Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3363–3367, April 1994
Biochemistry

The human splicing factor ASF/SF2 can specifically recognize pre-mRNA 5′ splice sites

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Communicated by Bruce S. Baker, December 3, 1993 (received for review August 19, 1993)

ABSTRACT **ASF/SF2 is a human protein previously shown to function in *in vitro* pre-mRNA splicing as an essential factor necessary for all splices and also as an alternative splicing factor, capable of switching selection of 5′ splice sites. To begin to study the protein's mechanism of action, we have investigated the RNA binding properties of purified recombinant ASF/SF2. Using UV crosslinking and gel shift assays, we demonstrate that the RNA binding region of ASF/SF2 can interact with RNA in a sequence-specific manner, recognizing the 5′ splice site in each of two different pre-mRNAs. Point mutations in the 5′ splice site consensus can reduce binding by as much as a factor of 100, with the largest effects observed in competition assays. These findings support a model in which ASF/SF2 aids in the recognition of pre-mRNA 5′ splice sites.**

Pre-mRNA splicing is a complex reaction that requires small nuclear ribonucleoprotein particles (snRNPs) and a large number of non-snRNP proteins (reviewed in refs. 1 and 2). While the four snRNPs U1, U2, U4/U6, and U5 have been well characterized (3, 4), much less is known about splicing proteins. In yeast, where introns are infrequent and alternative splicing is rare or nonexistent, a combination of genetic and biochemical approaches has led to the identification and characterization of a large number of non-snRNP proteins that are essential for splicing (reviewed in refs. 5 and 6). In higher eukaryotes, splicing proteins have been identified and characterized to date solely by biochemical assays. The sequences of about a half dozen of these proteins are known, and almost all contain one or more copy of the RNA recognition motif (7) or RNP-type RNA binding domain (RBD; see ref. 8). The RBD is an ≈80-amino acid domain found in a number of RNA binding proteins. It was first identified in poly(A) binding protein (9, 10), and one or more copies have since been found in many RNA binding proteins (reviewed in refs. 11 and 12). In several cases, RNA binding by these proteins has been shown to be sequence-specific, and it appears that different RBDs can recognize different RNA sequences and structures. The structures of two different RBDs have been determined (13–15), and each consists of a four-stranded antiparallel β-sheet and two α-helices. This arrangement places the two most highly conserved elements of the RBD, designated RNP-1 and RNP-2, adjacent to each other in the center of the β-sheet.

Several RBD-containing proteins have been shown to be essential for pre-mRNA splicing *in vitro*. $U2AF^{65}$ is an essential splicing factor that is required for U2 snRNP binding to the branch site region and that binds with specificity to the polypyrimidine tract of the 3′ splice site (ss) (16). $U2AF^{65}$ contains three regions that share sequence similarity to the RBD consensus, which are all required for sequence-specific binding (17). ASF/SF2, also an essential splicing factor, is required for a very early step in spliceosome assembly (18, 19) and contains two RBD-type sequences (20–22). SC-35 is similar in structure to ASF/SF2 (23) and is also essential for an early step in spliceosome assembly (24). Finally, several RBD-containing proteins have been shown, like U2AF, to bind with specificity to the polypyrimidine tract 3′ ss (25–30).

ASF/SF2 has a second activity *in vitro*, which is that it can modulate selection of alternative splice sites in a concentration-dependent manner (31, 32). Most frequently this results in a switch such that the 5′ ss most proximal to the 3′ ss is utilized almost exclusively at high concentrations of ASF/SF2 (33). These observations have led to the suggestion that one mechanism for modulating splice site selection *in vivo* might be to vary the concentration or activity of general splicing factors such as ASF/SF2. In this regard, it is noteworthy that the primary structure of ASF/SF2 shares similarity with known regulators of alternative splicing from *Drosophila*. Two of these proteins, Sex lethal (34) and Transformer-2 (35, 36), contain RBDs and each can bind specifically to target pre-mRNAs (37, 38). In addition, several of these regulators, including ASF/SF2, contain regions rich in repeats of arginine-serine dipeptides (RS domains).

