SR proteins are required for nematode *trans*-splicing in vitro

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ABSTRACT

SR (ser/arg) proteins have been shown to play roles in numerous aspects of pre-mRNA splicing, including modulation of alternative splicing, commitment of substrates to the splicing pathway, and splice site communication. The last of these, splice site communication, is particularly relevant to *trans*-splicing in which the 5' and 3' exons originate on separate molecules. The participation of SR proteins in naturally occurring, spliced leader RNA-dependent *trans*-splicing has not been examined. Here, we have isolated SR proteins from an organism that performs both *trans*- and *cis*-splicing, the nematode *Ascaris lumbricoides*. To examine their activity in in vitro splicing reactions, we have also developed and characterized an SR protein-depleted whole-cell extract. When tested in this extract, the nematode SR proteins are required for both *trans*- and *cis*-splicing. In addition, the state of phosphorylation of the nematode SR proteins is critical to their activity in vitro. Interestingly, mammalian (HeLa) and *A. lumbricoides* SR proteins exhibit equivalent activities in *cis*-splicing, while the nematode SR proteins are much more active in *trans*-splicing. Thus, it appears that SR proteins purified from an organism that naturally *trans*-splices its pre-mRNAs promote this reaction to a greater extent than do their mammalian counterparts.

Keywords: nematodes; splicing factors; SR proteins; *trans*-splicing

INTRODUCTION

Trans-splicing is a pre-mRNA splicing reaction that has been documented to naturally occur in several lower eukaryotes including trypanosomatids (Agabian, 1990), nematodes (Nilsen, 1997), trematodes (Rajkovic et al., 1990), and euglena (Tessier et al., 1991). In this reaction, the 5' exon is supplied by a small RNA termed the spliced leader RNA (SL RNA), which has similarities to the small nuclear RNAs (snRNAs) that participate in *cis*- or pre-mRNA intron splicing. The SL RNA in nematodes interacts with Sm antigens (Bruzik et al., 1988; Thomas et al., 1988; Van Doren & Hirsh, 1988; Nilsen et al., 1989) as well as with specific proteins (Denker et al., 1996) to form the spliced leader ribonucleoprotein particle (SL RNP), which is then competent to participate in *trans*-splicing (Maroney et al., 1990; Denker et al., 1996). In addition to the SL RNP and pre-mRNA 3' acceptor, other factors including U2, U4/U6, and U5 snRNPs are also required (Hannon et al., 1991; Maroney et al., 1996). Functional U1 snRNP is apparently not required for *trans*-splicing, as the 5' end of the U1

snRNA can be ablated by various means without affecting SL RNA utilization (Bruzik & Steitz, 1990; Hannon et al., 1991; Maroney et al., 1991; Seiwert & Steitz, 1993). Much of the work to date in the *trans*-splicing field has focused on the RNA moieties involved; however the wealth of recent data relating to SR proteins and their involvement in numerous splicing processes (for recent reviews, see Fu, 1995; Krämer, 1996; Manley & Tacke, 1996; Valcárcel & Green, 1996; Cáceres & Krainer, 1997) has suggested a role for these splicing factors in *trans*-splicing (Bruzik, 1996).

SR proteins are a family of related phosphoproteins that play numerous roles in both constitutive and regulated pre-mRNA splicing (for reviews, see Valcárcel & Green, 1996; Cáceres & Krainer, 1997; Wang & Manley, 1997; Will & Lührmann, 1997). Generally, SR proteins contain two major domains, an N-terminal region consisting of one or two RNA recognition motif(s) (RRM) and a C-terminal region rich in RS dipeptides. Each of these domains can function independently, with the RRM capable of binding RNA on its own (Cáceres & Krainer, 1993; Zuo & Manley, 1993) and the RS domain capable of activating pre-mRNA splicing so long as it is tethered to the transcript through a heterologous RNA binding protein (Graveley & Maniatis, 1998). In addition, the state of phosphorylation of SR proteins is crit-

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ical to their activity (Cao et al., 1997; Xiao & Manley, 1997; Du et al., 1998; Kanopka et al., 1998; Xiao & Manley, 1998; J.R. Sanford & J.P. Buzik, in press).

