MOLECULAR AND CELLULAR BIOLOGY, Nov. 2003, p. 7437–7447 Vol. 23, No. 21
0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.21.7437–7447.2003

Regulation of Alternative Splicing by SRrp86 and Its Interacting Proteins

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Received 29 April 2003/Returned for modification 2 June 2003/Accepted 29 July 2003

SRrp86 is a unique member of the SR protein superfamily containing one RNA recognition motif and two serine-arginine (SR)-rich domains separated by an unusual glutamic acid-lysine (EK)-rich region. Previously, we showed that SRrp86 could regulate alternative splicing by both positively and negatively modulating the activity of other SR proteins and that the unique EK domain could inhibit both constitutive and alternative splicing. These functions were most consistent with the model in which SRrp86 functions by interacting with and thereby modulating the activity of target proteins. To identify the specific proteins that interact with SRrp86, we used a yeast two-hybrid library screen and immunoprecipitation coupled to mass spectrometry. We show that SRrp86 interacts with all of the core SR proteins, as well as a subset of other splicing regulatory proteins, including SAF-B, hnRNP G, YB-1, and p72. In contrast to previous results that showed activation of SRp20 by SRrp86, we now show that SAF-B, hnRNP G, and 9G8 all antagonize the activity of SRrp86. Overall, we conclude that not only does SRrp86 regulate SR protein activity but that it is, in turn, regulated by other splicing factors to control alternative splice site selection.

In higher eukaryotes, the coding regions (exons) of nearly all genes are split, and the intervening sequences (introns) must be precisely and efficiently removed during splicing to allow correct protein expression (7). To add more complexity, a great number of genes contain multiple exons and introns and the choice of exon selection can vary in a tissue- or development-specific fashion (20, 43, 54). It has been estimated that about 60% of human genes undergo alternative splicing (6, 12), but even this number is likely to be an underestimate since cDNA databases are not complete and alternative splicing events in noncoding regions are relatively underreported.

Alternative splicing can be regulated by both *cis*-acting RNA elements, such as splicing enhancers and splicing silencers, and *trans*-acting factors, of which the SR protein family is perhaps the best studied. SR proteins are characterized by one or two RNA recognition motifs at the N terminus and a C-terminal region rich in arginine-serine dipeptides (RS domain). They are essential splicing factors that participate in multiple steps of splicing (19, 21, 38). In early spliceosome formation (E complex), SR proteins enhance the binding of U1 snRNP to 5′ splice sites through interaction with U1 snRNP U1-70K (30, 73). At later steps, SR proteins escort the U4/U6•U5 tri-snRNP into the spliceosome (50) and they play important roles in forming bridge complexes across exons and introns by mediating essential protein-protein interaction across splice sites (1, 29, 49, 66). For alternative splicing, SR proteins most often bind enhancer elements to facilitate the recognition and activation of weak splice sites (5).

The functions of SR proteins partially overlap in that most individual SR proteins can complement splicing-deficient S100 extracts. Consistent with partial redundancy, RNA interference-mediated knockdown of several SR proteins in *Caenorhabditis elegans* led to no observable phenotype (36). However, targeted disruption of ASF/SF2 in chicken DT40 cells (64), RNA interference with ASF/SF2 in *C. elegans* (36), and null alleles of *Drosophila* SR protein B52 all resulted in lethality (48). In addition, SR proteins display substrate specificity and have distinct functions in alternative splicing (19, 23, 61). Therefore, SR proteins do not simply have redundant functions but can act in a variety of ways to regulate splicing.

As key regulators of splicing, SR proteins themselves need to be regulated. The expression levels of some SR proteins are transcriptionally regulated (2, 52), but posttranscriptional control is also important. Many SR protein genes are themselves subject to alternative splicing, often in an autoregulatory manner (25, 59). Phosphorylation-dephosphorylation of the RS domain is also known to affect the activity and subnuclear localization of SR proteins (9, 28, 31, 40, 60, 62, 67, 68, 70). In addition, the effects of SR proteins can be counteracted by the action of other proteins, including the hnRNP A/B proteins (16, 39, 69), p32 (44), RSF1 (33), and two recently identified SR superfamily members, SRrp35 and SRrp40 (13, 53). These proteins inhibit SR proteins either by interfering with RNA binding or by disrupting crucial protein-protein interactions. The important point is that the activity of SR proteins is tightly controlled at multiple levels.

We have been studying SRrp86, a novel SR-related protein that can regulate the activity of other SR proteins both negatively and positively (3, 4, 34). In both in vitro and in vivo splicing assays, SRrp86 inhibits ASF/SF2, SC35, and SRp55 while activating SRp20. Domain analysis revealed that the C-terminal RS-EK-RS domain is required for full activity while the unique EK domain acts as a splicing inhibitor (4, 34). It appears that SRrp86 regulates SR proteins through direct protein-protein interaction, and we have identified some of the

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specific interaction targets, but the full range of proteins that interact with SRrp86 remain unknown.

To identify proteins that interact with SRrp86 in vivo, we performed a yeast two-hybrid library screen and coimmunoprecipitation experiments coupled to mass spectrometry. Consistent with the regulation of other SR proteins, we found that all of the core SR family members associated with SRrp86. In contrast, only SRp20 and SRp75 associated with a construct lacking the EK domain, indicating that this domain is an important modulator of protein-protein interaction. Besides SR proteins, other splicing factors and RNA-processing factors were also found to associate with SRrp86. In this study, five such proteins, SAF-B (scaffold attachment factor B), hnRNP G, YB-1, p72, and 9G8, were further investigated because they have been previously implicated in splicing regulation (11, 24, 41, 56, 63). By using a CD44 v5 minigene as a splicing reporter, we show that SAF-B, hnRNP G, and 9G8 antagonize the ability of SRrp86 to activate v5 inclusion. In the same assay, YB1 and p72 were found to have no effect. Our findings support the hypothesis that protein-protein interaction underlies the function of SRrp86 and reinforce the notion that combinatorial control of splicing by multiple factors allows precise regulation of alternative splicing.

MATERIALS AND METHODS

Yeast two-hybrid screening. Cloning and library screening were performed as previously described (14). Briefly, SRrp86 was cloned into the bait pBTM116 vector and a HeLa cDNA library was cloned into the pACT-2 vector (Clontech). After double selection based on growth on His^- plates, followed by β-galactosidase activity assays, positive clones were rescued in *Escherichia coli* and sequenced.

Plasmid constructs. pFlag-SRrp86, pFlag-SRrp86ΔEK, and pFlag-SRrp86ΔRS were subcloned from pcDNA-SRrp86, pcDNA-SRrp86ΔEK, and pcDNA-SRrp86ΔRS (4, 34) into the pFlag-CMV2 vector.

