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Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro

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ABSTRACT

The splicing reaction that removes introns from pre-messenger RNAs requires the assembly of the spliceosome on the nascent transcript, proper folding of the substrate–enzyme complex, and finally, two transesterification reactions. These stages in the splicing reaction must require careful orchestration. Here we show data that suggest that the sequential phosphorylation and dephosphorylation of SR proteins mark the transition between stages in the splicing reaction. Many data had already led to the idea that phosphorylation of SR proteins could modulate their activity, when we showed that dephosphorylation of these proteins abrogates their activity in a reaction measuring conversion of pre-spliceosomes to spliceosomes (Roscigno RF, Garcia-Blanco MA, 1995, *RNA 1*:692–706). Subsequently, Xiao and Manley (1997, *Genes & Dev 11*:334–344) showed that phosphorylated ASF/SF2, but not mock-phosphorylated ASF/SF2, activates the splicing of HIV tat pre-mRNA in reactions challenged with excess random RNA. Here we confirm and extend these two findings. Phosphorylated ASF/SF2 efficiently complemented an SR protein-deficient HeLa S100 extract in promoting the splicing of an adenovirus-2-derived pre-messenger RNA, whereas unphosphorylated ASF/SF2 did not. Moreover, we show that, whereas unphosphorylated ASF/SF2 inhibited splicing in HeLa nuclear extracts, phosphorylation of the ASF/SF2 reversed the inhibition and enhanced splicing. We also present data that shows that dephosphorylation of ASF/SF2 is required for the first transesterification reaction once the spliceosome has assembled. Thiophosphorylated ASF/SF2, which cannot be readily dephosphorylated, can promote spliceosome assembly, but cannot promote the first transesterification reaction. These data, together with other observations, indicate for the first time a requirement for SR protein dephosphorylation in pre-messenger RNA splicing in vitro.

Keywords: mRNA splicing mechanism; mRNA splicing signals; phosphorylation; SR domain

INTRODUCTION

SR proteins are a family of related factors that have been highly conserved from *Drosophila melanogaster* to humans (Zahler et al., 1992; Fu, 1995; Manley & Tacke, 1996; Valcarcel & Green, 1996). More distantly related proteins have been found in *Saccharomyces cerevisiae* and plants, suggesting a more extended evolutionary history to the family (Fu, 1995). Many SR proteins are splicing factors required for constitutive or alternative splicing (Ge & Manley, 1990; Krainer et al., 1990a, 1990b). SR proteins have a modular structure, beginning with one or two RNA binding domains near the amino terminus, usually followed by a glycine-rich region. A carboxy-terminal arginine/serine (RS) domain gives the proteins their name (Zahler et al., 1993a, 1993b). This RS domain contains runs of arginine–serine dipeptides of varying length, and the serine residues can be phosphorylated, creating the epitope recognized by monoclonal antibody mAb104 (Roth et al., 1991). SR proteins increase the apparent binding affinity of U1 snRNP for 5′ splice sites (Eperon et al., 1993; Kohtz et al., 1994; Jamison et al., 1995), and this effect is thought to be exerted at very early stages of spliceosome assembly (Krainer et al., 1990a; Fu, 1993; Kohtz et al., 1994; Staknis & Reed, 1994; Jamison et al., 1995). It is very likely that SR proteins bind the 70k

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protein of U1 snRNP (Wu & Maniatis, 1993; Kohtz et al., 1994) and pre-mRNA simultaneously, thus stabilizing the interaction of U1 snRNP with the 5′ splice site. Subsequent interactions involving U2 snRNP, and later U4/U6·U5 tri-snRNP, are also dependent on SR proteins (Roscigno & Garcia-Blanco, 1995; Tarn & Steitz, 1995).

ASF/SF2, a member of the SR protein family, plays a critical role in constitutive and alternative splicing of pre-mRNAs (Ge & Manley, 1990; Krainer et al., 1990a, 1990b). The constitutive splicing activity is strictly dependent on the RS domain in the carboxy terminus of the protein (Caceres & Krainer, 1993; Zuo & Manley, 1993). This domain mediates the interaction of ASF/SF2 with the U1 70k protein and is required for the assembly of a trimeric complex, including U1 snRNP, ASF/SF2, and the 5′ splice site of the pre-mRNA (Wu & Maniatis, 1993; Kohtz et al., 1994; Jamison et al., 1995).

SRPK1 and Clk, two kinases capable of phosphorylating RS domains, have been identified (Gui et al., 1994a, 1994b; Colwill et al., 1996). These kinases have dramatic effects on the subcellular localization of SR proteins (Gui et al., 1994a; Colwill et al., 1996); moreover, addition of high levels of SRPK1 to cell-free extracts of human HeLa cells inhibited splicing of a β-globin pre-mRNA (Gui et al., 1994a). These results suggest that phosphorylation of the RS domain of SR proteins could affect the cellular distribution of SR proteins and also modulate their activity. Tacke et al. (1997) showed that phosphorylation of SRp40, a member of the SR family, contributes to the RNA binding specificity of this protein. We previously demonstrated that phosphatase treatment abrogates the spliceosome-forming activity of SR proteins, indicating that phosphorylation was necessary for SR protein action (Roscigno & Garcia-Blanco, 1995). More recently, Xiao and Manley (1997) have observed that phosphorylated ASF/SF2, but not mock-phosphorylated ASF/SF2, rescues the splicing of HIV tat pre-mRNA in reactions challenged with excess random RNA. These findings indicate that phosphorylation of SR proteins may be critical for constitutive splicing activity in vitro.

Many prior data suggest roles for both protein phosphorylation and dephosphorylation in pre-mRNA splicing. Mermoud et al. (1994) showed that treatment of nuclear extracts with phosphatase blocked spliceosome formation. They further showed that a purified SR protein fraction could restore activity to phosphatase-treated extracts, implying that phosphorylated SR proteins are required for spliceosome assembly (Mermoud et al., 1994). Phosphatase inhibitors do not inhibit spliceosome formation, but block the transesterification reactions (Mermoud et al., 1992; Tazi et al., 1992). The same effect is observed when ATPγS is added to nuclear extracts or when the U1 70k protein is thiophosphorylated (Tazi et al., 1992). Taken together, these data suggest that phosphatase activity, possibly dephosphorylation of U1 70k, is required at a stage between spliceosome formation and the first transesterification reaction. Finally, Cardinali et al. (1994) showed that protein phosphatase 1 can alter 5′ splice site selection in vitro, again suggesting a link between protein phosphorylation and SR proteins.

