

The SR Protein SRp38 Represses Splicing in M Phase Cells

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Summary

SR proteins constitute a family of pre-mRNA splicing factors that play important roles in both constitutive and regulated splicing. Here, we describe one member of the family, which we call SRp38, with unexpected properties. Unlike other SR proteins, SRp38 cannot activate splicing and is essentially inactive in splicing assays. However, dephosphorylation converts SRp38 to a potent, general repressor that inhibits splicing at an early step. To investigate the cellular function of SRp38, we examined its possible role in cell cycle control. We show first that splicing, like other steps in gene expression, is inhibited in extracts of mitotic cells. Strikingly, SRp38 was found to be dephosphorylated specifically in mitotic cells, and we show that dephosphorylated SRp38 is required for the observed splicing repression.

Introduction

Splicing of mRNA precursors is not only a nearly ubiquitous and essential step in gene expression, but it is also an important mechanism for generation of protein diversity and regulation of gene expression. There are numerous examples where alternative splicing and its regulation play key roles in cell growth control, differentiation, and disease (reviewed by Graveley, 2001; Grabowski and Black, 2001). Indeed, it has been estimated that expression of 35% or more of human genes involves alternative splicing (e.g., Mironov et al., 1999). Given the frequency, importance, and complexity of alternative splicing, it is not surprising that the process is subject to diverse and sophisticated forms of regulation (reviewed by Smith and Valcarcel, 2000). An interesting principal that has emerged from studies to date is that regulation of splice site choice is frequently achieved by combinatorial interactions between RNA binding proteins that interact with sequences in the mRNA precursor to influence interactions involving the general splicing machinery. Unlike with transcription, where regulation is frequently initiated by high-affinity, sequence-specific DNA binding proteins, in many cases splicing regulation is brought about by cooperative interactions between factors that by themselves have more limited sequence specificity and affinity. As with transcription, however, these factors can act either positively or negatively. Key proteins in these regulatory complexes are frequently members of the SR and hnRNP protein families.

The SR proteins constitute a group of splicing factors that are highly conserved throughout metazoa (reviewed

in Manley and Tacke, 1996; Graveley, 2000). SR proteins, of which approximately a dozen have been described in mammalian systems, are modular, containing one or two N-terminal RNP-type RNA binding domains (RBD) and a C-terminal region consisting largely of multiple Arg-Ser dipeptide repeats (RS domain). They play multiple roles in splicing and have the unusual property of functioning as both general, essential factors in constitutive splicing and as regulators of alternative splicing. In constitutive splicing, the proteins play largely redundant functions such that any single SR protein is able to cooperate with the remainder of the splicing machinery to activate splicing in vitro. For this function, sequence-specific interactions with the pre-mRNA likely play a minimal role and instead, cooperative, RS domain-mediated protein-protein interactions facilitate and stabilize snRNP interactions both with the pre-mRNA and with each other (e.g., Wu and Maniatis, 1993; Kohtz et al., 1994). In alternative splicing, SR proteins can activate splice site selection by distinct mechanisms. In one, they function as in constitutive splicing, but if the mRNA precursor contains competing 5' and/or 3' splice sites, utilization of specific pairs is determined by a combination of the splice site sequence and position in the pre-mRNA and by the SR proteins' identity and concentration (e.g., Ge and Manley, 1990; Krainer et al., 1990; Zahler et al., 1993). Another mechanism involves sequences in mRNA called exonic splicing enhancers (ESEs; see Smith and Valcarcel, 2000; Blencowe, 2000, for reviews). ESEs function by binding combinations of SR proteins, which interact with general splicing factors to facilitate utilization of adjacent splice sites. Recognition of ESEs requires sequence-specific binding, and in keeping with this, SR protein function in ESE recognition is largely nonredundant. Although initially thought to be associated only with regulated splicing, more recent studies have indicated that ESEs can function as part of constitutively spliced exons (Schaal and Maniatis, 1999).

The RS domains of SR proteins are extensively modified by phosphorylation (reviewed by Misteli, 1999). At least two kinases, SRPK1 (Gui et al., 1994) and Clk/Sty (Colwill et al., 1996), have been shown to phosphorylate SR proteins. The phosphorylation status of SR proteins influences protein-protein interactions (Xiao and Manley, 1997, 1998) and, likely reflecting this, their subnuclear distribution (Misteli et al., 1997). Both hypo- and hyperphosphorylation can reduce splicing activity (Kanopka et al., 1998; Prasad et al., 1999), suggesting that RS domain phosphorylation is tightly controlled.

Analogous to the situation with transcription, repressor proteins also play significant roles in splicing control. And again as with transcription, several mechanisms seem to be utilized in splicing repression. The simplest is competitive binding, and the well-studied *Drosophila* Sex-lethal protein (e.g., Valcarcel et al., 1993) and the mammalian polypyrimidine tract binding protein (e.g., Chan and Black, 1997) provide examples of this mechanism. The hnRNP A proteins antagonize the activity of SR proteins in alternative splicing (Mayeda and Krainer,

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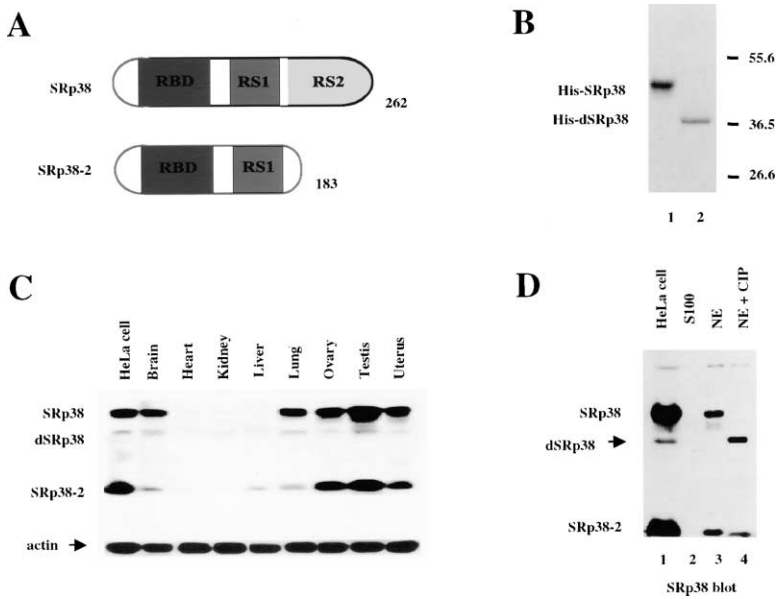


Figure 1. Isolation and Characterization of SRp38

(A) Diagram of SRp38 derivatives. Features of SRp38 and SRp38-2 are indicated. Numbers represent amino acid residues.

(B) Coomassie blue staining of purified recombinant proteins used in this study. His-tagged SRp38 (His-SRp38) was prepared from baculovirus-infected Sf9 cells (lane 1). Dephosphorylated His-tagged SRp38 (His-dSRp38) was prepared by treatment with CIP and repurified using Ni²⁺ beads (lane 2).