The CD44 v5 minigene, SAF-B, and hnRNP G cDNA clones for in vitro translation and in vivo expression were generously provided by S. Stamm (University of Erlangen, Erlangen, Germany). The 9G8 cDNA clone for in vitro translation was a gift from J. Stévenin (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France). The 9G8 cDNA clone for in vivo expression was a gift from J. F. Cáceres (MRC Human Genetics Unit, Edinburgh, United Kingdom). The YB-1 and p72 cDNA clones for both in vitro translation and in vivo expression were gifts from S. M. Berget (Baylor College of Medicine) and S. Kato (University of Tokyo).

Immunoprecipitation. Human embryonic kidney (HEK) 293T cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transfections were performed on 100-mm-diameter plates at 70% confluency with 10 μg of pFlag, pFlag-SRrp86, pFlag-SRrp86ΔEK, or pFlag-SRrp86ΔRS and 30 μl of Trans IT-LT2 (Mirus). At 24 h after transfection, cells were lysed in IP buffer (50 mM Tris [pH 7.4], 100 mM KCl, 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml) for 20 min on ice. About 850 μg of total cell lysate was incubated with 20 μl of M2 anti-Flag-conjugated agarose beads (Sigma) for 2 h at 4°C and washed three times in IP washing buffer (50 mM Tris [pH 7.4], 100 mM KCl, 0.1% NP-40). Proteins were eluted with 8 M urea–50 mM Tris, pH 8.0. One-third of the eluate was analyzed on 15% sodium dodecyl sulfate (SDS) gels; the rest was analyzed by mass spectrometry. For high-salt washes, 250 mM KCl was included.

Western blot assays. Proteins eluted from agarose beads were separated on 15% SDS gels and transferred to nitrocellulose membranes (Millipore). Western analyses were performed as previously described (4), except that the ani-SRp20 antibody (Zymed) was diluted 1:4,000.

Mass spectrometry. Direct analysis of large protein complexes was used to identify proteins copurifying with SRrp86 as described previously (51). Acquired tandem mass spectral data were searched against a human subset of the National Center for Biotechnology Information nonredundant protein database. Data processing of the SEQUEST output files into a list of proteins copurifying with SRrp86 was performed as described previously (35).

TABLE 1. Identification of SRrp86-interacting proteins by yeast two-hybrid library screen

Protein name	No. of clones
SC35	20
SRp46	1
9G8	1
Human immunodeficiency virus Tar RNA binding protein	1
Pin-1	1
Diacylglycerol kinase Δ	1
SH3-containing protein SH3GLB2	1
Unknown	4

Protein-protein interaction in vitro. Pulldown assays were performed as described previously (3). Briefly, 20 μl of rabbit immunoglobulin G (IgG)-conjugated agarose beads (Sigma) was incubated with in vitro-translated, ^{35}S-labeled proteins in the absence or presence of 2 μg of an SRrp86 construct containing two IgG binding sites from protein A fused to the carboxy terminus (47). Bound proteins were extensively washed and eluted in Laemmli loading buffer and resolved on 12% SDS gels.

In vivo splicing assays. COS7 cells were grown in DMEM/F12 medium with 10% FBS. Transfections were performed on 60-mm-diameter plates at 80% confluency with 1 μg of the CD44 v5 minigene (32) and the indicated cDNAs with 2 μl of Lipofectamine and 1 μl of plus reagent in accordance with the manufacturer's (Invitrogen) instructions. Cells were harvested after 48 h, and total RNA was isolated with Tri Reagent (Molecular Research Center). Reverse transcription (RT)-PCR was performed as previously described (3). Similarly, HeLa cells (grown in DMEM–10% FBS) were transfected with 1 μg of pMTE1A (71) and 200 ng of the indicated cDNA construct. Primer sequences and PCR conditions were the same as those previously described (10, 32), except that the PCR was limited to 18 cycles (CD44 v5) and 23 cycles (E1A) with end-labeled radioactive 5′ primers. The cycle numbers and starting RNA amounts for analysis of CD44 splicing were optimized in separate titration experiments (data not shown). Analysis of E1A splicing was done as previously described (8, 39). Products were resolved on 6% denaturing gels, visualized, and quantitated with a phosphorimager.

RESULTS

SC35 interacts with SRrp86. We previously showed that SRrp86 could regulate the activity of different SR proteins, and we hypothesized that such effects were due to protein-protein interaction (3, 4). To identify proteins that interact with SRrp86 in vivo, we performed a yeast two-hybrid cDNA library screen with full-length SRrp86 as bait. Approximately five million colonies were screened, representing five times the library size. One hundred fifteen positive clones were identified (β-galactosidase$^+$ His^+), and 30 random colonies were sequenced. Twenty-two of these were found to encode SR proteins, the great majority being SC35 with one SRp46 and one 9G8 clone (Table 1). Of note, the SC35 clones contained a mixture of different 5′ untranslated regions, excluding the possibility that a single SC35 cDNA might be overrepresented in the library. The prevalence of SR proteins in the two-hybrid library screen strongly indicates that these proteins indeed interact with SRrp86. Besides SR proteins, an SR-like protein, Pin1, was also identified. Pin-1 was first characterized as a cell adhesion protein and was recently reported to interact with RNPS1 and regulate alternative splicing in vivo (65). Interestingly, most of the proteins shown in Table 1 contain an RS-rich region, suggesting that the interaction with SRrp86 is likely mediated by such domains. Because the great majority of the two-hybrid clones encoded SC35 and because there are obvious technical limitations that could prevent identification of

