Human splicing factor ASF/SF2 encodes for a repressor domain required for its inhibitory activity on pre-mRNA splicing

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Abstract

The essential splicing factor ASF/SF2 activates or represses splicing depending on where on the pre-mRNA it binds. We have previously shown that ASF/SF2 inhibits adenovirus IIIa pre-mRNA splicing by binding to an intronic repressor element. Here we used MS2-ASF/SF2 fusion proteins to show that the second RNA binding domain (RBD2) is both necessary and sufficient for the splicing repressor function of ASF/SF2. Further, we show that the completely conserved SWQDLKD motif in ASF/SF2-RBD2 is essential for splicing repression. Importantly, this heptapeptide motif is unlikely to be directly involved in RNA binding given its position within the predicted structure of RBD2. The activity of the ASF/SF2-RBD2 domain in splicing was position dependent. Thus, tethering RBD2 to the IIIa intron resulted in splicing repression, whereas RBD2 binding at the second exon had no effect on IIIa splicing. The splicing repressor activity of RBD2 was not unique to the IIIa pre-mRNA, as binding of RBD2 at an intronic position in the rabbit ß-globin pre-mRNA also resulted in splicing inhibition. Taken together, our results suggest that ASF/SF2 encode distinct domains responsible for its function as a splicing enhancer or splicing repressor protein.
Introduction

The human ASF/SF2 protein is a member of the evolutionary conserved SR family of splicing factors (1). SR proteins are essential splicing factors required for constitutive splicing and, furthermore, play a regulatory role in alternative RNA splicing (reviewed in 2, 3, 4). SR proteins partake at multiple steps during spliceosome assembly. For example, they help to commit a pre-mRNA for splicing by stabilizing U1 snRNP and U2AF binding to the 5’ and 3’ splice sites, respectively (5-8). Also, they facilitate U4/U6-U5 snRNP recruitment to the spliceosome and are believed to bridge between splicing factors binding to the ends of the intron during spliceosome formation (9-11).

SR proteins contain one or two N-terminal RNP-type RNA binding domains (RBDs) and a highly charged variable-length C-terminal domain rich in arginine-serine dipeptide repeats (the RS domain), hence the name SR proteins (reviewed in 3). ASF/SF2 contain two RBDs, both that are required for high affinity RNA binding (12, 13). SR proteins are modular in structure with the RBDs making sequence specific contact with the RNA (14, 15) and the RS domain mediating protein-protein interactions with multiple general splicing factors (3, 6, 8, 16).

Previous studies have shown that SR proteins function as enhancer proteins, stimulating both constitutive and alternative splicing (reviewed in 3). SR proteins bind to splicing enhancer elements, which typically are located downstream of the affected intron (reviewed in 2). SR protein binding to enhancer elements help to recruit U2AF65 to a nearby weak 3’ splice site (8, 17, 18). Thus, the RS domain of ASF/SF2 appears to be required for U2AF recruitment to weak polypyrimidine tracts, whereas the RS domain is dispensable for ASF/SF2 activation of strong 3’ splice sites that bind U2AF.
efficiently (19). Interestingly, this study also suggested that the ASF/SF2-RBDs provide a specific splicing enhancer function and are not simply required for RNA binding. Collectively, available data suggests that the RBDs and the RS domains in SR proteins partake at distinct steps in splicing activation: the RS domain is sufficient for the second exon splicing enhancer dependent function (reviewed in 3) whereas the RBDs provide a distinct enhancer function besides U2AF recruitment (19).

In addition to functioning as splicing enhancer proteins SR proteins also have the capacity to repress splicing (20, 21). We have previously shown that SR proteins inhibit splicing of the regulated adenovirus L1 IIIa pre-mRNA (reviewed in 22) by binding to the, so-called, IIIa repressor element (3RE), located immediately upstream of the IIIa branch site (20). Mechanistic studies showed that SR proteins inhibit IIIa splicing by preventing U2 snRNP recruitment to the spliceosome (20, 23). The inhibitory effect of the 3RE on IIIa splicing is simply due to its high affinity for SR proteins. Thus, replacing the 3RE with consensus binding sites for ASF/SF2 resulted in splicing repression (20). Also, moving the 3RE to the second exon in the IIIa pre-mRNA converted the 3RE from a repressor element to a classical splicing enhancer element (20). Collectively, our results suggested that, dependent on where in the pre-mRNA SR proteins bind, they either activate or repress splicing.