(C) Expression pattern of SRp38 and SRp38-2 in selected tissues of adult mice. Equal amounts of total protein were loaded in each lane. HeLa cell lysate was also included. Proteins were analyzed by Western blotting with anti-SRp38 and anti-actin antibodies.

(D) SRp38, dSRp38 and SRp38-2 are present in HeLa nuclear extract. HeLa cell lysate (lane 1), S100 extract (lane 2), nuclear extract (lane 3), and CIP-treated nuclear extract (lane 4) were analyzed by Western blotting with anti-SRp38 antibody. dSRp38 is indicated by the arrow.

1992) and can also prevent splicing by binding to exonic elements known as exonic splicing silencers (ESSs; reviewed in Smith and Valcarcel, 2000), although the mechanism(s) involved are not known. In *Drosophila*, the P element somatic repressor (PSI) represses splicing by recruiting U1 snRNP to an upstream, pseudo 5' splice site, preventing recognition of the authentic site (Labourier et al., 2001). Two proteins with similarity to SR proteins, RSF1 in *Drosophila* (Labourier et al., 1999) and SRp86 in mammals (Barnard and Patton, 2000), function by interfering directly with other SR protein-mediated interactions. Finally, SR proteins themselves can under some conditions repress splicing, as with an adenovirus pre-mRNA, in which a binding site for the SR protein ASF2/SF2 is positioned such that it blocks U2 snRNP association with the pre-mRNA (Kanopka et al., 1996).

In this paper, we describe an SR protein, SRp38 (also known as NSSR-1 [Komatsu et al., 1999], TASR-2 [Yang et al., 2000], and SRp40 [Cowper et al., 2001]), with unusual properties. Most significantly, SRp38, despite a typical SR protein structure, is a splicing repressor that is strongly activated by dephosphorylation. Suggesting a possible physiological role for SRp38, we show that the protein becomes dephosphorylated during M phase of the cell cycle. We also show that splicing, like other processes in gene expression, is inhibited in extracts prepared from mitotic cells. Under these conditions, repression was found to be mediated by SRp38, indicating that cell cycle-specific dephosphorylation of SRp38 plays a role in gene silencing during mitosis.

Results

Isolation of SRp38

Previous work from our lab has described properties of the human splicing regulators Tra2 α and Tra2 β (Tacke et al., 1998). To extend these studies, we used a yeast two-hybrid screen to identify potential interacting proteins.

Among those uncovered was a protein with strong sequence similarity to SR proteins. Two forms of this protein, likely arising from alternative splicing, were recovered. Strengthening the similarity with SR proteins, the RBD showed the highest homology with that of the classical SR protein SC35 (46% identity). Based on the apparent size of the proteins, and in keeping with the naming system used for most SR proteins, we call the full-length protein SRp38 and the smaller form SRp38-2 (see Figure 1A). During the course of these studies, several other groups identified proteins that appear to be identical to SRp38 and SRp38-2, called TASR-2 and -1 (Yang et al., 1998, 2000), NSSR-1 and -2 (Komatsu et al., 1999), and SRp40 (Cowper et al., 2001). Consistent with the protein's sequence, these studies all suggested possible roles in splicing (see Discussion).

As a first step toward characterizing the function of SRp38, we prepared recombinant proteins and antibodies. Neither SRp38 nor SRp38-2 could be expressed efficiently in *E. coli*, but both were expressed to high levels in baculovirus-infected insect cells and were purified from these cells, either as his-tagged (Figure 1B) or GST fusion (not shown) proteins. Because of problems with solubility, we have not pursued studies with SRp38-2, and instead concentrated on full-length SRp38. Purified His-SRp38 was first used to produce rabbit anti-SRp38 antibodies, which were affinity purified with the SRp38 RBD. The purified antibodies were used to determine SRp38 expression patterns in various mouse tissues and in HeLa cells.

SRp38 is expressed in different tissues in distinct ways (Figure 1C). In ovary, testis, and uterus, both SRp38 and SRp38-2 are highly expressed, and at comparable levels. In brain and lung, on the other hand, SRp38 is strongly expressed, but SRp38-2 is barely detectable. Neither protein is produced at significant levels in heart, kidney, or liver. In all the expressing tissues, a faint species could be detected with gel mobility slightly greater than SRp38 (designated dSRp38), which we speculated might correspond to un-, or hypo-, phos-

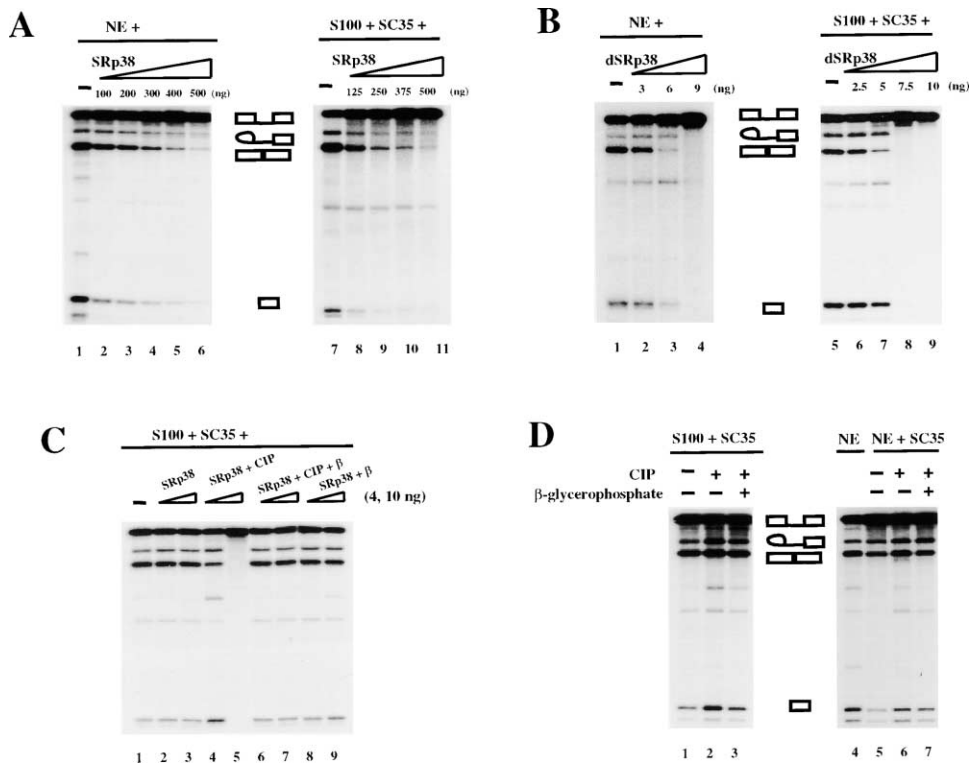


Figure 2. SRp38 Is a Splicing Repressor Activated by Dephosphorylation

(A) Inhibition of pre-mRNA splicing by SRp38. The indicated amounts of baculovirus-produced SRp38 (His-SRp38) were added to HeLa NE (lanes 2–6) and S100 complemented with 250 ng of SC35 (lanes 8–11) together with a ³²P-labeled β-globin pre-mRNA. Products of splicing were analyzed by denaturing PAGE and autoradiography. Splicing intermediates and products are indicated schematically.