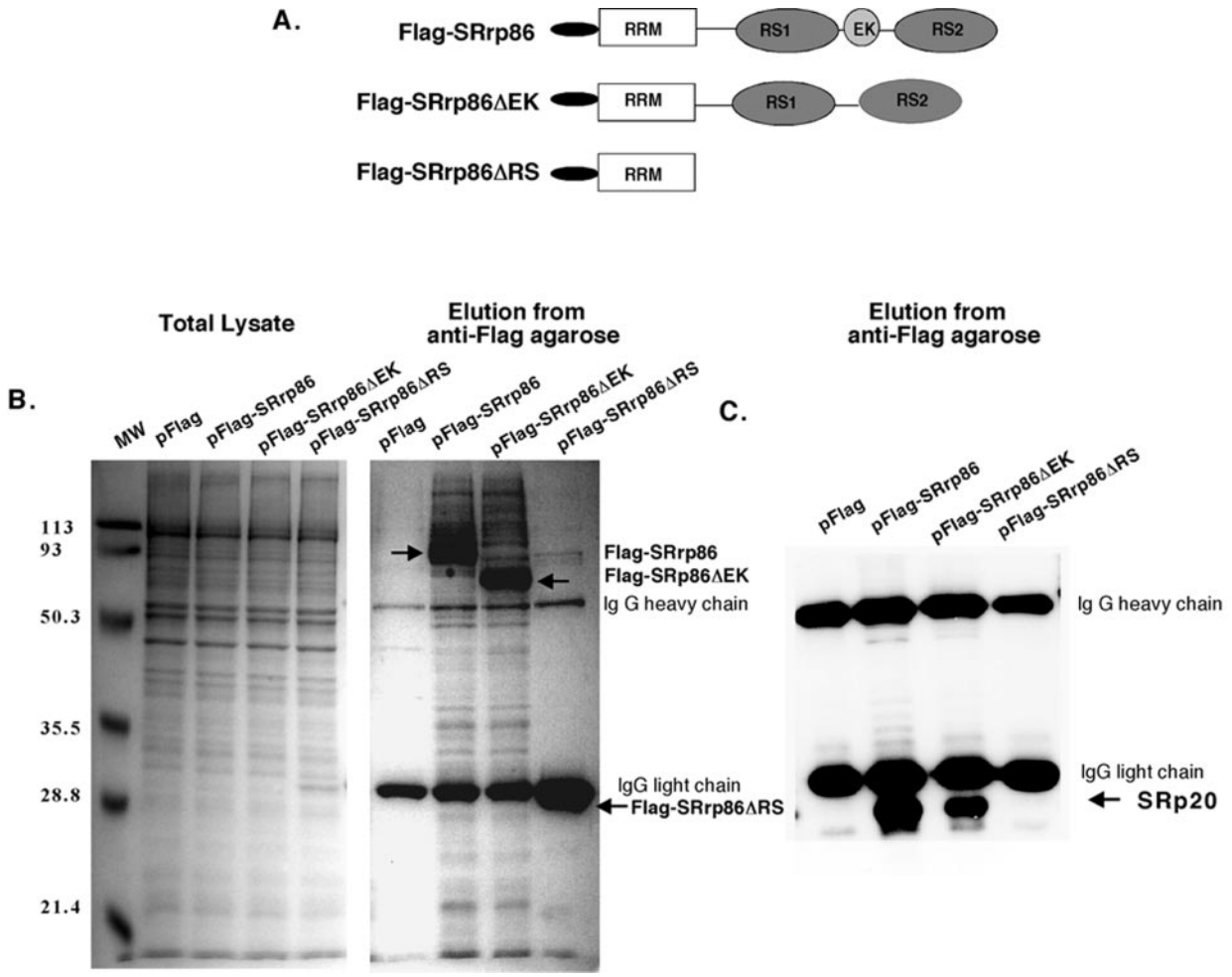

FIG. 1. Coimmunoprecipitation of SRrp86-associated proteins. HEK 293T cells were transfected with 10 μg of empty Flag vector or the different Flag-tagged SRrp86 constructs shown in panel A. Cells were harvested after 24 h, and about 850 μg of total cell lysate was immunoprecipitated with anti-Flag M2-conjugated agarose beads and washed in low-salt buffer containing 100 mM KCl. Proteins were eluted in 8 M urea and resolved on 15% SDS gels. (B) Five micrograms of total cell lysate (left side) and one-third of the bound proteins (right side) were resolved on 15% SDS gels and stained with Coomassie blue. Arrows mark the positions of the overexpressed constructs and anti-Flag IgG chains. Note that Flag-SRrp86ΔRS comigrates with the light chain of IgG. The values on the left are the sizes of the markers (lane MW) in kilodaltons. (C) One-third of the eluates was subjected to Western blot analysis with a monoclonal antibody against SRp20. The anti-Flag IgG chains were recognized by the secondary antibody because they are from a mouse.

other target proteins with such screens, we also used antibodies against epitope-tagged versions of SRrp86 and performed immunoprecipitation of cell lysates coupled to mass spectrometry to identify associated proteins.

SRp20 interacts with SRrp86 in vivo. Three epitope-tagged versions of SRrp86 were created with an N-terminal Flag tag. These include full-length SRrp86 and versions lacking the EK (Flag-SRrp86ΔEK) and RS-EK-RS (Flag-SRrp86ΔRS) domains (Fig. 1A). Addition of the Flag tag did not affect the function of these proteins, since all three constructs showed the same activity in splicing assays as did untagged versions (data not shown). To identify associated proteins, HEK 293T cells were transfected and immunoprecipitation experiments were carried out with anti-Flag M2-conjugated agarose beads. All three constructs were expressed at approximately similar levels, but it was clear that more proteins associated with SRrp86 and SRrp86ΔEK than with SRrp86ΔRS (Fig. 1B). Because we had previously shown that SRrp86, but not SRrp86ΔRS, interacts with and activates SRp20, we performed Western blot assays of the proteins associated with the epitope-tagged versions of SRrp86 with antibodies against SRp20. As shown in Fig. 1C, SRp20 associated with SRrp86 and SRrp86ΔEK but did not associate with SRrp86ΔRS. Thus, the coimmunoprecipitation experiments with epitope tags recapitulated previous results, enabling the use of mass spectrometry to further identify SRrp86-interacting proteins.

Proteins associated with SRrp86 in vivo. Immunoprecipitation reaction mixtures were washed under low-salt (100 mM KCl) and high-salt (250 mM KCl) conditions, and eluted proteins were subjected to mass spectrometry with direct analysis of large protein complexes (35). As controls, very few proteins were identified with either the SRrp86ΔRS construct or the empty vector (data not shown), even under low-salt washing conditions. In contrast, many proteins were found to associate

with full-length SRrp86. Some of these proteins were background proteins that could be detected even with the empty vector, whereas several other proteins were obviously unrelated to splicing or RNA processing and were often identified through the presence of only a single peptide. Multiple ribosomal proteins were also identified, although their relevance is unknown. Ribosomal proteins have also been identified in purified spliceosomes (27, 45). For our purposes, those SRrp86-associated proteins best supported by the tandem mass spectral data, including those identified in the two-hybrid screen or other assays (3), are shown in Table 2 grouped into seven classes: snRNPs, hnRNPs, SR proteins, helicases, RNA-processing factors, transcription factors, and kinases. Many of the associated proteins are either core SR proteins or members of the hnRNP family. These two groups of proteins are the most extensively studied proteins related to the control of alternative splicing. Their identification provides valuable support for the hypothesis that SRrp86 regulates splicing through protein-protein interaction with other regulatory proteins. Also, as described above, phosphorylation of SR proteins can dramatically alter protein activity and a well-characterized SR protein kinase (SRPK1) was also found to associate with SRrp86.