Here we show that phosphorylated ASF/SF2 (ASF/SF2~P) efficiently complemented a HeLa S100 extract in splicing of an adenovirus-derived pre-mRNA, whereas ASF/SF2 did not. Moreover, ASF/SF2 inhibited splicing when added to fully competent HeLa nuclear extract. Phosphorylation of ASF/SF2 not only reversed this inhibition, but enhanced splicing of this pre-mRNA over that observed in untreated nuclear extract. The requirement for phosphorylation of ASF/SF2 was likely at the formation of the commitment complex, the first stable intermediate in spliceosome formation. These data support and extend our prior work (Roscigno & Garcia-Blanco, 1995) and the work of Xiao and Manley (1997). We also show that thiophosphorylated ASF/SF2 (ASF/SF2~S) could complement a HeLa S100 extract to form spliceosomes, but was incapable of complementing the S100 extracts to undergo the first transesterification reaction of pre-mRNA splicing. These and other data lead us to postulate a requirement for dephosphorylation of ASF/SF2. Therefore, both phosphorylation and dephosphorylation of ASF/SF2 were required for pre-mRNA splicing in vitro.

RESULTS

Efficient splicing in HeLa S100 extracts required phosphorylation of ASF/SF2

The adenovirus-derived pre-mRNA PIP7.A (Gil et al., 1991) assembled into pre-spliceosomes and spliceosomes (Fig. 1, lane 1) and underwent splicing (Fig. 2, lane 1) upon incubation with HeLa nuclear extract (Dignam et al., 1983). As expected, incubation of pre-mRNA with HeLa S100 extract (S100B) did not result in the formation of spliceosomes (Fig. 1, lane 2) or in the appearance of spliced RNA (Fig. 2, lane 2). Surprisingly, pre-incubation with purified recombinant ASF/SF2 could not complement S100B extract to form pre-spliceosomes or spliceosomes (Fig. 1, lane 3) or to perform the splicing reaction (Fig. 2, lane 3). Previously, others had been able to complement S100 extracts with recombinant ASF/SF2 (Ge et al., 1991; Krainer et al., 1991). Although it is not certain why we observe this discrepancy, our complementation assay differed from those described previously. Two differences are worth noting: the levels of recombinant ASF/SF2 used by others were higher (see Ge et al., 1991), and, more importantly, in the complementation assay

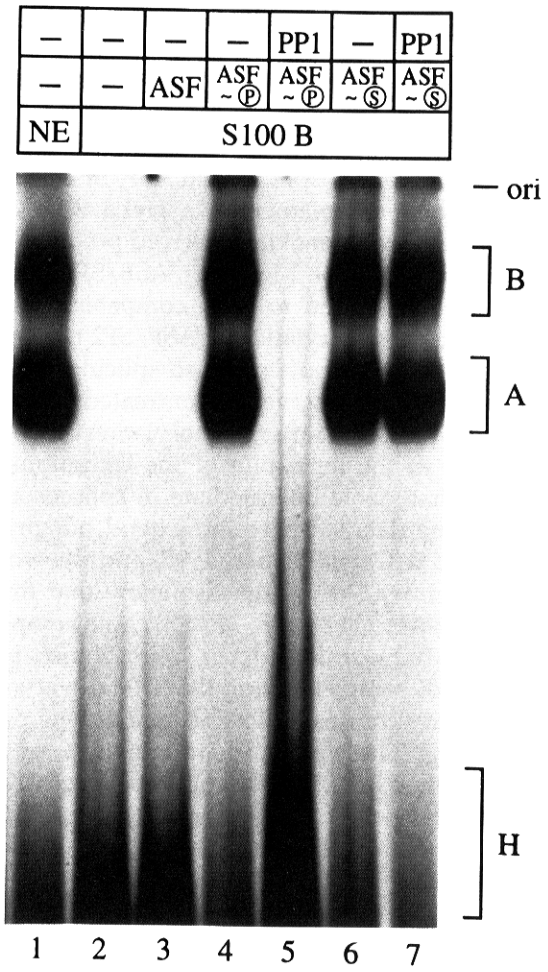

FIGURE 1. Phosphorylation of ASF/SF2 is required for spliceosome formation in HeLa S100 extracts. Uniformly labeled PIP7.A pre-mRNA was mock-pre-incubated or was pre-incubated with ASF/SF2. These reagents were then incubated under splicing conditions in the presence of HeLa cell nuclear extract (NE) or HeLa cell S100 extract (S100B). Formation of spliceosomes was assayed by native gel electrophoresis as described in Materials and Methods. NE promoted the formation of the nonspecific heterogeneous complexes (H), pre-spliceosomes (A), and spliceosomes (B) (lane 1), whereas only H complexes were formed with S100B (lane 2). Pre-mRNA was pre-incubated with 600 ng of: ASF/SF2 (lane 3); ASF/SF2~P (lane 4); ASF/SF2~P treated with rabbit muscle PP1 (lane 5); ASF/SF2~S (lane 6); or ASF/SF2~S treated with rabbit muscle PP1 (lane 7).

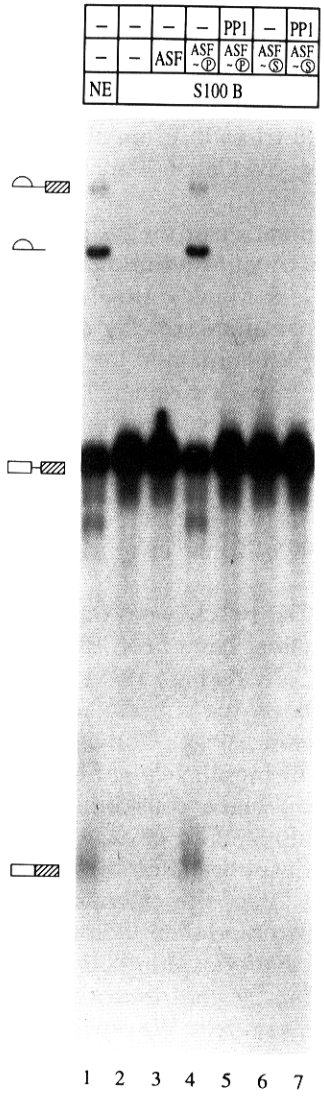

FIGURE 2. Phosphorylation of ASF/SF2 is required for pre-mRNA splicing of HeLa S100 extracts. Uniformly labeled PIP7.A pre-mRNA was mock-pre-incubated or was pre-incubated with preparations of ASF/SF2. These reagents were then incubated under splicing conditions in the presence of HeLa cell nuclear extract (NE) or HeLa cell S100 extract (S100B). The intermediates and products of the splicing reaction were detected using denaturing gel electrophoresis as described in Materials and Methods. NE was capable of pre-mRNA splicing (lane 1), whereas S100B was not (lane 2). Prior to splicing reactions, the RNA was pre-incubated with 600 ng of: ASF/SF2 (lane 3); ASF/SF2~P (lane 4); ASF/SF2~P treated with rabbit muscle PP1 (lane 5); ASF/SF2~S (lane 6); or ASF/SF2~S treated with rabbit muscle PP1 (lane 7). Icons, from top to bottom, indicate migration of the lariat intermediate, lariat product, pre-mRNA, and spliced product; the 5′ exon is not indicated because it was permitted to migrate out of the gel.

done in our experiments, ASF/SF2 was pre-incubated with pre-mRNA before addition to the S100 extract in a protocol similar to that described by Fu (1993) (see Materials and Methods). The conventional complementation assays were done by adding the ASF/SF2 directly to the S100 extract and then these supplemented extracts were tested for activity. It is likely that prebinding to pre-mRNA prevented phosphorylation of ASF/SF2 by kinases in the S100 extract, as was seen with random RNA by Xiao and Manley (1997).

