The splicing factor SRp20 modifies splicing of its own mRNA and ASF/SF2 antagonizes this regulation

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SRp20 is a member of the highly conserved SR family of splicing regulators. Using a variety of reporter gene constructs, we show that SRp20 regulates alternative splicing of its own mRNA. Overexpression of SRp20 results in a reduction in the level of exon 4-skipped SRp20 transcripts and activates the production of transcripts containing exon 4. These exon 4-included transcripts encode a truncated protein lacking the C-terminal RS domain. We provide evidence that SRp20 probably enhances the recognition of the otherwise unused, weak splice acceptor of exon 4. The recognition of exons with weak splice acceptor sites may be a general activity of SRp20. Unexpectedly, ASF/SF2, another member of the SR family, antagonizes the effect of SRp20 on SRp20 pre-mRNA splicing and suppresses the production of the exon 4-included form. Our results indicate that ASF/SF2 suppresses the use of the alternative exon 4, most likely by inhibiting the recognition of the splice donor of exon 4. These results demonstrate, for the first time, an auto-regulatory activity of an SR protein which is antagonized by a second SR protein.

Keywords: alternative splicing/ASF/gene expression/SF2/SR family

Introduction

Intron sequences that interrupt the coding region are characteristic of most eukaryotic genes. Consequently, the specific recognition of 5’ and 3’ splice sites by the splicing apparatus is an essential prerequisite for the accurate excision of introns from pre-mRNA (Berget, 1995; Black, 1995; Reed, 1996). This excision takes place in a multi-component protein–RNA complex termed the spliceosome, which consists of several small nuclear ribonucleoprotein particles (snRNPs) and a large number of additional factors, referred to collectively as non-snRNPs proteins (Moore et al., 1993; Madhani and Guthrie, 1994; Sharp, 1994). One class of non-snRNP factors important in constitutive and regulated splicing are the SR proteins (Zahler et al., 1992; Fu, 1995; Manley and Tacke, 1996). The SR proteins are highly conserved throughout the animal kingdom and were described originally based on a shared phospho-epitope recognized by the monoclonal antibody mAb104 (Roth et al., 1990, 1991; Zahler et al., 1992). All SR proteins contain at least one RNP-type RNA-binding domain (RBD), also called RNA recognition motif (RRM) (Kenan et al., 1991), at the N-terminus and a region of varying length rich in alternating serine and arginine residues at the C-terminus (RS domain). RRM are also found in other proteins which have been shown to bind RNA (Burd and Dreyfuss, 1994).

SR proteins are thought to be essential splicing factors, since SR proteins individually can complement splicing-deficient cytoplasmic S100 extracts, which lack SR proteins but contain all other factors necessary for splicing (Krainer et al., 1990a, 1991; Ge et al., 1991; Fu and Maniatis, 1992; Kim et al., 1992; Zahler et al., 1992; Cavaloc et al., 1994; Screaton et al., 1995). Despite the fact that these initial observations suggest partial redundancy of SR protein functions, in vitro and in vivo experiments clearly show that individual SR proteins have distinct specificity and efficiency in splicing different pre-mRNAs (Kim et al., 1992; Zahler et al., 1993; Screaton et al., 1995; Wang and Manley, 1995).

Further, SR proteins also function in the regulation of splice site selection. For example, SR proteins were shown to influence the selection of alternative splice sites in a concentration-dependent manner (Ge and Manley, 1990; Krainer et al., 1990b; Fu et al., 1992; Zahler et al., 1993).

In addition to the splice donor, the splice acceptor and the branch sites, another type of cis-acting element in pre-mRNA splicing has been described: the splicing enhancer. Splicing enhancers have been found in a number of mammalian pre-mRNAs and were shown to interact specifically with SR proteins, suggesting that SR proteins play an important role in the function of splicing enhancers (Lavigne et al., 1993; Sun et al., 1993a,b; Staknis and Reed, 1994; Dirksen et al., 1995; Ramchatesingh et al., 1995; Tacke and Manley, 1995).

