not result in inappropriate exon skipping of constitutively spliced pre-mRNAs *in vivo*, although they may be responsible for the altered patterns of alternative splicing of numerous cellular pre-mRNAs ob-

served in transformed cells, as previously suggested (3, 44). Although multiple copies of hnRNP A1 are probably bound to most, if not all, cellular pre-mRNAs in a ribonucleosome structure (9), the phasing of these ribonucleosomes on individual pre-mRNAs may influence splice site selection. High-affinity binding of hnRNP A1 to specific sequences that may be present on only certain pre-mRNAs could position the ribonucleosomes, resulting in the observed substrate specificity in hnRNP A1-mediated exon skipping. The kinetics of binding by hnRNP A1 and other hnRNP proteins (4, 44, 51) to different pre-mRNAs, relative to other splicing factors, may also determine the precise outcome of splice site selection for each substrate.

We propose that constitutive splice sites have evolved in conjunction with appropriate sequence and structural contexts to match exclusively appropriate pairs of 5' and 3' splice sites so as to avoid exon skipping, regardless of variations in the levels of SF2/ASF, hnRNP A1, SF7, and similar regulatory factors. In contrast, alternative splice sites that are *in cis* competition have more balanced relative strengths and are therefore susceptible to variations in the levels of such factors.

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