ASF/SF2 was phosphorylated, in a RS domain-dependent fashion, with SRPK1 (data not shown) using conditions described previously (Gui et al., 1994a, 1994b). ASF/SF2~P efficiently complemented the S100 extract in spliceosome formation (Fig. 1, lane 4) and splicing (Fig. 2, lane 4). The identical preparation of ASF/SF2 was used in lanes 3 and 4, and thus the differences obtained were not due to different specific activities of

the protein preparations. Moreover, the results were reproduced with several independent preparations of ASF/SF2; in all cases, activity was seen with ASF/SF2~P, but not with mock-phosphorylated ASF/SF2 or dephosphorylated preparations (see below, Fig. 1, lane 5; data not shown). Thus, we concluded that phosphorylation was required for the constitutive splicing activity of ASF/SF2 in vitro as defined by our complementation assays using S100 extracts. Moreover, the phosphorylation requirement had to be at the stage of pre-spliceosome assembly or at an earlier stage.

Phosphorylation status determines the action of ASF/SF2 in HeLa nuclear extract

We tested the effect of pre-incubating pre-mRNA with ASF/SF2~P or ASF/SF2 in a splicing-competent HeLa nuclear extract. Both complex formation (Fig. 3, lane 2) and splicing (Fig. 4, lane 2) were inhibited if ASF/SF2 was added to the pre-mRNA prior to incubation with nuclear extract. This inhibitory effect of ASF/SF2 always coincided with an increase in the levels of nonspecific complexes (complex H) (Fig. 3, lane 2; see also Fig. 5, lane 2). If, however, ASF/SF2 was added after pre-incubation of pre-mRNA and nuclear extract, spliceosomes formed normally (Fig. 5, lane 3). This finding suggested that the inhibitory effect of ASF/SF2 occurred at very early stages of spliceosome assembly. Indeed, pre-incubation of pre-mRNA with ASF/SF2 inhibited commitment complex formation measured using the functional assay first described by Legrain et al. (1988) as modified by Jamison et al. (1992) (data not shown). The inhibitory effect of ASF/SF2 depended on the presence of the RS domain. A mutant protein lacking this domain, ΔRS (Kohtz et al., 1994), whether pre-incubated or added late, was incapable of inhibiting spliceosome formation (Fig. 5, lanes 4 and 5).

We wondered whether or not the inhibitory action of ASF/SF2 on pre-mRNA was subject to regulation by phosphorylation of the RS domain, and thus we tested the effect of ASF/SF2 phosphorylated by SRPK1. Phosphorylation of ASF/SF2 resulted in complete abrogation of the inhibitory effect on spliceosome formation (Fig. 3, lane 3) and on pre-mRNA splicing (Fig. 4, lane 3). Moreover, ASF/SF2~P promoted the formation of spliceosomes (compare lanes 1 and 3 in Fig. 3) and modestly activated splicing (compare lanes 1 and 3 in Fig. 4). In suboptimal splicing reactions, ASF/SF2~P could markedly activate pre-mRNA splicing in a dose-dependent manner (Fig. 6). A clear enhancement over untreated nuclear extract was observed even at 5 ng of ASF/SF2~P (Fig. 6, lane 6). A slight preferential effect on the second phosphoryl transfer step in the splicing reaction, which can be observed in Figure 6, was not reproducible. Considering the data presented above, we concluded that phosphorylation by SRPK1 modulated ASF/SF2 action in HeLa nuclear extracts, reversing it from inhibition to activation.

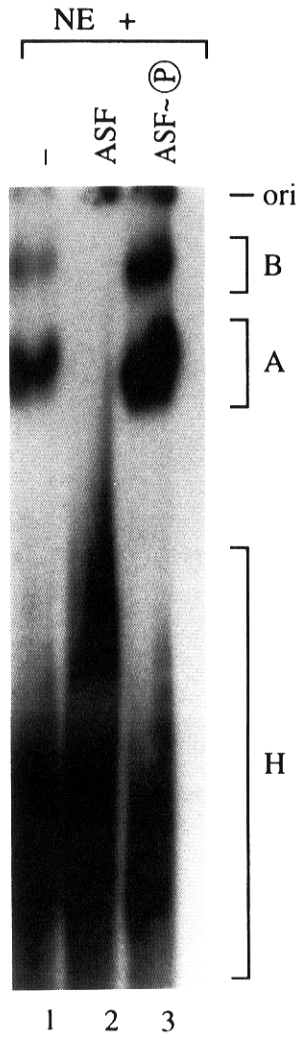

FIGURE 3. Unphosphorylated ASF/SF2 inhibits, whereas phosphorylated ASF/SF2 activates, spliceosome assembly in HeLa cell nuclear extracts. Uniformly labeled PIP7.A pre-mRNA was mock-pre-incubated or was pre-incubated with preparations of ASF/SF2. These reagents were then incubated under splicing conditions in the presence of HeLa cell nuclear extract (NE). Formation of spliceosomes was assayed by native gel electrophoresis as described in Materials and Methods. Nuclear extract promoted the formation of the nonspecific heterogeneous complexes (H), pre-spliceosomes (A), and spliceosomes (B) (lane 1). Prior to splicing reactions, the RNA was pre-incubated with 20 ng recombinant ASF/SF2 (lane 2) or 20 ng ASF/SF2~P (lane 3).

Phosphorylation of ASF/SF2 is not required for complex formation with pre-mRNA and purified U1 snRNP

Complexes form upon incubation of ASF/SF2, purified U1 snRNP, and PIP7.A pre-mRNA (Kohtz et al.,

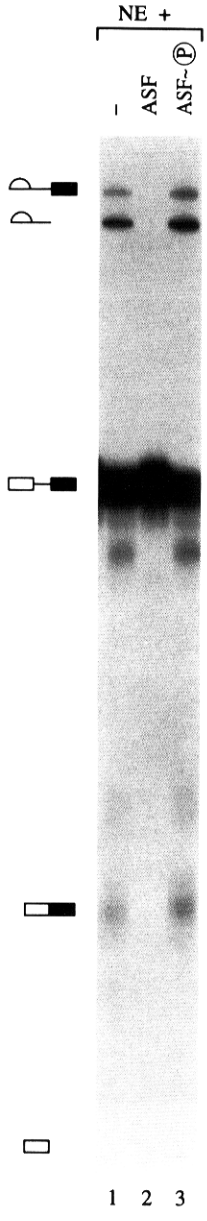

FIGURE 4. Unphosphorylated ASF/SF2 inhibits, whereas phosphorylated ASF/SF2 activates, pre-mRNA splicing in HeLa cell nuclear extracts. Uniformly labeled PIP7.A pre-mRNA was mock-pre-incubated or was pre-incubated with preparations of ASF/SF2. These reagents were then incubated under splicing conditions in the presence of HeLa cell nuclear extract (NE). The intermediates and products of the splicing reaction were detected using denaturing gel electrophoresis as described in Materials and Methods. HeLa nuclear extract was competent to perform pre-mRNA splicing (lane 1). Prior to splicing reactions, the RNA was pre-incubated with 20 ng recombinant ASF/SF2 (lane 2) or 20 ng ASF/SF2~P (lane 3). Icons, from top to bottom, indicate migration of the lariat intermediate, lariat product, pre-mRNA, spliced product, and 5′ exon.

