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Functional Properties of p54, a Novel SR Protein Active in Constitutive and Alternative Splicing

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The p54 protein was previously identified by its reactivity with an autoantiserum. We report here that p54 is a new member of the SR family of splicing factors, as judged from its structural, antigenic, and functional characteristics. Consistent with its identification as an SR protein, p54 can function as a constitutive splicing factor in complementing splicing-deficient HeLa cell S100 extract. However, p54 also shows properties distinct from those of other SR family members. p54 can directly interact with the 65-kDa subunit of U2 auxiliary factor ($U2AF^{65}$), a protein associated with the 3′ splice site. In addition, p54 interacts with other SR proteins but does not interact with the U1 small nuclear ribonucleoprotein U1-70K or the 35-kDa subunit of U2 auxiliary factor ($U2AF^{35}$). This protein-protein interaction profile is different from those of prototypical SR proteins SC35 and ASF/SF2, both of which interact with U1-70K and $U2AF^{35}$ but not with $U2AF^{65}$. p54 promotes the use of the distal 5′ splice site in E1A pre-mRNA alternative splicing, while the same site is suppressed by ASF/SF2 and SC35. These findings and the differential tissue distribution of p54 suggest that this novel SR protein may participate in regulation of alternative splicing in a tissue- and substrate-dependent manner.

Studies of pre-mRNA splicing and its regulation are essential for our understanding of mammalian gene expression (5, 7, 9, 34, 54, 60). Pre-mRNA splicing occurs in spliceosomes, which are RNA-protein complexes consisting of pre-mRNA, five small nuclear ribonucleoprotein (snRNP) particles, U1, U2, U4/U6, and U5, and a large number of protein factors (34, 54). Functions of many of these splicing factors are not well understood, although it is clear that they are essential for pre-mRNA splicing. In particular, recent work on a group of proteins containing arginine-serine-rich sequences (RS domain) has revealed that these proteins are critical for constitutive splicing and that their activities can regulate alternative splicing (9, 26).

The RS domain was first found in the *Drosophila* splicing regulators transformer (Tra), transformer-2 (Tra2), and suppressor of white apricot (2, 13, 33, 48, 52). Molecular cloning of mammalian spliceosomal proteins U1-70K, both subunits of U2 auxiliary factor ($U2AF^{65}$ and $U2AF^{35}$), SC35, and ASF/SF2 revealed that each of these proteins also contains an RS domain (28, 31, 44, 56, 66, 72, 85, 87). A subset of these proteins have been defined as the SR family of splicing factors on the basis of their structural, antigenic, and functional features (79). This family includes ASF/SF2 (or SRp30a) (31, 44), SC35 (also called SRp30b or PR264) (28, 75), SRp20 (X16 or RBP1), SRp55 (B52) and SRp75 (4, 17, 37, 65, 79–81), SRp40 (HRS) (8, 21, 65), 9G8 (16), and SRp30c (65). Many of the SR proteins share an antigenic epitope recognized by monoclonal antibody (MAb) 104 (63) and copurify in a two-step purification procedure (79). Their primary sequences contain two structural motifs: an RNA recognition motif (RRM) of the RNP type and an RS domain. Functionally, each individual SR protein can complement HeLa cell S100 extract for splicing activity. Most of the known SR proteins play important roles not only in constitutive splicing but also in alternative splicing (15, 25, 29, 30, 41, 46, 50, 51, 55, 57, 65, 68, 76, 80, 82). In *Drosophila melanogaster*, SRp55 (B52) has been found to be critical for viability and development of the organism (45, 59). Therefore, it is essential to understand the functional properties of SR proteins.

We report here characterization of a new member of the SR family, p54, which is a protein previously identified by its reactivity with an autoantiserum (18). We show that recombinant p54 is recognized by MAb 104 and can complement S100 extract for splicing activity. However, effects of p54 on regulation of alternative splicing of certain pre-mRNAs are different from those of the prototypical SR proteins ASF/SF2 and SC35. Furthermore, we find that p54 has a profile of protein-protein interactions distinct from those of ASF/SF2 and SC35. Because protein-protein interactions are crucial for the functioning of SR proteins and other splicing factors containing RS domains (3, 14, 19, 39, 67, 77), our results indicate that the protein-protein interaction profile can be a distinguishing feature of individual SR proteins. Finally, we demonstrate that p54 is differentially expressed in different tissues. The differential distribution of SR proteins and their distinct functional properties suggest that the regulation of alternative splicing of different pre-mRNAs may be determined by relative activities of multiple SR proteins and their interactions with different components of the splicing machinery.

MATERIALS AND METHODS

Expression and purification of recombinant p54 in the baculovirus system. The cDNA fragment containing the full-length coding region of the p54 gene was amplified by PCR and inserted in-frame into baculovirus expression vector BacHisA (Invitrogen) at the *Bam*HI site. The sequence of the full-length p54 cDNA fragment was verified by DNA sequencing using Sequenase (U.S. Biochemical Corp.). The baculovirus expression plasmid was cotransfected with linearized *Autographa californica* nuclear polyhedrosis virus DNA purchased from Invitrogen. Transfection and recombinant virus isolation were carried out as instructed by the manufacturer.

Sf9 cells were cultured in IPL41 medium (Gibco-BRL) supplemented with 10% fetal calf serum in suspension to 2.5×10^6/ml and infected with the recombinant virus expressing p54. The following purification procedure was modified from the published protocol (84), and all the steps were carried out at 4°C. Approximately 72 h postinfection, cells were lysed with buffer A (20 mM Tris HCl [pH 7.5], 100 mM KCl, 5 mM EDTA, 5 mM KF, 5 mM glycerol phosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol [DTT])

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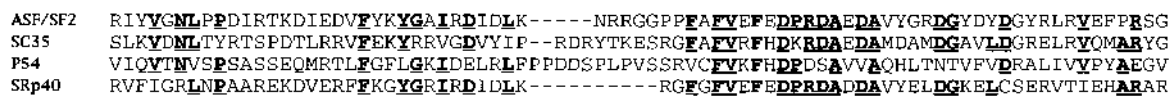

