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Regulation of alternative splicing by SRrp86 through coactivation and repression of specific SR proteins

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ABSTRACT

SRrp86 is an 86-kDa member of the SR protein superfamily that is unique in that it can alter splice site selection by regulating the activity of other SR proteins. To study the function of SRrp86, inducible cell lines were created in which the concentration of SRrp86 could be varied and its effects on alternative splicing determined. Here, we show that SRrp86 can activate SRp20 and repress SC35 in a dose-dependent manner both in vitro and in vivo. These effects are apparently mediated through direct protein–protein interaction, as pull-down assays showed that SRrp86 interacts with both SRp20 and SC35. Consistent with the hypothesis that relatively modest changes in the concentration or activity of one or more splicing factors can combinatorially regulate overall splicing, protein expression patterns of SRrp86, SRp20, and SC35 reveal that each tissue maintains a unique ratio of these factors. Regulation of SR protein activity, coupled with regulated protein expression, suggest that SRrp86 may play a crucial role in determining tissue specific patterns of alternative splicing.

Keywords: alternative splicing; pre-mRNA splicing; SR proteins; SRrp86

INTRODUCTION

The coding regions of nearly all eukaryotic genes contain intervening sequences (introns) that must be efficiently and precisely removed to allow translation of functional proteins (Burge et al., 1999; Hastings & Krainer, 2001). For many genes, splicing results in the joining of all exons, but recent genomic analyses suggest that greater than 60% of the known genes in higher eukaryotes utilize alternative splicing to generate multiple protein products from a single gene (International Human Genome Sequencing Consortium, 2001). Further, it has been estimated that up to 15% of characterized genetic diseases involve mutations that cause defects in splicing (Krawczak et al., 1992). Most commonly, such mutations disrupt splice sites and lead to exon skipping; however, in other cases, changes in alternative splicing patterns are sufficient to cause disease (Phillips & Cooper, 2000; Patton & Smith, 2001). For such a widespread phenomenon, precious little is known about how alternative splicing is regulated (Lopez, 1998; Smith & Valcárcel, 2000).

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The simplest scenario to account for regulated alternative splicing is that the interaction of the general splicing machinery with a particular pre-mRNA defines a characteristic splicing pattern, and that cell-specific regulatory factors are needed to switch the splicing pattern. However, with the possible exception of Nova-1 (Jensen et al., 2000), no clear-cut examples of splicing decisions determined by the presence of a single cell-specific regulator have been identified in mammalian cells. More commonly, regulation of splicing has been found to be due to the combinatorial actions of fairly widely expressed regulatory factors. The two most well-characterized families of proteins that have been implicated in splice site regulation are the hnRNP family and SR proteins. hnRNPs (heterogeneous ribonucleoproteins) are involved in packaging of RNA transcripts, play a crucial role in the transport of spliced mRNAs out of the nucleus, and can regulate alternative splicing, usually by antagonizing other factors or repressing specific splice sites (Mayeda & Krainer, 1992; Valcárcel & Gebauer, 1997; Krecic & Swanson, 1999; Reed & Magni, 2001). SR proteins are characterized by the presence of one or two N-terminal RNA recognition motifs (RRMs) and a C-terminal region rich in serine–arginine (SR) dipeptides (Gravely, 2000). SR proteins are important for both constitutive and alternative splic-

ing and can regulate splice site selection, both positively and negatively (Manley & Tacke, 1996; Cáceres & Krainer, 1997; Gravely, 2000). Because many SR family members can individually complement splicing-deficient S100 cytoplasmic extracts, it first appeared that they might be functionally redundant. Consistent with partial redundancy, blocking the expression of six different SR proteins in *Caenorhabditis elegans* using RNA interference (RNAi) resulted in no observable phenotype (Longman et al., 2000). However, RNAi inhibition of ASF/SF2 in *C. elegans*, targeted disruption of ASF/SF2 in chicken DT40 cells, and null alleles of B52, a *Drosophila* SR protein, all resulted in lethality (Wang et al., 1996; Hoffman & Lis, 2000; Longman et al., 2000). In addition, multiple studies have shown that individual SR proteins display substrate specificity and have distinct functions in alternative splicing (Fu, 1995; Tacke & Manley, 1999; Hastings & Krainer, 2001). Most commonly, SR proteins have been shown to bind to splicing enhancer elements and activate the splicing of exons flanked by weak splice sites (Blencowe, 2000).