Accumulating evidence supports the view that SR proteins function at multiple steps during splicing and that protein–protein interactions among SR proteins and between SR proteins and other essential splicing components (e.g. snRNPs) mediate this function. For example, the SR proteins ASF/SF2 and SC35 were shown to interact simultaneously and specifically with U1snRNP70k, associated with the 5’ splice donor, and the 35 kDa subunit of the splicing factor U2AF, associated with the 3’ splice acceptor (Wu and Maniatis, 1993; Kohtz et al., 1994) (Throughout this manuscript, the terms ASF/SF2 and ASF-1 are used interchangeably). In addition, high concentrations of SR proteins can even replace the necessity for U1snRNP (Crispino et al., 1994; Tarn and Steitz, 1994), indicating that during splicing, SR proteins interact very early with the pre-mRNA. Together, these and additional studies (reviewed by Fu, 1995; Manley and Tacke, 1996) support the idea that during splicing, SR proteins function by bridging components bound to the 5’ and 3’ splice sites.

Relatively little information is available regarding the regulation of SR protein gene expression. At the transcrip-
tional level, significant differences in expression of different SR proteins have been observed in various cell types or tissues. SRp20, for example, is highly expressed in thymus, testis and spleen, but its mRNA is low or undetectable in liver, lung and kidney (Ayane et al., 1991). SC35 expression is also highly variable in cell lines (Fu and Maniatis, 1992; Vellard et al., 1992). Interestingly, expression of SRp40 and SRp30c seems to be regulated by mitogens (Diamond et al., 1993; Screaton et al., 1995), raising the possibility that splicing may be coupled to the signal transduction pathways. Another interesting control mechanism is the regulation of splicing factor expression by alternative splicing. Sex determination in Drosophila is the best studied example for the crucial role of alternative splicing in the production of functionally different transcripts in the two sexes (Baker, 1989).

In mammalian cells, cDNAs corresponding to alternatively spliced forms of SR protein mRNAs, including ASF/SF2, SRp40 and SRp55, have been isolated (Ge et al., 1991; Screaton et al., 1995). In most cases, the alternative isoforms would encode truncated, RS domain-lacking proteins of still unknown function. We have reported previously the existence of alternatively spliced SRp20 mRNA and showed that this alternative form (exon 4-included) is elevated in starved cells and disappears as soon as cells are growth stimulated (Jumaa et al., 1997). Here, we show that SRp20 protein auto-regulates the alternative splicing of its own pre-mRNA by promoting the inclusion of exon 4. Further, we show that ASF/SF2 represses the production of the exon 4-included SRp20 form, thus acting antagonistically to SRp20.

Results

Overexpression of SRp20 genomic DNA mainly produces the exon 4-included form of SRp20 mRNA

To learn more about SRp20 function in vivo, expression vectors using the mouse SRp20 gene (Jumaa et al., 1997) driven by three different promoters [cytomegalovirus (CMV), SRα and μ] were constructed (Figure 1A). The murine B-cell lymphoma K46 was transfected with these vectors and total RNA was isolated 1 day later. Following Northern blotting, an additional band migrating more slowly than endogenous SRp20 mRNA was observed (Figure 1B). Since we have shown previously that SRp20 can be alternatively spliced to give transcripts either containing or lacking exon 4 (Jumaa et al., 1997), we suspected that the more slowly migrating band on Northern blots from cells transfected with genomic SRp20 DNA was the exon 4-included form. This was confirmed by re-probing the Northern blot with an exon 4-specific probe (Figure 1B). The slowly migrating band which hybridized with the cDNA probe also hybridized with an exon 4 probe. In contrast, the control transfection (Figure 1B, lane 4) showed no hybridization with the exon 4 probe, confirming previous data that the exon 4-included form is very rare in proliferating cells (Jumaa et al., 1997). The two SRp20 splice forms can also be detected by an RT–PCR reaction using RNA from transfected cells and primers corresponding to sequences from the translational start and stop codons of SRp20 (Figure 1C, lanes 1–3). As expected, only one band, corresponding to the exon 4-skipped form of SRp20, was obtained from the control transfection (Figure 1C, lane 4).