FIG. 1. Alignment of ASF/SF2, SC35, p54, and SRp40 sequences in the RRM region. The RNP2 and RNP1 elements of the RRMs are shown. The amino acid residues which are identical among three or more proteins are shown in boldface and underlined.

by sonication. Following centrifugation, the supernatant was saved and ammonium sulfate was added to a final concentration of 1.8 M. After being stirred for 90 min, the mixture was spun in an SS34 rotor for 30 min at 10,000 rpm. The supernatant was loaded onto a phenyl-Sepharose CL-4B column (Pharmacia) equilibrated with buffer C (20 mM Tris HCl [pH 7.5], 0.2 mM EDTA, 1 mM DTT) containing 1.8 M ammonium sulfate. Protein was eluted in buffer C containing 0.9 M ammonium sulfate. After dialysis using BC100 (20 mM Tris HCl [pH 7.5], 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT), protein was precipitated by $MgCl_2$ precipitation (92). The pellet was resuspended in BC100 and further purified by using a nickel-agarose column (Qiagen) under native conditions as specified by the manufacturer. Protein was eluted with 250 mM imidazole, dialyzed against BC100 containing 10% glycerol, and stored at −70°C in small aliquots. In some experiments, p54 was further purified by elution from a polyacrylamide gel after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and renatured as described previously (79, 82).

In vitro splicing and complementation of S100 extract. In vitro splicing experiments were carried out with capped, ^{32}P-labeled β-globin pre-mRNA substrate which was transcribed in vitro from a β-globin plasmid (HβΔ6) as described previously (29, 43). Three and a half microliters of HeLa nuclear extract or 7 μl of S100 cytoplasmic extract was used for each reaction in the presence or absence of recombinant SC35 or p54. SC35 was purified as described previously (77). Splicing reaction products were separated on a urea-polyacrylamide gel and detected by autoradiography.

Construction of splicing substrate and SR protein expression plasmids. Mammalian expression plasmids containing duplicated 5′ or 3′ splice sites were constructed by inserting *Hin*dIII-*Bam*HI fragments of plasmids 5′D16X and 3′D115 (29, 58) into pcDNAIII and named 5′D16XpcDNA and 3′D115pcDNA, respectively. E1A pre-mRNA-expressing plasmid pMTE1A was kindly provided by J. Cáceres and A. Krainer (Cold Spring Harbor Laboratory). SR protein-expressing plasmids were constructed by cloning SC35, ASF/SF2, p54, and SRp40 cDNAs into pcDNAIII. SRp40 cDNA was obtained by PCR from HeLa cDNA, and the full-length sequence was confirmed by sequencing. Plasmid DNA for transfection was prepared by using Qiagen columns (Tip-500).

Transfection and RT-PCR. HeLa cells were cultured in 60-mm-diameter dishes to 60 to 70% confluence. Transfection was carried out with 12 μg of Lipofectin (Gibco-BRL) with 6 μg of splicing substrate plasmid (5′D16XpcDNA, 3′D115pcDNA, or pMTE1A). Cells were cotransfected with 1 μg of either vector plasmid pcDNAIII or a plasmid expressing SC35, p54, ASF/SF2, or SRp40. Under these conditions, approximately 10% of the cells were transfected, as estimated by cotransfection of a green fluorescent protein-expressing plasmid. At 48 h after transfection, cells were harvested and total RNA was prepared as described previously (32). Reverse transcription was carried out with Moloney murine leukemia virus superscript reverse transcriptase (Gibco-BRL). PCR was performed with *Taq* DNA polymerase in the presence of 1 μCi of [^{32}P]dCTP (Amersham) per reaction. The cycle numbers of PCR were kept to a minimum to detect signals in the linear range. Expression of transfected SC35, p54, ASF/SF2, and SRp40 was detected by reverse transcription-PCR (RT-PCR) using a forward primer corresponding to the vector 5′ untranslated region sequence and reverse primers specific to each of the SR protein-encoding cDNAs. RT-PCR products were analyzed on a polyacrylamide gel and detected by autoradiography. Experiments were repeated three times or more.

Yeast two-hybrid protein-protein interaction assay. The protein-protein interactions were assayed in the yeast two-hybrid system, using yeast bait plasmids expressing LexA fusion proteins of SC35, ASF/SF2, and p54 and prey plasmids expressing B42 activation domain fusion proteins of U1-70K, SC35, ASF/SF2, U2AF^{65}, and U2AF^{35} (77, 86). The liquid β-galactosidase assay was performed as previously described previously (77). The β-galactosidase activity was normalized with protein concentrations of the corresponding yeast extracts. Background is defined as the amount of β-galactosidase activity detected with cotransformation of the bait plasmid and the vector plasmid containing only the activation domain without other cDNA sequences.

Coimmunoprecipitation experiments. Coimmunoprecipitation assays were carried out with in vitro-translated p54, U2AF^{35}, and U2AF^{65}. These in vitro-translated proteins were prepared by using a coupled in vitro transcription-translation system (Promega) in the presence of [^{35}S]methionine. The U2AF^{35} and U2AF^{65} proteins were designed to contain a flag epitope (10), and coimmunoprecipitation experiments were performed with antiflag antibody as described previously (77).

Northern analyses. Total RNA was extracted from different tissues of a young adult mouse as described by Kingston (38). Poly(A)$^+$ RNA was prepared by using the PolyATract mRNA isolation system (Promega). Approximately 2 μg of poly(A)$^+$ RNA was loaded in each lane, separated by formaldehyde-agarose gel, and transferred to a Hybond filter (Amersham). The filter was probed with random-primed ^{32}P-labeled p54, SC35, U2AF^{35}, or γ-actin (24) probe.