Despite the growing body of knowledge concerning the effects of SR proteins on constitutive and alternative splicing, very little is known about how these factors are themselves regulated. SR proteins are subject to phosphorylation and it appears that their phosphorylation state changes as splicing proceeds (Zahler et al., 1993; Mermoud et al., 1994; Roscigno & Garcia-Blanco, 1995; Tacke et al., 1997; Xiao & Manley, 1997; Kanopka et al., 1998). The effects of SR proteins can also be counteracted by the action of the hnRNP A/B proteins, p32, and RSF1 (Mayeda & Krainer, 1992; Mayeda et al., 1994; Yang et al., 1994; Labourier et al., 1999; Petersen-Mahrt et al., 1999). More recently, two additional repressors of SR protein activity have been isolated that are themselves members of the SR protein superfamily (Cowper et al., 2001).

Previously, we identified a novel member of the SR protein superfamily, SRrp86, that is similar to SR proteins in primary amino acid sequence but that lacks activities common to other SR proteins (Barnard & Patton, 2000). Here we show that SRrp86, although unable to affect alternative splicing patterns by itself, can regulate the activity of two other SR proteins (SRp20 and SC35) in a dose-dependent manner by direct protein–protein interaction. Western blots showed that the ratio of these factors changes between tissues, allowing for fine tuned control of tissue-specific alternative splicing by varying the levels of activators and repressors of splice site selection.

RESULTS

SRrp86 activates SRp20 in vitro

Previously, we showed that SRrp86 could inhibit the ability of ASF/SF2, SC35, and SRp55 to rescue splicing deficient S100 extracts and promote alternative splice site selection (Barnard & Patton, 2000). In contrast, SRrp86 activated splicing in S100 extracts when combined with SRp20. To study the effects of SRrp86 and SRp20 on alternative splice site selection, we utilized a pre-mRNA substrate with competing 3′ splice sites and performed in vitro splicing assays in the presence of recombinant SR proteins (Fig. 1). Splicing in HeLa nuclear extract with this substrate resulted primarily in the selection of the distal 3′ splice site (Fig. 1). However, when extracts were supplemented with SRp20, the level of proximal splice site selection increased from 20% to 37%. Further addition of SRrp86 to these reactions caused proximal splice site selection to increase from 37% to 55%. In contrast, supplementation with ASF/SF2 had no further effect and the individual addition of either ASF/SF2 or SRrp86 did not alter proximal splice site selection. Thus, at least for this substrate, SRp20 is able to activate proximal splice site selection and SRrp86 can combine to increase such selection. From multiple supplementation experiments, the relative levels of SRp20 and SRrp86 combined to determine the overall level of in vitro proximal splice site selection (data not shown).

In vivo activation of SRp20 by SRrp86

To test whether SRrp86 might regulate the activity of SR proteins in cells, the Tet-on system (Clontech) was utilized to generate cell lines in which the level of SRrp86 could be controlled by the presence or absence of tetracycline or doxycycline. Figure 2A demonstrates that incubation of one of these cell lines (DBUT5) in the presence of doxycycline resulted in a significant induction of SRrp86 mRNA from the Tet-On allele. Western blots of lysates from these cells showed that a coordinate increase in protein synthesis accompanied the induction of mRNA synthesis in the presence of doxycycline (Fig. 2B). To assay splice site selection in these cells, a minigene containing exons 3–7 of the SRp20 gene was transfected into the DBUT5 cells and the level of exon 4 inclusion was monitored by RT/PCR (Fig. 2C). Exon 4 of the SRp20 gene contains an in frame, premature stop codon and is subject to autoregulatory alternative splicing such that excess SRp20 promotes inclusion of exon 4 and feedback regulates the production of SRp20 protein (Jumaa & Nielsen, 1997). When the reporter minigene containing exons 3–7 was transfected into the DBUT5 cell line, exon 4 was mostly skipped, regardless of the presence or absence of doxycycline (Fig. 2D, left panel). However, DBUT5 cells transfected with both the reporter minigene and a plasmid encoding SRp20 cDNA showed an increase in exon 4 inclusion (to 45%), consistent with autoregulatory control. To determine whether coexpression of SRrp86 and SRp20 would result in increased inclusion of exon 4, DBUT5 cells were transfected with