In our previous study (20) we noted that a truncated ASF/SF2 protein, lacking the RS domain, was capable of inhibiting IIIa splicing. We proposed that binding of SR proteins to the 3RE blocked IIIa splicing by sterically interfering with U2 snRNP recruitment to the IIIa branch site. Here we tested this model. The results strongly argue against this simplistic model. Thus, an MS2-LacZ fusion protein, with a size exceeding that of MS2-ASF/SF2, did not interfere with IIIa splicing. We further show that ASF/SF2 encodes for a specific splicing
repressor domain required for its inhibitory effect on IIIa pre-mRNA splicing. Thus, we show that the second RNA binding domain (RBD2) of ASF/SF2 is both necessary, and sufficient for ASF/SF2 mediated repression of IIIa splicing. Furthermore, we show that the SWQDLKD motif conserved in all SR proteins containing an RBD2 is required for ASF/SF2-RBD2 repression of splicing. Our results also confirm that the ASF/SF2-RS domain functions as a splicing enhancer domain (24), although its activity was very weak under our experimental conditions. However, our results further suggest that RBD2 may augment the splicing enhancer function of the RS domain. Interestingly, the functions of RBD2 and the RS domains in splicing regulation are opposite and position dependent. Thus, RBD2 inhibits IIIa splicing when tethered to the IIIa intron, but has no effect when bound to the second exon. In contrast, the ASF/SF2-RS domain activates IIIa splicing weakly when tethered to the IIIa second exon, but has no effect on splicing when bound at an intronic position. Importantly, the repressor activity of ASF/SF2-RBD2 was not unique to the IIIa pre-mRNA. Thus, tethering ASF/SF2-RBD2 to the intron of the rabbit β-globin pre-mRNA similarly results in splicing inhibition, suggesting that the repressor activity of RBD2 may be a general feature of ASF/SF2.

**EXPERIMENTAL PROCEDURES**

**Construction of plasmids encoding MS2-ASF/SF2 hybrid proteins**

The gene encoding the MS2 coat protein was cloned by reverse transcription of the genomic MS2 RNA followed by PCR amplification using primers 5´-GTGACATATGGCTTCTAACTTTAC-3´ and 5´-CGGGATCCGTAGTAGCCGGAGTTTGCTGC-3´. The PCR product was cloned as
a NdeI-BamHI fragment into the pET15b vector (Novagen), generating plasmid pET15b-H$_6$MS2 (N-terminal 6-His tag). The ASF/SF2 gene was excised from plasmid pAdG5Trip(His)-ASF/SF2 (25) as a BamHI fragment and cloned into the BamHI site in pET15b-H$_6$MS2, generating plasmid pET15b-H$_6$MS2-ASF/SF2. Fragments encoding different domains of ASF/SF2 (Fig. 1A) were generated by cleavage with suitable restriction enzymes; 5´-ends were converted to BamHI sites by linker addition and ligated into the BamHI site in pET15b-H$_6$MS2. Substitution mutants of MS2-RBD2+RS (Fig. 6A) were constructed by standard PCR mutagenesis using oligonucleotide primers with the desired amino acid changes (details available upon request). The carboxy-terminal part of the LacZ gene, a.a. 711-1023, was taken from plasmid pCH110 (Pharmacia) and cloned as a BamHI fragment into pET15b-H$_6$MS2. The gene encoding SRPK1 (26) was cloned into pET24a (Novagen) to create pET24a-T7-SRPK1 (N-terminal T7 tag). Sequences of all hybrid proteins or junction points were confirmed by DNA sequencing.

**Cloning of reporter plasmids for transcript synthesis**

To generate the IIIa-MS2I and IIIa-MS2I(inv) constructs two self complementary oligonucleotides (5´-GATCTCCGGTTGAGGATCACCCAACCGGT-3´ and 5´-GATCACCGGTTGGTGA7CTCAACCGGA-3´), encoding one MS2 operator sequence (27), were annealed, the ends filled-in with the Klenow polymerase and ligated in the direct or inverted orientations into the blunt-ended EagI site in plasmid pGDIIIaß(Eag-Sca) (20). In IIIa-MS2(-15) the same oligonucleotide was ligated into the BglII site positioned just upstream of the IIIa branch site in pGDIIIaß(Eag-Sca). To generate the IIIa-MS2E and IIIa-MS2E(inv) constructs the same double-stranded oligonucleotide was ligated into the blunt-ended MluI site in pGDIIIaß(Eag-Sca). Plasmid glob-MS2I was generated by PCR.
amplification, using as an upstream primer 5´-
GAAGATCTCCGGTTGAGGATCACCCAACCGGTGATCAAGAAACTG-3´ (which
positions an MS2 operator site 53 nucleotides upstream of the ß-globin branch
site) and downstream primer 5´-CAAGGGTCCCCAAACTCA-3´. The PCR
product was cloned as a BglII/BamHI fragment into plasmid
pSPß(IIIaHindIII/Sca)ß (20). IIIa-3RE(-53) was constructed by introducing a
double-stranded oligonucleotide encoding the 49 nucleotide long 3RE (20) into at
the Eag site in plasmid pGDIIIaß(Eag-Sca). The relevant sequences in all
constructs were confirmed by DNA sequencing. Capped and 32P-labeled pre-
mRNAs were made by runoff transcription from purified PCR products with T7
RNA polymerase, as previously described (28).