Besides SR proteins and hnRNPs, a variety of other proteins implicated in splicing were also identified, including snRNPs and RNA helicases. Several RNA-processing factors and transcription factors were also identified, some of which have been linked to alternative splicing regulation (see below). The exact relevance of association between SRrp86 and some of these proteins remains to be determined, but the fact that some of these proteins are apparently involved in other nuclear events supports the notion that many nuclear events are coupled (37). Overall, the interaction data strongly suggest that a network of interactions between SRrp86 and other factors could play a key role in the regulation of splice site selection.

Protein-protein interactions involving the EK domain. Since the EK domain was shown to inhibit splicing by apparently titrating one or more splicing factors (34), we compared proteins associated with the SRrp86 and SRrp86ΔEK constructs. Strikingly, only 15 proteins (Table 2, footnote *a*) were found to associate with SRrp86ΔEK under low-salt washing conditions. In contrast to the situation with the full-length protein, only two SR core proteins (SRp20 and SRp75) and three hnRNPs associated with SRrp86ΔEK. Interestingly, one protein, Tra2α, was found to interact with only the SRrp86ΔEK construct whereas the closely related protein Tra2β3 was identified with SRrp86. The change in the number and identity of proteins that associate with the full-length and ΔEK constructs suggests that the EK domain might serve in some way to modulate the interaction of SRrp86 with splicing regulatory proteins. Incubation of splicing extracts with peptides derived from the EK region inhibited splicing (34), and it appears that such inhibition is due to titration of one or more SR proteins.

SRrp86 stably associates with SAF-B, hnRNP G, YB-1, p72, SRp20, and 9G8. The large number of proteins found to interact with SRrp86 under low-salt conditions suggests that SRrp86 might be involved in multiple pathways but could also reflect possible nonspecific interactions or multiple weak interactions. To increase the stringency required for interaction, immunoprecipitation pellets were washed in buffer containing 250 mM KCl. As expected, fewer proteins and lower overall concentrations of each protein were found to associate with SRrp86 under these conditions (Table 3). Nevertheless, even when identified with only a single peptide, each of the proteins shown in Table 3 showed a statistically significant association

TABLE 2. Proteins associated with SRrp86 (low salt)

Group and accession no.	Protein (no. of peptide hits)
snRNPs	
P13641	Sm D1 (1)
P43331	Sm D3 (1)
Q12874	SF3a60 (SAP 61) (1)
Q15393	SF3b130 (SAP 130) (2)
Q15029	U5 snRNP 116KD (1)
hnRNPs	
P09651	hnRNP A1[a] (3)
P22626	hnRNP A2/B1 (6)
P51991	hnRNP A3 (2)
P07910	hnRNP C1/C2 (4)
Q14103	hnRNP D0 (2)
P38159	hnRNP G[a] (2)
Q07244	hnRNP K (3)
Q00839	hnRNP U (SAF-A)[a] (2)
P31943	hnRNP H (4)
SR proteins	
AF459094	SRrp86/SRrp508[a] (5)
Q07955	ASF/SF2 (8)
Q01130	SC35 (1)
P23152	SRp20[a] (3)
Q08170	SRp75[a] (3)
Q13243	SRp40 (4)
Q13247	SRp55 (5)
Q13242	SRp30c (3)
Q16629	9G8 (5)
AF057159	Tra2-β3[a,b] (2)
Helicases	
Q13838	Probable ATP-dependent RNA helicase p47 (3)
Q92841	DEAD-box protein p72 (1)
O00571	Helicase-like protein 2 (3)
RNA-processing factors	
AY048592	TLS-associated serine-arginine protein (2)
Q07021	p32 (1)
P11940	PABP 1[a] (10)
Q13310	PABP 4[a] (3)
P09874	PARP 1 (2)
S64732	SAF-B (3)
P23246	PSF[a] (3)
AL590446	Pin-1 (1)
Q15717	Hu-R (2)
O95218	Zinc finger protein 265[a] (2)
P98175	RNA binding protein 10 (6)
P06748	Nucleophosmin (3)
P19338	Nucleolin (11)
Transcription factors	
AF152961	FACTp140 (3)
A54857	NF-AT 45K[a] (5)
Q08945	SSRP1[a] (5)
P16991	YB-1[a] (2)
AF117756	TRAP150[a] (3)
Kinases and associated factors	
S45337	SRPK1 (3)
P19138	Casein kinase II (3)
P25388	Rack-1 (7)
Q9upe1	Serine/threonine protein kinase 23 (3)

[a] Also found to associate with SRrp86ΔEK.
[b] Tra2-β3 associated with SRrp86, and Tra2-α associated with SRrp86ΔEK.

TABLE 3. Proteins associated with SRrp86 (high salt)

Group and accession no.	Protein (no. of peptide hits)
hnRNPs	
P38159	hnRNP G (1)
O60812	hnRNP C like (2)
Q00839	hnRNP U (SAF-A) (1)
SR proteins	
AF459094	SRrp86/SRrp508 (2)
P23152	SRp20 (1)
Q16629	9G8 (1)
Helicase	
Q92841	DEAD-box protein p72 (1)
RNA-processing factors	
S64732	SAF-B (1)
P11940	PABP1 (1)
Transcription factors	
P16991	YB-1 (2)
AF117756	TRAP150 (2)

with SRrp86. Among these, six proteins (SAF-B, hnRNP G, YB-1, p72, SRp20, and 9G8) were especially interesting because they have been previously implicated in splicing regulation (11, 15, 24, 41, 56). Before proceeding with functional splicing assays, we first confirmed the immunoprecipitation results with in vitro pulldown assays with an epitope-tagged version of SRrp86 containing two copies of an IgG binding site from protein A (47). Individual ^{35}S-labeled proteins were incubated with recombinant, tagged SRrp86, and interacting proteins were captured with IgG-conjugated beads. As shown in Fig. 2, no interaction between the IgG-conjugated beads and

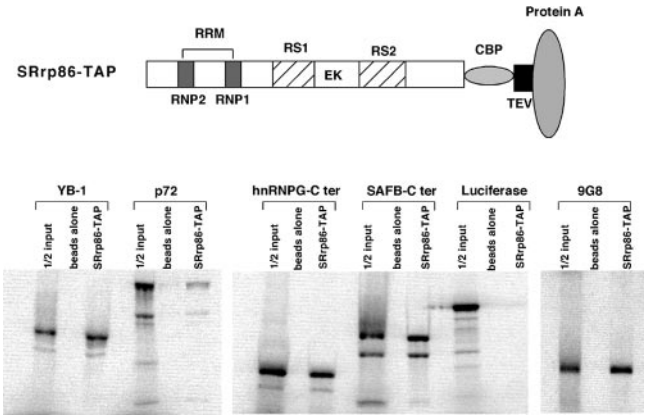

FIG. 2. In vitro interaction between SRrp86 and SAF-B, hnRNP G, YB-1, p72, or 9G8. Epitope-tagged SRrp86 was created by fusing the TAP tag (47) to the carboxy terminus of SRrp86. This tag consists of a calmodulin binding peptide and two IgG binding sites from protein A separated by a tobacco etch virus protease site. For our purposes, only the IgG binding sites were used. TAP-tagged SRrp86 was purified from baculovirus-infected Hi5 cells and incubated with the indicated ^{35}S-labeled protein. Associated proteins were captured by passage over IgG-conjugated agarose beads. Bound proteins were eluted and analyzed on SDS gels. As controls, in vitro-translated luciferase was also incubated with SRrp86-TAP and background binding to the beads alone was determined for each reaction.