In vitro, SR proteins have been shown to regulate splice-site switching in natural or artificial constructs with duplicated splice junctions (Ge & Manley, 1990; Krainer et al., 1990a; Fu et al., 1992). They have also been demonstrated to commit pre-mRNAs to the splicing pathway (Fu, 1993) and participate in alternative splicing decisions both in vitro (Sun et al., 1993; Tian & Maniatis, 1993; Ramchatesingh et al., 1995) and in vivo (Cáceres et al., 1994; Wang & Manley, 1995). In addition to these global activities, several roles for SR proteins have been described that illustrate discrete activities in relation to other spliceosomal constituents. SR proteins can regulate the interaction of the U1 snRNP with different 5' splice sites (Eperon et al., 1993; Tarn & Steitz, 1995; Zahler & Roth, 1995) and in fact can compensate for the U1 snRNP in extracts that have either had their U1 5' end blocked by a 2'-OME oligonucleotide (Tarn & Steitz, 1994), or have been depleted of U1 (Crispino et al., 1994). The demonstration of a preferred binding site for ASF/SF2 that includes a 5' splice site (Zuo & Manley, 1994) may well be related to this activity. In addition to these U1 snRNP-related activities of SR proteins, they have been seen to promote U2 snRNP binding to the branchpoint sequence (Lavigne et al., 1993; Tarn & Steitz, 1995) and to participate in the recruitment of the U4/6•5 tri-snRNP to the spliceosome (Roscinio & Garcia-Blanco, 1995). Finally, SR proteins have also been shown to promote activities that impact on non-snRNP splicing factors. Several studies have examined the role that SR proteins play in the assembly of complexes at the 3' splice site (Hoffman & Grabowski, 1992; Staknis & Reed, 1994; Wang et al., 1995; Zuo & Maniatis, 1996). In these experiments, SR proteins were able to promote the formation of stable complexes at the 3' splice site by enhancing the interaction of U2AF with the polypyrimidine tract. More recently, the SR protein SC35 has been shown to functionally substitute for U2AF⁶⁵ in U2AF-depleted extracts (MacMillan et al., 1997).

The activity of SR proteins that is most pertinent to *trans*-splicing is their ability to promote splice-site interaction. Several reviews have examined this subject in detail (Berget, 1995; Black, 1995; Buzik, 1996; Reed, 1996). Briefly, with the U1 snRNP containing the 70-kDa protein bound at the 5' splice site and the U2AF heterodimer at the polypyrimidine tract, SR proteins including ASF/SF2 and SC35 can interact with 70 kDa and U2AF³⁵ thereby allowing for a potential interaction between the two ends of the intron (Wu & Maniatis, 1993; Kohtz et al., 1994). In addition, SC35 has been shown to mediate an ATP-dependent interaction between U1 and U2 snRNPs that is independent of the 5' splice site (Fu & Maniatis, 1992). These experiments led to the finding that 5' and 3' splice sites on separate

RNA molecules could productively interact to generate spliced product in mammalian extracts dependent upon the presence of either a splicing enhancer or 5' splice site downstream of the 3' splice site (Buzik & Maniatis, 1995; Chiara & Reed, 1995). This bimolecular reaction was shown to require SR proteins by splicing in cytoplasmic, S100 extract (Buzik & Maniatis, 1995). In addition, recent results suggest that SR proteins can directly promote interaction between two naturally *cis*-spliced exons (Stark et al., 1998).

A separate interaction involving splicing factor BBP (SF1; Arning et al., 1996) and its interaction with both the branchpoint sequence and a U1 snRNP component (Prp40p) was identified that also allows for communication between the two ends of the intron (Abovich & Rosbash, 1997; Berglund et al., 1997). The conservation of components of this interaction between yeast and mammals argues for its importance; however, it is not incompatible with the previous cross-intron bridging model that has been extensively studied in mammalian systems.