**Purification of SR proteins**
MS2 hybrid proteins were coexpressed in E. coli BL21(DE3) (Novagen) together
with SRPK1, as previously described (29). His-tagged proteins were purified
under native conditions by standard Ni-column chromatography, as described by
the manufacturer (Novagen). The eluted proteins were dialyzed against 20 mM
HEPES (pH 7.9), 100 mM KCl, 20% glycerol, 0.2 mM EDTA and stored at −70°C.
The final protein concentration was determined by the Bradford method (30),
with BSA as a standard. Native SR proteins were purified from HeLa cells as
previously described (1, 31).

**In vitro splicing**
HeLa nuclear extracts (HeLa-NE) was prepared and in vitro splicing reactions
performed as described (28). Briefly, splicing reactions contained 15-20% of
HeLa-NE, 2.6% polyvinyl alcohol, 20 mM creatine phosphate, 2 mM ATP, 12%
glycerol, 12 mM HEPES (pH 7.9), 60 mM KCl and 15 fmol of 32P-labelled pre-
mRNA in a total volume 25 µl. The final MgCl$_2$ concentration varied between 2.5–3.5 mM, depending on the substrate [3.2 mM for IIIa-MS2I and IIIa-MS2I(inv) transcripts; 3.5 mM for glob-MS2I transcript; 2.5 mM for IIIa-MS2E and IIIa-MS2E(inv) transcripts]. The indicated amounts (see figure legends) of MS2 hybrid proteins were preincubated for 10 min at 30°C with the pre-mRNA in a reaction mixture containing all components except the nuclear extract. Splicing was initiated by addition of HeLa-NE and reaction mixtures incubated at 30°C for 1-3 h. Reaction products were resolved on denaturing 8% polyacrylamide denaturing gels and visualized by autoradiography. The identity of spliced products and intermediates were assigned based on size. Dried gels were subjected to PhosphorImager quantification as previously described (23). Quantitative data given in the text were based on three, or more, independent experiments.

**Mobility shift assay**

Short $^{32}$P-labeled RNA transcripts containing the MS2 operator site in a direct or inverted orientation were generated by T7 transcription (28). Binding conditions and gel analysis was essentially as previously described (24). Briefly, 15 fmol $^{32}$P-labelled RNA, 0.8 µg BSA, 0.25 µg tRNA, 3.2 mM MgCl$_2$, 1.3% polyvinyl alcohol, 1 mM DTT, 6 µl of buffer D or MS2-ASF hybrid protein in buffer D (1 pmol) was incubated for 10 min at 30°C in a 10 µl reaction mixture, heparin added (final concentration 0.5 µg/µl) and resolved on a 3.5% (60:1 acrylamide/bisacrylamide) - 0.4% agarose native gel. The gel was run in a cold room at 350V for 3 h in a 75 mM Tris-glycine buffer.

**RESULTS**
An MS2-ASF/SF2 fusion protein inhibits IIIa splicing

We previously showed that the ASF/SF2ΔRS protein efficiently represses IIIa splicing (20). The aim of this study was to determine whether a specific sequence element within the RBDs was necessary for the splicing repressor phenotype of ASF/SF2. However, mutating the ASF/SF2-RBDs would be expected to impair the RNA binding capacity of the ASF/SF2 mutant proteins. We therefore reconstructed the ASF/SF2 deletion mutants as fusion proteins with the MS2 coat protein (Fig. 1A), which binds to a well defined RNA stem loop structure, the MS2 operator (32).

However, replacing the 3RE in the IIIa pre-mRNA with an MS2 operator site positioned fifteen nucleotides upstream of the IIIa branch site (IIIa-MS2(-15); Fig. 2A) resulted in inhibition of constitutive IIIa splicing (Fig. 2B, lane 1), most likely because the MS2 site sequesters the so called anchoring sequence as a double-stranded RNA (see Discussion). In agreement with this hypothesis inverting the MS2 operator site resulted in a restoration of constitutive IIIa splicing (Fig. 2B, lane 2). To overcome this negative effect of the MS operator a modified IIIa reporter construct was generated by moving the MS2 operator site to a position located 53 nucleotides upstream of the IIIa branch site (IIIa-MS2I; Fig. 2A). At this position the MS2 operator does not interfere with basal IIIa splicing, and functions as an MS2-ASF/SF2 responsive splicing repressor element (see below). However, since we shifted the position of the repressor element in the IIIa-MS2I pre-mRNA it became important to show that the 3RE also functions as splicing repressor element when moved further away from the IIIa branch site. We therefore constructed a control transcript in which the 3RE was moved from its natural position at –6 relative to the IIIa branch site to position –53 (IIIa-3RE(-53); Fig. 2A). As shown in Fig. 2C, addition of purified HeLa SR proteins
efficiently repressed IIIa-3RE(-53) splicing (lanes 1-4). This inhibition was specific since SR proteins did not inhibit splicing of the IIIa-MS2I transcript (lanes 5-8), here used as a control. We conclude that the 3RE, also at a distance from the branch site, functions as a splicing repressor element (see also Discussion).