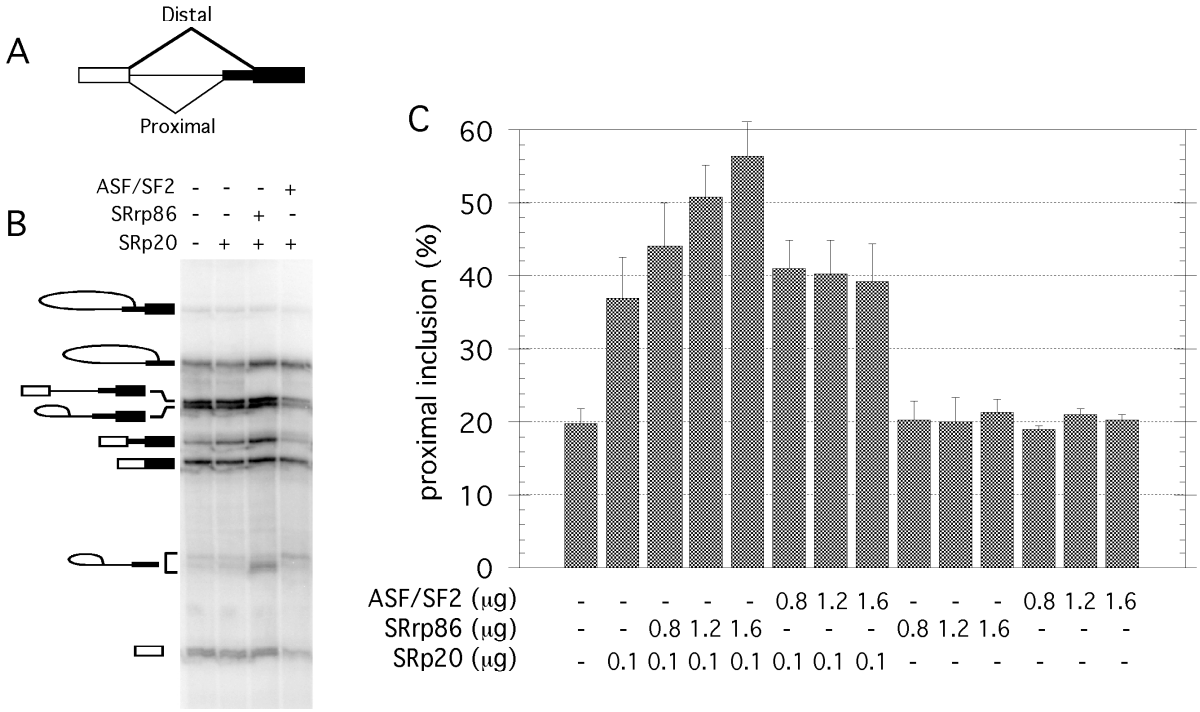

FIGURE 1. In vitro activation of SRp20 by SRrp86. **A:** Schematic diagram of an α-tropomyosin-derived pre-mRNA substrate containing competing 3′ splice sites. The 3′ splice site closest to the 5′ splice site is referred to as the proximal splice site with the distal splice site located 81 nt downstream. **B:** In vitro splicing of the substrate diagramed above in HeLa nuclear extract supplemented with the indicated SR proteins. Recombinant proteins (SRp20, 125 ng; SRrp86, 1.2 μg; or ASF/SF2, 1.1 μg) were added to splicing reactions for 1 h at 30 °C. RNAs were then isolated and subjected to electrophoresis on 8% polyacrylamide gels. The identity of each band is indicated to the right. **C:** In vitro splicing reactions were supplemented with the indicated recombinant proteins before analysis as in **B**. Percent proximal splice site usage was determined by phosphorimager analysis.

the plasmid encoding SRp20, incubated with increasing concentrations of doxycycline, and splicing patterns analyzed. As shown in Figure 2D, expression of SRrp86 in the presence of SRp20 resulted in a nearly complete switch in the levels of exon 4 inclusion from 25% to 77%. Thus, both in vitro and in vivo, SRrp86 can activate SRp20.