The ability of SR proteins to promote the association of separate RNAs containing either a 5' or a 3' splice site is likely to be germane to the process of *trans*-splicing, in which the natural situation involves the interaction of two molecules, leading to a *trans*-spliced product. Although numerous RNA-RNA interactions and their associated rearrangements are intimately involved in the splicing reaction itself (Nilsen, 1998), RNA-protein and protein-protein interactions probably play a large role in the initial formation of the spliceosome (Buzik, 1996; Reed, 1996). Here, we have prepared SR proteins from the nematode *Ascaris lumbricoides*, an organism whose transcripts can participate in both *trans*- and *cis*-splicing. To address questions relating to SR protein activity in these two splicing reactions, we have developed an SR protein-depleted whole-cell extract. When *A. lumbricoides* SR proteins were added to splicing assays in SR protein-depleted whole-cell extracts, they were seen to be required for both *trans*- and *cis*-splicing and to depend on a discrete level of SR protein phosphorylation. Perhaps most intriguingly, when the activities of HeLa and *A. lumbricoides* SR proteins were compared in *A. lumbricoides* SR protein-depleted whole-cell extract, the nematode SR proteins displayed significantly more activity at promoting *trans*-splicing, whereas mammalian and nematode SR proteins were interchangeable in *cis*-splicing reactions.

RESULTS

Analysis of SR proteins from the nematode *A. lumbricoides*

To examine what role SR proteins might play in authentic *trans*-splicing reactions, we purified them from an organism that endogenously performs both *trans*-

and *cis*-splicing, the nematode *A. lumbricoides*. This helminth is particularly amenable to biochemical and molecular investigation of pre-mRNA splicing because of the availability of an *in vitro* system. Previous studies have shown that if fertilized eggs are allowed to develop for 5 days at 30 °C, reaching the ~32-cell stage, then extracts made at this point in development are competent to perform both *trans*- and *cis*-splicing (Hannon et al., 1990). This *in vitro* system therefore offers a unique opportunity to examine factors that are required for *trans*-splicing, *cis*-splicing, or both.

SR proteins were isolated from ~32-cell-stage *A. lumbricoides* embryos using a two-step ammonium sulfate cut followed by precipitation in MgCl₂, the standard methodology in other systems (Zahler et al., 1992). Figure 1A shows the profile of SR proteins purified from *A. lumbricoides* alongside a parallel SR preparation from HeLa cells. Several proteins are enriched in the *A. lumbricoides* lane (Fig. 1A, lane 4). These include proteins with apparent molecular weights of 70 kDa, 38 kDa, 30 kDa, and 17 kDa as well as several other faint bands between the 17 kDa and 30 kDa species. When compared to HeLa SR proteins (Fig. 1A, lane 2) the only protein(s) that appear to comigrate are the protein(s) comprising the 30-kDa band. In mammals, this band is actually made up of several SR proteins, including SC35, SF2/ASF, SRp30c, and 9G8 (Cáceres & Krainer, 1997). Lanes 3 and 5 of Figure 1A contain HeLa and *A. lumbricoides* SR proteins, respectively, that have been dephosphorylated with alkaline phosphatase. Most species exhibit increased mobility after phosphatase treatment except for the *A. lumbricoides*

coides protein at 38 kDa, which appears to decrease mobility. It is apparent that dephosphorylation leads to shifts in apparent molecular weight, consistent with the *A. lumbricoides* SR proteins existing as phosphoproteins.