RESULTS

Sequence features of p54. The cDNA for p54 was originally cloned by screening a human hepatoma cDNA expression library with an autoantiserum enriched in anti-lamin B antibodies (18). The molecular mass of the protein predicted from the cDNA sequence was 54 kDa; hence, the protein was named p54. Sequence analysis of p54 cDNA suggests that it encodes a protein containing an RS domain. Immunocytochemical studies suggest that intracellular localization of p54 is similar to that of splicing factor SC35 (18). As observed by Birney et al. (8), we noted that p54 also contains an RRM of the RNP consensus sequence type as well as an overlapping octapeptide motif present in several SR proteins (Fig. 1). While the amino acid identities in the RRM region between SC35, ASF/SF2, and SRp40 range from 27 to 45% (with similarities from 38 to 63%), the sequence identities between p54 and other SR proteins are lower, ranging from 17 to 21% (with similarities from 44 to 46%). Therefore, the RRM of p54 is more distantly related to that of other SR proteins than other known SR family members are to each other.

In the RS domain of p54, there are 10 imperfect RRSRS XSX repeats, the motif found in other SR proteins, including SC35, SRp40, SRp75, and 9G8, spliceosomal protein U1-70K (16, 21, 28, 56, 65, 66, 72), and *Drosophila* splicing regulators Tra and suppressor of white apricot (52, 48).

Thus, p54 has the overall structural features of the SR family of splicing factors. We therefore tested whether p54 could function as a splicing factor.

p54 shares a common antigenic epitope with SR proteins. We have used the baculovirus system to express recombinant p54 after several unsuccessful attempts to express p54 in *Escherichia coli*. We constructed a recombinant baculovirus expressing p54 fused to a histidine tag. Recombinant p54 from Sf9 insect cells infected with the recombinant baculovirus was purified by using nickel-agarose. Purified p54 has an apparent molecular mass of 69 to 70 kDa (Fig. 2A, lane 2), similar to that of the p54 translated in vitro (data not shown) (18). This aberrant gel mobility is most likely due to phosphorylation, since the RS domain of p54 contains at least five sites with the predicted consensus substrate sequence for protein kinases. Other SR proteins also have apparent molecular masses higher than the predicted values as a result of phosphorylation (31, 35, 44, 79).

By Western blotting (immunoblotting), we found that p54 is recognized not only by a p54-specific antipeptide antibody (Fig. 2B, lane 3) but also by MAb 104 (lanes 4 and 5), which recognizes an epitope shared by several members of the SR family of splicing factors (63, 79). When equal amounts of purified recombinant p54 and SC35 were used in Western blotting experiments, both proteins were detected by MAb 104 at similar intensities (data not shown).

p54 can complement splicing-deficient S100 for splicing activity. To test the function of p54, SR protein-deficient HeLa

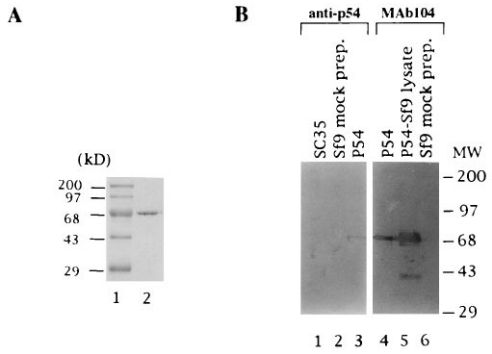

FIG. 2. p54 is recognized by its antipeptide antibody and by MAb 104. (A) Purified recombinant p54 (lane 2) was analyzed by SDS-PAGE followed by Coomassie blue staining. Lane 1 contains size markers. (B) Purified recombinant SC35 (lane 1), p54 (lanes 3 and 4), the mock protein preparation from uninfected Sf9 cells (lanes 2 and 6), and the total cell lysate of Sf9 cells expressing p54 (lane 5) were separated by SDS-PAGE and transferred to nitrocellulose filters. Anti-p54 peptide antibody (lanes 1 to 3) or MAb 104 (lanes 4 to 6) was used for the Western blotting.

cell S100 extract was used in an in vitro splicing assay (Fig. 3). As shown by previous studies, S100 extract cannot splice pre-mRNA efficiently (42) (Fig. 3B, lane 1). SR proteins such as SC35, however, can complement S100 extract for splicing activity (29) (Fig. 3A, lane 2). Similarly, p54 protein was capable of complementing the S100 extract for splicing activity (lane 3). This effect is due to the activity of p54 and not due to any contaminating proteins in the preparation, because p54 further purified by separation on SDS-PAGE and elution from the gel had the same activity (lane 4) whereas the control protein preparation from uninfected Sf9 cells did not show any complementation activity (lane 5). The restoration of splicing

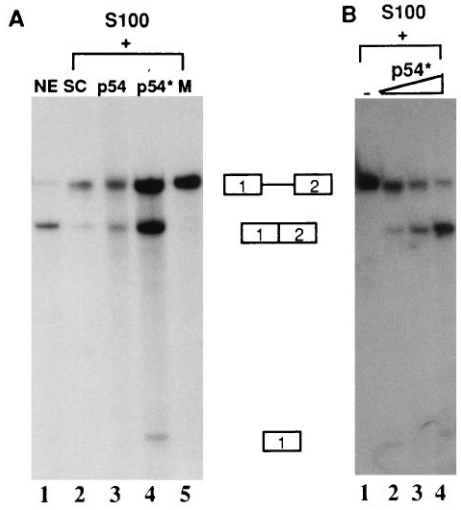

FIG. 3. p54 can complement splicing-deficient S100 extract for splicing activity. (A) Purified recombinant SC35 or p54 was used in an S100 complementation assay. ^{32}P-labeled human β-globin pre-mRNA was prepared by in vitro transcription and incubated under standard splicing condition with HeLa cell nuclear extract (NE; 3.5 μl; lane 1) or with S100 extract (7 μl; lanes 2 to 5) supplemented with SC35 (lane 2) or mock protein preparation from uninfected Sf9 cells (lane 5) or p54 (lanes 3 and 4). Lane 3 contains purified p54, and lane 4 contains p54 further purified by elution from SDS-PAGE followed by renaturation. The amount of recombinant proteins used was approximately 200 ng for each reaction. (B) Different amounts of purified p54 (as described for lane 4 of panel A) were used for the S100 complementation assay as described above. Lanes 1 to 4 contain S100 supplemented with 0, 50, 150, and 300 ng of p54.