An important goal in understanding how ASF/SF2 functions, as both an essential and an alternative splicing factor, is to understand how it interacts with RNA. Here we present data that ASF/SF2 binds specifically to pre-mRNA fragments containing an intact 5′ ss.

MATERIALS AND METHODS

DNA Plasmids. The simian virus 40 (SV40) small t intron-containing fragment, from the plasmid pHd (39), was inserted into the *Hin*dIII–*Pst* I sites of pGem in the orientation of the SP6 promoter, producing pGt. All pGt derivatives contain 11 nucleotides (nts) from the pGem3 vector at their 5′ end. Mutants 50–511 were made by *in vitro* mutagenesis of pGt using appropriate oligonucleotides. Mutant t31 was made with an oligonucleotide that introduced a 5-base change at the 3′ ss of the small t intron. The 5′ ss mutant tΔ5 was derived from mutant 51, resulting in deletion of sequences upstream of position +4 relative to the 5′ ss. The 3′ ss deletion mutant tΔ3 was made by deleting sequences downstream from position −4 relative to the 3′ ss of pGt. The pGe plasmid containing the wild-type (wt) 13S intron of E1a was constructed by inserting a 230-bp fragment from the adenovirus E1a gene (33) into the *Hin*dIII–*Xba* I sites of pGem. The mutant pGe51, containing 4 base changes at the 5′ ss of the 13S intron, was constructed from pGe.

RNA and Protein Preparations. *In vitro* run-off transcription was performed with SP6 polymerase (unless indicated) as described (31). To synthesize radiolabeled transcripts, [α-^{32}P]GTP and [α-^{32}P]UTP (650 Ci/mmol; 1 Ci = 37 GBq)

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Abbreviations: snRNP, small nuclear ribonucleoprotein; RBD, RNA binding domain; ss, splice site(s); wt, wild-type; SV40, simian virus 40.

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were included in synthesis reactions. Transcripts were purified on denaturing polyacrylamide gels. Expression and purification of ASF/SF2 proteins in *Escherichia coli* were as described (20). Protein concentrations were determined by the Bradford method, and proteins were monitored by Coomassie blue staining of SDS/polyacrylamide gels.

RNA Binding Assays. The same RNA binding buffer was used for UV crosslinking and gel shift assays and contained 10 mM Hepes (pH 7.9), 200 mM KCl, 20 mM NaCl, 0.025% Nonidet P-40, 1 mM dithiothreitol, 15 μg of bovine serum albumin per ml, and 10% glycerol. For UV crosslinking, 1–3 × 10^4 cpm (10–50 fmol) of each RNA was added per 25-μl reaction mixture, which also contained 5 μg of tRNA (and unlabeled competitor RNA where indicated). Twenty to 200 pmol of purified recombinant protein (see figure legends) was incubated with RNA in binding buffer for 20 min at room temperature, and the RNA–protein complexes were irradiated at a distance of 6 cm with a hand-held mineral light (model UVG-11; 254 nm) for 10 min. Ten micrograms of RNase A was added and the samples were incubated for 30 min at 30°C. An equal volume of 2 × SDS gel loading buffer was added to each sample, and aliquots were analyzed by electrophoresis through an SDS/15% polyacrylamide gel. Gel shift assays were performed with smaller amounts of ^{32}P-labeled RNA (1–5 fmol). One to 400 pmol of ASF/SF2 derivatives as indicated and 5 μg of tRNA (plus unlabeled competitor where indicated) were added to 25-μl reaction mixtures prior to addition of the labeled probes. Samples were incubated for 30 min at room temperature and analyzed on 6% polyacrylamide (acrylamide:bisacrylamide ratio, 40:1) gels. Gels were electrophoresed in 0.5× TBE buffer at 3.5 W for 4 hr.