To further verify the identity of these proteins as authentic SR proteins, we next performed a Western blot with MAb 104, a monoclonal antibody that recognizes a phosphoepitope common to SR protein family members (Roth et al., 1990; Zahler et al., 1992). As in Figure 1A, the blot contains both native phosphorylation state (Fig. 1B, HeLa, lane 2; *A. lumbricoides*, lane 4) as well as dephosphorylated (Fig. 1B, HeLa, lane 3; *A. lumbricoides*, lane 5) SR proteins. The native HeLa proteins generate the characteristic pattern of reactive bands including SRp75, SRp55, SRp40, SRp30(s), and SRp20. In the *A. lumbricoides* lane, the proteins that are the most enhanced in the Coomassie blue-stained gel (Fig. 1A, lane 4) are the ones that cross-react with MAb 104. The single exception is aSRp70, which is not visible on this blot, but has reacted weakly with MAb 104 in separate experiments. Other evidence, however, suggests that it is an authentic SR protein (see below, Fig. 4A). In addition, prior dephosphorylation with alkaline phosphatase leads to both a decrease in overall signal intensity as well as the above mentioned mobility shifts (Fig. 1B, lane 5, *A. lumbricoides* and lane 3, HeLa). The 30-kDa protein(s) reproducibly generate more intense signals than do the other cross-reacting bands. This could be due to either their greater abundance, their higher level of phosphorylation, or differences in the sequence context of the epitope relative to the other SR protein species at this particular

FIGURE 1. Isolation of SR proteins from the nematode *Ascaris lumbricoides*. **A:** Coomassie blue stained protein gel comparing the SR proteins purified from HeLa cells (H) to those from *A. lumbricoides* (A). Aliquots of SR proteins were either unmodified (–) or pre-treated (+) with alkaline phosphatase (AP) prior to loading them on the gel. For comparison, AP alone was loaded as a control. Stars represent AP either alone or mixed with the SR protein samples. **B:** Western blot with MAb 104 (Roth et al., 1990) of SR proteins as in **A**. The 70-kDa *A. lumbricoides* SR protein is not detected on this Western blot, but has been seen in other cases (data not shown).

stage of development. We have recently shown that during early development in *A. lumbricoides* (from fertilized eggs to ~32-cell embryos) the level of phosphorylation of SR proteins changes concomitant with their activity in *in vitro* splicing reactions (J.R. Sanford & J.P. Bruzik, in press). In addition, the *A. lumbricoides* SR proteins can serve as substrates for the SR protein kinase SRPK1 (Gui et al., 1994a) (data contained in Fig. 4A). Thus, by several criteria, the proteins that we have isolated from *A. lumbricoides* are bona fide SR proteins.

Development and characterization of an SR protein-depleted whole-cell extract from *A. lumbricoides*

In mammalian systems, the supernatant from the nuclear pellet, spun at $100,000 \times g$ or S100 fraction, contains all of the necessary components for *in vitro* pre-mRNA splicing except SR proteins (Krainer et al., 1990b; Zahler et al., 1992). Thus addition of SR proteins allows for the rescue of splicing activity in an S100. *A. lumbricoides* splicing extracts are whole cell in nature because the nuclei are too fragile to withstand dounce homogenization, and therefore an S100-like fraction is not available (Hannon et al., 1990). To generate a whole-cell splicing extract that was depleted of

SR proteins, we took advantage of their insolubility in $MgCl_2$ (Zahler et al., 1992). Prior to the $100,000 \times g$ clearing spin in the *A. lumbricoides* whole-cell extract procedure (Hannon et al., 1990), we titrated in $MgCl_2$. At an optimal concentration of 10 mM $MgCl_2$, we were able to generate a reproducibly splicing-deficient whole-cell extract. The important question was then whether addition of purified SR proteins would restore splicing in this extract.

To characterize this extract, we began by examining the whole-cell extract's sensitivity to treatment with micrococcal nuclease (MN). As one would expect, *trans*- and *cis*-splicing in *A. lumbricoides* whole-cell extract is sensitive to treatment of the extract with MN (Fig. 2A, compare lanes 2 and 4, *trans*-splicing; compare lanes 6 and 8, *cis*-splicing). The splicing defect in the $MgCl_2$ -depleted extract can be overcome by supplementation with resuspended pellet from the 10 mM $MgCl_2$ centrifugation (Fig. 2B, lane 4, *trans*-splicing; lane 9, *cis*-splicing). In addition, this pellet is insensitive to treatment with MN, as prior MN treatment of the pellet does not affect its ability to rescue splicing (Fig. 2B, *trans*-splicing, lane 5; *cis*-splicing, lane 10).