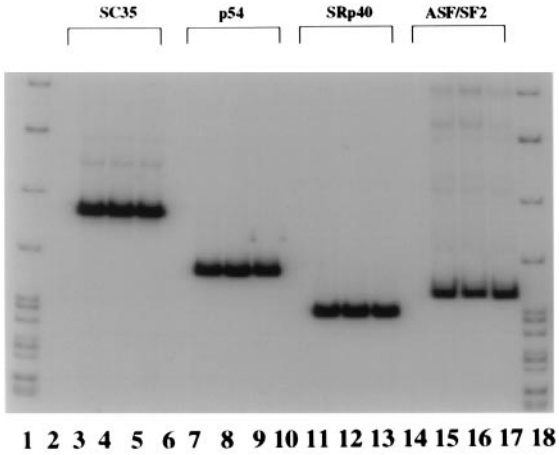

FIG. 4. Expression of SC35, p54, SRp40, and ASF/SF2 when cotransfected with different pre-mRNAs. HeLa cells were cotransfected with each of three alternative splicing pre-mRNA constructs and each of the SR protein-expressing plasmids. Expression of the corresponding SR proteins was detected by RT-PCR using total RNAs extracted from the transfected cells. In lanes 3, 7, 11, and 15, the 5′D16pcDNA construct was used together with plasmids expressing SC35, p54, SRp40, and ASF/SF2, respectively. In lanes 4, 8, 12, and 16, the E1A pre-mRNA construct was cotransfected with plasmids expressing SC35, p54, SRp40, and ASF/SF2, respectively. In lanes 5, 9, 13, and 17, 3′D115pcDNA was cotransfected with plasmids expressing SC35, p54, SRp40, and ASF/SF2, respectively. Lanes 2, 6, 10, and 14 are the controls transfected with the vector plasmid alone. The radiolabeled pBR322 *Msp*I-cut DNA size markers are shown in lanes 1 and 18.

activity in S100 extract was dependent on the concentration of p54 added to the reaction (Fig. 3B, lanes 2 to 4). Thus, p54 is similar to other known SR proteins in its ability to complement S100 extract for splicing activity. The structural, antigenic, and functional similarities between p54 and other SR proteins indicate that p54 is a new member of the SR family.

p54 affects alternative splice site selection in vivo. We have examined the role of p54 in regulating alternative splicing in vivo and compared it with the roles of SC35, SRp40, and ASF/SF2. We used three splicing substrates containing competing 5′ or 3′ splice sites and characterized the splicing pattern in transiently transfected HeLa cells (Fig. 4 and 5). Individual pre-mRNA splicing substrate constructs were cotransfected with either the vector plasmid alone, a plasmid expressing p54, or each of plasmids expressing the SR proteins. Splicing products were detected by RT-PCR. Using Western blotting assays with MAb 104 to examine the cell lysates of the transfected HeLa cells, we did not observe a significant increase of the corresponding SR proteins (data not shown). This was likely due to the fact that the transfection efficiency was only 10% under our conditions (see Materials and Methods). However, expression of the SR proteins SC35, p54, SRp40, and ASF/SF2 from the transfected plasmids was detectable by using RT-PCR with primers which were able to detect transfected but not endogenous SR proteins. The expression levels of these SR proteins from corresponding plasmids were found to be equivalent when the plasmids were cotransfected with different splicing substrates (Fig. 4).

We first tested a β-globin construct containing competing 5′ splice sites which has been well characterized in vitro (29, 41, 43, 57). When this β-globin construct (5′D16pcDNA) was transfected into HeLa cells, the distal 5′ splice site was much more efficiently used than the proximal 5′ splice site (Fig. 5A), as was observed in vitro when nuclear extract was used (29, 41, 42, 58). A low level of spliced product corresponding to the