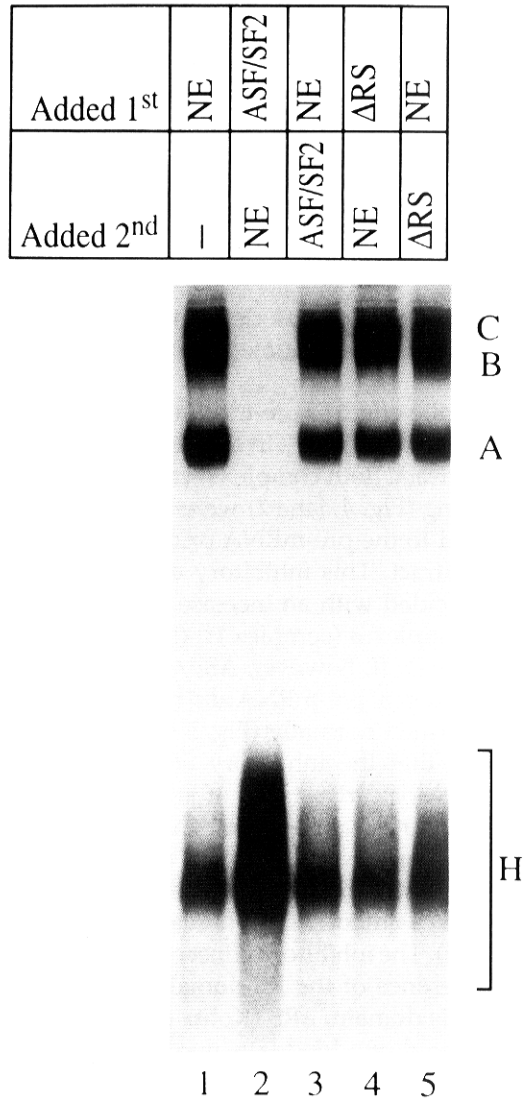

FIGURE 5. ASF/SF2 inhibits spliceosome formation in an RS domain-dependent manner. Formation of spliceosomes was assayed by native gel electrophoresis. Uniformly labeled pre-mRNA was incubated with HeLa cell nuclear extract (NE) under splicing conditions and this led to the efficient formation of the nonspecific heterogeneous complexes (H), pre-spliceosomes (A), and spliceosomes (B, C) (lane 1). Prior to splicing reactions, the RNA was: pre-incubated with 20 ng recombinant ASF/SF2 (lane 2); pre-incubated with nuclear extract and subsequently incubated with ASF/SF2 (lane 3); pre-incubated with 20 ng of a mutant of ASF/SF2 missing the RS domain (ΔRS) (lane 4); or pre-incubated with nuclear extract and subsequently incubated with ΔRS (lane 5). The migration of complexes A, B, C, and H is indicated. Complex C, which is observed as a heterogeneous set of complexes migrating slower than B, is not easily detectable and thus could not be identified in all experiments.

1994; Jamison et al., 1995). We asked whether or not ASF/SF2~P could collaborate with pre-mRNA and U1 snRNP to form these trimeric complexes, which are likely to represent pre-commitment complexes. Indeed, ASF/SF2~P formed complexes with pre-mRNA and U1 snRNP as described previously for ASF/SF2 (data not shown). In these experiments, ASF/SF2 was pre-incubated with pre-mRNA as described above for experiments performed in S100 or nuclear extracts. Complexes formed with similar efficiency, whether

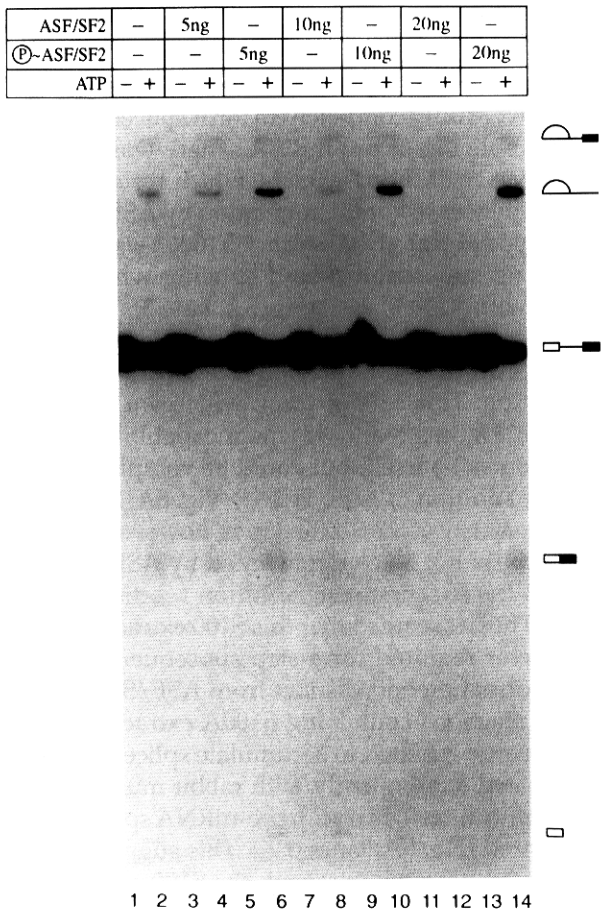

FIGURE 6. Phosphorylation of ASF/SF2 by SRPK1 reverses the inhibition and activates pre-mRNA splicing in vitro. Splicing of PIP7.A pre-mRNA was performed as described in Figure 4, without added ATP (odd-numbered lanes) or supplemented with 1 mM ATP (even-numbered lanes) as indicated. pre-mRNA was incubated directly under splicing conditions (lanes 1 and 2) or was pre-incubated with ASF/SF2 or with of ASF/SF2~P, prior to the incubation under splicing conditions at a concentration of 5 ng, 10 ng, or 20 ng, respectively, as indicated (lanes 3–14). Icons, from top to bottom, indicate migration of the lariat intermediate, lariat product, pre-mRNA, spliced product, and 5′ exon.

ASF/SF2 or ASF/SF2~P was used; however, the latter formed complexes with slower migration (data not shown). We take these data to indicate that phosphorylation of ASF/SF2 is not required for the earliest action of this protein, the promotion of 5′ splice site recognition by the U1 snRNP (Kohtz et al., 1994; Jamison et al., 1995).