MS2-ASF/SF2 hybrid proteins were expressed and purified from E. coli as 6-His tagged proteins together with SR protein kinase 1 (SRPK1), as previously described (29). As shown in Fig. 3A, coexpression with SRPK1 was necessary for the MS2-ASF/SF2 protein to function as a splicing repressor protein. Thus, addition of increasing amounts of an unphosphorylated MS2-ASF/SF2 protein did not inhibit IIIa-MS2I splicing (Fig. 3A, lanes 1-5). In contrast, addition of the same molar amount of an MS2-ASF/SF2 protein purified from SRPK1 coexpressing cells, resulted in a more than 5-fold repression of IIIa splicing (Fig. 3A, lanes 6-10). Unexpectedly, SRPK1 coexpression resulted in an increase in the RNA binding capacity of the MS2 fusion proteins (data not shown). Thus, the lack of activity of the unphosphorylated MS2-ASF/SF2 fusion protein appears to result from a failure to bind efficiently to the MS2 operator. Although SRPK1 is not expected to phosphorylate all MS2-ASF/SF2 hybrid proteins used in this study (Fig. 1A) we choose to purify all hybrid proteins from SRPK1 coexpressing cells. Fig. 1B shows a Coomassie stained gel of typical batches of MS2-ASF/SF2 hybrid proteins used in the experiments presented here.

As shown in Fig. 4, the inhibitory effect of MS2-ASF/SF2 requires an MS2 operator site. Thus, using a IIIa-MS2I transcript with an inverted MS2 operator site IIIa-MS2I(inv) completely annulled the inhibitory effect of MS2-ASF/SF2 on IIIa splicing (Fig. 4, lanes 9-12). This loss of splicing inhibition results from the fact that the MS2-ASF/SF2 protein does not bind to the inverted MS2 operator site (Fig. 3B, lane 9). Fig. 3B further shows that all tested MS2-ASF/SF2 hybrid
proteins bind efficiently (lanes 1-8) and specifically (lanes 9-15) to the MS2 operator site. The appearance of multiple complexes most likely reflects the capacity of the MS2 protein to multimerize (see 33). It is interesting to note that the complexes formed were not retarded in migration, as one would have predicted from the relative size of the MS2 fusion proteins (Fig. 1B), a result suggesting conformational variations.

**MS2-ASF/SF2 does not inhibit IIIa splicing by steric interference**

We have previously shown that ASF/SF2 inhibits IIIa splicing by preventing U2 snRNP recruitment to the IIIa branch site (20, 23). Since the ASF/SF2ΔRS protein also inhibited IIIa splicing we determined whether ASF/SF2 inhibition of splicing resulted from a steric interference. As shown in Fig. 4, the wild type MS2 coat protein did not interfere with IIIa-MS2I splicing (lanes 1-4), arguing that protein interaction at position –53 in the IIIa intron is not enough to cause splicing repression. Potentially, MS2-ASF/SF2 inhibits IIIa splicing because binding of a bulky protein near the IIIa branch site physically excludes U2 snRNP recruitment to the IIIa 3’ splice site. Such a model predicts that the size of the protein, binding to the intron, is the critical parameter. To test this hypothesis we constructed an MS2-LacZ fusion protein that was engineered to have a size exceeding that of the full length MS2-ASF/SF2 protein (445 amino acids compared to 382; Fig. 1). Importantly, the MS2-LacZ fusion protein did not inhibit IIIa-MS2I splicing (Fig. 4, lanes 5-8).