Translation of the exon 4-included form would result in a truncated protein lacking the RS domain. Since it is conceivable that this truncated protein has no, different or even antagonistic function compared with SRp20, we speculated that alternative splicing of SRp20 could be a cellular mechanism to avoid excessive amounts of SRp20. To test this, K46 cells were transfected with the SRp20 expression construct driven by the CMV promoter and RNA was isolated from the cells at various times after transfection. Figure 1D shows that 1 h after transfection, only the exon 4-skipped form is produced and in low amounts. With time, the amount of the exon 4-included form increases and the skipped form decreases. This result is consistent with the idea that SRp20 protein derived from
the skipped form mRNA accumulates after transfection and causes a switch to the exon 4-included form.

**SRp20 promotes the inclusion of exon 4 in its own mRNA and ASF antagonizes this function**

The results in Figure 1 demonstrate that increasing amounts of SRp20 are correlated temporally with increased levels of alternatively spliced SRp20 pre-mRNA. Since SRp20 is itself a member of the SR family of splicing factors, which are known to influence alternative splicing, it was conceivable that SRp20 was involved in regulating the splicing of its own pre-mRNA in a feedback loop. To show that this regulation in fact needs a functional SRp20 protein, a frameshift mutation was introduced at amino acid 55 in SRp20 by cleaving with the restriction enzyme *Bam*HI in exon 2, filling in the protruding ends and religating the construct shown in Figure 1A. The frameshift construct produces the same pre-mRNA as the constructs shown in Figure 1, except that the additional 4 bp resulting from the fill-in destroy the open reading frame (ORF) of SRp20 and introduce a stop codon six amino acids after the fill-in. Figure 2A demonstrates that, in contrast to the unmodified genomic SRp20 construct (lane 1), the majority of the RNA from the frameshift construct is spliced to the exon 4-skipped form of SRp20 mRNA (lane 3). Co-transfection of an SRp20 cDNA expression vector with the frameshift construct causes the mRNA from the frameshift construct to shift to the included form (lane 4). This confirms that functional SRp20 is required to induce the alternative splicing of its own pre-mRNA. Since SR proteins have similar structure and behave, more or less, similarly in most *in vitro* splicing reactions, we wanted to test the function of another SR protein on SRp20 exon 4 splicing. We transfected an ASF/SF2 expression vector (a gift from J. Wang) together with the unmodified and frameshift SRp20 constructs. Surprisingly, ASF/SF2 causes the exon 4-included form to disappear, without increasing the amount of the skipped form (Figure 2A, lanes 2 and 5). These results indicate that ASF/SF2 and SRp20 have antagonistic effects on SRp20 exon 4 splicing, with SRp20 acting as an activator and ASF/SF2 as a repressor. The production of the included form is inhibited by ASF/SF2, even if the wild-type genomic SRp20 expression construct is transfected (Figure 2A, lane 2), suggesting that ASF/SF2 is dominant over SRp20 in this system.

To identify the regions of SRp20 pre-mRNA required for this regulation, two minigene constructs were generated. The XB minigene contains the entire 3′ region of the SRp20 gene starting at the *Xho*I site in exon 3. The second construct, the XH minigene, is similar to the XB minigene except for a deletion which removes all SRp20-related sequences beginning from the middle of exon 5 and extending downstream (Figure 2B). Transfection of these minigenes produced transcripts spliced mainly in the exon 4-skipped form (Figure 2B, lanes 1 and 4). Co-transfection with an SRp20 cDNA expression vector stimulated the inclusion of exon 4 and strongly reduced the amounts of the skipped form from both minigenes (Figure 2B, lanes 2 and 5). Following co-transfection with an ASF/SF2 expression vector, no included form was detected and the amount of the skipped form did not increase (lanes 3 and 6). These experiments show that the sequences between exons 3 and 5 of the SRp20 gene contain the signals necessary for the activation and repression of exon 4 splicing by SRp20 and ASF/SF2 respectively.