Protein phosphatase 1 (PP1) dephosphorylates ASF/SF2 and eliminates the activation of splicing mediated by ASF/SF2~P

In order to show that phosphorylation of ASF/SF2 was responsible for the observed activity, we performed the following experiments. First, we repeated our observations using two different forms of SRPK1 kinase, a baculovirus-derived SRPK1 and an *Escherichia coli*-derived Gst-SRPK1 fusion protein that retains SRPK1 activity (see Materials and Methods). The results obtained were the same regardless of the kinase preparation used (data not shown). Second, ASF/SF2~P was incubated with 0.4 U/μL of purified rabbit muscle PP1, a serine-threonine phosphatase (see Materials and Methods), which resulted in complete dephosphorylation of the ASF/SF2 (data not shown). Dephosphorylated ASF/SF2 was unable to complement S100 extract in spliceosome formation (compare lanes 4 and 5, in Fig. 1) or in pre-mRNA splicing (compare lanes 4 and 5 in Fig. 2). Moreover, dephosphorylated ASF/SF2 inhibited splicing as efficiently as ASF/SF2 that had never been phosphorylated (Fig. 7, compare lanes 3 and 5). Third, we incubated ASF/SF2~P with PP1 in the presence of the phosphatase inhibitor microcystin-LR (MacKintosh et al., 1990). The ASF/SF2 remained phosphorylated and, as expected, could complement an S100 extract (data not shown) and did not inhibit splicing in HeLa nuclear extract, but rather modestly activated it (Fig. 7, lane 6). Because of the dilution of the treated ASF/SF2 preparations after treatment, the levels of microcystin-LR carried into the splicing reactions were not expected to inhibit splicing. Nonetheless, controls were done to insure that the observed effects were not due to the presence of low levels of SRPK1, PP1, or microcystin-LR in the splicing reactions (Fig. 7, lanes 7 and 8, and data not shown). These data demonstrate that the observed activation of splicing was due indeed to phosphorylation of ASF/SF2.

Thiophosphorylated ASF/SF2 can complement S100 extracts to form spliceosomes but not to perform the first step of splicing

Gst-SRPK1 and ATPγS were used to thiophosphorylate ASF/SF2, a modification that is not readily reversed by phosphatases. Thiophosphorylated ASF/SF2 (ASF/SF2~S) could complement S100B extract to form spliceosomes (Fig. 1, lane 6) as efficiently as ASF/SF2~P, and, as expected, PP1 treatment did not affect the action of ASF/SF2~S (Fig. 1, lane 7). ASF/SF2~S, however, could not complement the S100 extract to complete the first transesterification reaction (Fig. 2, lane 6).

Pre-incubation of pre-mRNA with ASF/SF2~S inhibited splicing (Fig. 7, lane 9), whereas the components of a mock thiophosphorylation reaction, in which ASF/SF2 was omitted, did not (Fig. 7, lane 10). Interestingly, pre-incubation of pre-mRNA with ASF/SF2~S did not inhibit spliceosome formation under conditions in which pre-incubation with ASF/SF2 led to complete inhibition (data not shown). These data suggested a requirement for dephosphorylation of ASF/SF2 at a stage subsequent to spliceosome formation

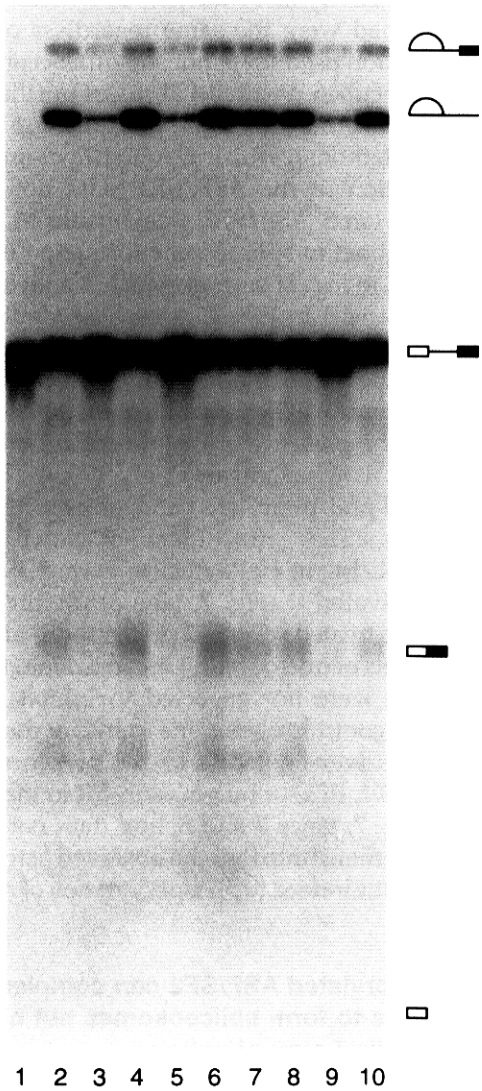

FIGURE 7. Both phosphorylation and dephosphorylation of ASF/SF2 are required for activation of splicing in vitro. Splicing of PIP7.A pre-mRNA was performed as described in Figure 4, in the absence of added ATP (lane 1) or in the presence of 1 mM ATP (lanes 2–10). Pre-mRNA was pre-incubated with: 20 ng of ASF/SF2 (lane 3); 20 ng of ASF/SF2~P (lane 4); 20 ng of ASF/SF2~P that had been treated with 0.4 U/μL of rabbit muscle PP1 (lane 5); 20 ng of ASF/SF2~P that had been treated with 0.4 U/μL of rabbit muscle PP1 but in the presence of 1 μM microcystin-LR (lane 6); PP1 [0.0016 U/μL final concentration] (lane 7); microcystin-LR [4 nM final concentration] (lane 8); 20 ng of ASF/SF2~S (lane 9); or 4 μM ATP-γS (lane 10). Icons, from top to bottom, indicate migration of the lariat intermediate, lariat product, pre-mRNA, spliced product, and 5′ exon.

but prior to the first transesterification step in pre-mRNA splicing. The unlikely possibility that the block seen with ASF/SF2~S was mediated by binding of a phosphatase and steric hindrance to the reaction has not been formally excluded.

PP1 can activate splicing in an S100 extract that cannot be complemented with ASF/SF2~P

In the presence of ASF/SF2~P, the majority of the S100 extracts were fully competent to form spliceosomes and perform splicing of pre-mRNA. There was, however, heterogeneity of these extracts, which resulted in two other responses to complementation by ASF/SF2~P. A second type of HeLa S100 extract (S100C) was incapable of forming spliceosomes and splicing when supplemented with ASF/SF2~P (Fig. 8A, lane 3). This extract has not been investigated further and may be uninformative. A third S100 extract led us to a very interesting observation. This S100 extract, which we designated S100A in Figures 8 and 9, was incapable of forming spliceosomes by itself, but could be complemented to do so by addition of ASF/SF2~P (Fig. 8A, lane 1). Unlike the majority of the S100 extracts, however, the S100A extract could not be complemented by ASF/SF2~P to undergo the first transesterification reaction (Fig. 8B, lane 3). Thus, it seemed that this S100 extract was missing a factor required for a step subsequent to spliceosome formation and distinct from ASF/SF2~P.