RESULTS

Our previous studies showed that ASF/SF2 can influence splicing of SV40 early (20, 31) and adenovirus E1a (33) pre-mRNAs. The protein activated splicing of each RNA when added to extracts lacking ASF/SF2 and favored use of the downstream most 5′ ss in each. To determine whether this activity might reflect sequence-specific binding of ASF/SF2, we first performed a series of UV crosslinking experiments with purified HeLa cell ASF/SF2 and RNA fragments spanning the entire early SV40 transcript used in the splicing experiments. The results indicated that the strongest crosslinking occurred to an ≈100-nt RNA encompassing the 66-nt small t intron (H. Ge and J.L.M., unpublished data). Here we have examined binding to a similar fragment, using recombinant ASF/SF2. In most experiments, an ASF/SF2 mutant, ASFΔRS, was used (22). This derivative lacks 50 C-terminal residues, which correspond almost precisely to the RS domain. A similar region in U2AF^{65} has been shown to increase affinity but reduce sequence-specific RNA binding *in vitro* (17), and a similar situation occurred with ASF/SF2 (result not shown).

ASFΔRS UV Crosslinks Preferentially to 5′ ss-Containing RNAs. We first tested the ability of ASFΔRS to crosslink to each of four different ^{32}P-labeled RNA transcripts (see Fig. 1*E*). One (t intron) was a 95-nt RNA that spanned the entire small t intron. A second (13S) contained the E1a 13S 5′ ss, but not the 3′ ss, in a 125-nt transcript. pGEM and sk were similarly sized (104 and 115 nt) transcripts of plasmid sequences. Identical concentrations of each were incubated with equal amounts of ASFΔRS, UV irradiated, treated with RNase A, and subjected to SDS/polyacrylamide gel electrophoresis. Fig. 1*A* shows that significant crosslinking of the small t RNA to ASFΔRS occurred. While the 13S RNA also crosslinked efficiently (albeit less well than small t), crosslinking of the two nonspecific RNAs was at most barely detectable. Fig. 1*B* shows a Coomassie blue stain of the same gel, which indicates that the amount of ASFΔRS in each sample was identical. To demonstrate that the observed variations in crosslinking were due to differences in RNA binding, as opposed to differences in crosslinking efficiencies, the competition experiments shown in Fig. 1 *C* and *D* were performed. ^{32}P-labeled small t (Fig. 1*C*) and 13S RNAs (Fig. 1*D*) were each incubated with ASFΔRS plus increasing amounts of the indicated unlabeled RNAs. In each case, only the homologous RNA reduced crosslinking significantly.