Inhibition of SC35 by SRrp86

We next tested the effects of induction of SRrp86 on splice site selection using the SRp20 minigene in the presence of ASF/SF2, SC35, and SRp75 in DBUT5 cells (Fig. 3A). For ASF/SF2 and SRp75, little, if any, change in the amount of exon 4 inclusion could be detected, regardless of whether SRrp86 was induced or not. In contrast, overexpression of SC35 by itself caused activation of a cryptic splicing event in the SRp20 minigene. This mRNA resulted from the activation of a cryptic 5′ splice site in the intron downstream of exon 5 generating a spliced mRNA that skips all of exon 4 but includes exon 5 and 157 nt of intron 5 (Fig. 3B). Importantly, induction of SRrp86 in the presence of SC35 caused a reversal of this effect and returned the splicing pattern to near normal. Repression of SC35 activity was again dependent on the dose of SRrp86 (data not shown). Thus, SRrp86 can inhibit SC35 in vivo, consistent with previous in vitro analyses (Barnard & Patton, 2000).

SRrp86 interacts with SRp20 and SC35

From the above, SRrp86 is able to activate SRp20 and inhibit SC35. Although the exact mechanism whereby SRrp86 can act both positively and negatively to control splice site selection is unknown, we wished to determine whether these effects might be mediated by protein–protein interaction. To accomplish this, a tagged version of SRrp86 (SRrp86-TAP) was created and pull-down experiments were performed using ^{35}S-labeled, in vitro-translated proteins. The epitope tag consisted of two IgG-binding domains from Protein A fused to SRrp86, overexpressed and purified from Baculovirus-infected Hi5 cells. Candidate SR proteins were incubated with SRrp86-TAP and interacting proteins were recovered by passage over IgG beads. As shown in Figure 4, both SRp20 and SC35 interacted with tagged SRrp86, but did not associate with the IgG beads alone. In contrast, ASF/SF2 and SRp75 only marginally interacted with SRrp86. This agrees with the functional

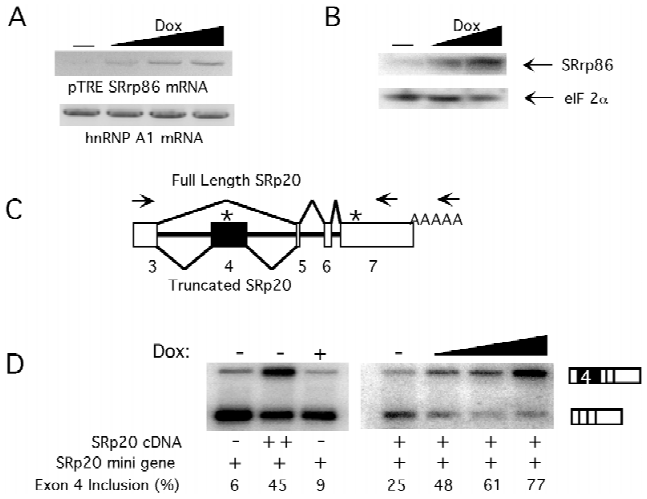

FIGURE 2. Dose dependent regulation of splice site selection by SRrp86. **A:** The Tet-On cell line DBUT5 was grown in the presence of increasing concentrations of doxycycline (0, 2, 4, or 8 μg/mL) for 48 h. RNA was then isolated and RT/PCR was performed to analyze the expression of SRrp86. As a control, RT-PCR of hnRNP A1 mRNA was performed in parallel at each doxycycline dose. PCR DNAs were resolved on agarose gels, stained with ethidium bromide, and photographed as a negative image. **B:** As in **A**, the DBUT5 cell line was grown in the presence of 0, 5, or 10 μg/mL doxycycline for 48 h. Cells were then lysed in loading buffer and proteins separated on 12% Laemmli gels before transfer to PVDF membranes. Western blots were probed with affinity-purified antibodies against SRrp86 or a monoclonal antibody against the eukaryotic translation initiation factor eIF 2α. **C:** Structure and alternative splicing of the SRp20 minigene. Exon 4 (solid rectangle) is subject to autoregulatory alternative splicing. Asterisks indicate the positions of stop codons and arrows mark the position of primers used in RT/PCR. **D:** Regulation of splicing by SRrp86. DBUT5 cells were transfected with the SRp20 genomic minigene and 100 ng (right panel) or 200 ng (left panel) of an SRp20 cDNA in the presence or absence of 0, 2.5, 5, or 7.5 μg/mL doxycycline (right panel) or 8 μg/mL doxycycline (left panel). RT/PCR was performed using the primers indicated in **C**. PCR primers were radiolabeled with ^{32}P and DNAs were separated on 2% agarose gels, dried, and subjected to autoradiography. Inclusion or skipping of exon 4 is diagramed to the right of the gel and the levels of exon 4 inclusion were quantitated by phosphorimager analysis.