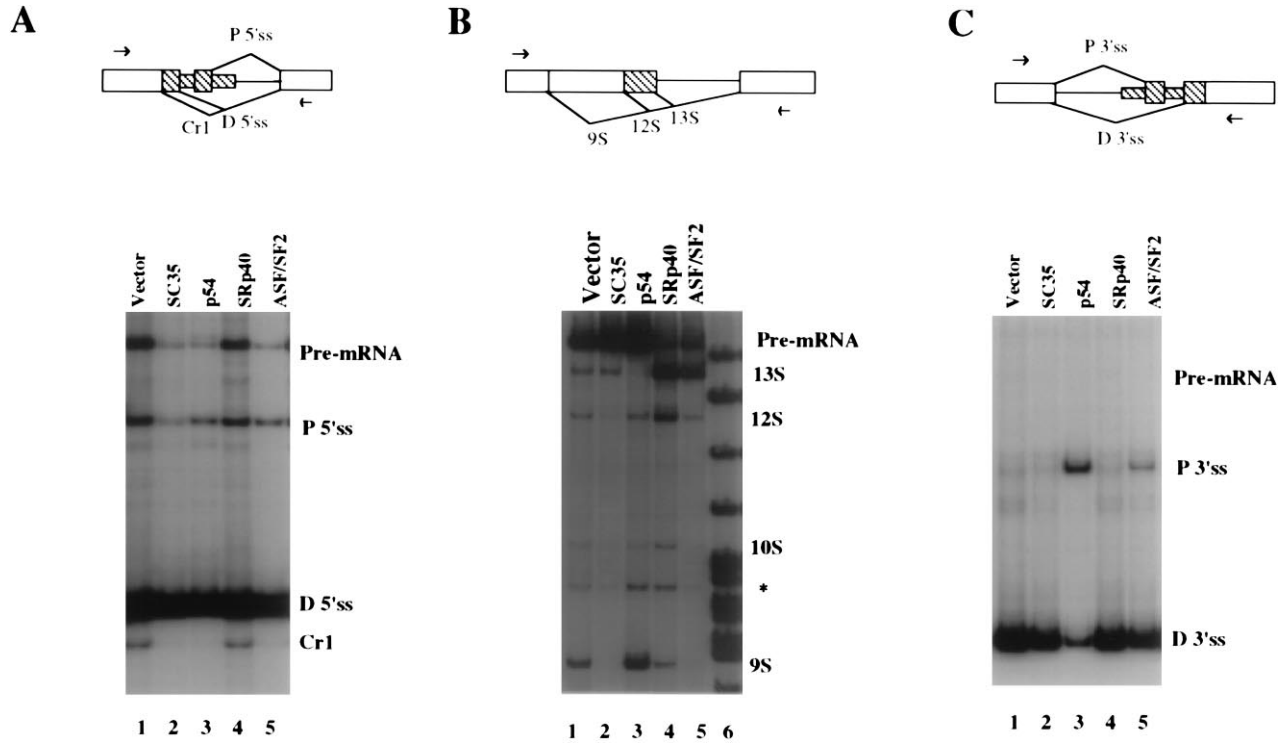

FIG. 5. p54 regulates alternative 5′ and 3′ splice site selection in vivo in comparison with SC35, SRp40, and ASF/SF2. HeLa cells were cotransfected with alternative splicing pre-mRNA construct 5′D16pcDNA (A), the E1A gene (B), or 3′D115pcDNA (C) and each of the SR protein-expressing plasmids. The splicing products were detected by RT-PCR using total RNA prepared from the transfected cells and primers (indicated by the arrows) specific to upstream and downstream exon sequences of the corresponding pre-mRNA. (A) Effects of the individual SR proteins on alternative 5′ splice site selection in the pre-mRNA containing duplicated 5′ splice sites. 5′D16pcDNA was cotransfected with vector plasmid alone or the plasmid expressing SC35, p54, SRp40, or ASF/SF2. The DNA bands corresponding to unspliced pre-mRNA and spliced products using the proximal or distal 5′ splice site (P5′ss or D 5′ss) or the upstream cryptic 5′ splice site (Cr1) are marked on the right. (B) Effects of the SR proteins on alternative splicing of the E1A gene. The vector plasmid and each of the plasmids expressing SC35, p54, SRp40, and ASF/SF2 (lanes 1 to 5, respectively) were cotransfected with the E1A gene. Lane 6 shows pBR322 *Msp*I-cut DNA size markers. Positions of pre-mRNA and different splicing products are indicated. 10S mRNA is a product of a double-splicing event. The band marked by the asterisk may be a PCR artifact and did not consistently appear in different experiments. (C) Effects of the SR proteins on alternative 3′ splice site selection. The β-globin pre-mRNA construct 3′D115pcDNA with duplicated 3′ splice sites was cotransfected with vector plasmid alone or the plasmid expressing SC35, p54, SRp40, or ASF/SF2. The positions of the pre-mRNA and spliced products corresponding to usage of the proximal or distal 3′ splice site are indicated.

usage of a cryptic 5′ splice site upstream of the distal 5′ splice site was also detected (indicated by Cr1 in Fig. 5A). The identities of these PCR products were confirmed by direct DNA sequence analyses of the corresponding bands isolated from the gel. Cr1 is the same as the previously described cryptic 1 exon1-105 site, whose sequence is AAG/GTGAAC, while the sequence for the normal 5′ splice site is CAG/GTTGGT (15, 74). None of the SR proteins tested, including p54, SC35, SRp40, and ASF/SF2, could activate the proximal 5′ splice site (Fig. 5A), even when the amounts of SR-expressing plasmids cotransfected were increased fivefold (data not shown). With p54, SC35, and ASF/SF2, a small but consistent increase in overall splicing efficiency was observed, as evidenced by a decrease in the ratio of the pre-mRNA to spliced products quantitated by a PhosphorImager (Fig. 5A, lanes 2, 3, and 5, and data not shown). Interestingly, in these cases, the usage of the cryptic splice site was suppressed (Fig. 5A; compare the band marked Cr1 in lanes 2, 3, and 5 with that in lane 1). This finding suggests that p54, SC35, and ASF/SF2 can differentiate the authentic splice site from the cryptic splice site and may play a role in inhibiting cryptic splice site usage in vivo. The effect of ASF/SF2 on inhibiting cryptic sites is different from its activation of cryptic site usage in a mutant E1A pre-mRNA (76), showing that the effect on cryptic sites is substrate dependent.

The effect of p54 and other SR proteins on 5′ alternative splice site selection was also examined by using adenovirus E1A pre-mRNA. Transfection of the E1A gene into HeLa cells results in formation of 9S, 12S, and 13S mRNAs as a result of usage of different 5′ splice sites (15) (Fig. 5B, lane 1). As observed previously (87), SC35 decreased 9S and 12S mRNA formation but had no effect on 13S mRNA production (Fig. 5B, lane 2). By contrast, p54 caused a significant increase of 9S mRNA formation and concomitant decrease of 13S mRNA without affecting 12S mRNA (lane 3). This result indicates that p54 facilitated the distal, but not the proximal, 5′ splice site usage in E1A pre-mRNA splicing. SRp40 increased usage of both the 13S and 12S 5′ splice sites and slightly decreased production of 9S mRNA (lane 4). ASF/SF2, on the other hand, increased 13S mRNA production and decreased 9S mRNA production, indicating a shift from distal 5′ splice site to the most proximal 5′ splice site (lane 5) (15, 64). Therefore, in the case of E1A pre-mRNA splicing, SC35, p54, SRp40, and ASF/SF2 clearly have different effects on alternative 5′ splice site selection. ASF/SF2 and SRp40 activate the most proximal 5′ splice site. ASF/SF2 and SC35 inhibit usage of the most distal 5′ splice site, whereas p54 facilitates usage of the most distal 5′ splice site.