If the reactions containing S100A extract and ASF/SF2~P were permitted to accumulate spliceosomes and were treated subsequently with rabbit muscle PP1 (final concentration 0.4 U/μL), pre-mRNA splicing could be activated (Fig. 9A, lanes 3–6). This suggested that a phosphatase was missing from the S100 extract and it could be substituted by PP1, or, alternatively, that this extract contained very high levels of an SRPK1-like kinase. Treatment with PP1 in the absence of ASF/SF2~P did not result in splicing (Fig. 9A, lane 2). We wondered whether the missing activity could be acting on the ASF/SF2~P or on other components of the splicing machinery. Reactions containing S100A extract and 600 ng ASF/SF2~P were permitted to accumulate spliceosomes (1st incubation) and were supplemented subsequently with another 600 ng ASF/SF2~P or ASF/SF2 (2nd incubation) (Fig. 9B). In 2nd incubation reactions supplemented with ASF/SF2~P, no splicing was observed (Fig. 9B, lane 4), however, if ASF/SF2 was added, splicing was activated (Fig. 9B, lane 5) to a similar degree as if PP1 was added (Fig. 9B, lane 6). The experiments above suggested that the missing component in S100A was an activity that could convert the added ASF/SF2~P to the dephosphorylated form. The data above further support a requirement for dephosphorylation of ASF/SF2 prior to the first transesterification reaction in splicing in vitro.

DISCUSSION

Phosphorylation and dephosphorylation act as signals that demarcate stages in the splicing reaction in vitro

SR proteins have been shown to activate splicing (Ge & Manley, 1990; Krainer et al., 1990a; Fu, 1993), to

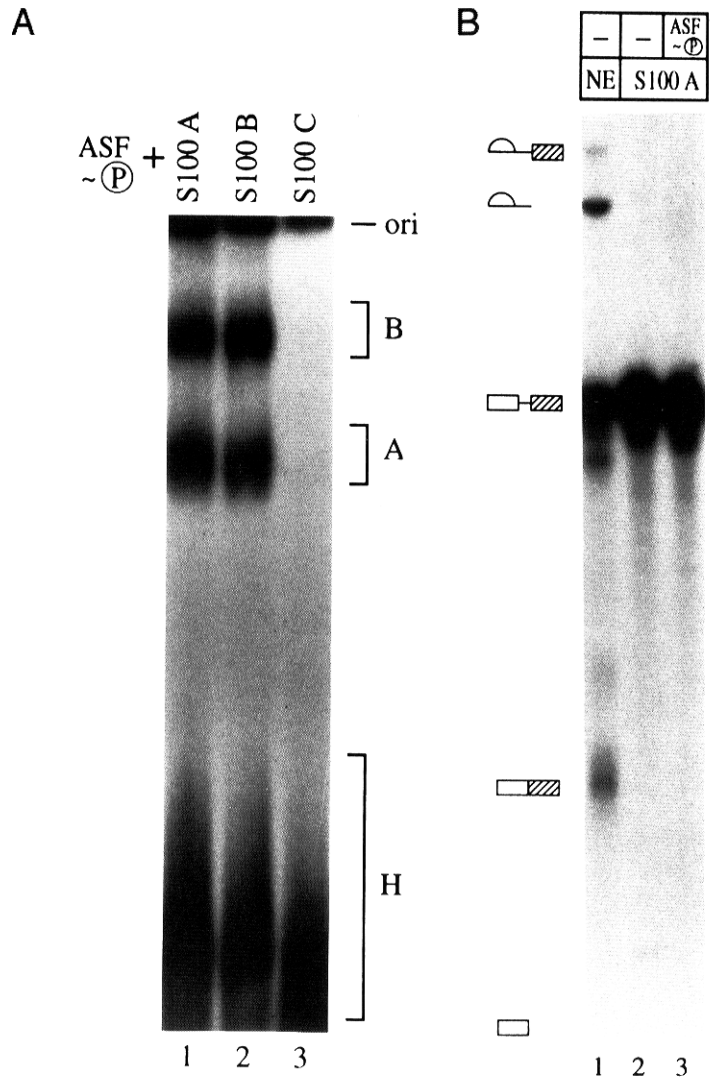

FIGURE 8. Phosphorylated ASF/SF2 can complement S100A extract in spliceosome formation but not in splicing. **A:** Multiple S100 extracts were tested in the complementation assay described in Materials and Methods. Uniformly labeled PIP7.A pre-mRNA was pre-incubated with 600 ng of ASF/SF2~P and subsequently incubated under splicing conditions with HeLa cell S100 extracts, S100A (lane 1), S100B (lane 2), and S100C (lane 3). Formation of spliceosomes was assayed by native gel electrophoresis as described in Materials and Methods. Nonspecific heterogeneous complexes (H), pre-spliceosomes (A), and spliceosomes (B) are indicated. **B:** Uniformly labeled PIP7.A pre-mRNA was incubated under splicing conditions with HeLa cell nuclear extract (NE) or HeLa cell S100A extract. Intermediates and products of the splicing reaction were detected using denaturing gel electrophoresis as described in Materials and Methods. NE was capable of supporting pre-mRNA splicing (lane 1), whereas S100A was not (lane 2), even if the pre-mRNA had been pre-incubated with 600 ng of ASF/SF2~P (lane 3). Icons, from top to bottom, indicate migration of the lariat intermediate, lariat product, pre-mRNA, spliced product, and 5′ exon.

selectively enhance splicing of proximal 5′ alternative splice sites (Ge & Manley, 1990; Krainer et al., 1990b), and, more recently, to inhibit splicing (Kanopka et al., 1996). In agreement with our prior data (Roscigno & Garcia-Blanco, 1995) and that of Xiao and Manley (1997), we conclude that phosphorylation of ASF/SF2 is required for efficient spliceosome formation and splicing in vitro. We speculate that this requirement will be observed with other SR proteins, such as SC35, and, moreover, that this requirement will be important for in vivo activity. We also conclude that dephosphorylation of ASF/SF2 is equally critical, and is required for the first transesterification reaction of pre-mRNA splicing. Our data agree with, and may explain, the effect of phosphatases (Cardinali et al., 1994; Mermoud et al., 1994) and their inhibitors (Mermoud et al., 1992; Tazi et al., 1992) on pre-mRNA splicing and spliceosome formation. Phosphorylation of ASF/SF2 at the commitment step may serve as the signal, which is possibly transduced via a modified interaction between ASF/SF2 and U1 snRNP, as suggested by the data of Xiao and Manley (1997), for further assembly of the spliceosome. A second signal provided by the dephosphorylation of ASF/SF2 is required once splice-