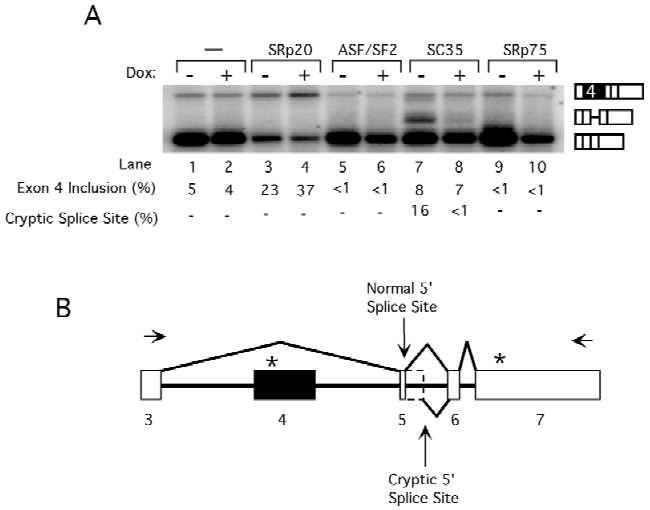

FIGURE 3. Activation and repression of splicing by SRrp86. **A:** Different SR proteins were cotransfected with the SRp20 genomic minigene in the presence (10 μg/mL) or absence of doxycycline. Inclusion or exclusion of exon 4 was monitored as in Fig. 2. In the presence of SC35, a cryptic splice site was activated resulting in an increase in the size of exon 5 (dotted lines) and the splicing pattern as shown in **B**. Induction of SRrp86 abolished cryptic splice site activation by SC35.

splicing analyses above and suggest that SRrp86 preferentially interacts with only a subset of SR proteins. Further, it appears that the effects of SRrp86 on SRp20 and SC35 are apparently mediated by direct protein–protein interaction.

Expression pattern of SRrp86

Given that SRrp86 can alter the activity of SR proteins, it was of interest to discern the expression pattern of SRrp86 compared to core SR proteins. At the RNA level, SRrp86 is expressed in all tissues examined (data not shown). However, at the protein level, SRrp86 is most efficiently translated in testis and brain and was undetectable in kidney (Fig. 5A). In contrast, protein expression of SRp20 was detected in all tissues examined with the highest levels in testis and spleen and the lowest levels in pancreas. For SC35, previous analyses have shown that the phosphorylated form varies between tissues with the highest levels in thymus and spleen and the lowest levels in heart and skeletal muscle tissues (Hanamura et al., 1998). Comparison of the ratio of these factors (and the control proteins hnRNP C and eIF 2α) between tissues demonstrates that the relative levels of these proteins differ from tissue to tissue. Specific changes in the ratio of these factors likely allows precise control of alternative splicing patterns by varying the levels of activators and repressors of splicing, or both, in the case of SRrp86.

The fact that SRrp86 is constitutively expressed at the RNA level yet restricted at the protein level sug-

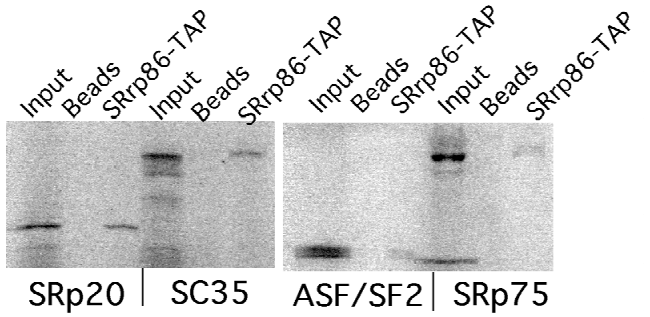

FIGURE 4. SRrp86 interacts with SRp20 and SC35. SRrp86 tagged at the C-terminus with two copies of the IgG-binding domain from Protein A was expressed and purified from Baculovirus-infected Hi5 cells. IgG-agarose beads, or beads coated with SRrp86 (SRrp86-TAP), were incubated with the indicated ^{35}S-labeled SR proteins. Bound proteins were eluted and analyzed on SDS gels and compared to the levels of input protein as well as control reactions using beads alone.