**Both exon 4-included and skipped SRp20 mRNA forms are translated into protein**

The consequences of SRp20 splicing regulation on the production of SRp20 protein were tested by constructing an expression vector (Figure 3A) using genomic SRp20 DNA with an N-terminal haemagglutinin epitope tag derived from influenza virus (Field et al., 1988). In addition, control expression vectors were also tested which carried tagged versions of the exon 4-included and skipped SRp20 cDNA. No difference in function between tagged and untagged proteins was observed using any of the substrates described above (not shown), indicating that the tag does not interfere with SRp20 function.

Following transfection of a tagged genomic SRp20
Fig. 3. Both exon 4-included and skipped SRp20 mRNA forms are translated into protein. (A) The structure of the influenza haemagglutinin-tagged SRp20 genomic construct is shown; the tag is shown immediately downstream of the CMV promoter, followed by the SRp20 gene starting at the ATG in exon 2. (B) Western blot analysis with the anti-tag antibody 12CA5 of cells transfected with tagged exon 4-included SRp20 (ti-SRp20, lane 1); the tag-containing parental expression vector (PV, lane 2); tagged genomic SRp20 (tg-SRp20, lanes 3–5) co-transfected either with parental vector (lane 3), untagged exon 4-skipped SRp20 (s-SRp20, lane 4) or tagged ASF-1 (lane 5) expression vector; tagged exon 4-skipped SRp20 (ts-SRp20, lane 6). The positions of ASF1, SRp20 and truncated SRp20 (from exon 4-included transcripts, SRp20trunc) are indicated to the right. (C) Western blot analysis of cell extracts prepared 24 h after transfection of K46 cells either with tagged skipped-SRp20 cDNA (lanes 1 and 5), tagged ASF-1 cDNA (lanes 2 and 6) or the tag-parental vector (PV in lanes 3 and 7). In lane 4, protein extract from untransfected cells was loaded. Lanes 1–4 were reacted with the monoclonal antibody mAb104 recognizing phosphorylated SR proteins. Bands corresponding to known SR proteins are labelled to the left of the blot and the transfected SRp20 in lane 1 is marked by an arrow. The asterisk indicates that other SR proteins co-migrate with ASF1 (e.g. SC35, 9G8 and SRp30c). In lanes 5–7, the monoclonal antibody 12CA5 was used. Equal amounts of protein were applied to all lanes, based on the signal obtained with a control monoclonal antibody recognizing the eukaryotic initiation factor 4A (not shown).

Construct into K46 cells and subsequent Western blotting, two protein bands reacting with the anti-tag antibody are observed (Figure 3B, lane 3) which co-migrate with the respective tagged proteins produced by the included (lane 1) and skipped (lane 6) SRp20 cDNAs. As expected, co-expression of SRp20 cDNA down-regulates the amount of SRp20 protein derived from the exon 4-skipped form (Figure 3B, lane 4), whereas co-expression of ASF/SF2 cDNA down-regulates the amount of protein from the included form (Figure 3B, lane 5).

Phosphorylation of SR proteins is thought to be important for their activity (Fu, 1995). The transfected SRp20 is also phosphorylated because it reacts with mAb104 (Figure 3C, lane 1, arrow), which was shown to react with SR proteins when they are phosphorylated (Roth et al., 1990). As expected, Figure 3C shows that when compared side-by-side, the transfected, tagged SRp20 (detected with the anti-tag antibody 12CA5 in lane 5) migrates slightly more slowly than the endogenous SRp20 detected with antibody 104 (lanes 1–4). The amount of tagged SRp20 detected with mAb104 is considerably lower than the endogenous SRp20 signal, suggesting that transient transfection does not significantly change the overall pool size of phosphorylated SRp20. Similar observations have been made by other investigators (Zhang and Wu, 1996). The implications of this observation are not clear since neither the relationship between mAb104 reactivity and SR protein activity nor the ratio of phosphorlated to total endogenous SRp20 have been established.