(B) Potent inhibition of splicing by dSRp38. Increasing amounts of His-dSRp38 (2.5 ng to 10 ng) were incubated with NE (lanes 2–4) and with S100 complemented with 125 ng of SC35 (lanes 6–9).

(C) Characterization of splicing inhibition by dSRp38. His-dSRp38 (4 or 10 ng) was added to splicing reactions as follows: untreated (lanes 2 and 3, respectively), CIP-treated and repurified (lanes 4 and 5), CIP-treated in the presence of β-glycerophosphate (β) and repurified (lanes 6 and 7), and mock-treated in the presence of β and repurified (lanes 8 and 9).

(D) CIP treatment does not affect SC35-activated splicing. SC35 was added to splicing reactions in S100 extracts (lanes 1–3) or in NE (lanes 5–7) as follows: untreated (lanes 1 and 5), CIP-treated and repurified (lanes 2 and 6), and CIP-treated and repurified in the presence of β (lanes 3 and 7).

phorylated SRp38. To test this, and also to elucidate SRp38 expression levels in HeLa cells, we performed Western blots with HeLa whole-cell lysate, cytoplasmic extracts (S100), and nuclear extract either untreated (NE) or treated with calf intestinal phosphatase (NE+CIP) (Figure 1D). The pattern of expression in HeLa cells was essentially identical to the mouse ovary/testis/uterus pattern. Like other SR proteins, SRp38 was not detected in S100. In NE, CIP treatment converted SRp38 to a form with mobility identical to dSRp38, strongly suggesting that this form in fact corresponds to dephosphorylated SRp38.

SRp38 Is a Splicing Repressor Activated by Dephosphorylation

We next wished to determine whether SRp38 behaves like a typical SR protein; e.g., whether it can activate splicing in extracts lacking SR proteins. Using an S100 complementation assay, purified SRp38 (His- or GST-tagged) was unable to activate splicing of several different pre-mRNAs (results not shown). However, during the course of these experiments, we noticed that SRp38

in fact repressed splicing of a human β-globin pre-mRNA (or other substrates), either when added to NE or to S100 supplemented with SC35 or ASF/SF2 (Figure 2A; results with other substrates and ASF/SF2 not shown). Although near-complete inhibition could be observed, relatively large amounts of recombinant protein (~500 ng) were required, and the significance of this repression was therefore unclear (see also below). Similar levels of repression were typically detected whether His-tagged SRp38 or GST-SRp38 (not shown) was employed. However, some preparations of GST-SRp38 required significantly less protein to achieve comparable repression, and this enhanced activity seemed to correlate with the presence of small amounts of a species with SDS gel mobility that suggests that it might correspond to unphosphorylated GST-SRp38.

In light of the above results, we tested directly whether the phosphorylation status of SRp38 affects the protein's activity in splicing assays. Purified SRp38 was treated with CIP and repurified (Figure 1B; see Experimental Procedures), and increasing amounts were added to splicing reactions with NE or S100 plus SC35 (Figure 2B). Splicing inhibition was again observed, but

strikingly, the amounts of dSRp38 required for full repression were greatly reduced. In both NE and S100, less than 10 ng resulted in complete inhibition, suggesting that dephosphorylated SRp38 was at least 50-fold more effective at splicing repression than was phosphorylated SRp38. Identical results (not shown) were obtained with two additional substrates, HIV-tat- and IgM H-chain-derived pre-mRNAs, suggesting that dSRp38 acts as a general splicing repressor.

Especially in light of the unexpected magnitude of the CIP-induced activation of splicing repression, it was essential to rule out the possibility that inhibition might have been due to an artifact of the CIP treatment. We therefore performed several control experiments to address this. In one, purified SRp38 was first incubated alone, with CIP, with CIP plus the phosphatase inhibitor β -glycerophosphate (β), or with β alone, and all four preparations of SRp38 were then repurified. Only the sample treated with CIP alone was dephosphorylated, as judged by SDS-PAGE (results not shown), and only this sample gave rise to splicing repression at the concentrations tested (4 and 10 ng; Figure 2C, lanes 4 and 5). In another experiment, SC35 (his-tagged, produced from recombinant baculovirus-infected cells) was incubated alone, with CIP, or with CIP plus β , and repurified in a manner identical to that used with SRp38. In this case, a larger amount of each sample (100 ng) was added either to S100 or to NE, and effects on splicing were analyzed (Figure 2D). Not only did CIP-treated SC35 show no inhibitory effect in NE (lanes 4–7), but it also retained full activity in activating splicing in S100 (lanes 1–3). We conclude that the observed activation of SRp38 as a splicing repressor by CIP was indeed due to dephosphorylation.

Characteristics of dSRp38-Mediated Splicing Repression

To gain insight into the mechanism by which dSRp38 inhibits splicing, we first determined at which step splicing was blocked. To this end, we performed spliceosome assembly assays, utilizing the β -globin pre-mRNA substrate and S100 extract activated with SC35, plus or minus 7.5 ng dSRp38. The results of a time course (Figure 3A) show that this amount of dSRp38 is sufficient to prevent formation of complex A, which represents the earliest step in spliceosome assembly detectable by this method. We conclude that dSRp38 interferes with interactions involving early acting factors, such as U1 or U2 snRNP, branchsite recognition factors, and/or SR proteins.

We next examined whether dSRp38 activity involves interactions with SR protein splicing activators. This was tested first by asking whether increasing amounts of SC35 could restore splicing inhibited by a small amount of dSRp38. The results (Figure 3B) show that splicing inhibition was not restored by increased levels of SC35, indicating that SR proteins are not the targets of dSRp38 repression activity. This is consistent with the fact that dSRp38 was found to interact at most weakly with other SR proteins in GST “pull-down”-type assays (although phosphorylated SRp38 interacts strongly; see Discussion) (data not shown). Moreover, the amount of SR protein required to activate splicing is an order of magni-