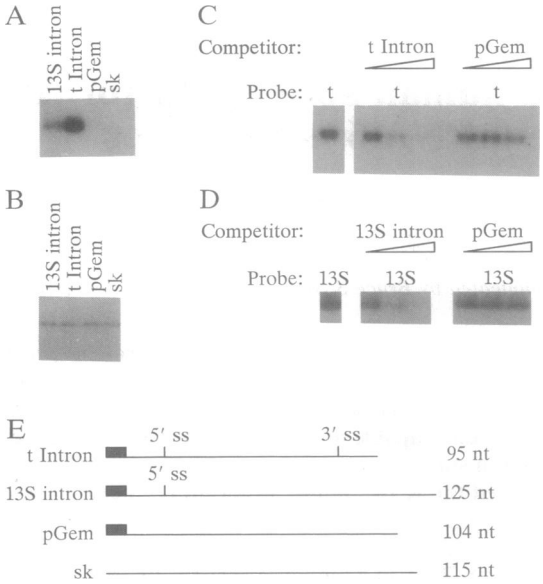

FIG. 1. ASFΔRS binds preferentially to pre-mRNAs relative to nonspecific RNAs. RNA binding was measured using a UV crosslinking assay, in which ASFΔRS (80 pmol) and the indicated ^{32}P-labeled RNA substrates (30 fmol) were incubated and analyzed. (*A*) UV crosslinking of ASFΔRS to four RNA fragments. The indicated RNAs were incubated with ASFΔRS and analyzed. (*B*) The same gel as in *A* was stained with Coomassie blue, destained, and photographed prior to exposure to x-ray film. (*C*) Competition with small t RNA. The t intron-containing RNA fragment was labeled and mixed with either unlabeled t RNA or unlabeled nonspecific RNA (pGEM) under the same conditions as in *A*, except that 5-, 25-, or 125-fold molar excess of the competitor was added, as indicated. (*D*) Competition with adenovirus E1a RNA. This experiment was identical to that shown in *C* except the 13S intron RNA was the labeled probe and was also used as the specific competitor. (*E*) Schematic diagram of the RNAs used in UV crosslinking; 5′ ss and 3′ ss are indicated. The t intron, 13S intron, and pGEM RNAs each contain 11 nts from the pGEM vector (shaded box). The size of each transcript is indicated.

The above experiments show that ASFΔRS crosslinked more efficiently to the small t RNA than to the 13S RNA. A possible explanation for this was that the former contained all signals essential for splicing, while the latter contained only the 5′ ss. To test whether the 3′ and/or the 5′ ss in the small t RNA might be important for efficient crosslinking, we compared crosslinking of the full-length RNA with derivatives lacking either the 5′ or the 3′ ss. The results (Fig. 2) show that removal of the 3′ ss had at most a small effect on crosslinking. However, the deletion that removed the 5′ ss (Δ 5′) essentially eliminated crosslinking. Competition experiments analogous to those in Fig. 1 confirmed that the differences in crosslinking were in fact due to differences in binding (Fig. 2). These findings suggest that the 5′ ss may be essential for efficient binding of ASFΔRS to the RNAs tested.

Point Mutations in the Small t Intron 5′ ss Significantly Reduce ASFΔRS Binding. To extend our analysis of ASF/SF2 RNA binding, we examined the interaction of ASFΔRS with the small t RNA using a gel shift assay. Experiments employing the four RNAs used in the crosslinking experi-

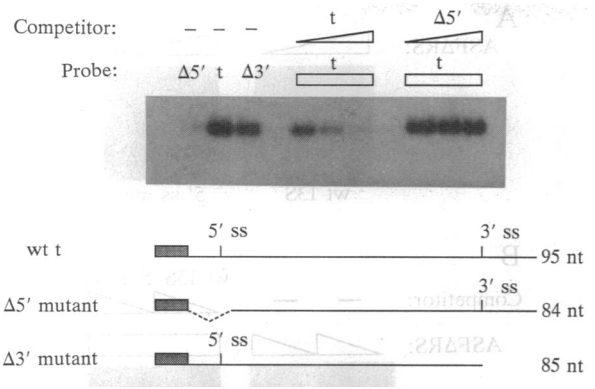

FIG. 2. The 5′ ss may be essential for efficient binding of ASFΔRS to RNA. The sizes and features of the Δ5′ and Δ3′ RNAs are indicated at the bottom. The shaded boxes indicate 11 nts from the pGEM vector. The left 3 lanes show the results of UV crosslinking with the indicated RNAs plus ASFΔRS, performed exactly as in Fig. 1A. The right 6 lanes show the results of competition experiments in which wild-type t RNA (wt t) was labeled and mixed with increasing amounts of either the wt t or 5′ ss mutant unlabeled competitor. The labeled substrate (probe) and competitors are indicated above each lane. As in Fig. 1C, 5-, 25-, or 125-fold molar excess of each competitor was used.