We then investigated the effect of p54 on alternative 3′ splice site selection. When the β-globin plasmid containing duplicated 3′ splice sites (3′D115pcDNA) was transfected into

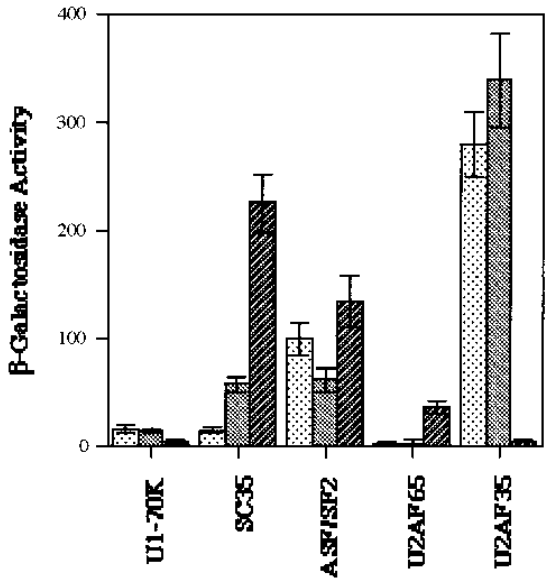

FIG. 6. Pairwise interactions between p54 and other RS domain-containing spliceosomal proteins in comparison with SC35 and ASF/SF2 as detected by the yeast two-hybrid interaction assay. Quantitative liquid β-galactosidase assays were performed on at least three independent yeast isolates for each combination. Relative β-galactosidase activities shown represent the fold activation above background (see Materials and Methods). ▨, SC35; ▤, ASF/SF2; ▨, p54.

the HeLa cells, highly efficient splicing was observed, with almost exclusive usage of the distal 3′ splice site (Fig. 5C, lane 1). This splicing was so efficient that only spliced products, not precursor mRNA, were detected. This was not due to an artifact of PCR amplification, because under the same PCR conditions, the corresponding PCR product was easily detectable from a very small amount of the 3′D115pcDNA plasmid DNA (data not shown). In addition, the RT-PCR product corresponding to the mRNA precursor expressed from the transfected 5′D16pcDNA, which had a size similar to that of the product expressed from plasmid 3′D115pcDNA, was also detectable (Fig. 5A). p54 and ASF/SF2 had significant effects on activating the proximal 3′ splice sites (Fig. 5C, lanes 3 and 5), while SC35 and SRp40 had no obvious effect on the splicing of this pre-mRNA substrate (lanes 2 and 4). In these experiments, the level of SC35 and SRp40 expression was found to be equivalent to that of p54 and ASF/SF2 expression (Fig. 4).

By comparing the effects of p54, SC35, ASF/SF2, and SRp40 on splicing of different pre-mRNA substrates containing competing 5′ or 3′ splice sites, we conclude that p54 has distinct activities in regulating alternative splice site selection in a substrate-dependent manner.

p54 has a unique profile in protein-protein interactions. Previous studies have shown that an important mechanism of SR protein function relates to interactions with other splicing factors (3, 39, 77). We have examined the interaction between p54 and other RS domain-containing splicing factors. We first tested these interactions in the yeast two-hybrid system (86), which has been shown to be a reliable assay for protein-protein interactions among splicing factors (1, 47, 77). The two prototypical SR proteins, SC35 and ASF/SF2, behave similarly in their protein-protein interaction profiles. Surprisingly, we found that the spectrum of p54-interacting proteins was different from those of SC35 and ASF/SF2 (Fig. 6). We have shown previously in multiple assays that both SC35 and ASF/SF2 interact with SC35, ASF/SF2, U1-70K, and U2AF^{35} but not with U2AF^{65} (77). We have now demonstrated that p54 interacts not only with SC35 and ASF/SF2 but also with U2AF^{65}. On the other hand, p54 does not interact directly with U1-70K or U2AF^{35}.

To confirm the interaction between p54 and U2AF^{65} observed in the yeast two-hybrid system, we performed coimmunoprecipitation assays using in vitro-translated and ^{35}S-labeled p54 and U2AF^{65} proteins. When the flag epitope-tagged U2AF^{65} protein was precipitated with the antiflag antibody, p54 was coprecipitated (Fig. 7, lane 2). This coprecipitation is due to the association between p54 and U2AF^{65}, because in vitro-translated p54 by itself was not precipitated by the antiflag antibody (lane 1). The association between p54 and U2AF^{65} was unlikely to be mediated by RNA because RNase treatment following in vitro translation did not affect coprecipitation of the two proteins (data not shown). In the coimmunoprecipitation assay, there was no detectable interaction between p54 and U2AF^{35} (lane 3), again confirming the result from the yeast two-hybrid system.

Both SC35 and p54 can complement S100, but they behave differently in mediating interactions among the RS domain-containing splicing factors. This observation suggests that different members of SR family may function in 5′ and 3′ splice site association by mediating interactions among different components of the spliceosome.

p54 is differentially expressed in different tissues. The tissue distribution of p54 mRNA was compared with those of SC35 and U2AF^{35} by Northern analyses (Fig. 8). Each of these splicing factors has several transcripts with differential distribution in different tissues, indicating that these splicing factors are differentially expressed and that the splicing of their pre-mRNAs may also be regulated. Although the expression level of U2AF^{35} is relatively uniform in most tissues examined (except muscle), both SC35 and p54 have different levels of expression in different tissues. Curiously, all three splicing factors showed much lower expression in skeletal muscle than in other tissues (Fig. 8, lane 8). In skeletal muscle, it was previously

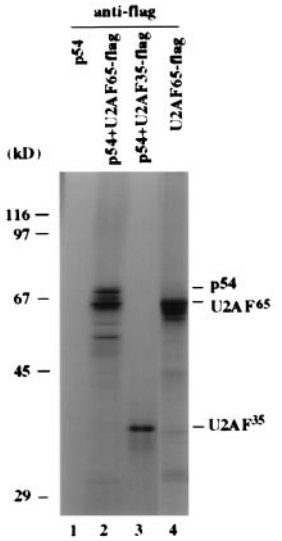

FIG. 7. p54 interacts with U2AF^{65} but not with U2AF^{35} in coimmunoprecipitation experiments. In vitro-translated ^{35}S-labeled p54 alone (lane 1) or p54 coincubated with flag epitope-tagged in vitro-translated U2AF^{65} or U2AF^{35} (lane 2 or 3, respectively) was immunoprecipitated with an antiflag antibody. The precipitated proteins were separated by SDS-PAGE, and an autoradiogram of the gel is shown. Positions of protein molecular mass size markers are indicated on the left.