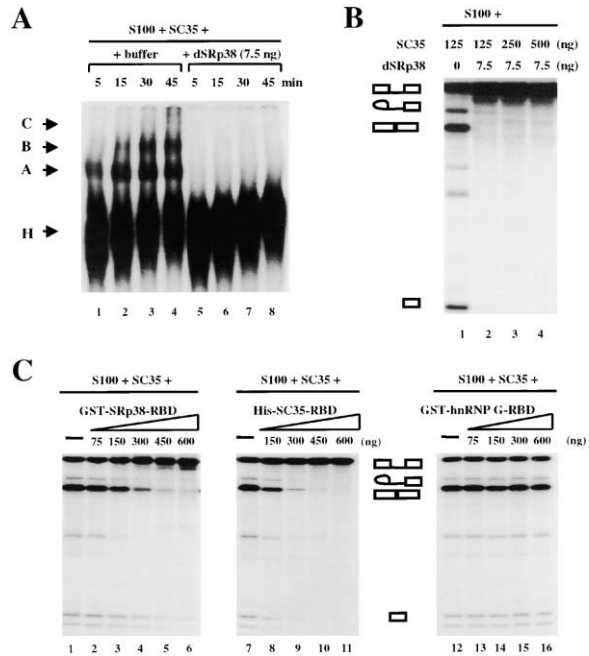


Figure 3. Characteristics of dSRp38-Mediated Splicing Repression (A) dSRp38 inhibits an early step in spliceosome assembly. Spliceosome assembly assays were carried out in S100 extract complemented with 125 ng of SC35 in the presence (lanes 5–8) or absence (lanes 1–4) of 7.5 ng of His-dSRp38. Splicing complexes were resolved by nondenaturing PAGE. (B) The target of splicing inhibition by dSRp38 is not an SR protein. Increasing amounts of SC35 (250 ng and 500 ng) were added to dSRp38-inhibited reactions as indicated (lanes 3 and 4). (C) The RS domain of SRp38 is required for potent repression. The indicated amounts of purified GST-SRp38 RBD (lanes 1–6), His-SC35 RBD (lanes 7–11), and GST-hnRNP G-RBD (lanes 12–16) were added to splicing reactions containing β -globin pre-mRNA substrate in S100 extract plus SC35.

tude higher than the concentration of dSRp38 required for inhibition (e.g., Figure 3B).

Given that interactions with other SR proteins (which are mediated by RS domains) are not the basis of dSRp38 repression, it was conceivable that splicing inhibition was brought about by the RBD. To determine whether the SRp38 RBD was sufficient to repress splicing, we purified from *E. coli* fusion proteins containing the related RBDs from SRp38 and SC35, as well as from an hnRNP protein, hnRNP G. The proteins were added to splicing reactions performed in S100 activated by SC35, and the results (Figure 3C) show that both the SRp38 and SC35 RBDs, but not the hnRNP G RBD, inhibited splicing, although only at high concentrations. Notably, the amounts of either RBD and of full-length phosphorylated SRp38 (Figure 2A) required for splicing inhibition were essentially identical. As full-length SC35 is a splicing activator, repression brought about by the inactive SC35 RBD likely reflects competitive interactions that interfere with association of full-length SC35 with the pre-mRNA. It is likely that the similar weak repression observed with both phosphorylated SRp38 and the isolated RBD reflect the same mechanism. Such “passive” repression, which was also described recently by Cowper et al. (2001), may result only from the large excess

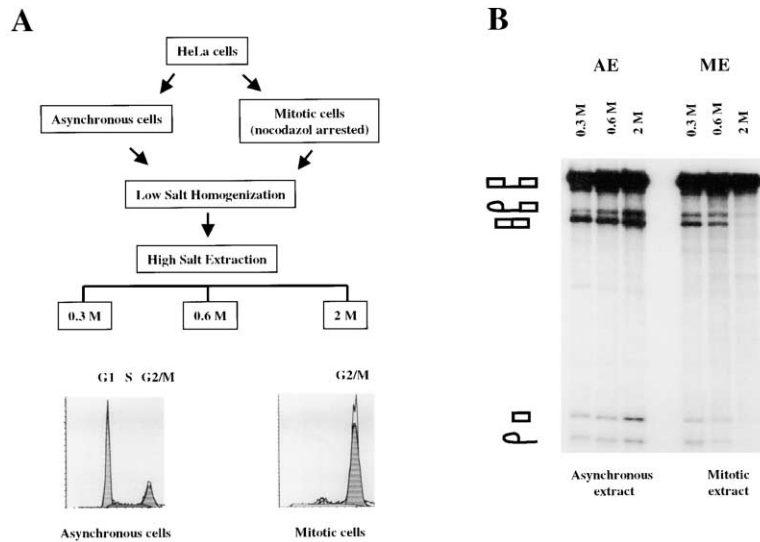


Figure 4. Pre-mRNA Splicing Is Inhibited in Mitotic Cell Extracts

(A) Whole-cell extract preparation scheme from asynchronous and nocodazol-arrested (mitotic) HeLa cells. Untreated HeLa cells or cells arrested in M phase were harvested, suspended in low salt buffer, and homogenized. Lysates were extracted with the indicated NaCl concentrations, and extracts were centrifuged and dialyzed. FACS results of cells used in this preparation are shown below the scheme.

(B) Inhibition of pre-mRNA splicing in mitotic, but not asynchronous, high salt extracts. Equal amounts of each whole-cell extract prepared from asynchronous and mitotic cells were tested for splicing activity with β -globin pre-mRNA.

of protein required, and thus may not be physiologically relevant. If this were the case, then phosphorylated SRp38 would be inactive as a repressor, and phosphorylation/dephosphorylation would serve as an on/off switch.

Pre-mRNA Splicing Is Inhibited in Mitotic Cell Extracts

The results described above indicate that SRp38 has the potential to be a strong, general repressor of splicing that is activated by dephosphorylation. We next wished to determine how these properties of SRp38 might contribute to splicing control *in vivo*. One situation where a phosphorylation-regulatable splicing repressor might potentially play a significant role is in M phase of the cell cycle. It is well known that gene expression in mammalian cells is generally inhibited during mitosis. Both transcription (Segil et al., 1996; Akoulitchev and Reinberg, 1998; Long et al., 1998) and polyadenylation (Colgan et al., 1996) of mRNA precursors are inhibited by phosphorylation of key components, and cap-dependent translation is also repressed, interestingly in this case by a mechanism involving dephosphorylation (Pyrtonnet et al., 2001). However, whether or not the splicing machinery is also inhibited in mitosis has not been investigated.

To examine possible splicing inhibition during M phase, we wished to prepare extracts from asynchronously growing HeLa cells and from M phase cells (arrested with nocodazol). However, a potential complicating factor is the fact that the nuclear envelope breaks down during mitosis, and thus the standard nuclear extract typically used to assay pre-mRNA splicing could give misleading results. We therefore developed a whole-cell extract procedure that should not be affected by the status of the nuclear envelope (Figure 4A; see Experimental Procedures). As part of the optimization of the extract protocol, samples of the asynchronous and mitotic cell lysates were extracted with different NaCl concentrations, 0.3, 0.6, and 2 M, and the resultant extracts were used in splicing reactions, again with the β -globin

pre-mRNA (Figure 4B). With the asynchronous cells, all three salt extractions gave rise to splicing competent extracts, with the 2 M extract displaying the highest activity. With the mitotic extracts, a strikingly different pattern was observed. At 0.3 M, activity was essentially the same as in the asynchronous extract. But activity was significantly reduced in the 0.6 M extract and barely detectable in the 2 M extract. These results indicate both that the pre-mRNA splicing machinery is repressed in extracts of mitotic cells, and that inhibition is mediated by (a) factor(s) that, in contrast with all the factors required for splicing, require(s) high salt for solubilization.