ments in Figs. 1 and 2 revealed a specificity similar to that described above (results not shown). To examine in more detail the role of the 5′ ss in binding of ASFΔRS, we analyzed the effects of a number of point mutations in the 5′ ss consensus of the small t RNA. Fig. 3 shows the results of a gel shift experiment in which decreasing amounts of ASFΔRS were incubated with small t RNA and with each of three mutated derivatives. These RNAs contained 3 (mutant 51), 1 (52), or 3 (53) base change(s) in the 5′ ss consensus, as shown at the bottom of Fig. 3. The mutant RNAs were all bound less efficiently by ASFΔRS than was the wt RNA. By comparing

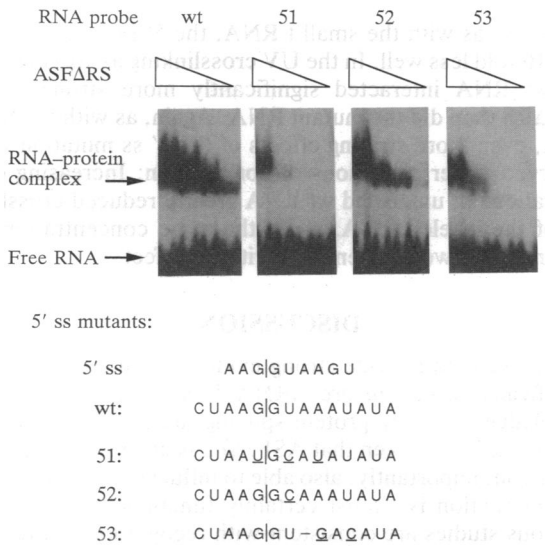

FIG. 3. Point mutations in the small t intron 5′ ss significantly reduce ASFΔRS binding. Binding of ASFΔRS to the t intron RNA described in the legend to Fig. 1 was analyzed by gel shift assays under the same conditions employed in the UV crosslinking experiments, except that only 3 fmol of labeled RNA was used. Three mutant RNAs containing base changes at the 5′ ss are shown at the bottom. Mutated nucleotides are underlined and aligned with the wt and 5′ ss consensus sequences. wt small t RNA and the 5′ ss mutants were incubated with increasing amounts of ASFΔRS (1.2, 4, 12, and 40 pmol). The free RNA and RNA–protein complexes were separate on 6% native polyacrylamide gels. RNA substrates used in binding reactions are indicated above the lanes.

the amounts of RNA shifted at different protein concentrations, we estimate that the affinity of ASFΔRS, relative to the wt RNA, was reduced by a factor of ≈25 for mutant 51, by ≈3–5-fold for mutant 52, and by 15-fold for mutant 53. In contrast, a mutation changing 5 nts at the 3′ ss had no significant effect on binding (results not shown; see Table 1).

We also tested the effects of 5′ ss mutations on ASFΔRS binding in competition assays. For this, an amount of ASFΔRS sufficient to shift ≈50% of the small t RNA was incubated with ^{32}P-labeled wt RNA plus increasing amounts of the indicated unlabeled competitor RNAs (Fig. 4A). As expected, the wt RNA functioned very effectively as a competitor, reducing binding to the labeled RNA significantly at even the lowest concentration of competitor, a 5-fold excess, and almost eliminating binding at the highest concentration (125-fold excess). In contrast, a nonspecific RNA (pGEM) and mutant 51 RNA were essentially inactive as competitors. Inefficient competition was also observed when the RNA containing the single base change at position +2 of the 5′ ss (mutant 52) was used. Significant competition was detected only with the highest amount of competitor tested, indicating that at least 10-fold more 52 RNA was required to obtain competition comparable to that observed with the wt RNA. We also examined the ability of wt and mutant 52 RNAs to compete when mutant 52 RNA was used as the labeled substrate (Fig. 4B). wt RNA was again an excellent competitor, greatly reducing binding of the labeled RNA at even the lowest concentration. When mutant 52 RNA was used as a competitor, binding of the labeled RNA was also reduced, although significantly less effectively than with wt RNA. Together these results strongly suggest that the 5′ ss is critical for efficient binding of ASFΔRS to the small t RNA.