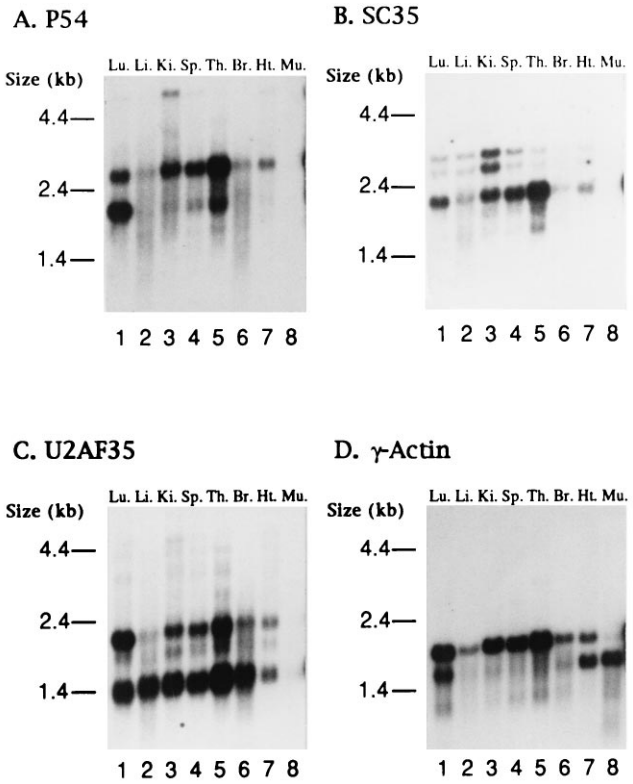

FIG. 8. Northern analyses of P54, SC35, and U2AF^{35}. Approximately 2 μg of poly(A)-selected RNA from different murine tissues (lanes 1 to 8 correspond to lung, liver, kidney, spleen, thymus, brain, heart, and muscle, respectively) was loaded in each lane. After Northern transfer, the filter was probed with a ^{32}P-labeled probe prepared from p54 (A), SC35 (B), U2AF^{35} (C), or γ-actin (D) cDNA. γ-Actin was used as a control for RNA quantity. The same nitrocellulose filter was used for hybridization with different probes. The autoradiograms of the filter are shown.

noticed that SC35 and ASF/SF2 were almost undetectable at the protein level and that SRp75 and SRp55 were the major SR proteins detected by Western blotting using MAb 104 (92, 93). The differential distribution of SR proteins, together with their different functional properties, suggests that the overall balance of activities of different SR proteins may contribute to regulation of alternative splicing of different pre-mRNAs.

DISCUSSION

p54 as a new member of the SR family of splicing factors. p54 was initially identified by an antibody that was thought to recognize nuclear protein lamin B. Although p54 was shown to be a nuclear protein with a speckled pattern of localization similar to that of SC35 (18), there was no direct evidence that p54 could function as a splicing factor. We have presented here several lines of evidence showing that p54 is a new member of the SR family of splicing factors. First, p54 has sequence similarity in both the RS domain and RNA binding domain to other SR proteins. Second, p54 protein is recognized by MAb 104, another hallmark of the SR proteins (79). Third, p54 can complement the SR protein-deficient S100 extract for splicing activity. Finally, p54 can regulate alternative 5′ and 3′ splice site selection.

Therefore, by structural, antigenic, and functional criteria, p54 is a previously unrecognized splicing factor of the SR family, which now includes at least nine different proteins: SRp20 (X16 or RBP1), ASF/SF2 (SRp30a), SC35 (SRp30b or PR264), SRp30c, SRp40 (HRS), SRp55 (B52), SRp75, 9G8, and p54 (4, 16–18, 21, 28, 31, 37, 65, 75, 81). Recent studies suggest that there exist another set of SR-related proteins associated with splicing complexes (11, 12). Functions of these proteins are not clear yet.

Different SR proteins. The discovery of an increasing number of SR proteins indicates their importance and also raises the question of why there are such a large number of different SR proteins. This issue can be discussed in the context of our results for p54.

As shown by sequence comparison in the RNA binding domain, p54 is a divergent member of the SR family. It may have an RNA binding specificity different from those of other SR proteins. Even the more closely related SC35 and ASF/SF2 have been shown to have different RNA binding properties (69). Therefore, different SR family members may have distinct spectra of in vivo pre-mRNA targets.

p54 has unique properties in regulating alternative splicing of different pre-mRNAs. For example, p54 is the only SR protein studied so far which promotes the most distal 5′ splice site usage on the E1A pre-mRNA. The only other protein reported which facilitates selection of this splice site in vivo is heterogeneous nuclear RNP (hnRNP) A1, which does not belong to the SR family (15). In that case, overexpression of hnRNP A1 increased 9S mRNA formation without much effect on 13S mRNA production (15). p54, however, significantly reduced 13S mRNA and at the same time increased production of 9S mRNA (Fig. 5B, lane 3). Therefore, p54 is also different from hnRNP A1 in regulation of splice site selection in E1A pre-mRNA splicing. Our experiments show that p54 behaves the same as either ASF/SF2 or SC35 on some pre-mRNA substrates but has different activities on other pre-mRNAs (15, 29, 50, 76, 80, 82). These results suggest that different SR proteins have different roles in regulating alternative splicing of different pre-mRNAs and that combinatorial activities of SR proteins may determine the exact splicing pattern of each pre-mRNA. Further studies will be carried out to determine mechanisms of different functions of different SR proteins in regulating alternative splicing using both in vivo and in vitro assays.

Our Northern analysis of SC35 and p54 showed the presence of several different transcripts with different distributions among different tissues. These different transcripts may encode different isoforms of SR proteins with different functions, as has been shown for ASF/SF2 (34; reviewed in reference 26 for other SR proteins). The differential tissue distributions of several other SR proteins have also been observed (65, 80). These studies suggest that the pattern of pre-mRNA alternative splicing in different tissues can be regulated by the relative contribution of several SR proteins.