any of the constructs could be detected (beads alone), and further, no interaction was detected between SRrp86 and a luciferase control protein. In contrast, SAF-B, hnRNP G, YB-1, and 9G8 all interacted strongly with SRrp86 TAP while p72 interacted somewhat more weakly. The weaker association between p72 and SRrp86 is consistent with the fact that even under low-salt washing conditions, only a single p72 peptide was identified. This suggests that p72 does indeed interact but that the association is weak, barely above the background. None of the interactions described above appear to be mediated by RNA, since pretreatment of extracts with RNase A had little effect (data not shown). Note that only the C termini of hnRNP G and SAF-B were used in the pulldown assays since they were more efficiently translated (personal communication), and most importantly, the C terminus of SAF-B had previously been shown to mediate the interaction with RNA polymerase II-CTD and a subset of SR proteins in both yeast two-hybrid and in vitro pulldown assays (41). The C-terminal domains of both SAF-B and hnRNP G also interact with SRrp86.

Regulation of alternative splicing by SRrp86 and interacting proteins. To examine whether SAF-B, hnRNP G, YB-1, p72, or 9G8 might act in concert with SRrp86 to regulate alternative splicing, in vivo splicing assays were performed with a CD44 v5 minigene. This construct contains an alternatively spliced variant exon (exon v5) flanked by two constitutively spliced insulin exons (Fig. 3A) (32). Cells were cotransfected with different combinations of these factors, and inclusion of the v5 exon was analyzed by RT-PCR. As shown in Fig. 3B, cotransfection of increasing amounts of SRrp86 cDNA (50 to 800 ng) resulted in a steady increase in v5 inclusion, showing that SRrp86 can individually activate CD44 v5 inclusion in a dose-dependent manner. Activation of v5 inclusion was dependent on the integrity of the RS domains, since no activation was observed with the ΔRS construct, even at much higher concentrations (Fig. 3C). In contrast, deletion of the EK domain (ΔEK) reduced, but did not abolish, such activity.

We next tested whether v5 inclusion would be affected by proteins that interact with SRrp86. Accordingly, SRrp86 (200 ng) was cotransfected with SAF-B, hnRNP G, YB-1, p72, or 9G8, and v5 inclusion was determined. At the concentrations tested, neither YB-1 nor p72 affected v5 inclusion alone or in combination with SRrp86 (data not shown). Similarly, SAF-B alone did not alter v5 inclusion (Fig. 4A), consistent with previous results (57). However, when cotransfected with SRrp86, SAF-B was able to antagonize the effect of SRrp86 in a dose-dependent manner, completely blocking activation at the highest doses tested (Fig. 4A). This suggests not only that can SRrp86 alter SR protein activity but that other splicing regulatory proteins can, in turn, act on SRrp86.

Transfection of 9G8 alone inhibited CD44 v5 inclusion, with significant inhibition observed with as little as 50 ng (Fig. 4B). When 9G8 was cotransfected with SRrp86, a decrease in v5 inclusion was again observed in a dose-dependent manner. Thus, 9G8 can both inhibit CD44 v5 inclusion and antagonize the activity of SRrp86.

The effect of hnRNP G on CD44 v5 inclusion was very similar to that of 9G8, except that only 5 ng of hnRNP G cDNA was needed to inhibit v5 inclusion by about 50% (Fig. 4C). Cotransfection of SRrp86 and hnRNP G showed that