SRp38 Is Dephosphorylated in M Phase

Having obtained evidence that the splicing machinery is repressed in mitotic extracts, we next wished to determine the phosphorylation status of SRp38 in mitotic cells. We first treated HeLa cells with nocodazol for 24 hr (as in Figure 4) and prepared whole-cell lysates from the mitotic cells and from asynchronous cells. These were analyzed by Western blotting with the anti-SRp38 antibodies used above, with mAb104, which recognizes a common phosphoepitope in the RS domain of SR proteins (Roth et al., 1991), and with a phosphorylation-insensitive anti-ASF/SF2 polyclonal antibody (Figure 5A). Strikingly, the anti-SRp38 antibodies revealed accumulation in the mitotic extract of a species with mobility consistent with it corresponding to dSRp38. Somewhat unexpectedly (e.g., Gui et al., 1994), little change was detected in the pattern of classical SR proteins, suggesting that the phosphorylation status of these proteins is not significantly altered as cells accumulate in M phase. (SRp38 is recognized by mAb104, albeit more weakly than classical SR proteins. It was not resolved from SRp40 in the gel shown.) To extend these results, extracts prepared from HeLa cells at different times following release from a double-thymidine block were analyzed by Western blotting, which revealed accumulation of dSRp38 only in mitotic cells (Figure 5B).

To confirm that the species detected in the M phase lysates was in fact dSRp38, we analyzed by Western

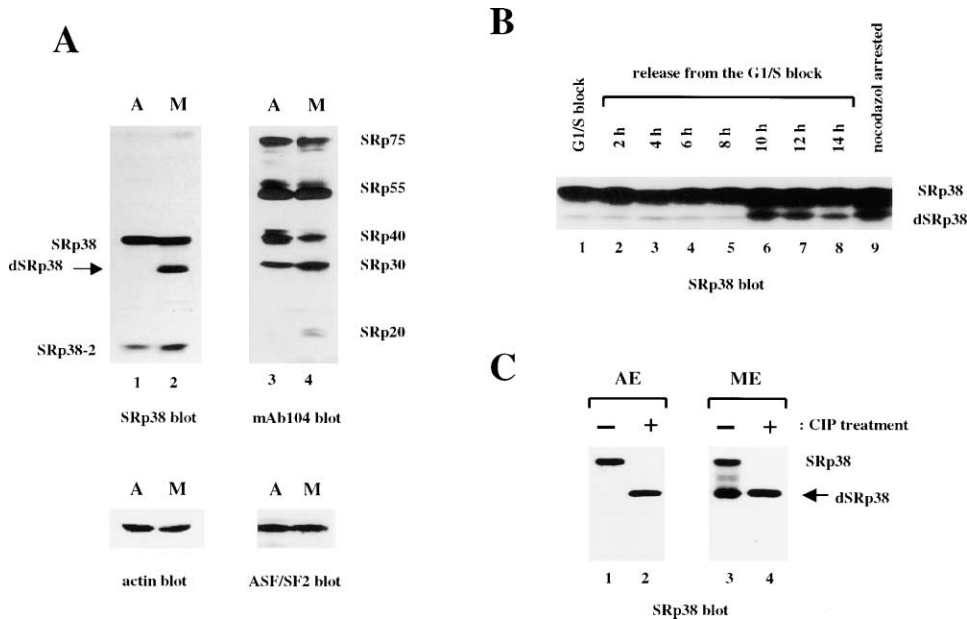


Figure 5. SRp38 Is Dephosphorylated in M Phase

(A) dSRp38 accumulates in M phase. Lysates of asynchronously grown HeLa cells (lanes 1 and 3) and mitotic cells arrested with nocodazol (lanes 2 and 4) were analyzed by Western blotting with anti-SRp38, mAb104, anti-ASF/SF2 and anti-actin antibodies. A, asynchronous cell lysates; M, mitotic cell lysates. Positions of SRp38 proteins and SR proteins are indicated.

(B) SRp38 expression through the cell cycle. Extracts from HeLa cells arrested at G1/S by double-thymidine block (lane 1), from cells released from the block (lanes 2–8), and from cells arrested at M phase by 4 hr nocodazol treatment 8 hr after release (lane 9) were analyzed by Western blotting with anti-SRp38 antibodies. FACS analysis indicated that mitotic cells accumulated from 10 to 14 hr after release (lanes 6–8).

(C) SRp38 is dephosphorylated in M phase cells. Whole-cell extracts from asynchronous (AE) and M phase (ME) cells were analyzed by Western blotting with anti-SRp38 antibodies without (lanes 1 and 3) or with (lanes 2 and 4) treatment with CIP.

blotting asynchronous and M phase cell extracts treated or not treated with CIP. The results (Figure 5C) show that in the asynchronous cell extracts, CIP treatment completely converted SRp38 to a more rapidly migrating form with identical mobility to the species detected in M phase cells. Likewise, in M phase extracts, SRp38 was converted to the same rapidly migrating form, and no additional species were detected. (Blots with purified recombinant SRp38 and dSRp38 indicated the antibodies used in this experiment recognized dSRp38 ~2-fold more efficiently than SRp38 [results not shown], and therefore, to facilitate comparisons of gel mobility, lower amounts of the CIP-treated extracts were analyzed.) Quantitation of these and additional blots indicated that the amount of dSRp38 increased from about 2% of the total SRp38 protein in asynchronous cells to 30% in mitotic cells.

Presence of dSRp38 Correlates with Splicing Inhibition

The above results provided evidence that splicing is inhibited in mitotic cell extracts by (a) factor(s) extracted at high salt, and that SRp38 becomes dephosphorylated in M phase cells. If dSRp38 is responsible for M phase splicing repression, then it should be extracted from mitotic extracts at high salt but not at low salt. To test this, we first examined extracts prepared as in Figure 4, i.e., at 0.3, 0.6, and 2 M NaCl, both for splicing activity (Figure 6A) and for the presence of dSRp38 (Figure 6B). As above, splicing was normal in the 0.3 M mitotic ex-

tracts, partially blocked at 0.6 M, and nearly entirely inhibited at 2 M. Remarkably, Western blotting revealed that, while SRp38 and SRp38-2 were extracted equally at all salt concentrations, dSRp38 was not present in the 0.3 M extract, but fully extracted in the 2.0 M extract (compare with whole-cell lysate, Figure 5A) and present at intermediate levels in the 0.6 M extract. To confirm and extend these results, we focused on the fact that 0.6 M NaCl gave an intermediate level of splicing inhibition and dSRp38 extraction. We therefore repeated the above experiment, except using 0.7 M NaCl for the intermediate salt concentration. Strikingly, splicing was significantly more inhibited at 0.7 M than at 0.6 M (Figure 6A; compare ME1 and ME2) and dSRp38 was also more efficiently extracted (Figure 6B). Together, these results provide a perfect correlation between splicing inhibition in mitotic cell extracts and the presence of dSRp38.