Finally, we have found that the profile of protein-protein interactions of p54 is different from those of the other SR proteins. This finding not only offers a possible explanation for the different behavior of the p54 protein in regulation of alternative splicing but also suggests that the protein-protein interaction profile is a distinguishing feature of individual SR proteins.

Implications of the protein-protein interaction profile of p54. Both ASF/SF2 and SC35 are essential for early steps of spliceosome assembly (27, 40) and are capable of committing pre-mRNAs into the splicing pathway (25). During early commitment complex formation, ASF/SF2 can facilitate U1 snRNP binding to the 5′ splice site (23, 82) by interacting with U1-70K (39). U2AF binds to the polypyrimidine tract near the 3′ splice site and then promotes U2 snRNP binding to the branch site

(6, 53, 63, 83, 84). Interaction between the 5′ and 3′ splice sites is established during the early step of spliceosome assembly (53). Studies on protein-protein interactions among the RS domain-containing splicing factors demonstrated that SC35 and ASF/SF2 can interact with both U1-70K (35, 77) and $U2AF^{35}$ but not $U2AF^{65}$ (77). These studies led to the proposal of a model that SR proteins mediate interactions between 5′ and 3′ splice sites by directly interacting with spliceosomal proteins associated with the two splice sites (77).

Our current study shows that p54 cannot interact directly with U1-70K or $U2AF^{35}$ but can interact with other SR proteins (ASF/SF2 and SC35) and with $U2AF^{65}$. This finding has several functional implications.

First, it suggests possible variations of the components in the multiprotein complex that mediates interaction between 5′ and 3′ splice sites. It is conceivable that p54 can mediate 5′ and 3′ splice site interaction by interacting directly with $U2AF^{65}$ associated with the 3′ splice site and at the same time interact with other SR proteins, such as ASF/SF2 and SC35, which in turn interact with U1-70K. In this scenario, p54 is different from SC35 or ASF/SF2 in that it cannot directly interact with the 5′ component (U1-70K) but can interact with the protein associated with the 3′ splice site ($U2AF^{65}$).

Second, the protein-protein interaction profile of p54 is different from those of the SR proteins SC35 and ASF/SF2 and is actually similar to that of $U2AF^{35}$. $U2AF^{35}$ is the small subunit of U2AF which binds to the polypyrimidine tract near the 3′ splice site. The large subunit of U2AF, $U2AF^{65}$, comes in direct contact with pre-mRNA (83–85). $U2AF^{35}$ and $U2AF^{65}$ are tightly associated with each other. This association is conserved through evolution (84). Both subunits of U2AF are conserved between *D. melanogaster* and humans (36, 61). The finding of apparent functional dispensability of $U2AF^{35}$ observed in the earlier studies is therefore puzzling (83, 84). In those studies, when poly(U) was used to deplete both $U2AF^{65}$ and $U2AF^{35}$ from the nuclear extract, the extract was deficient in splicing activity. Addition of $U2AF^{65}$ alone was sufficient to reconstitute splicing activity of the depleted extract (84), suggesting that $U2AF^{35}$ was not essential for splicing. There are two possible explanations for this observation. One is that $U2AF^{35}$ is not required for constitutive splicing and may be required only for alternative splicing; the other is that it is functionally redundant with other proteins. A recent study by Zuo and Maniatis (88) demonstrates that $U2AF^{35}$ is important for both constitutive and enhancer-dependent splicing. Our observation here suggests that p54 may be one of the proteins which could functionally replace $U2AF^{35}$ by virtue of its ability to interact with $U2AF^{65}$ and SR proteins to perform the bridging function of $U2AF^{35}$ (77).

Third, SR proteins function in multiple processes of spliceosome assembly (19, 27, 37, 40–43, 62, 67, 70) and can even mediate the interaction between 5′ and 3′ splice sites when they are separately contained in two RNA molecules (14, 19). They interact with exonic splicing enhancer elements important for splicing (14, 22, 46, 57, 68, 71, 76, 78). SR proteins also participate in regulation of alternative splicing. They interact directly with alternative splicing regulators such as Tra and Tra2 (3, 77). SR proteins are found to be present in the *doublesex* pre-mRNA enhancer complex (73) and bind to the *doublesex* enhancer element cooperatively with Tra and Tra2 (49). Our finding of distinct protein-protein interaction profile suggests that individual SR proteins may play different roles in these processes which require protein-protein interactions.

Differences of SR functions in vivo and in vitro. Some of the effects of SR proteins on splice site selection were different in vivo and in vitro. The β-globin pre-mRNAs containing duplicated 5′ splice sites are well characterized (29, 41, 43, 58). In vitro, the distal 5′ splice site was predominantly used when HeLa cell nuclear extracts were added, similar to our result obtained with the transfected HeLa cells. Addition of SR proteins such as ASF/SF2 or SC35 enhanced the proximal 5′ splice site in vitro. This is different from our results with the transfected HeLa cells. The most obvious effect of SC35, ASF/SF2, and p54 was found to be suppression of cryptic splice site usage without enhancing proximal 5′ splice site usage. This difference is not due to inability of transfected SR proteins to function in HeLa cells, since they have an effect on cryptic splice site selection and they also regulate alternative splicing on other pre-mRNA substrates when expressed at similar levels (Fig. 4 and 5). The difference between in vivo and in vitro effects of SC35 and ASF/SF2 can be explained by differences in overall balance of different splicing factors present in the live nuclei and in the nuclear extracts.

Function of SR proteins in differential recognition of authentic and cryptic splice sites. Our results show that SR proteins SC35, p54, and ASF/SF2 can inhibit cryptic splice site selection on the β-globin pre-mRNA containing competing 5′ splice sites. This observation suggests that SR proteins may play a role in enhancing the fidelity of splicing in vivo by differentiating authentic splice sites from cryptic splice sites and inhibiting the use of cryptic splice sites. It is possible that SR proteins also interact with sequence elements outside the 5′ splice site consensus region and interact with other protein factors and that combinatorial effects of both RNA-protein and protein-protein interactions determine the usage efficiency of a certain splice site. The role of SR proteins in maintaining splicing fidelity in vivo needs to be further studied to determine sequence elements and protein factors involved.

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