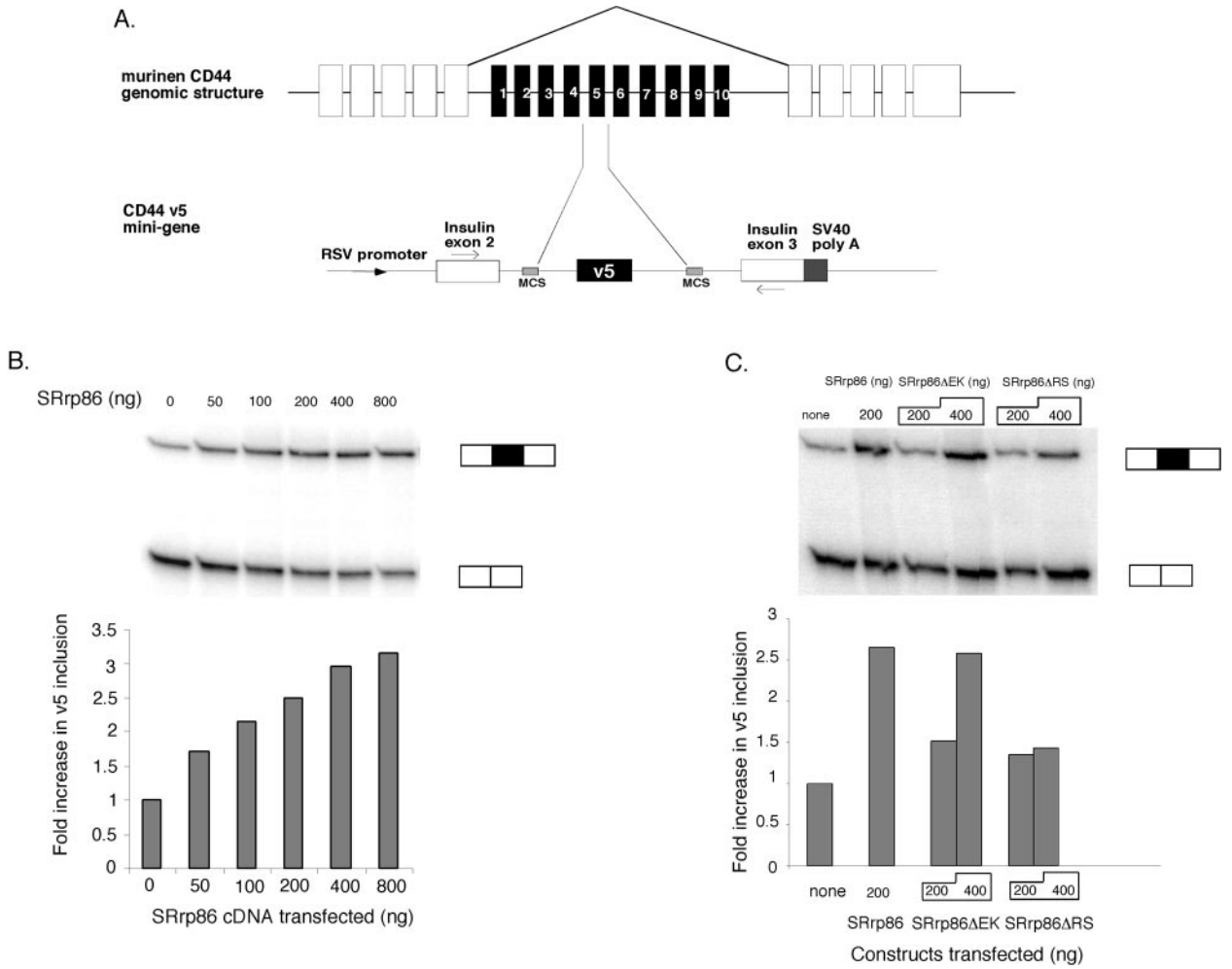

FIG. 3. SRrp86 activates CD44 v5 inclusion in vivo. (A) Schematic diagram of the murine CD44 gene (top) and CD44 v5 minigene (bottom; RSV, Rous sarcoma virus; MCS, multiple cloning site; SV40 poly A, simian virus 40 polyadenylation site). An arrowhead indicates the position of the promoter, and arrows indicate the positions of the PCR primers. (B) Increasing amounts of SRrp86 cDNA were cotransfected with the CD44 v5 minigene into COS7 cells. Spliced products were analyzed by RT-PCR, separated on 6% denaturing gels, and quantified by phosphorimager analysis. The increase in v5 inclusion is graphed below. Exon skipping and inclusion products are diagramed at the right. (C) As in panel B, the effects of increasing amounts of SRrp86ΔEK and SRrp86ΔRS on v5 inclusion were examined in transfected COS7 cells.

hnRNP G also antagonized the effect of SRrp86 in a dose-dependent manner, with almost complete inhibition observed at only 50 ng of hnRNP G. To test whether such inhibition could be reversed by adding more SRrp86, increasing amounts of SRrp86 cDNA were cotransfected with 50 ng of hnRNP G (Fig. 4D), and as expected, more v5 inclusion was observed as more and more SRrp86 was cotransfected. Therefore, among the different proteins tested, CD44 v5 inclusion seems most sensitive to the levels of hnRNP G.

Antagonism between SRrp86 and 9G8 is not substrate specific. The finding that 9G8 could antagonize SRrp86 was very interesting since we previously showed that SRrp86 could negatively regulate SR proteins whereas the converse had not been observed. To examine whether such regulation of SRrp86 might be substrate specific, we used a second substrate. The E1A pre-mRNA contains three alternative 5′ splice sites leading to 13S, 12S, and 9S mRNA. As shown in Fig. 5, cotransfection of increasing amounts of SRrp86 caused a steady increase in the level of 9S mRNA because of increasing use of the most distal 5′ splice site. Concomitantly, use of the most proximal 5′ splice site was reduced, as reflected by the 12S/13S ratio.

To test the effects of individual and combinations of factors on 5′ splice site selection with the E1A substrate, cotransfection experiments were again performed (Fig. 6). In complete contrast to the effects observed with SRrp86, transfection of 9G8 significantly decreased 9S and 12S splicing and activated 13S. When SRrp86 was cotransfected with 9G8, the ability of SRrp86 to activate 9S splicing was greatly reduced, with 13S mRNA being the predominant product, showing that antagonism between SRrp86 and 9G8 is not substrate specific. For YB1 and p72 alone, no effects on either 9S production or the 12S:13S ratio were observed (Fig. 6). When YB-1 was cotransfected with SRrp86, there was a slight reduction in the stimulation of the 12S/13S ratio by SRrp86, but no effect on 9S production was observed. Similarly, no effect of p72 on SRrp86