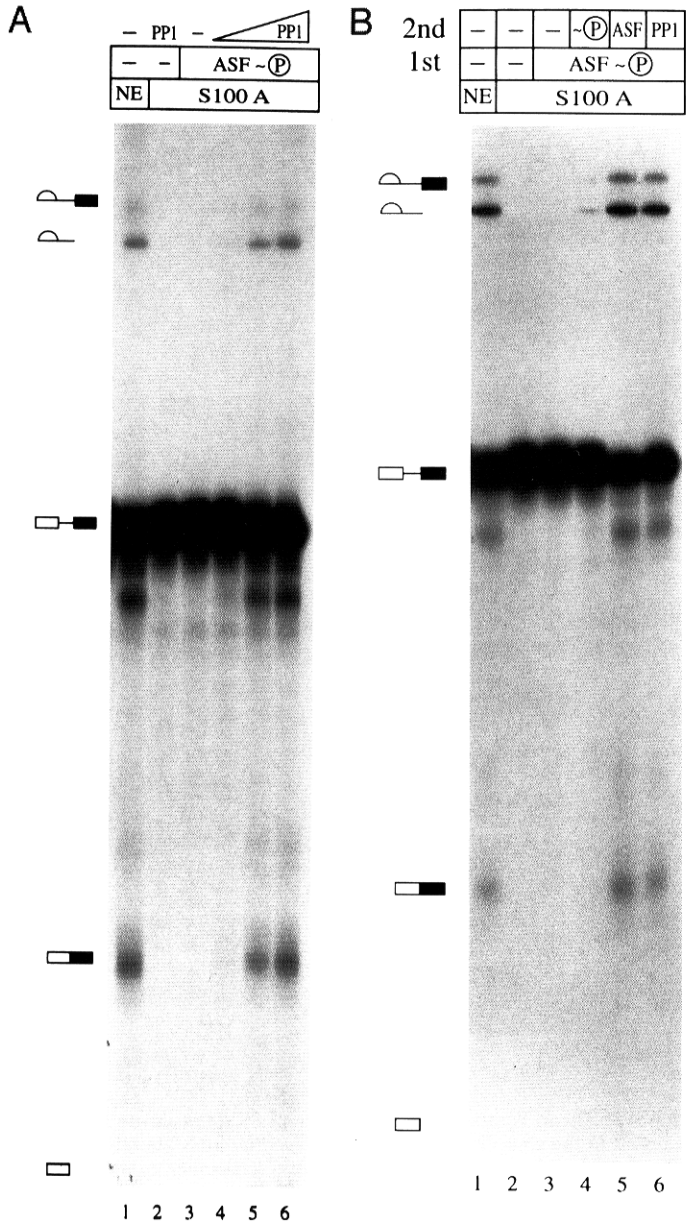

FIGURE 9. S100A extract can be rescued by incubating with rabbit muscle PP1. **A:** Uniformly labeled pre-mRNA was pre-incubated with a mock buffer (lanes 1 and 2), or 600 ng ASF/SF2~P (lanes 3–6), and subsequently incubated with HeLa cell nuclear extract (NE) or HeLa cell S100A extract under splicing conditions. After a 20-min incubation, some of the reactions were supplemented with 0.1 U, 0.2 U, or 0.4 U of rabbit muscle PP1 (lane 4–6) and incubated again for an additional 20 min (see Materials and Methods). **B:** Uniformly labeled pre-mRNA was pre-incubated with a mock buffer (lanes 1 and 2) or 600 ng ASF/SF2~P (lanes 3–6), and subsequently incubated with HeLa cell nuclear extract (NE) or HeLa cell S100A extract under splicing conditions (1st incubation). After a 15-min incubation, some of the reactions were supplemented with an additional 600 ng ASF/SF2~P (lane 4), 600 ng ASF/SF2 (lane 5), or 0.4 U of rabbit muscle PP1 (lane 6) and incubated again for an additional 30 min (2nd incubation). Splicing of pre-mRNA was determined by separation of intermediates and products using denaturing gel electrophoresis as described in Materials and Methods. Icons, from top to bottom, indicate migration of the lariat intermediate, lariat product, pre-mRNA, spliced product, and 5′ exon.

osome assembly is complete, and perhaps after all conformational changes within it have taken place, but before the first phosphoryl transfer reaction. Thus, phosphorylation and dephosphorylation of ASF/SF2, and by extension of other SR proteins, may be a timing mechanism that coordinates the complex series of interactions that must be made and unmade during splicing of pre-mRNA.

A direct role for phosphorylation and dephosphorylation on the splicing reaction

The fact that both phosphorylation and dephosphorylation of an SR protein are required to proceed through the splicing reaction argues for a direct role of phosphorylation, rather than a role secondary to effects of nuclear localization or sequestration. It is more likely

that the nuclear changes observed by addition of SRPK1 of Clk kinases (Gui et al., 1994a; Colwill et al., 1996) or phosphatases (Misteli & Spector, 1996) are due to inhibition of splicing activity, similar to nuclear changes observed upon inhibition of transcription (Misteli et al., 1997). This is not to say that phosphorylation may not also play a role in the cell cycle regulation of the nuclear distribution of SR proteins (see discussion in Fu, 1995).

Phosphorylation of ASF/SF2 is required for spliceosome assembly in vitro

Mermoud et al. (1994) showed that phosphatase treatment of nuclear extracts inhibited spliceosome formation, but did not affect an early interaction between the U1 snRNP and pre-mRNA. In agreement with this, we have observed that both ASF/SF2 and ASF/SF2~P can interact with U1 snRNP and pre-mRNA to form complexes. We postulate that an early pre-commitment complex between SR proteins, U1 snRNP, and the 5′ splice site of the pre-mRNA may form with dephosphorylated SR proteins. One can envision that phosphorylation of SR proteins is required to drive commitment complex formation. This could be accomplished by increasing the affinity of SR proteins for the U1 70k protein (Xiao & Manley, 1997), and by modifying the RNA binding properties of the phosphorylated SR proteins (Tacke et al., 1997; Xiao & Manley, 1997). It is also possible that phosphorylation of ASF/SF2 enhances binding to factors at the 3′ end of the intron, such as U2AF (Wu & Maniatis, 1993).

We have shown previously that SR proteins are required for efficient conversion of pre-spliceosomes to spliceosomes (Roscigno & Garcia-Blanco, 1995). Dephosphorylation of the SR proteins inhibited this activity (Roscigno & Garcia-Blanco, 1995). Consistent with this, we observed here that ASF/SF2~P promoted higher spliceosome to pre-spliceosome ratios in HeLa nuclear extracts. We postulated that formation of spliceosomes from spliceosomes requires SR protein–snRNP interactions similar to those observed between ASF/SF2 and U1 70k, and predicted the existence of 70k-like or SR-like proteins in U4/U6·U5 tri-snRNP (ibid). Recently, Fetzer et al. (1997) demonstrated that the tri-snRNP-associated 27k protein is an SR protein.

Dephosphorylation of ASF/SF2 is required for the first transesterification reaction in vitro

The fact that ASF/SF2~S could substitute ASF/SF2~P in activating spliceosome formation in S100 extracts suggests that dephosphorylation of ASF/SF2 is not required for spliceosome assembly. ASF/SF2~S, however, could not complement S100 extracts to perform the first transesterification reaction. Moreover, ASF/SF2~S inhibited the first transesterification in HeLa nuclear extracts. These data indicate that dephosphorylation of this protein is required for this splicing reaction. This finding is consistent with three observations of others. First, inhibitors of phosphatase activity block the phosphoryl transfer reactions of splicing (Mermoud et al., 1992; Tazi et al., 1992). In particular, it was suggested that a form of PP1 is involved in the first step of splicing. Second, high levels of SRPK1, which could overwhelm endogenous phosphatase activity, inhibited splicing of an HIV tat pre-mRNA (Gui et al., 1994b). Third, high levels of Clk kinase, but not of an inactive mutant, could inhibit splicing, again possibly by overwhelming native phosphatases (J. Manley, pers. comm.).