To extend this analysis, we examined a number of additional 5′ ss mutations, using the competition assay shown in Fig. 4. Specifically, we determined the ability of unlabeled mutant RNAs to compete with ^{32}P-labeled wt small t RNA in gel shift assays and compared this with the amount of unlabeled wt RNA required to achieve a similar level of competition. The results obtained are presented in Table 1

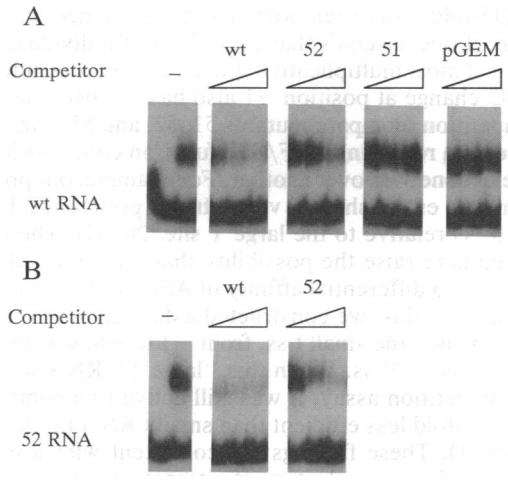

FIG. 4. A single base change at the 5′ ss reduces ASFΔRS binding at least 10-fold in a competition assay. Gel shift assays were performed under the conditions indicated in Fig. 3, except that the indicated competitors were mixed with the radiolabeled RNA prior to incubation with ASFΔRS (20 pmol). (A) Labeled small t RNA was incubated with ASFΔRS and the competitors are listed above the lanes. Molar ratios of competitor to substrate were 5, 25, and 125. The left two lanes are controls containing labeled small t RNA only or small t RNA plus ASFΔRS, but no competitor. (B) Similar experiment in which mutant 52 RNA was labeled and mixed with unlabeled small t or mutant 52 RNA competitors.

Table 1. RNA binding competition by mutant pre-mRNAs

	Mutant	Relative amount required for 50% reduction in ASFΔRS binding
5′ ss		
wt	AAG|GUAAAU	1
50*	AAG|GUAAGU	<2
51	AAU|GCAUAU	>100
52	AAG|GCAAAU	10
53	AAG|GUGACA	25
54	UCG|GUAAAU	20
55	AAG|GUAUAU	5
56	AAG|GUUAGU	25
57	AAG|GUGAAU	5
58	AAG|GUCGAC	10
59	AAG|GUUAUA	5
510	AAG|CUAAAU	15
511 (T)†	GAG|GUAUUU	7
3′ ss		
wt	AUUUUAG|AUU	1
31	AUUACGC|GUU	<2

RNA binding was measured by gel shift assays as in Fig. 3. Three femtomoles of ^{32}P-labeled SV40 t RNA (wt) and 40 pmol of ASFΔRS were present in all reaction mixtures, together with increasing amounts of the unlabeled competitor RNAs. Fifteen femtomoles of wt RNA was required to reduce binding ≈50%, and this value was taken as 1. Mutated bases are underlined. Vertical lines represent the positions of splice sites.
*Matches the consensus 5′ ss.
†Matches the SV40 large T 5′ ss.

and allow several conclusions. The first addresses the question of whether a perfect 5′ ss consensus might be the optimal target for binding. While the small t site is an exceptionally strong match to consensus, it is not perfect (8/9; see Fig. 3). Mutant 50 contains a perfect consensus, changing the A to a G at position +5. The behavior of this RNA as a competitor was nearly identical to that of wt, indicating that a perfect consensus is not a better binding site. Second, single base changes at positions +1 through +4 all had significant effects on competition (mutants 510, 52, 57, and 55). The largest effect (15-fold) was seen with a G to C change at +1. In addition, if one assumes that the effects of individual mutations are at most multiplicative, then it can be concluded that a G to U change at position −1 also has a substantial effect on competition (compare mutants 51, 52, and 55). An important question regarding ASF/SF2 function concerns its ability to select one 5′ ss over another. For example, our previous experiments established a very strong preference for the small t 5′ ss relative to the large T site (20, 31). The results presented here raise the possibility that this may reflect, at least in part, a differential affinity of ASF/SF2 for the two 5′ ss. To address this, we constructed a derivative of the small t RNA in which the small t ss, from −3 to +6, was replaced with the large T 5′ ss. When this "large T" RNA was used in the competition assay, it was still active as a competitor, but was ≈7-fold less efficient than small t RNA (mutant 511; see Table 1). These findings are consistent with a role for ASF/SF2 affinity in selection of alternative splice sites.