The sequences required for alternative splicing of SRp20 mRNA are located within or immediately flanking exon 4

To localize the sequences responsible for regulating SRp20 exon 4 splicing more precisely, a 614 bp genomic fragment containing exon 4 with short flanking intron sequences (Figure 4) was cloned into an exon trap expression construct. This exon trap vector includes a CMV promoter and a downstream expression cassette consisting of two constitutive exons derived from the rat insulin gene, separated by an intron which contains a multiple cloning site (Figure 5B, construct a). The first exon contains an ATG and the second a polyadenylation signal. The multicloning site in the intron was used to insert modified fragments derived from the SRp20 alternative exon (Figure 5B).

The different constructs in Figure 5 were transiently transfected into K46 cells, either with a control vector (– lanes) or with an expression vector for SRp20 (p20 lanes) or ASF/SF2 (ASF1 lanes). One day after transfection, the use of exon 4 was analysed by RT–PCR...
using primers complementary to the common 5’ and 3’ exons. Figure 5A (panel a) shows that co-transfection with SRp20 or ASF/SF2 expression constructs has no effect on the constitutive splicing of the 5’ to 3’ parental vector exons. In the construct carrying an intact exon 4 with flanking intron sequences (b), exon 4 is not recognized at all in the transfected cells and co-transfection with an SRp20 expression construct strongly promotes exon 4 inclusion (panel b). Thus, the sequences necessary for SRp20-mediated promotion of exon 4 inclusion are present on this 614 bp fragment.

In an attempt to localize further the sequences necessary for exon 4 splice regulation, a series of deletions in this fragment were constructed (Figure 5B, constructs c–f). The skipping of exon 4 seen in construct b in the absence of co-transfected SR protein was not affected appreciably by deletions in the upstream intron or in the first half of exon 4 (panels b–e, − lanes). However, deletion of 303 bases in the second half of exon 4 (construct f) leads to partial inclusion of exon 4 (panel f, − lane), which can be inhibited by ASF/SF2 co-expression (panel f, ASF1 lane). The results also show that none of the deletions blocked the ability of co-expressed SRp20 to stimulate exon 4 inclusion. Although minor changes in the efficiency of stimulation are seen, more precise conclusions will require a more quantitative approach such as RNase protection.

When the constitutive exon 3 from the SRp20 gene is inserted into the exon trap vector (Figure 5B, construct g), it is exclusively recognized (panel g). Co-transfection with either SRp20 or ASF/SF2 vectors had no influence on its splicing, demonstrating that their effects on exon 4

![Image](image-url)

**Fig. 4.** The nucleotide sequence of the murine SRp20 genomic fragment containing exon 4 and a portion of the flanking introns. Exon sequences are in upper and intron sequences in lower case letters.

![Image](image-url)

**Fig. 5.** Localization of sequences involved in exon 4 splicing regulation. (A) RT–PCR analysis of cells co-transfected with the constructs shown in (B) and either parental vector as a control (lanes marked with ‘-‘), exon 4-skipped SRp20 (lanes marked with ‘p20’) or ASF-1 (lanes marked with ‘ASF1’) expression vectors. On the right, the structure of the mRNA detected by RT–PCR using the primers indicated by bars under the exons in construct ‘a’ is shown. (B) Exon trap constructs: construct a represents the exon trap cassette with 5’ and 3’ flanking exons. Construct b shows the exon trap cassette with the alternative exon 4 of SRp20 cloned into the intron polylinker. Open boxes are exon trap-derived exons and exon 4 is shown as a hatched box. The lines are exon trap-derived introns. Thin filled boxes are intron sequences derived from sequences flanking exon 4 (see also Figure 4). Constructs c–f carry deletions shown to the right of the respective constructs (numbering is according to Figure 4). In construct g, the constitutive exon 3 (filled box) with adjacent intron sequences (hatched thin boxes) from the SRp20 gene were cloned into the exon trap vector. Constructs h and i represent mixed exons between exon 3 and exon 4 of SRp20. In construct h, exon 4 provides the 5’ splice donor site and SRp20 exon 3 the 3’ splice acceptor site. Construct i is the opposite. Restriction sites indicated in construct b were used to generate the deletion constructs. H, HincII; M, MscI; S, SpeI. The XhoI site (X) in construct g and the PvuII site (not shown) in exon 4 were used to generate the mixed exons in constructs h and i.
Fig. 6. Mutations in the SRp20 exon 4 splice sites modify the regulation of exon 4 splicing. (A) RT–PCR analysis, as described in Figure 5, was performed on various exon 4 mutants. (B) Construct b shows the sequence of the unmodified splice construct. Exon sequences are in upper case and intron sequences are in lower case letters. The solid lines (lengths given below the line in construct b) indicate unchanged intron sequences flanking exon 4. The dashed lines indicate unchanged exon 4 sequences. In construct j, multiple point mutations (underlined) were introduced into the 3' splice acceptor site. In construct k, only the splice donor was modified. In construct l, both splice sites were modified and in construct m, intron sequences (nucleotides 1–95 and 565–614 in Figure 4) were also deleted, in addition to the modifications in both splice sites.