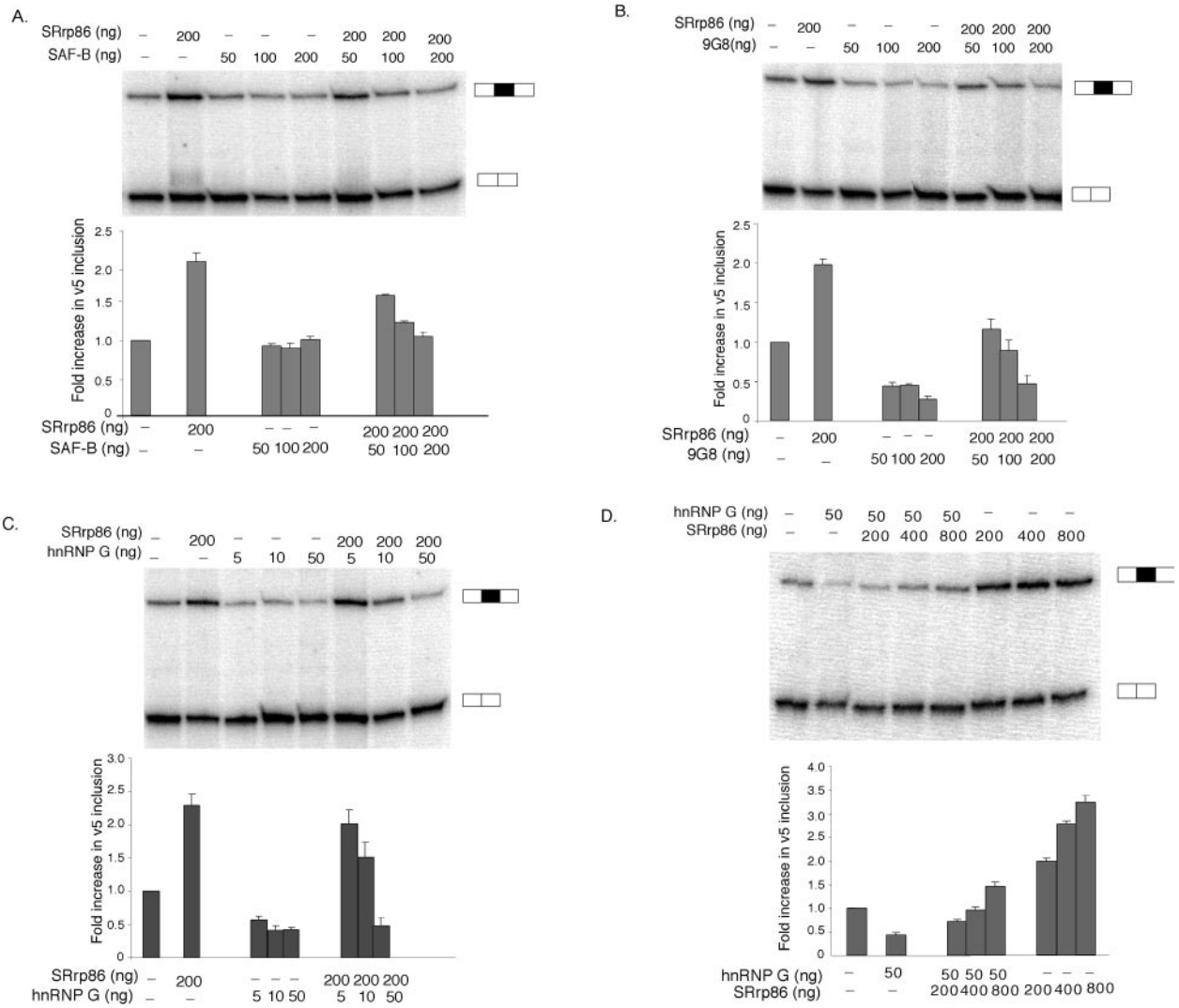

FIG. 4. SAF-B, hnRNP G, and 9G8 antagonize SRrp86 in promoting v5 inclusion. (A to D) Increasing amounts of the indicated constructs were cotransfected with the CD44 v5 minigene into COS7 cells, and v5 inclusion was analyzed as described in the legend to Fig. 3. Representative gels are shown, and the increase in v5 inclusion was determined from multiple independent transfection experiments.

was observed. Neither YB-1 nor p72 is therefore able to alter SRrp86 activity, at least for the two splicing substrates tested. In contrast, 9G8 acts opposite to SRrp86 and can even antagonize the effect of SRrp86.

DISCUSSION

Most commonly, alternative splicing is controlled by coordinate regulation between positive and negative factors. We first identified SRrp86 as a splicing regulator able to both activate and inhibit the activity of other SR proteins. In this report, we identify proteins that interact with SRrp86 with a yeast two-hybrid cDNA library screen and coimmunoprecipitation coupled to mass spectrometry. We found that all of the core SR proteins, many hnRNPs, and a limited number of other splicing regulatory proteins associate with SRrp86. The most stable interacting proteins, at least as defined by the ability to withstand increasing salt concentrations, include SAF-B, hnRNP G, YB-1, SRp20, and 9G8. We had previously shown that SRrp86 could activate SRp20, and we now show that SAF-B, hnRNP G, and 9G8 can antagonize SRrp86. Even though YB-1 and, to a much lesser extent, p72 interact with SRrp86, we were unable to observe any effect of these proteins on SRrp86. From the effects observed with SRp20, SAF-B, hnRNP G, and 9G8, we conclude that SRrp86 not only regulates SR protein activity but is, in turn, regulated by other splicing factors. This supports the idea that overall regulation of alternative splicing is mostly combinatorial, mediated by multiple protein-protein and, from other studies, protein-RNA interactions. In this manner, control of gene expression by alternative splicing generates great protein diversity without the need for transcript-specific regulatory proteins, allowing complexity without an overly burdensome genome size.

SRrp86-SR protein interaction. To identify the full range of proteins that might interact with SRrp86, we first set up a yeast two-hybrid cDNA library screen with full-length SRrp86 as

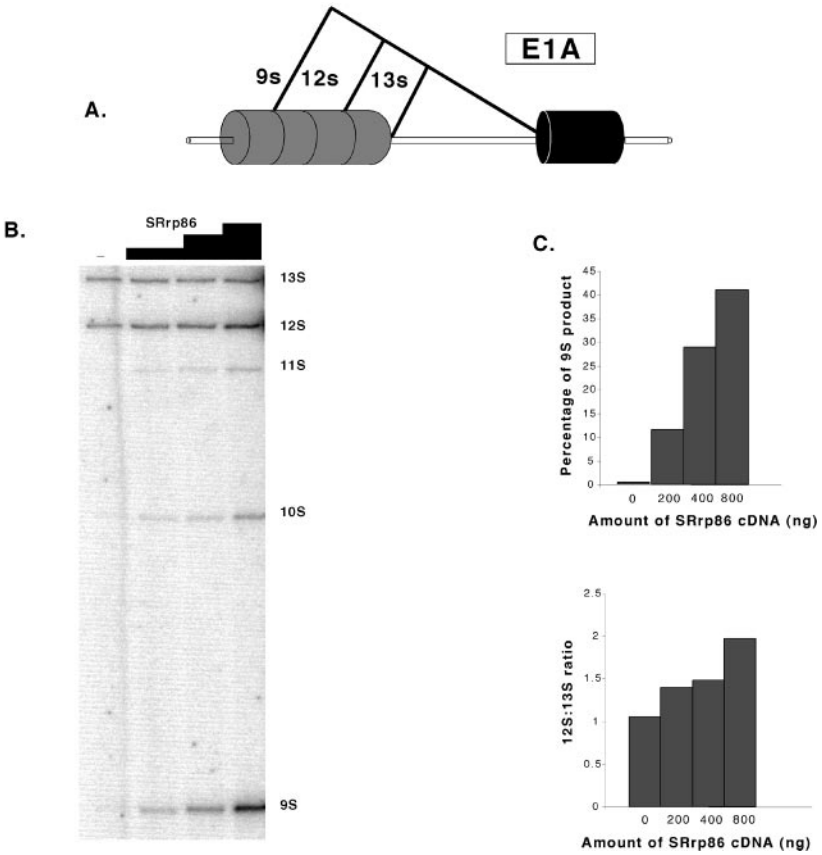

FIG. 5. SRrp86 activates the distal 5′ splice site of the E1A minigene. (A) The E1A minigene contains three alternative 5′ splice sites and a common 3′ splice site to generate 9S, 12S, and 13S mRNAs, as shown. (B) Increasing amounts of SRrp86 cDNA were cotransfected with the E1A minigene, and spliced products were analyzed by RT-PCR, separated on 6% denaturing gels, and quantified by phosphorimager analysis. The values on the right indicate the sedimentation values for adenovirus E1a mRNAs. The increase in 9S mRNA and the 12S/13S ratio are shown in panel C.

bait. Three SR proteins were identified, SC35, 9G8, and SRp46, although the overwhelming interacting partner was clearly SC35. Interestingly, of the few non-SR proteins identified in the two-hybrid screen, most contained extensive RS repeats, implying that such regions are central to protein-protein interactions involving SRrp86. Consistent with this, all of the core SR proteins were identified by coimmunoprecipitation coupled to mass spectrometry. An open question is whether all of these interactions are direct or possibly mediated by RNA. We favor the former because treatment with RNase did not abolish in vitro interaction and previous experiments have also supported direct interaction (3, 4). Nevertheless, it remains possible that additional interactions mediated by RNA could expand the range of proteins that interact with SRrp86.