Efficient Binding of ASFΔRS to Adenovirus E1a Pre-mRNA Also Requires an Intact 5′ ss. To test whether ASFΔRS could bind another pre-mRNA in a 5′ ss-dependent manner, we used the adenovirus E1a pre-mRNA fragment that was employed in the crosslinking experiments described above. Binding of ASFΔRS to the wt RNA and to a mutant containing four base changes in the 5′ ss was examined using gel shift and UV crosslinking assays. Consistent with the crosslinking experiment in Fig. 1, the E1a RNA (wt 13S) was shifted less efficiently than was the SV40 RNA (Fig. 5*A*).

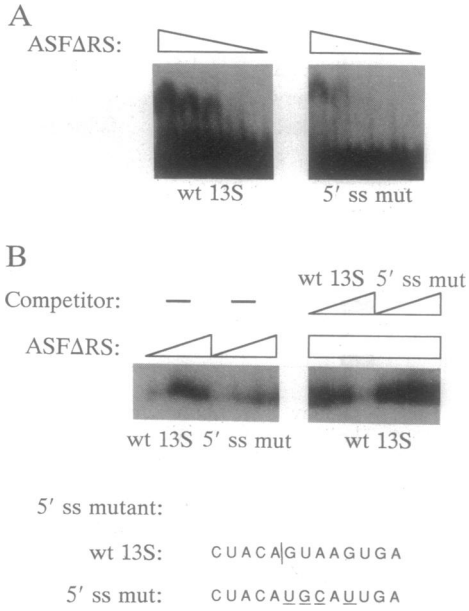

FIG. 5. Efficient binding of ASFΔRS to E1a pre-mRNA required an intact 5′ ss. The E1A 13S RNA shown in Fig. 1 was utilized in binding assays. The nucleotide sequences encompassing the 5′ ss of the wt RNA and a mutant derivative are shown at the bottom. Binding of ASFΔRS to wt and mutant RNAs was analyzed by gel shift (*A*) and UV crosslinking (*B*) assays. (*A*) Increasing amounts (0.8, 2.0, 4.0, 12, and 28 pmol) of ASFΔRS were incubated as in Fig. 3 with either wt or mutant RNAs, and the RNA–protein complexes were separated on a 6% native polyacrylamide gel. (*B*) The radiolabeled RNA substrates used are indicated below the lanes, and the unlabeled competitors are shown above. In the left panel, wt 13S and the 5′ ss mutant RNAs were labeled and incubated with increasing amounts (12, 40, and 120 pmol) of ASFΔRS under the conditions employed in Fig. 1. In the right panel, wt 13S RNA was labeled and mixed with excess amounts (5-, 25-, and 125-fold) of unlabeled 13S or 5′ ss mutant RNAs in the presence of 60 pmol of ASFΔRS.

However, as with the small t RNA, the 5′ ss mutant bound 5- to 10-fold less well. In the UV crosslinking assay (Fig. 5*B*), the wt RNA interacted significantly more strongly with ASFΔRS than did the mutant RNA. Again, as with the SV40 RNA, even more striking effects of the 5′ ss mutation were observed under conditions of competition: Increasing concentrations of unlabeled wt RNA greatly reduced crosslinking of the labeled RNA, while the same concentrations of mutant RNA were essentially without effect.