are not general. Replacement of the exon 4 splice acceptor with sequences from a constitutive exon (construct h) results in constitutive use of exon 4. Interestingly, as was seen for the genomic expression constructs and the minigene constructs, co-transfection of ASF/SF2 reduces the amount of exon 4-included product without increasing the amount of exon 4-skipped transcripts (panel h).

In a construct containing a hybrid exon 4 with the 5' splice donor derived from a constitutive exon (construct i), the majority of the transcripts include exon 4 and, following co-transfection with SRp20, only exon 4-included transcripts are seen (panel i). Interestingly, ASF/SF2 has little effect on the splicing of this construct.

The regulation of exon 4 splicing requires suboptimal splice sites

Comparison of exon 4 splice donor and acceptor sites with the consensus sequences reveals that they are both suboptimal (Figure 4). The splice acceptor has a very weak polypyrimidine tract and the last two nucleotides of exon 4 at the splice donor site do not fit the AG consensus. When the splice acceptor was modified to give a strong polypyrimidine tract by replacing all purines with pyrimidines (Figure 6B, construct j), primarily exon 4-included transcripts were produced (Figure 6A, panel j), even in the absence of co-transfected SRp20. However, co-expression of ASF/SF2 resulted in a major band migrating more slowly than the exon 4-included product. Subsequent cloning and sequencing of this band revealed that it is the product of a partially spliced transcript where the 5' vector exons is joined to exon 4 and the downstream intron is retained (we will refer to this as the partially spliced transcript). Another band just below the partially spliced transcript was often observed, and subsequent experiments (not shown) revealed that it is a heteroduplex formed during the PCR between partially spliced and exon 4-included transcripts.

Modifying the splice donor to fit the consensus AGgt resulted in major band migrating more slowly than the exon 4-included product. Subsequent cloning and sequencing of this band revealed that it is the product of a partially spliced transcript where the 5' vector exons is joined to exon 4 and the downstream intron is retained (we will refer to this as the partially spliced transcript). Another band just below the partially spliced transcript was often observed, and subsequent experiments (not shown) revealed that it is a heteroduplex formed during the PCR between partially spliced and exon 4-included transcripts.
Discussion

Numerous studies have contributed to the view that SR proteins play a general role in splicing and can modulate splice site selection in a concentration-dependent manner (reviewed by Fu, 1995; Manley and Tacke, 1996). One imaginable consequence of this is that cells may regulate the expression or activity of individual SR proteins, or their antagonists, to control the expression of one or more target genes in a tissue-specific and/or developmentally regulated fashion. For example, Screaton et al. (1995) reported that following T-cell activation, alternative splicing of CD44 and CD45 is accompanied by changes in the levels of several SR proteins.

The experiments described in this study demonstrate that genomic SRp20 constructs can express two different forms of SRp20 resulting from the alternative splicing of exon 4. Further, overexpression of SRp20 itself results in the accumulation of SRp20 mRNA containing exon 4, and overexpression of ASF/SF2 blocks this form. The consequence of exon 4 inclusion is an interruption of the ORF of SRp20 and the production of a protein lacking an RS domain.

The fact that exon 4 and very little adjacent intron sequences are sufficient to reproduce this regulation, even when placed in the context of unrelated flanking exons, indicates that the cis-elements required for SRp20 and ASF/SF2 regulation are located within these sequences. Deletion of almost all exon 4 sequences does not eliminate the effects of SRp20 and ASF/SF2 on exon 4 splicing. This suggests either that there are multiple, redundant targets, or that the splice sites themselves are the targets.