Collectively, these results suggest that ASF/SF2 repression of IIIa splicing is specific, and does not simply result from a steric interference, caused by binding of a large protein near the IIIa branch site.
**ASF/SF2-RBD2 is necessary and sufficient for ASF/SF2 repression of IIIa splicing**

Since the RBDs of ASF/SF2 are sufficient for IIIa splicing inhibition (20) we next sought to identify the sequence element in the ASF/SF2-RBDs required for splicing repression. As shown in Fig. 5, the MS2-RBD2+RS protein repressed IIIa-MS2I splicing (lanes 13-16). A further dissection of ASF/SF2 demonstrated that the ASF/SF2-RS domain did not inhibit IIIa-MS2I splicing (Fig. 5, lanes 9-12), whereas the MS2 fusion protein encoding for ASF/SF2-RBD2 inhibited IIIa-MS2I splicing approximately 5-fold (Fig. 5, lanes 5-8). The inhibitory effect of RBD2 was specific since the MS2-ASF/SF2-RBD1 protein did not block IIIa-MS2I splicing (Fig. 5, lanes 1-4). Taken together these results suggest that the second RNA binding domain of ASF/SF2 encodes for a splicing repressor domain, which is both necessary (see also Fig. 6) and sufficient for repression of IIIa splicing.

**The putative α-helix 1 in ASF/SF2-RBD2 is required for splicing repression**

Systematic analysis of sequence motifs in pre-mRNA splicing factors have shown that many contain one or two RNP-type RNA binding domains (RBDs or RRMs, 34). The crystal structure of the RBDs in several proteins suggests a common $\beta_1$, $\alpha_1$, $\beta_2$, $\beta_3$, $\alpha_2$, $\beta_4$ organization with the RNP2 and RNP1 motifs in the anti-parallel $\beta_1$ and $\beta_3$ strands providing the essential residues for RNA binding (reviewed in 35).

To further characterize the sequences in ASF/SF2-RBD2 required for splicing repression we constructed four mutants of MS2-RBD2+RS (Fig. 6A). We choose MS2-RBD2+RS as the parental protein for mutant construction since this fusion protein was more robust, retaining the splicing regulatory activity better than MS2-RBD2; i.e. after multiple thawings or storage. The ASF/SF2 pre-mRNA
is alternative spliced in vivo, generating three protein isoforms, which differ at their C-terminus (36). Two of the proteins lack the RS domain and approximately 12 amino acid residues from the carboxy-terminus of RBD2: amino acids corresponding to the β4 strand of RBD2 (Fig. 6A). Removal of these residues inactivates ASF/SF2 as a constitutive splicing factor and regulator of alternative splicing (13). As shown in Fig. 6B, deletion of the β4 strand abolished the inhibitory effect of MS2-RBD2+RS on IIIa-MS2I splicing. This result argues that the carboxy-terminus of RBD2 is required for the repressor function of ASF/SF2. Interestingly, the SWQDLKD motif contained in α-helix 1 is completely conserved in the RBD2 in all SR proteins containing two RBDs (see 37). In the predicted structure of ASF/SF2-RBD2 this helix is positioned opposite to the β1 and β3 strands making contact with the RNA. As shown in Fig. 6B, introduction of conservative amino acid changes, which are predicted to preserve the α-helical structure of the motif (37), annulled the splicing repressor activity of the MS2-RBD2+RS protein. In contrast, mutating the RNP-1 motif or the highly conserved KLD motif at the carboxy-terminus of α-helix 2 (see 37) did not significantly affect the splicing repressor phenotype of MS2-RBD2+RS. Collectively these results suggest that the conserved α-helix 1 and the integrity of the β4 strand are critical for ASF/SF2 function as a splicing repressor protein.

**Tethering the ASF/SF2-RBD2 domain to the intron of the β-globin pre-mRNA results in splicing inhibition**

We previously showed that inserting the SR protein responsive 3RE element at an intronic position in the rabbit β-globin pre-mRNA converted this transcript from an SR-protein-activated to an SR-protein-repressed transcript (20). Collectively, our results suggested that the inhibitory effect of SR protein may be
a general feature of SR protein, binding to the intron of a pre-mRNA, and not restricted to the viral IIIa pre-mRNA.

To determine whether the ASF/SF2-RBD2 also was sufficient to inhibit β-globin splicing we inserted an MS2 operator site at position -53 in the rabbit β-globin first intron (transcript glob-MS2I). As shown in Fig. 7, MS2-RBD2 repressed glob-MS2I splicing approximately 5-fold, whereas the MS2-RS protein failed to block splicing. In contrast, MS2-RBD2 or MS2-RS did not have any negative effects on wild type β-globin splicing (data not shown). Collectively, these results suggest that the inhibitory effect of the ASF/SF2-RBD2 domain on splicing, when tethered to the intron, may be a general feature and not restricted to the adenoviral IIIa pre-mRNA.

The repressor function of ASF/SF2-RBD2 is position dependent

Previous studies have shown that tethering the ASF/SF2-RS domain to the downstream exon is sufficient to reproduce the exonic splicing enhancer activity of ASF/SF2 (24, 38). As we show here, tethering the ASF/SF2-RBD2 to the intron results in repression of splicing (Figs. 5 and 7).