dSRp38 Is Necessary for Mitotic Splicing Repression

The correlation between the presence of dSRp38 and mitotic splicing repression, coupled with the fact that SRp38 is abundant in HeLa cells (~3 × 10⁶ molecules per cell; data not shown), strongly suggests that dSRp38 functions to repress the splicing machinery during mitosis. Consistent with this, addition of classical SR proteins to mitotic extracts was unable to restore splicing (results not shown), strengthening the conclusion that they are not inactivated during mitosis. But to provide more conclusive evidence for the role of dSRp38, we

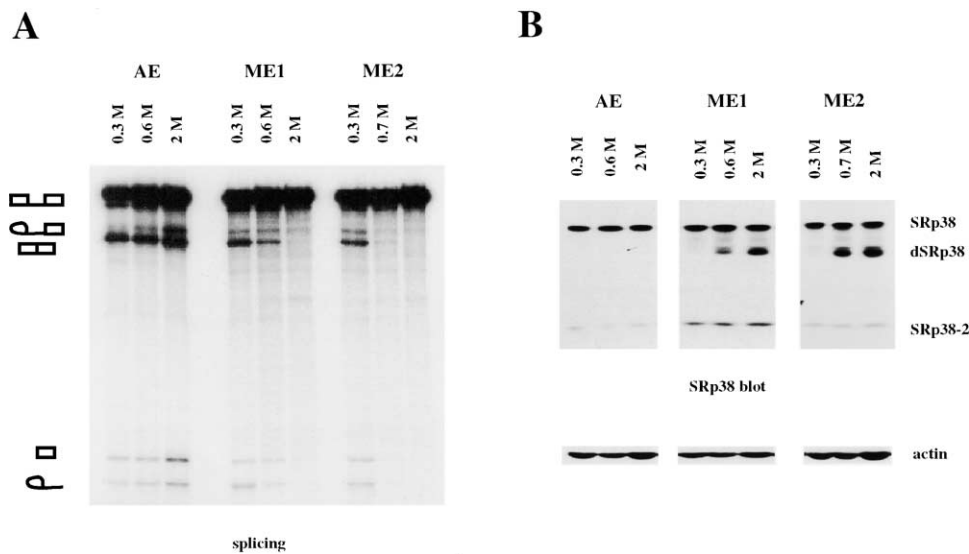


Figure 6. Presence of dSRp38 Correlates with Splicing Inhibition

(A) Splicing activity of different whole-cell extracts. Equal amounts of whole-cell extracts prepared from asynchronous cells (AE) and two preparations of mitotic cells (ME1 and ME2) extracted with the indicated salt concentrations were tested for splicing activity using β -globin pre-mRNA

(B) Extraction of dSRp38 from whole-cell extracts is salt sensitive. Extracts used in (A) were analyzed by Western blot with anti-SRp38 and anti-actin antibodies.

wished to deplete repressed (i.e., high salt) mitotic extracts of dSRp38 and to determine whether such a depleted extract becomes competent for splicing. Our initial attempts to do this using anti-SRp38 antibodies were unsuccessful, as we were unable to deplete the protein efficiently (results not shown). We therefore decided to attempt to deplete the SRp38 proteins by another approach, RNA affinity. This method has been used before, for example, to deplete the abundant hnRNP A1 proteins from NE (Caputi et al., 1999), but requires knowledge of the protein's high-affinity RNA binding site. To determine this for SRp38, we performed SELEX (Tuerk and Gold, 1990) with the GST-SRp38 RBD protein (see Experimental Procedures; Y. Feng, C.S., and J.L.M., unpublished). The consensus sequence that emerged was used to produce an 84 nucleotide biotinylated RNA containing three copies of the consensus motif (bio-C3), and this was used to deplete SRp38 from mitotic extract by binding to avidin beads. Figure 7A displays a Western blot indicating that SRp38, dSRp38, and SRp38-2 were all depleted from the bio-C3-treated extract, but not from a mock-treated extract. Importantly, depletion was specific, as standard SR proteins were not significantly removed (Figure 7A).

We next compared the splicing activity of the mock- and bio-C3-depleted extracts with untreated mitotic extract (Figure 7B). Strikingly, the bio-C3-depleted extract now displayed significant activity (lane 4), while the mock (lane 3) and untreated (lane 2) extracts remained essentially inactive. To show that activation was due specifically to removal of dSRp38, increasing amounts of dSRp38 were added to the bio-C3-depleted extract (Figure 7C). Splicing was again strongly repressed and, in keeping with the fact that dSRp38 was not quantitatively removed from the mitotic extract (Figure 7A), the

amount of dSRp38 necessary to effect complete inhibition (~ 4 ng) was even less than observed with comparable asynchronous extract (~ 7.5 ng; see above). Addition of phosphorylated SRp38 or other SR proteins (e.g., ASF/SF2) was without effect (Figure 7D), confirming that inhibition was specific to dSRp38.

Discussion

The experiments presented here provide evidence that activation of a splicing repressor by dephosphorylation is important in inhibition of the splicing machinery during M phase of the cell cycle. SRp38 thus behaves in a distinctive manner, unlike previously characterized SR proteins. Below we discuss the features of SRp38 that might underlie these properties, the significance of mitotic splicing repression more generally, and finally the role of dephosphorylation as an activating mechanism.

SRp38 has an overall primary structure typical of SR proteins, but its properties are quite atypical. Biochemically, SRp38 does not copurify with other SR proteins in the classical $(\text{NH}_4)_2\text{SO}_4$ -MgCl₂ purification protocol (Zahler et al., 1992; our unpublished results). Functionally, other known SR proteins are individually able to activate splicing in extracts lacking SR proteins; SRp38 has no detectable activating ability. The basis for these differences is currently unknown, but likely reflects differences in the RS domain. However, there is one property that we believe SRp38 likely shares with other SR proteins, which is the ability to affect splicing both generally and in a sequence-specific manner. The general, or sequence-independent, activation function of SR proteins, also referred to as exon-independent (Hertel and Maniatis, 1999), is driven by cooperative interactions involving RS domain-mediated interactions with other