A prerequisite for the influence of SRp20 and ASF/SF2 on exon 4 splicing appears to be the suboptimal exon 4 splice acceptor and donor sites. Nucleotide changes which generate a better polypyrimidine tract in the splice acceptor site render exon 4 constitutive and eliminate the need for SRp20. This suggests that SRp20 may be involved in the recognition of exons with weak splice acceptors. Consistent with this idea, the Drosophila homologue of SRp20, RBP1, can activate the weak polypyrimidine tract or repress, and the question of whether SRp20 and ASF/SF2 auto-regulate the splicing of their pre-mRNAs. Alternatively spliced isoforms of ASF/SF2 (Ge et al., 1991), SC35 (Sureau and Perbal, 1994), SRp40 and SRp55 (Screaton et al., 1995) have been isolated and, in most cases, these isoforms would encode truncated proteins similar to alternative SRp20. As yet, SRp20 is the only SR protein that has been shown to auto-regulate alternative splicing of its pre-mRNA and to be regulated by another member of the SR family. Although we did not test this, it is also possible that the splicing of SRp20 mRNA is regulated by other SR proteins as well.

Also, the experiments presented here do not address the mechanism by which exon 4 recognition is enhanced or repressed, and the question of whether SRp20 and ASF/SF2 bind directly to specific sequences within exon 4 remains to be answered.

Materials and methods

Plasmid construction

Expression plasmids were based on the CMV promoter in the mammalian expression vector pcRneo (a kind gift from H.Eibel), the SRα (Takebe et al., 1988) promoter or the μ enhancer/promoter. An 11 kb SRp20 genomic fragment starting at the Bsrfl site in exon 1 and containing the entire gene was cloned downstream of each of these three promoters. Expression constructs with these promoters and the SRp20 exon 4-skipped and included cDNAs were also made. The ASF/SF2 expression plasmid (pCGNF1) was a kind gift from J.Wang. Using the NsiI site at the SRp20 ATG, the genomic DNA, the exon 4-included cDNA or exon 4-skipped cDNA of SRp20 were subcloned into the PstI site of the ASF/SF2 expression vector pCGN (Tanaka and Herr, 1990) to generate tagged SRp20 proteins. For splicing constructs carrying the exon 4 region, a PCR fragment amplified from mouse genomic DNA with the primers αE5 (5'-CAGTGTGAGTCCTCTATG-3') and αE7 (5'- AAGCCGTTACTCTTCCATG-3') was cloned into Bluescript (Stratagene) for sequencing and modification by deletion and site-directed mutagenesis. Subsequently, the exon 4 fragments were subcloned into the SalI– BamHI sites of the intron polylinker of the mammalian expression vector exon trap (MoBiTec). The XB and XH mingenes were constructed...
by subcloning the Xho–BamHI or Xho–HindIII fragments from the exon 4 region of SRp20, respectively, into pcRneo.

**Cell culture and transfection**

Cell lines were cultured in Iscove's MDM (Gibco-BRL) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM β-mercaptoethanol at 37°C and 7.5% CO2. For transfection, the cells were harvested and plated onto 5084 streptomycin and 50 μg/ml oleic acid at 37°C for 60 min. Then, the reaction was used to amplify specifically the SRp20 and SRp20-related transcripts using 0.2 U of Super Taq DNA polymerase (HT Biotechnology), 10 μmol of each dNTP and 25 pmol of primers in a 50 μl reaction. In the case of the full-length SRp20 genomic DNA constructs, the PCR was performed either with the primer pairs T7/SP6 or X16F/X16R in a 12 μl reaction with 100 ng of supercoiled plasmid DNA. Electroporation was performed at 225 V and 950 μF in 250 μl of IMDM without serum. Control transfections with reporter constructs showed that 50–70% of the cells transiently express the electroporated DNA.