The reason for generating the 10 mM $MgCl_2$ -pelleted extract was to establish an SR protein-depleted system in which to study the activities of SR proteins in *trans*- and *cis*-splicing. We therefore next asked whether pu-

FIGURE 2. *Trans*- and *cis*-splicing in *A. lumbricoides* whole-cell extract and SR protein-depleted whole-cell extract. **A:** *Trans*- and *cis*-splicing reactions in both untreated and micrococcal-nuclease (MN)-treated whole-cell extract. **B:** SR protein-depleted whole-cell extract can be rescued for both *trans*- and *cis*-splicing activity upon addition of resuspended pellet from the $MgCl_2$ precipitation. Splicing reactions proceeded for the indicated time in minutes. Shaded boxes represent *trans*-acceptor exon, striped boxes, the *trans*-spliced leader exon, and open boxes the *cis*-spliced exons.

FIGURE 4. *Trans*- and *cis*-splicing activity are sensitive to the state of phosphorylation of exogenously supplied SR proteins. **A:** Purified SR proteins were either mock treated or dephosphorylated with protein phosphatase 1 (PP1) prior to labeling reactions employing SRPK1 and $\gamma^{32}\text{P}$ -ATP. A lane containing SRPK1 and $\gamma^{32}\text{P}$ -ATP alone was run for comparison. Prior dephosphorylation of the SR proteins leads to an increased incorporation of label. **B:** Aliquots of phosphatase treated or mock phosphatase treated SR proteins were added to SR protein-depleted whole-cell extract. Splicing substrates are denoted as in Figure 2. Splicing reactions proceeded for the indicated time in minutes.

dephosphorylation equally inhibited each reaction (Fig. 4B, lane 5, *trans*-splicing; lane 10, *cis*-splicing). Mock treated SR proteins in their native state of phosphorylation are able to rescue both *trans*- (Fig. 4B, lane 4) and *cis*-splicing (Fig. 4B, lane 9). Equal low concentrations of PP1 added to whole-cell extract have no effect on *trans*- and *cis*-splicing activities (data not shown). Pretreatment of the SR proteins with alkaline phosphatase also decreases their activity to the same level (data not shown). Alkaline phosphatase, however, is much more effective at dephosphorylation as judged by both mobility shift assays and subsequent rephosphorylation with $\gamma^{32}\text{P}$ -ATP and SRPK1 (J.R. Sanford & J.P. Bruzik, unpubl. observation). Since both the subtle (PP1) and harsh (AP) dephosphorylation treatments equally affect SR protein activity, this raises the possibility of nonequivalent sites of phosphorylation in SR proteins. These data indicate that both *trans*- and *cis*-splicing reactions are sensitive to alterations in the state of phosphorylation of SR proteins that are added to the SR protein-depleted whole-cell extract.

Nematode and mammalian SR proteins exhibit different activities in *trans*- and *cis*-splicing

One of the reasons that nematodes are interesting from an RNA processing standpoint is that they perform both *trans*- and *cis*-splicing, and in fact, single transcripts can undergo both reactions (Krause & Hirsh, 1987). Mammals, on the other hand, do not appear to naturally perform SL RNA-mediated *trans*-splicing, although reactions utilizing exogenously supplied substrates are able to proceed (Bruzik & Maniatis, 1992). Because of the ability of SR proteins to promote splice site interactions (for reviews, see Bruzik, 1996; Reed, 1996), we asked whether the SR proteins isolated from *A. lumbricoides* and mammalian cells would display any specificity for these two reactions when tested in both *trans*- and *cis*-splicing in SR protein-depleted whole-cell extract (Fig. 5). In *cis*-splicing, nematode (Fig. 5, lane 6) and HeLa (Fig. 5, lane 5) SR proteins exhibit equivalent activity in *A. lumbricoides* SR protein-depleted extract. SR proteins isolated from both sources were able to