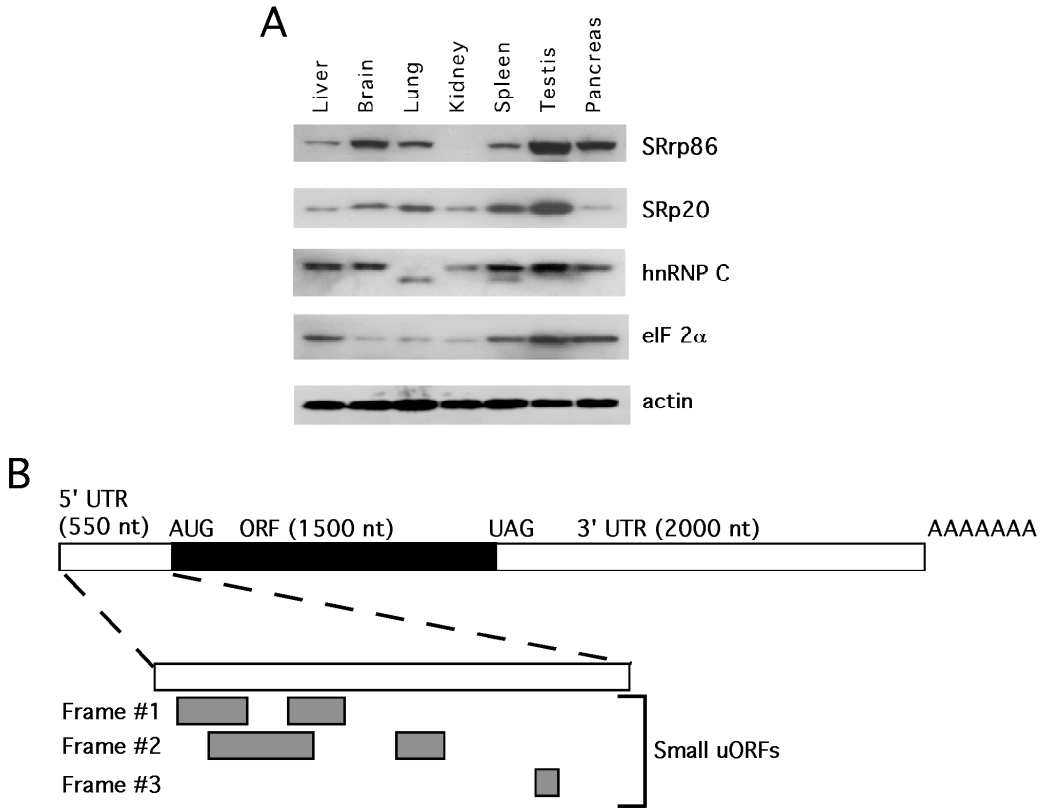

FIGURE 5. Tissue distribution of SRrp86. **A:** A multiple tissue western blot with 75 μg of rat tissue protein in each lane was immunoblotted with affinity-purified antibodies against SRrp86 as well as antibodies against SRp20, hnRNP C, eIF 2α, and actin. **B:** cDNA structure of SRrp86. The full-length SRrp86 cDNA is shown with five small uORFs preceding the complete open reading frame (ORF) for SRrp86 (black rectangle).

gests translational regulation. This is consistent with the finding that the 5′ untranslated region (UTR) is unusually long (549 nt) and contains several small upstream open reading frames (Fig. 5B). Such upstream ORFs (uORFs) have been shown to be responsible for establishing translational control (Morris & Geballe, 2000). Interestingly, the 5′ UTR of SRrp86 is highly conserved between human and rat (95% homology), and incredibly, the amino sequences of the short uORFs are also conserved, suggesting that the resulting peptides may function in some manner.