Next we asked whether the opposite activity of the ASF/SF2-RS and RBD2 domains on splicing was position dependent. For this experiment the MS2 operator was inserted at the IIIa second exon, 131 nucleotides downstream of the IIIa 3’ splice site. In agreement with previous results (24, 38), an MS2-RS protein stimulated IIIa-MS2E splicing, although very inefficiently in our experimental system (approximately 2-fold; Fig. 8, lanes 5-8). In contrast, tethering the MS2-ASF/SF2-RBD2 protein to the second exon had essentially no effect on IIIa-MS2E splicing (Fig. 8, lanes 1-4). Interestingly, the MS2-RBD2+RS protein activated IIIa-MS2E splicing slightly better (approximately 4-fold) compared to the MS2-RS protein, suggesting that the RS domain may be the minimal domain required...
for the ASF/SF2 splicing enhancer function, but that RBD2 augments the enhancer activity of the RS domain.

Taken together, our results suggest that the activity of the RBD2 and the RS domains on splicing is opposite and position dependent.

**DISCUSSION**

Here we present evidence suggesting that the splicing enhancer and splicing repressor functions of ASF/SF2 can be separated and attributed to distinct domains of the protein. Thus the RS domain has been shown to function as a splicing enhancer domain (24, 39), whereas we show that RBD2 encodes for a splicing repressor domain (Figs. 5 and 7). Under our experimental conditions the ASF/SF2-RS domain functioned very inefficiently as a splicing enhancer domain. However, our results indicate that RBD2 may augment the splicing enhancer activity of the RS domain. Thus, MS2-RBD2+RS functioned approximately 2-fold better as a splicing enhancer protein compared to MS2-RS (Fig. 8). Furthermore, we show that the effects of the RS and RBD2 domains in splicing are position dependent and opposite. Thus, RBD2 inhibits splicing when tethered to the IIIa or the β-globin introns (Fig. 5 and 7), but has no negative effects when bound to the IIIa second exon (Fig. 8). Conversely, the ASF/SF2-RS domain activates splicing slightly when tethered to the IIIa second exon (Fig. 8), but has no effect when bound to the intron (Fig. 5).

Why does ASF/SF2 binding to an intron inhibit splicing? Our previous studies have shown that ASF/SF2 binding to the 3RE inhibits IIIa splicing by preventing U2 snRNP recruitment to the spliceosome (20, 23). Theoretically, this may be accomplished by, at least, three different mechanisms. First, binding of a large protein to a site close to the branch point in a pre-mRNA may not be compatible with the simultaneous binding of U2 snRNP; i.e. a simple steric
interference. The results presented here clearly argue against this model, since an MS2-LacZ fusion protein, with a size approximately 15% larger than MS2-ASF/SF2, did not inhibit splicing (Fig. 4). Second, protein binding to the so-called anchoring sequence (40) may preclude U2 snRNP recruitment to the branch site. The anchoring sequence is the RNA sequence located immediately upstream of the branch site which make contact with the SF3 component of U2 snRNP (40).

In the wild type IIIa transcript, the anchoring sequence is contained within the 3RE, likely contributing to the inhibitory effect of the 3RE on IIIa splicing. Such a conclusion is supported by our finding that positioning the highly structured MS2 operator site 15 nucleotides upstream of the IIIa branch site completely ablates IIIa splicing in the absence of any MS2 proteins (Fig. 2B). This finding is compatible with the previous study (40), which suggested that the single-stranded nature of the anchoring sequence is required for SF3 interaction with RNA. However, in the IIIa-MS2I transcript, the MS2 operator sequence was moved further upstream to position -53 relative to the IIIa branch site. At this location the MS2 operator does not interfere with basal IIIa splicing.

Importantly, at this position MS2-ASF/SF2 inhibited IIIa splicing (Figs. 4 and 6) whereas MS2-ASF/SF2 hybrid proteins lacking RBD2 did not block splicing (Fig. 5), strongly arguing that ASF/SF2 coverage of the anchoring sequence is not the mechanism by which ASF/SF2 inhibits splicing. This conclusion is further supported by the observation that moving the 3RE to position -53 (Fig. 2C), or the MS2 operator site to position -127 (data not shown) relative to the IIIa branch site, still results in SR or MS2-ASF/SF2 protein mediated inhibition of splicing. Collectively, our data suggests a third model, namely that ASF/SF2-RBD2 functions as a specific splicing repressor domain. Potentially, ASF/SF2-RBD2 interacts directly with a component(s) required for early splice site recognition, or alternatively functions as a nucleation site for recruitment of
factors that negatively affects splice site recognition. Currently we have no data that discriminate between these or other alternative mechanisms. However, our data clearly shows that ASF/SF2-RBD2 indeed functions as a splicing repressor domain when tethered to an intronic position in the 111a or the β-globin pre-mRNAs. Obviously, characterization of the mechanism by which ASF/SF2-RBD2 inhibits pre-mRNA splicing is a major goal in our future research. Our initial characterization of ASF/SF2-RBD2 mutant proteins provides some interesting clues that will help in this characterization. Thus, we show that mutating the SWQDLKD motif in the putative α-helix 1 or deletion of the β4 strand of RBD2 destroys the repressor activity of ASF/SF2-RBD2, while mutations that affects the RNP1 motif or α-helix 2 has no, or minor, effect on the splicing repressor activity of RBD2. Since neither α-helix 1 nor the β4 strand is expected to be directly involved in RNA binding they may provide an essential surface for ASF/SF2-RBD2 interaction with splicing components mediating splicing repression.