Multiple phosphatases and multiple targets for phosphatases?

Our data show an interesting parallel to prior work of Tazi et al. (1993), who showed that thiophosphorylation of the U1 70k protein did not affect spliceosome formation but inhibited the splicing reaction prior to the first step. Thus, it seems that phosphorylated 70k and phosphorylated SR proteins are both critical targets of phosphatases. It is intriguing to speculate on the temporal relationship between phosphatase action and the conformational changes that take place in the spliceosome prior to the first transesterification reaction. Much like the fact that phosphorylation of SR proteins is required for these proteins to promote snRNP–snRNP assembly, dephosphorylation may be required to permit dynamic snRNP–snRNP disassembly and movement within the spliceosome. In particular, it is possible that releasing the pairing between U1 snRNA and the 5′ splice site requires a weakening of the interaction between 70k protein and SR proteins such as that caused by dephosphorylation (Xiao & Manley, 1997).

The differential sensitivity of the two transesterification steps to okadaic acid (Mermoud et al., 1992) suggests two distinct forms of phosphatase and at least two different targets of dephosphorylation. It is very likely that our data and that on 70k may explain, at least in part, the requirement for a phosphatase in the first step of splicing. It is not clear whether or not this phosphatase is assembled with the spliceosome. An association between PSF, a splicing factor, and PP1 was noted in a two-hybrid screen using the catalytic subunit of PP1 as bait, and was confirmed in vitro (Hirano et al., 1996). PSF has been described as required for an early step in spliceosome assembly (Patton et al., 1993) and as a factor required for the second step (Gozani et al., 1994). The interaction between PSF and PP1 may provide a clue for the identity of one form of the phosphatase associated with the spliceosome. The fact that both ASF/SF2 and PP1 treatment can rescue the arrested spliceosomes in reactions performed in S100A extracts suggests that the endog-

enous SR protein phosphatase does not have to be tightly associated with spliceosomes. This finding also implies that spliceosome-bound SR proteins can exchange with SR proteins in the extract.

MATERIALS AND METHODS

Extracts, proteins, and vectors

HeLa nuclear extracts and S100 extracts were prepared as described by Dignam et al. (1983). S100 extracts made at different dates were given letters to identify them (e.g., S100A). Eight S100 extracts were tested, two showed complementation by ASF/SF2~P as seen with S100B, three showed complementation, but with significantly weaker activity, one (S100A) showed complementation of spliceosome formation but not of splicing, and two could not be complemented at all (S100C). This heterogeneity in the preparations of S100 extracts, which contrasted with nuclear extract preparations, has been observed by others.

The His-tagged ASF/SF2 and ΔRS were prepared and purified using Ni columns as described by Ge et al. (1991) and Kohtz et al. (1994). Baculovirus-derived SRPK1 was a kind gift from X.-D. Fu (U.C.S.D.). The vector Gst-SRPK1 was a gift from J. Fleckner (Aarhus, Denmark) and Gst-SRPK1 fusion protein was prepared from *E. coli* as described by Suñe and Garcia-Blanco (1995). Purified rabbit muscle PP1 was a gift from S. Shenolikar (Duke University). The PIP7.A RNA has been used before (Khotz et al., 1994) and is derived from adenovirus-2.

Phosphorylation and thiophosphorylation of ASF/SF2

The phosphorylation reaction of ASF/SF2 was performed in the presence of 200 ng/μL ASF/SF2, 15 ng/μL SRPK1, 50 mM Tris, pH 7.6, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP. Reactions were performed for 90 min at 30 °C as described by Gui et al. (1994b). Thiophosphorylation of ASF/SF2 was performed under the same conditions except that 1 mM ATP-γS (Sigma) was added into the reaction instead of ATP. Parallel reactions with radiolabeled [^{32}P] γATP (final concentration of 0.8 μM) or [^{35}S] γATP (final concentration of 0.2 μM) (NEN) were performed to check the efficiency of the phosphorylation reactions. For the experiments showing complementation of S100 extracts, the phosphorylated or thiophosphorylated ASF/SF2 was purified through Ni columns as described by Ge et al. (1991). Mock-phosphorylation reactions were performed in the absence of ATP. The concentration of ASF/SF2 in these reactions was high enough so that the protein preparations could be diluted in buffer to prevent effects from SRPK1 or ATPγS.

Dephosphorylation of ASF/SF2

Phosphorylated or thiophosphorylated ASF/SF2 was incubated with 0.4 U/μL purified rabbit muscle PP1 at 30 °C for 30 min. Microcystin-LR, at 1 μM final concentration, was added to inhibit the phosphatase. Mock-dephosphorylation was performed by adding the Microcystin-LR with the rabbit muscle PP1.

Splicing reactions in HeLa nuclear extracts

Four nanograms (2 pmol) of uniformly [^{32}P]-labeled PIP7.A pre-mRNA was pre-incubated with either buffer D (Dignam et al., 1983) or the indicated amount of ASF/SF2 (see figure legends) in this buffer for 5 min at 30 °C. The RNA-ASF/SF2 mixtures were then placed on ice for 5 min. Splicing reactions were performed as described previously (Jamison et al., 1992). Final concentrations were 33% (v/v) for nuclear extract, 64 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 5 mM creatine phosphate. Splicing reactions were incubated at 30 °C for 15 min, placed on ice, and supplemented with heparin to a final concentration of 0.5 mg/mL and further incubated at 30 °C for 5 min, then loaded on nondenaturing polyacrylamide gels for visualization of splicing complexes (Konarska & Sharp, 1987). To identify splicing intermediates and products, the reactions were incubated for 45 min at 30 °C, RNA was extracted and loaded on a 15% denaturing polyacrylamide gel, and visualized by autoradiography (Grabowski et al., 1985). All pre-incubations of pre-mRNA with ASF/SF2 and other reagents were performed at 30 °C for 5 min.

Splicing reactions in S100 extracts

Complementation of S100 extracts with ASF/SF2~P was performed in a final volume of 33 μL. Each reaction contained 500–600 ng (660–792 pmol) ASF/SF2 or ASF/SF2~P, which was pre-incubated with 4 ng (2 pmol) of uniformly [^{32}P]-labeled PIP7.A pre-mRNA, 72 mM KCl, 0.8 mM ATP, 3.8 mM creatine phosphate, 1.2 mM $MgCl_2$, 8 μL of S100, and 16 μL of buffer D (Dignam et al., 1983). Splicing reactions were incubated and resolved as described above. All pre-incubations of pre-mRNA with ASF/SF2 and other reagents were performed at 30 °C for 5 min.

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