**Analysis of alternative splicing**

Constructs (10 μg) expressing transcripts containing exons 4 or co-transfected into K46 cells with either 10 μg of empty expression vector as a control or with 10 μg of either SRp20 or ASR/SF2 expression vectors. At 20–24 h after transfection, the cells were harvested and 5 μg of total RNA were used for cDNA synthesis with 20 μl of RT (RT Biotechnology) and 5 μg of oligo(dT) at 37°C for 60 min. Then, 1/20 of the reaction was used to amplify specifically the SRp20 and SRp20-related transcripts using 0.2 U of Super Taq DNA polymerase (HT Biotechnology), 10 μmol of each dNTP and 25 pmol of primers in a 50 μl reaction. In the case of the full-length SRp20 genomic DNA constructs, the PCR was performed either with the primer pairs T7/SP6 or X16F/X16R in a 12 μl reaction. The PCR conditions for the primer pairs T7/X16R or T7/Sp6 were 20 cycles of 50 s at 94°C, 20 s at 60°C for annealing and 50 s at 72°C for extension. Detection was by ethidium bromide staining and Southern blotting and hybridization with a SRp20 cDNA probe. Both methods gave the same results. The PCR analysis for the experiments with the exon trap vector (MoBrTec) was performed with primers 2 (5'-GAGGATCCCTTCCCCCACCACC-3') and 3 (5'-CTCTAGACACCATGCATCGTGATTCC-3') and X16F (5'-CCCCCAGCAGACCATGCTGTTCC-3') or with X16R (5'-CCCCCGAGGACCATGCTGTTCC-3'). The PCR conditions for the primer pairs T7/X16R or T7/Sp6 were 20 cycles of 50 s at 94°C for denaturation, 50 s at 55°C for annealing and 1 min at 72°C for extension. Detection was by ethidium bromide staining and Southern blotting using a probe derived from the 5' exon.

The transfection experiments were repeated at least three times for each construct combination. Two additional cell lines (the murine hybridoma X63Ag8 and the murine fibroblast LTK- ) were also tested for the auto-regulation of SRp20 splicing and the results were similar to those with K46 cells (not shown).

**Northern blot analysis**

For Northern blots, 10 μg of total cellular RNA, prepared as described by Chomczynski (1987), were separated electrophoretically on 1% agarose/formaldehyde gels, transferred to Nylon membranes (GeneScreen, NEN or Biodyne A, Pall) and hybridized with 32P-labelled DNA probes using standard methods (Sambrook, 1989).

**Western blot analysis**

Cell pellets were lysed by resuspension at a concentration of 2×106 cells/ml in lysis buffer [0.5% NP-40, 10 mM Tris 7.5, 1 mM MgCl2, 1 mM dithiothreitol (DTT), 10 mM KCl, and 0.4 M NaCl]. After 5 min on ice, the samples were centrifuged at 11 000 g for 5 min. Protein in the supernatant (corresponding to 1×106 cells/lane) was separated by SDS-PAGE (15% acrylamide) under reducing conditions and transferred to a nitrocellulose membrane. Following blocking with 5% milk powder, the membrane was incubated first with the primary antibodies mAb104 (mouse IgM, ATCC 2067-CRL) or biotinylated 12CA5 (anti-haemagglutinin, Boehhringer Mannheiem). Following incubation with peroxidase-coupled goat anti-mouse antibodies (Southern Biotechnology) for recognizing RBP1 RNA target sequences. The membrane was incubated first with the primary antibodies mAb104 (mouse IgM, ATCC 2067-CRL) or biotinylated 12CA5 (anti-haemagglutinin, Boehhringer Mannheiem). Following incubation with peroxidase-coupled goat anti-mouse antibodies (Southern Biotechnology) for recognizing RBP1 RNA target sequences. The membrane was incubated first with the primary antibodies mAb104 (mouse IgM, ATCC 2067-CRL) or biotinylated 12CA5 (anti-haemagglutinin, Boehhringer Mannheiem). Following incubation with peroxidase-coupled goat anti-mouse antibodies (Southern Biotechnology) for recognizing RBP1 RNA target sequences.


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Auto-regulatory splicing of SRp20