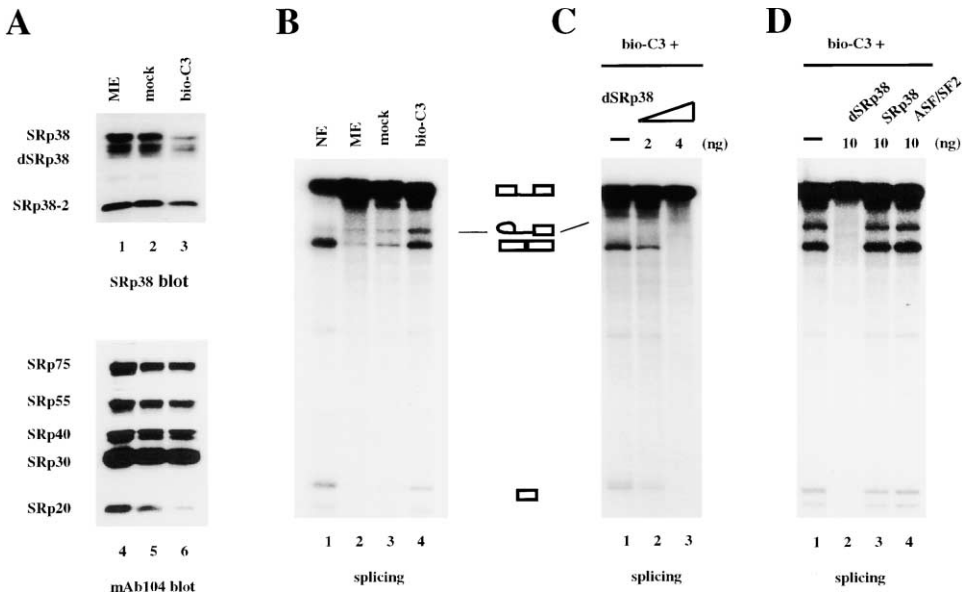


Figure 7. dSRp38 Is Necessary for Mitotic Splicing Repression

(A) Depletion of dSRp38 using RNA affinity. A biotinylated RNA containing three copies of the SRp38-selected consensus sequence (bio-C3) and used to deplete endogenous dSRp38 from mitotic extract (lane 1). Bio-C3 RNA was immobilized with avidin agarose and incubated with mitotic extracts (lane 3). Mock depletion was done with avidin agarose (lane 2). The resultant extracts were analyzed by Western blotting with anti-SRp38 (lanes 1–3) and mAb104 (lanes 4–6) antibodies. (Detection of SRp20 is variable due to inconsistent transfer during blotting.) ME, mitotic extract; mock, mock-depleted mitotic extract; bio-C3, mitotic extract depleted using biotinylated C3 RNA.

(B) Mitotic extract depleted of dSRp38 is competent for splicing. Extracts from (A) were tested for splicing activity using β -globin pre-mRNA. Mitotic extract, lane 2; mock-depleted mitotic extract, lane 3; dSRp38-depleted mitotic extract, lane 4. Standard nuclear extract (NE) was used for comparison (lane 1).

(C) dSRp38 restores splicing repression to depleted mitotic extract. SRp38-depleted mitotic extract was supplemented with the indicated amounts of His-dSRp38 (lanes 2 and 3) and analyzed for splicing activity.

(D) Restoration of splicing repression by dSRp38 is specific. The indicated proteins were added to SRp38-depleted extract and splicing analyzed as above.

RS domain-containing general splicing factors. RBD-mediated interactions with the pre-mRNA, while necessary (Zuo and Manley, 1993; Caceres and Krainer, 1993), are of less importance, and likely display relaxed sequence specificity. Given the general nature of repression mediated by SRp38, it is likely that similar principles underlie its mechanism of action. It has also been shown that the phosphorylation status of the splicing activator ASF/SF2 strongly influences interactions with other proteins (Xiao and Manley, 1997, 1998), and it is therefore reasonable to believe that dephosphorylation of SRp38 activates splicing repression by influencing protein-protein interactions. It is noteworthy that dephosphorylation of SRp38 weakens interactions with other SR proteins, as this is opposite to the response observed with ASF/SF2 (Xiao and Manley, 1998). This could contribute to repression, for example by disrupting required SR protein interactions.

It is likely that SRp38, like other SR proteins, possesses sequence-specific activities in addition to its general repression function. This is consistent with the high-affinity, sequence-specific RNA binding we detected by SELEX, and with the ability of phosphorylated SRp38 to interact with classical SR proteins and the Tra2 splicing regulators (unpublished data). It is possible that SRp38 cooperates with SR/Tra2 proteins to regulate (positively and/or negatively) splicing of specific target transcripts. Perhaps this reflects functions for example

in brain (e.g., Komatsu et al., 1999), which has high SRp38 levels but few mitotic cells.

SRp38 was identified previously by several different approaches. TLS/FUS-associated SR proteins TASR-2 and -1, which correspond to SRp38 and SRp38-2, were isolated from a yeast two-hybrid screen with TLS/FUS as bait (Yang et al., 1998, 2000). TLS/FUS is a member of a family of proteins that associate with general transcription factors such as TFIID and RNA polymerase II (RNAP II), as well as with SR protein splicing factors, and may thus function in linking transcription and RNA processing (reviewed in Hirose and Manley, 2000). Neural salient SR proteins 1 and 2, corresponding to SRp38 and SRp38-2, were discovered with degenerate RBD and RS domain primers from a neural-enriched cDNA library (Komatsu et al., 1999). In both the above studies, transient cotransfection assays suggested that overexpression of the proteins influenced alternative splicing of reporter transcripts. SRp40 (identical to SRp38) was uncovered by database searching (Cowper et al., 2001). As referred to above, recombinant SRp40 displayed repression activity at high concentrations, likely reflecting competition with activating SR proteins.

Our studies have shown that pre-mRNA splicing can be strongly inhibited in mitotic cell extracts. Splicing thus joins the other major steps in gene expression, transcription, polyadenylation, and translation, known to be silenced in M phase. However, there is an intriguing

difference between the mechanisms by which splicing and these other processes are repressed. In the other cases, specific essential general factors are inhibited: subunits of TFIID (Segil et al., 1996) and TFIIF (Akoulitchev and Reinberg, 1998; Long et al., 1998) in RNAP II-mediated transcription, poly(A) polymerase in 3' end formation (Colgan et al., 1996), and eIF4G in cap-dependent translation (Pyronnet et al., 2001). In splicing, our data suggest that the general splicing machinery is largely if not entirely intact, as it can be extracted from mitotic cells in active form at low salt. The inhibition we observed was mediated instead by activation of a specific repressor, SRp38. Given that the concentration of SRp38 is high and roughly equivalent to that of snRNPs ($>10^6$ /cell), dSRp38-induced repression is capable of effectively silencing the splicing machinery during M phase. Why the cell would use a dedicated repressor to inhibit splicing is unclear. One possibility reflects the fact that the massive, complex splicing machinery relies on numerous redundant components (e.g., SR proteins) to guarantee its precise functioning. It might therefore be difficult to silence splicing without inactivating many components; activation of a single repressor could be more efficient. Another possibility is that leaving the splicing machinery intact facilitates processing of a possible class of mRNAs that must be produced during M phase; i.e., that are SRp38 resistant.