In retrospect the finding that the ASF/SF2-RBD2 is sufficient for repression of 111a splicing may provide a logical explanation to our previous observation that all tested SR proteins, except SRp20, inhibited 111a splicing efficiently (20). Thus, SRp20, which is the shortest SR protein, encodes for only one RBD. All other tested SR proteins (SRp75, SRp55, SRp40 and SRp30 encode for two RBDs. The SRp30 fraction, used in this study, contained a mixture of SR proteins with one RBD (SC35 and 9G8) or two RBDs (ASF/SF2 and SRp30c) (1, 41, 42). Potentially the RBD2 in other SR proteins may similarly be splicing repressor domains. From this point it is interesting to note that the SWQDLKD motif here shown to be required for ASF/SF2-RBD2 repression of splicing is conserved in the RBD2 of all SR proteins containing two RBDs (see 37). The significance of the SWQDLKD motif rather than the predicted structure of
ASF/SF2-RBD2 is indicated by the observation that MS2-RBD1 generates distinct complexes in a gel retardation assay (Fig. 3B, lane 3), whereas MS2-RBD2, which is of the same size as MS2-RBD1 (Fig. 1B), generates smaller complexes with a fuzzy appearance (Fig. 3B, lane 4). These results may be interpreted to indicate that ASF/SF2-RBD2 does not adopt the predicted structure of an RNP-type RBD.

The finding that ASF/SF2 encodes for separate domains, functioning as splicing enhancer and splicing repressor domains, adds another level by which ASF/SF2 may partake in constitutive and alternative RNA splicing. Potentially, this modular structure of ASF/SF2 may contribute to the splice site switching activity of this protein. Previous studies have shown that ASF/SF2 promotes proximal 5’ and 3’ splice site selection in model transcripts (12, 13, 43). Also, ASF/SF2-RBD1 + RBD2 was confirmed to be sufficient to promote proximal 5’ splice site selection both in vitro and in vivo (12, 13). In a recent study (44) it was demonstrated that RBD2 of ASF/SF2 plays a dominant role in determining the alternative splicing specificity of chimeric SR proteins. This result may be related to our finding that ASF/SF-RBD2 has a distinct function as a repressor domain in splicing regulation. In fact, our recent studies have shown that, under our experimental conditions, ASF/SF2-RBD2 is sufficient to induce proximal 5’ splice site selection in the adenovirus E1A pre-mRNA (manuscript in preparation). However, the effect of ASF/SF2-RBD2 on alternative 3’ splice site selection appears more complex. Preliminary data suggests that RBD2 or RBD2 + RS does not reproduce the effect of the wild-type ASF/SF2 protein on proximal 3’ splice site selection (unpublished observations).

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REFERENCES

FIGURE LEGENDS

**Fig. 1.** Structure of MS2-ASF/SF2 hybrid proteins used to map the ASF/SF2 splicing repressor domain. A, The schematic structure of the wild type ASF/SF2 protein is shown at the top. Hybrid proteins encode for the following ASF/SF2 sequences: MS2-ASF/SF2, amino acids (a.a.) 1-248; MS2-RBD1, a.a. 1-92; MS2-RBD2, a.a. 92-197; MS2-RBD2+RS, a.a. 92-248 and MS2-RS, a.a. 196-248. MS2-LacZ encode for the carboxy-terminus of the LacZ protein; a.a. 711-1023. B, Coomassie stained gel of typical batches of MS2-ASF/SF2 hybrid proteins used in this study.