A network of splicing regulators. Six proteins (SAF-B, hnRNP G, YB-1, p72, SRp20, and 9G8) that interact with SRrp86 were examined in more detail since they have been proposed to be involved in splicing regulation. SAF-B was first identified on the basis of binding to scaffold or matrix attachment regions (S/MAR) (46). It colocalizes with splicing factors in nuclear speckles and interacts with several SR proteins through its C-terminal domain (41). YB-1 is a multifunctional protein that binds to promoter regions in the nucleus to activate transcription (42), binds to mRNA in the cytoplasm, regulating stability and translation efficiency (17, 18), and can also bind splicing enhancer elements to stimulate CD44 exon v5 inclusion (56). hnRNP G is a member of the hnRNP family and has been shown to interact with Tra2β and SRp30c (15). p72, although it interacted the weakest with SRrp86, is an ATP-dependent DEAD-box RNA helicase that can activate inclusion of CD44 variant exons 4 and 5 (24). Both SRp20 and 9G8 are canonical SR proteins that play multiple roles in both constitutive and alternative splicing (11, 26). With the exception of YB-1 and p72, we show that coexpression of each of these proteins with SRrp86 leads to differential effects on alternative splicing. SAF-B, hnRNP G, and 9G8 were all able to inhibit SRrp86, whereas SRrp86 activates SRp20 (3). Altogether, the data suggest a network of protein-protein interactions that lead to precise fine tuning of alternative splicing events.

Mechanistically, determination of the exact function of SRrp86 requires additional work but it appears to modulate protein-protein interactions that define bridge complexes across introns and exons. Such complexes have been proposed to help define exons and introns linking protein complexes across splice sites. For alternative splicing events, defining such exons can be difficult because the accompanying splice sites are often weak. Splicing enhancers apparently exert their effects by

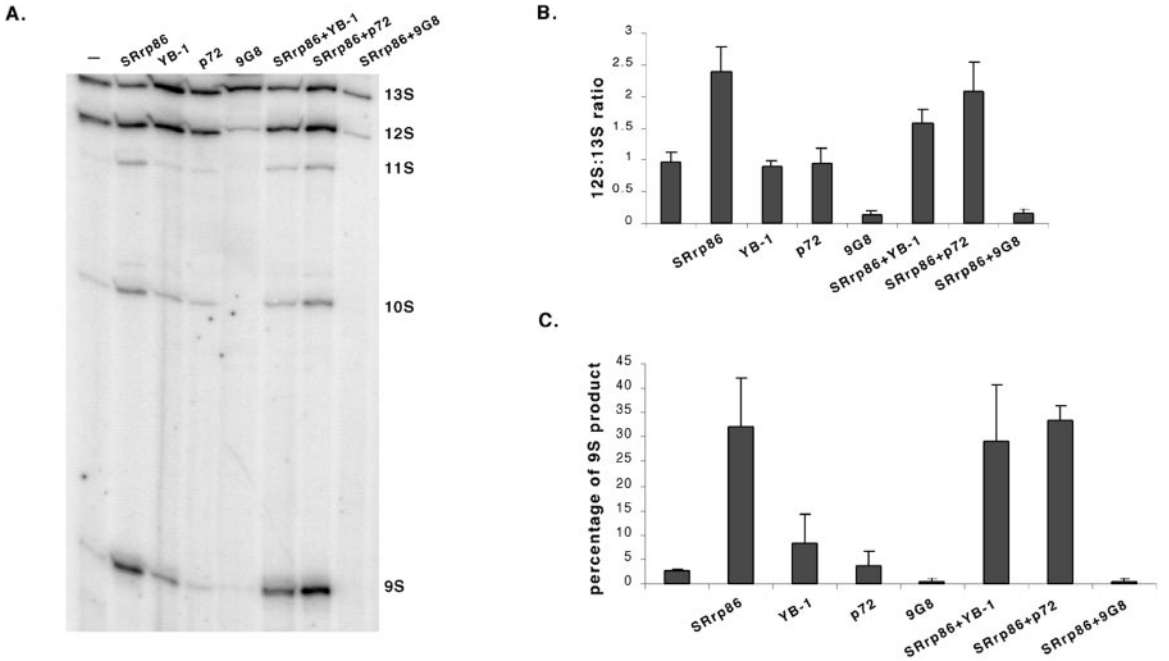

FIG. 6. 9G8 antagonizes SRrp86. (A) HeLa cells were transfected with the E1A minigene and 200 ng of the indicated constructs. Spliced products were analyzed by RT-PCR, separated on 6% denaturing gels, and quantified by phosphorimager analysis. The fold increase in the 12S/13S mRNA ratio is graphed in panel B, and the increase in 9S mRNA is shown in panel C. Averages and standard deviations were determined from multiple independent transfection experiments.

helping to overcome weak splicing signals through the recruitment of SR proteins, ultimately enabling tissue-, cell-, or development-specific activation of splicing. SRrp86 may exert its effects by activating or repressing the formation of bridge complexes by modulating interactions between splicing regulatory proteins at specific splice sites and/or enhancer elements.

SRrp86: a component of the spliceosome? Under low-salt washing conditions, we found that a few snRNPs associated with SRrp86 although when we lowered our standards to include single peptide hits, many more were identified (data not shown). Several recent studies have analyzed the protein composition of purified spliceosomes by mass spectrometry (27, 45, 72). Although many SR proteins have been identified in active spliceosomes, only one group identified SRrp508, the human homolog of SRrp86 (45). It is possible that SRrp86 only transiently associates with the spliceosome. We favor this possibility because it is supported by the fact that antibodies against SRrp86 are not able to immunoprecipitate any of the U snRNAs (data not shown).

The EK domain modulates protein-protein interaction. Previously, we found that deletion of the EK domain converted SRrp86 from a splicing inhibitor to a splicing activator (34). The EK domain is highly polar, with alternating positively and negatively charged amino acids mimicking hyperphosphorylated RS domains. Similar domains have been found in proteins involved in splicing, such as the RNA helicase Hel117 (58), U1-70K (55), YT521-B (22), and SAF-B (41). However, none of these proteins are EK rich and instead are DR or ER rich. The exact function of D/ER regions is not known, but it has been shown that they contribute to protein-protein interactions, at least for YT521-B and SAF-B (22, 41). Consistent with this, SAF-B was found to interact with SRrp86, but not with SRrp86ΔEK. The EK domain might provide a secondary level of regulation to define the scope and strength of protein-protein interactions involving SRrp86.

ACKNOWLEDGMENTS

We thank Sue Berget, Javier Cáceres, James Stévenin, Stefan Stamm, and Shigeaki Kato for vectors and constructs.

This work was supported by National Institutes of Health grant GM62487.

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