DISCUSSION

Given the prevalence of alternative splicing in higher eukaryotes (Croft et al., 2000; International Human Genome Sequencing Consortium, 2001), it is important to understand the mechanisms that determine tissue-specific alternative splicing. Members of the SR protein family have been shown to play an important role in regulating splice site selection, but how these proteins are themselves regulated remains unclear. Regulated phosphorylation of SR proteins is clearly important and characterization of several kinases and phosphatases involved in SR protein modification is underway (reviewed in Gravely, 2000). However, the identification of proteins such as p32 (Petersen-Mahrt et al., 1999), RSF1 (Labourier et al., 1999), SRrp35, SRrp40 (Cowper et al., 2001), and now SRrp86 indicates that SR proteins are subject to multiple modes of regulation, consistent with an important role in controlling splicing patterns.

Dose-dependent regulation of alternative splicing in cells

Previous attempts to investigate the role of SR proteins on in vivo alternative splicing typically relied on transient transfection experiments (Cáceres et al., 1994; Screaton et al., 1995; Wang & Manley, 1995; Cramer et al., 1999; Barnard & Patton, 2000). Results from these studies generally agree with in vitro splicing assays, but at the same time, completely opposite effects, including repression of splicing, have been observed (Wang & Manley, 1995), probably due to the fact that it is difficult to control the levels of the overexpressed proteins. Cell line knock-outs enabled analysis of splicing following repression of an inducible allele of ASF/SF2 and showed that genetic depletion altered splicing patterns (Wang et al., 1996). The generation of Tet-on

cells in which the levels of SRrp86 could be carefully regulated allowed us to precisely control the expression of SRrp86 and monitor its effects on splicing. Importantly, raising the concentration of SRrp86 alone had no detectable effect on splice site selection in the DBUT5 cells with the SRp20 minigene or with substrates derived from human growth hormone and adenovirus E1A (data not shown). However, in combination, induction of SRrp86 resulted in a clear activation of SRp20 and repression of SC35. Therefore, SRrp86 can act as a regulator of SR protein function in vivo.

Potentially, the effects of SRrp86 could be substrate specific, accounting for the lack of an effect on SRp75 or ASF/SF2. To test this, we utilized constructs derived from adenovirus E1A, human growth hormone, and the Ich-1 (Caspase-2) genes. For E1A, ASF/SF2 caused a consistent increase in proximal splice site selection but induction of SRrp86 caused no further changes in splice site selection (data not shown). We also observed inhibition of SC35 by SRrp86 using the Ich-1 gene, but the changes were fairly modest (data not shown). We have not yet identified a substrate that responds well to SRp75. Thus, for the substrates we have tested, the effects of SRrp86 are consistent with the experiments included in this report, but it remains possible that regulation of SR proteins by SRrp86 could extend to other members of the family in a substrate specific manner.

Regulation of SR proteins by SRrp86

Changes in the cellular concentration of SRrp86 would be expected to alter the global activity of SR proteins, down-regulating some while up-regulating others. Although the inhibitory activity of SRrp86 is similar to p32, RSF1, SRrp35, and SRrp40, it appears to be a broader regulator due to its ability to both activate and repress SR protein activity. It is thought that splicing repression by RSF1 is caused by sequence-specific binding and subsequent interference with the normal protein–protein interactions of SR proteins (Labourier et al., 1999). Inhibition by p32 appears to be due to repression of ASF/SF2 phosphorylation, consistent with p32 binding to only hypophosphorylated ASF/SF2 (Petersen-Mahrt et al., 1999). For SRrp35 and SRrp40, it is not yet known how these proteins repress splicing but their effects are consistent with competition for RNA-binding sites (Cowper et al., 2001). In contrast, SRrp86 directly interacts with target SR proteins (Barnard & Patton, 2000; J. Li, D.C. Barnard, & J.G. Patton, submitted). It should be stressed that each of the multiple assays that have been used to examine interaction between SR proteins and SRrp86 suffer from potential pitfalls, making it difficult to quantitate the strength of interaction. Nevertheless, regulation of SR proteins by SRrp86 appears to be distinct from simple disruption of the normal phosphorylation/dephosphorylation cycle of SR proteins, as the phosphorylation state of the target SR protein does not apparently alter interaction nor does SRrp86 appear to affect the ability of SR proteins to be phosphorylated (Barnard & Patton, 2000; D.C. Barnard & J.G. Patton, unpubl.). It is still not clear how SRrp86 is able to both activate and repress target SR proteins, but such regulation appears to be distinct from the mechanisms invoked for p32, RSF1, SRrp35, and SRrp40. The overall picture that emerges is that a variety of mechanisms are utilized to regulate the activity of SR proteins, consistent with an important role for these proteins in controlling splice site selection.