Regardless of the rationale for the existence of a mechanism employing an M phase-specific repressor, a more general question is why must the splicing machinery be actively repressed during mitosis? Might not it be sufficient to block transcription to prevent synthesis of new mRNAs, and polyadenylation to prevent maturation of newly synthesized transcripts? One possibility is that splicing repression is a redundancy to ensure that inappropriate gene expression does not occur during mitosis. Another is that it is advantageous to the cell to shut down all processes that could compete or interfere with the essential events of mitosis. Repression could also reflect a mechanism to prevent deleterious association of nuclear spliceosomes with cytoplasmic components, including mature mRNA, as a consequence of nuclear envelope breakdown. Any or all of these scenarios could explain the importance of mitotic splicing repression.

Activation of a mitotic regulator by dephosphorylation is relatively unusual; more typical are phosphorylation-mediated mechanisms involving direct phosphorylation by *cdc2/cyclin B*. In addition to the factors mentioned above that are important for synthesis of mRNA, factors required for RNAP I and RNAP III transcription are inactivated by *cdc2/cyclin B* phosphorylation (reviewed by Gottesfeld and Forbes, 1997), and phosphorylation of histones is important for proper chromosome condensation (e.g., Van Hooser et al., 1998). However, as mentioned above, cap-dependent translation is inhibited during mitosis by dephosphorylation (Pyronnet et al., 2001). It is unclear why translation and splicing utilize dephosphorylation as the final step in mitotic repression. It is notable, however, that SRp38 contains only two SP (or TP) dipeptides, required for cdk phosphorylation, and neither of these match the cdk consensus, S/TPXR/K. It will be important in the future to identify the M phase-specific phosphatase responsible for SRp38

dephosphorylation, as well as to understand in more detail how dSRp38 mediates such efficient repression. Our results have provided evidence that dephosphorylation of a splicing regulator can play an important role in cell cycle-dependent control of gene expression.

Experimental Procedures

SRp38 cDNA Isolation

Yeast two-hybrid screening was performed as described (Okamoto et al., 1996). To isolate Tra2 α interacting proteins, $\sim 10^7$ transformants from cDNA libraries of human lymphocyte, HeLa, and mouse fibroblast were screened. Database homology searching revealed ten different clones, including two at the time unknown proteins with structures typical of SR proteins, which we named SRp38 and SRp38-2. PCR cloning was used to construct expression plasmids and these were confirmed by full-length sequencing.

Antibody Production

Polyclonal antibodies were raised in rabbits (Cocalico Biologicals) against full-length SRp38 or the RBD (used in Figure 5B). Both antisera were affinity purified with the SRp38 RBD coupled to Affi-Prep 10 Support (Bio-Rad) beads using standard procedures (Harlow and Lane, 1988). The anti-SRp38, but not the anti-RBD, antibodies recognized dSRp38 ~ 2 -fold more efficiently than SRp38.

Mouse Tissues and HeLa Cells Western Blotting

Mice (male and female) tissues were sectioned and immediately frozen. Preparation of tissue samples was performed essentially as described (Hanamura et al., 1998). HeLa cells grown on plates were washed with cold PBS and lysed on the plates with protein SDS sample buffer. Whole-cell extracts prepared from HeLa cells released from a double-thymidine block were the kind gift of Y. Xu.

Recombinant Proteins

His-SRp38, SC35 and ASF/SF2 and GST-SRp38 were prepared from recombinant baculovirus-infected Sf9 cells. His-tagged recombinant proteins were purified under denaturing conditions by Ni²⁺ agarose chromatography and renatured by dialysis (Tacke and Manley, 1995). GST-SRp38 RBD and hnRNP G RBD proteins were prepared from *E. coli* JM101 using glutathione-Sepharose 4B. His-SC35 RBD was prepared from *E. coli* BL21 (Tacke and Manley, 1995). Purity and concentration of proteins were determined by Coomassie blue staining of SDS gels.

Dephosphorylation of Recombinant Proteins and Extracts

One hundred micrograms of His-tagged protein was incubated with 100 units of CIP (NEB) for 30 min at 37°C in phosphatase buffer (10 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 70 mM NaCl, and 1 mM DTT), where indicated in the presence of 50 mM β -glycerophosphate, and repurified by Ni²⁺ agarose chromatography as described above. Quantitation of repurified proteins was carried out by silver staining and comparison with known standards and by Western blotting using anti-histidine antibody (H-15, Santa Cruz Biotechnology) with known amounts of His-tagged control proteins. Fifty micrograms of nuclear extracts, from asynchronous or mitotic cell extracts, was dephosphorylated by incubation with 30 units of CIP for 30 min at 37°C in phosphatase buffer and analyzed by Western blotting.

In Vitro Splicing Assays

In vitro splicing was performed essentially as described (Tacke and Manley, 1995). Spliceosome assembly assays were performed as described (Hirose et al., 1999).

Whole-Cell Extract Preparation

For isolation of M phase HeLa cells, nocodazol (Sigma) was added to a final concentration of 40 ng ml⁻¹ as described (Colgan et al., 1996). After 24 hr, 95% of the cells were arrested in M phase, as determined by fluorescence-activated cell sorting. Whole-cell extracts were prepared from asynchronous and mitotic cells as described in Manley et al. (1980) with modifications. HeLa cells ($\sim 10^9$ cells) were collected, washed with cold PBS, and swollen in 18 ml

of Buffer A (Dignam et al., 1983) for 10 min on ice. Swollen cells were lysed by homogenization with 10 strokes using a tight pestle (Kontes). Six milliliter aliquots of each cell lysate were mixed with 3 ml Buffer C (Dignam et al., 1983), except containing NaCl to give final concentrations of 0.3, 0.6, 0.7, or 2 M for 30 min at 4°C. The resultant extracts were centrifuged at 40,000 rpm in a Beckman 50 Ti rotor for 1 hr at 4°C, and supernatants were dialyzed against buffer D (Dignam et al., 1983). Extracts were quick-frozen and stored at -80°C.

Depletion of dSRp38 Using RNA Affinity

SELEX with purified GST-SRp38 RBD was performed for eight rounds essentially as described (Tacke and Manley, 1995). Three copies of the DNA sequence corresponding to the consensus (5'-ACAAAGACAAA) were subcloned into pGEM3 as described (Tacke and Manley, 1995), and biotinylated C3 RNA (bio-C3 RNA) was transcribed in the presence of 0.4 mM biotin-16-UTP (Enzo Life Sciences) and 1.5 mM UTP with SP6 RNA polymerase. Fifty micrograms of bio-C3 RNA was bound to 100 μ l of avidin-agarose beads (Sigma). After washing with buffer D, the beads were divided into two aliquots. Two tubes of beads were prepared for mock depletion; 300 μ l of mitotic extract was added to the first batch of mock beads or bio-C3 RNA beads and rocked for 4 hr at 4°C. After a brief spin, the supernatant was transferred to the next tube and the process repeated. Depletion experiments were repeated three times with variations in splicing activity of no more than 20%.

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