FIGURE 5. Comparison of mammalian and nematode SR protein activity in *trans*- and *cis*-splicing reactions. *Trans*- and *cis*-splicing reactions were performed in SR protein-depleted whole-cell extract supplemented with identical amounts of HeLa (H) or *A. lumbricoides* (A) SR proteins. While virtually identical activities were seen in *cis*-splicing reactions, *trans*-splicing was much more responsive to nematode SR proteins. Lanes without SR proteins added (–) received SR protein buffer (BC100) instead. Splicing substrates are denoted as in Figure 2. All splicing reactions were incubated for 90 min.

rescue *cis*-splicing approximately fivefold over the addition of buffer alone (Fig. 5, lane 4). When rescue with *A. lumbricoides* SR proteins is compared to rescue with HeLa SR proteins, the ratio is ~1:1. The results with *trans*-splicing were quite different. In this case, nematode SR proteins displayed 11-fold activation (Fig. 5, lane 3) compared to twofold with mammalian SR proteins (Fig. 5, lane 2). The ratio between these two activities is greater than fivefold. These two experiments were performed using the same extract, the same protein stocks, and were run on the same gel; therefore there is no variation in the amounts of protein from the two sources added to the different reactions. This result raises the possibility that the SR proteins present in organisms that endogenously perform *trans*-splicing might be specialized for this reaction.

DISCUSSION

We have demonstrated that SR proteins purified from the nematode *A. lumbricoides* contain an array of sizes, ranging from 70 kDa to 17 kDa. When compared to mammalian SR proteins isolated from HeLa cells, only

the protein(s) at 30 kDa comigrate. All of these species cross-react with MAb 104, a monoclonal antibody that recognizes a phosphoepitope common to each of the SR proteins. In addition, they can serve as substrates for the SR protein kinase SRPK1. When tested in an SR protein-depleted whole-cell extract, *A. lumbricoides* SR proteins can rescue both *trans*- and *cis*-splicing reactions, dependent upon their state of phosphorylation. Furthermore, whereas mammalian and nematode SR proteins display identical activity in *cis*-splicing reactions, the *A. lumbricoides* SR proteins are much more active at promoting *trans*-splicing.

Early complexes involved in *trans*- and *cis*-splicing

One of the questions that has been posed regarding *trans*-splicing is: How do the SL RNP, containing the 5' splice site, and the pre-mRNA, containing the 3' splice site, initially interact? This communication would be at the earliest stage of *trans*-spliceosome assembly, presumably prior to establishment of the precise RNA–RNA interactions required for catalysis (Nilsen, 1997). A priori, this interaction would seem to pose more of a problem for the cell than the interaction of the splice sites in constitutive *cis*-splicing because the splice sites are present on separate transcripts. A body of work has examined the question of early spliceosome assembly in both yeast and mammalian *cis*-splicing systems (Rymond & Rosbash, 1992; Reed & Palandjian, 1997). In particular the dissection of the early (E) complex in mammalian systems has demonstrated that, in addition to the U1 snRNP and the heterodimeric splicing factor U2AF, SR proteins are present in and actually promote the formation of this presplicing complex (Staknis & Reed, 1994).

Trans-splicing in *A. lumbricoides* seems to involve a very different scenario with regard to the composition of the early *trans*-spliceosomal constituents. For some time the role, or lack thereof, of U1 RNP in *trans*-splicing has been questioned. Early proposals suggested that the SL RNP might be able to supply the function(s) of U1 to its own 5' splice site (Bruzik et al., 1988). Further experiments demonstrated that either with bona fide *in vitro trans*-splicing in *A. lumbricoides* whole-cell extract or utilizing tethered SL RNA–3' exon constructs in mammalian cell nuclear extract, the functional 5' end of the U1 snRNA was not required for splicing activity (Bruzik & Steitz, 1990; Hannon et al., 1991). More recent work has demonstrated that, although there is a small amount of U1 present in purified *trans*-spliceosomes, it is probably not functionally significant (Maroney et al., 1996).