Combinatorial control of alternative splicing

Combinatorial control is currently the most favored explanation for the regulation of alternative splicing. If SRrp86 levels between cells and tissues remained constant in relation to SRp20, SC35, or other target SR proteins, it would be difficult to propose a significant role for SRrp86 in differential splicing. However, the expression patterns of these proteins clearly differs between tissues, creating unique ratios of alternative splicing factors. The differences in expression of SRrp86 are likely due to its unusual cDNA structure. The presence of several conserved uORFs suggests that translational control might affect the level of SRrp86. In addition, alternatively spliced forms of SRrp86 have been identified (D.C. Barnard & J.G. Patton, unpubl.) that utilize a cryptic splice site within intron 5, generating mRNAs containing a premature stop codon. Thus, just as SRp20 production is autoregulated (Jumaa & Nielsen, 1997), so too might SRrp86 alter its own pre-mRNA splicing to precisely control protein levels, allowing for differential expression between cells and tissues. In turn, the ratio of SRrp86 to target SR proteins would change, which, when combined with phosphorylation control of SR protein activity, would provide sufficient precision to regulate gene expression through alternative splicing.

MATERIALS AND METHODS

In vitro splicing

In vitro transcription and splicing reactions were carried out as previously described (Patton et al., 1991, 1993). Briefly, the α-TM derived *cis*-parent substrate (Coolidge et al., 1997) was linearized with *Bam*HI and transcribed with SP6 RNA polymerase. RNA was spliced in the presence of HeLa nuclear extract and baculovirus-expressed recombinant proteins (Barnard & Patton, 2000). RNAs were resolved on 8% denaturing gels.

Cell culture and transfection

HeLa Tet-on cell lines were grown in DMEM with 10% Tet System Approved Fetal Bovine Serum (Clontech), 1×

penicillin/streptomycin, and 100 μg/mL G418. Transfections were carried out as previously described (Barnard & Patton, 2000) with TransIT LT1 liposome (Mirus). For generating stable Tet-on cell lines, cells were transfected with 20 μg of pTRE-SRrp86 vector and 1 μg of pTK-Hygromycin. After 48 h, cells were split 1 to 5 in the presence of 200 μg/mL hygromycin. Clonal cell lines were ring cloned and tested for SRrp86 overexpression. Cells were maintained in the presence of 100 μg/mL G418 and 100 μg/mL hygromycin. For transient transfections, 1 μg of the SRp20 minigene (XB minigene; Jumaa & Nielsen, 1997) was used with 100–200 ng of pcDNA vector (empty) or with the indicated SR protein cDNA vector.

Analysis of in vivo splicing

Cells were harvested and RNAs were isolated 48 h after transfection using TRI Reagent (Molecular Research Center, Inc.). RT-PCR analysis was used to analyze splicing products. Briefly, oligo dT was used to prime reverse transcription reactions followed by amplification with gene-specific primers. The PCR primers were: XB exon 3 (5′-GGAGTCCTCC ACCTCGGC-3′) and XB exon 7 (5′-TCACGTGCCTCTTAT GATCC-3′). PCR was carried out in the presence of ^{32}P-labeled XB exon 7 primer for 25 cycles and products were separated on 2% agarose gels and dried.

Protein–protein interaction

Tagged SRrp86 was created by fusing the TAP tag (Rigaut et al., 1999) onto the C-terminus of SRrp86. Baculovirus-expressed SRrp86-TAP was incubated with rabbit IgG-conjugated agarose beads (Sigma), mixed, and washed with a buffer containing 20 mM Tris, pH 8.0, 0.5% NP-40, 1 M NaCl, and 1 mM EDTA. Bound beads were equilibrated in the same buffer with 100 mM NaCl before incubation with in vitro-translated ^{35}S-labeled SR proteins. Complexes were then washed three times in the low-salt buffer above before separation on 12 or 15% SDS gels.

Cloning and expression analysis

Affinity purified α-SRrp86 as well as α-SRp20 (Zymed), α-hnRNP C, and α-eIF 2α, were used to probe a multiple tissue western blot (Genotech). Seventy-five micrograms of protein were loaded in each lane and probed with anti-actin antibodies to control for even transfer. Cloning of human and rat SRrp86 cDNAs was as previously described (Barnard & Patton, 2000).

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