**Fig. 2.** The MS2 operator inhibits constitutive IIIa splicing when positioned close to the IIIa branch site. A, Schematic picture of IIIa hybrid pre-mRNAs. Note that in all transcripts the 3RE was deleted from its natural position and replaced by a β-globin sequence (thin line) that does not bind SR proteins (20). The MS2 operator is depicted as a steam-loop structure, while the inverted MS2 operator is shown as a wavy line. The SR responsive 3RE element is shown as a black box. B, Positioning the MS2 operator 15 nucleotides upstream of the IIIa branch site inhibits IIIa splicing. The indicated pre-mRNAs were incubated in HeLa-NE in the absence of any MS2 proteins. C, The indicated pre-mRNAs were incubated in HeLa-NE with increasing amounts of SR proteins purified from HeLa cells (0.2, 0.4, and 0.6 µg). Products were resolved by gel electrophoresis and visualized by autoradiography. Positions of the pre-mRNA, splicing intermediates and splicing products are indicated in the center.
**Fig. 3.** A, Coexpression of MS2-ASF/SF2 with SRPK1 enhances the splicing repressor activity of the MS2-ASF/SF2 protein. Splicing reactions were incubated with the IIIa-MS2I pre-mRNA and increasing amounts (1-6 pmol) of unphosphorylated (lanes 1-5) or SRPK1 phosphorylated MS2-ASF/SF2 (lanes 6-10). Products were resolved by gel electrophoresis and visualized by autoradiography. Positions of the pre-mRNA, splicing intermediates and splicing products are indicated on the left. B, MS2-ASF/SF2 hybrid protein binds specifically to the MS2 operator sequence. Two short RNAs containing the MS2 operator sequence in a direct (MS2 OP) or inverted (MS2 OP(inv)) orientation were incubated with 1 pmol of the phosphorylated form of the indicated MS2-ASF/SF2 hybrid proteins. Products were resolved on a native polyacrylamide gel.

**Fig. 4.** MS2-ASF/SF2 does not inhibit splicing by steric interference. Splicing reactions were carried out using the IIIa-MS2I or IIIa-MS2I(inv) pre-mRNAs (depicted at the top) and minus/plus 2 pmol of wild-type MS2 (lanes 2 and 4), MS2-LacZ (lanes 6 and 8) or MS2-ASF/SF2 proteins (lanes 10 and 12). Products were resolved by gel electrophoresis and visualized by autoradiography. Positions of pre-mRNA, splicing intermediates and splicing products are indicated on the left.

**Fig. 5.** ASF/SF2-RBD2 functions as a splicing repressor domain. Splicing reactions were carried out in HeLa-NE using the IIIa-MS2I or IIIa-MS2I(inv) pre-mRNAs (depicted at the top) and minus/plus 2 pmol of the indicated MS2-ASF/SF2 fusion proteins. Products were resolved by gel electrophoresis and visualized by autoradiography. Positions of pre-mRNA, splicing intermediates and splicing products are indicated on the left.
**Fig. 6.** The conserved SWQDLKD motif contained in the putative α-helix 1 of ASF/SF2-RBD2 is essential for splicing repression. A, Schematic picture showing the structure of MS2-RBD2+RS and MS2-RBD2+RS mutant proteins. The amino acid sequence of RBD2 is shown expanded and the residues changed in each mutant protein are indicated. The completely conserved SWQDLKD motif is shown boxed in the sequence. B, Splicing reactions were carried out in HeLa-NE using IIIa-MS2I pre-mRNA (depicted at the top) minus/plus 4 pmol of MS2-RBD2+RS (wt) or mutant proteins. Products were resolved by gel electrophoresis and visualized by autoradiography. Positions of pre-mRNA, splicing intermediates and splicing products are indicated on the left.

**Fig. 7.** ASF/SF2-RBD2 is sufficient to inhibit β-globin splicing. Splicing reactions were carried out in HeLa-NE using the glob-MS2I pre-mRNA (depicted at the top) and increasing amounts (1 and 4 pmol) of the MS2-RBD2 or MS2-RS proteins. Products were resolved by gel electrophoresis and visualized by autoradiography. Positions of pre-mRNA, splicing intermediates and splicing products are indicated on the left.

**Fig. 8.** The ASF/SF2-RBD2 domain stimulates the splicing enhancer function of the ASF/SF2-RS domain. Splicing reactions were carried out in HeLa-NE using the IIIa-MS2E or IIIa-MS2E(inv) pre-mRNAs (depicted at the top) and minus/plus 2 pmol of the indicated MS2-ASF/SF2 fusion proteins. Products were resolved by gel electrophoresis and visualized by autoradiography. Positions of pre-mRNA and splicing products are indicated on the left. Note that the pre-mRNA, and to a lesser extent the spliced product, migrates as multiple species probably because of the MS2 operator appended at the second exon. Further, note that the pre-mRNAs and the spliced products form slowly migrating dimers in
some experiments (lanes 1-8), probably through base-pairing between the MS2 operator sites (correct or inverted) during the electrophoretic separation of splicing products. Dimer formation is an artifact that in an un reproducible way occurred in some experiments.
Fig. 1
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Fig. 4
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Fig. 5
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Fig. 8
Human splicing factor ASF/SF2 encodes for a repressor domain required for its inhibitory activity on pre-mRNA splicing
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