DISCUSSION

The above data provide strong evidence that ASF/SF2 can specifically recognize pre-mRNA 5′ ss, making it, to our knowledge, the only protein splicing factor capable of recognizing a 5′ ss. Given that ASF/SF2 is an essential splicing factor and, importantly, also able to influence 5′ ss selection, this interaction is almost certainly functionally significant. Previous studies are consistent with recognition of the 5′ ss by a factor other than U1 snRNP. First, the original suppression experiments that proved the base-pairing interaction between U1 snRNA and the 5′ ss also revealed that not all 5′ ss mutations tested could be suppressed (40). This finding suggested that these nonsuppressible bases might serve a function in splicing other than pairing with U1 snRNA, and our results are consistent with the idea that this could be binding of ASF/SF2. Second, UV crosslinking experiments employing nuclear extracts identified an ≈35-kDa protein whose crosslinking to pre-mRNA depended on an intact 5′ ss and which was approximately the size of ASF/SF2 (41).

Finally, in the *Drosophila* P element 5′ exon, a multiprotein complex has been shown to bind a 5′ ss-like sequence (42).

How could 5′ ss binding play a role in ASF/SF2 function? An attractive model is that the protein functions in recruitment of U1 snRNP. A factor that can facilitate U1 snRNP binding to 5′ ss has been detected in nuclear extracts, although its identity was not determined (43). In addition, our recent experiments have detected direct interactions between ASF/SF2 and U1 snRNP that likely contribute to 5′ ss recognition (44). U5 (45) and U6 (46) snRNPs have also been shown to crosslink to pre-mRNAs near the 5′ ss. Although these interactions both appear to require ASF/SF2 activity, they may occur subsequent to ASF/SF2's role in splicing.

With respect to the role of ASF/SF2 in alternative 5′ ss selection, a simple model can be presented. At low concentrations, ASF/SF2 preferentially binds to and activates the 5′ ss with the highest affinity for the protein. At higher concentrations, additional 5′ ss could be recognized and activated, and under these conditions we suggest that the 5′ ss closest to the 3′ ss will be preferentially utilized, due simply to its proximal location. By this model, distal sites of equal on lesser affinity for ASF/SF2 would not be used if ASF/SF2 was the sole determining factor. In such cases, we suggest that an additional factor(s) is required for use of the upstream sites. Indeed, two proteins capable of activating distal 5′ ss *in vitro* have been described (47, 48).

This general model for ASF/SF2 function raises an important question—i.e., what is a "high-affinity" ASF/SF2 binding site? Although our data have shown that many 5′ ss mutations can reduce binding, they do not address the question of what is the optimal ASF/SF2 binding site. An intriguing possibility is suggested by findings that efficient splicing of specific introns in several different pre-mRNAs requires purine-rich sequences located in the downstream exons (ref. 49 and references therein). Indeed, we have recently found that ASFΔRS can bind similar sequences (R. Tacke and J.L.M., unpublished data). Given that 5′ ss are themselves generally purine rich (the small t site contains 7 of 9 purines), it could be that certain 5′ ss are targets for ASF/SF2 binding because they fit a purine-rich consensus. ASF/SF2 could then function in splicing by binding either at or near the 5′ ss to be activated or, perhaps in the case of low-affinity 5′ ss, at a distant site in the pre-mRNA.

Although ASF/SF2 is, to our knowledge, the only protein that can recognize 5′ ss, there are likely to be others. The strongest candidates are additional members of the SR protein family, which is a conserved group of proteins that share structural similarities and that appear to have related activities *in vitro* (50, 51). However, significant variations in the behavior of these proteins have recently been detected (52–55). It will be of interest to determine if the RNA binding specificity of other SR proteins is similar to that of ASF/SF2.

We thank H. Ge, J. Harper, R. Tacke, and J. Kohtz for helpful discussions and M. Wang for technical assistance. This work was supported by National Institutes of Health Grant GM 48259.

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