The early complex in *trans*-spliceosomal assembly does not appear to require U1. This initiation of *trans*-splicing is even more difficult to envision than the reaction of two half molecules in bimolecular splicing

(Bruzik & Maniatis, 1995; Chiara & Reed, 1995; Ghetti & Abelson, 1995; Anderson & Moore, 1997), where a normally *cis*-spliced intron has been cut into pieces. In the case of authentic SL RNA mediated *trans*-splicing, the SL RNA appears to enter the spliceosome in the form of a tetra-snRNP particle containing SL•U4/6•5 (Maroney et al., 1996), which would occur at an early point in assembly. In mammals, the U4/6•5 tri-snRNP is escorted to the spliceosome by SR proteins (Roscigno & Garcia-Blanco, 1995), however, this occurs later in the assembly pathway at the point of transition from prespliceosome to spliceosome. Thus, the timing of entry of at least several components of the *trans*-spliceosome does not correlate with the temporal order established in *cis*-splicing mammalian systems. SR proteins may therefore promote the early assembly of *trans*-spliceosomes; however, the snRNPs involved at discrete points appear quite different than the snRNP(s) involved in *cis*-splicing.

Possible protein–protein interactions in *trans*-splicing

Purified mammalian spliceosomes contain the well-studied snRNPs and in addition, myriad protein factors (Krämer, 1996). Through the work of several different groups, distinct protein–protein interactions involved in pre-mRNA splicing have come to light. These include the interaction of SR proteins with the U1 70-kDa protein (Kohtz et al., 1994), as well as their simultaneous interaction with U1 70 kDa and U2AF³⁵ (Wu & Maniatis, 1993). This set of protein–protein interactions could allow for splice-site communication across the intron. In reactions that are relevant to *trans*-splicing, SR proteins are able to promote spliced-product formation even when the 5' and 3' splice sites are located on separate transcripts (Bruzik & Maniatis, 1995; Chiara & Reed, 1995). SR proteins are also able to mediate interactions across exons (for review, see Berget, 1995). Either a 5' splice site or a splicing enhancer downstream of the 3' splice site to be activated can lead to increased splicing activity of the upstream intron (for reviews, see Bruzik, 1996; Reed, 1996).

Trans-splicing reactions possess an inherent need for interactions through three dimensions as the 5' and 3' splice sites are not tethered to one another through a continuous intron. The SL RNA and pre-mRNA 3' acceptor therefore must rely on a defined set of interactions for productive association or *trans*-splicing to occur. Prime candidates to play a major role in this process are the SR proteins. Their documented ability to participate in splice site association, either in *cis*-splicing or in artificial bimolecular splicing systems, has demonstrated a role for them in bridging the gap between 5' and 3' splice sites. In authentic, SL RNA-dependent *trans*-splicing systems, this ability might be key to *trans*-spliceosomal assembly. In this case, SR

proteins would have to display a specific interaction with some component(s) of the pre-mRNA 3' acceptor as well as with factor(s) associated with the SL RNP. The first of these might be U2AF³⁵, as SR proteins have been shown to specifically contact this splicing factor in *cis*-splicing systems (Wu & Maniatis, 1993). At the 5' splice site, SR proteins might interact with SL RNP specific proteins (Denker et al., 1996) or with SL•U4/6•5 multi-snRNP components. A further dissection of the protein–protein interactions involved in *trans*-splicing should provide an answer to this question.

Differences in the activities of mammalian and nematode SR proteins

Many different activities of SR proteins have been examined *in vitro*. The ability to promote constitutive splicing has been assayed in S100 fractions, with different SR proteins able to rescue splicing of several different introns (Zahler et al., 1992, 1993a). In addition, SR proteins isolated from different sources (mammals vs. insects) display similar rescue of constitutively spliced substrates (Mayeda et al., 1992). Alternative splicing assays have demonstrated that different SR proteins from the same organism can display markedly distinct activities (Zahler et al., 1993a), whereas single SR proteins from vertebrate and nonvertebrate sources can generate identical alternative splicing patterns on multiple substrates (Mayeda et al., 1992). In the case of *trans*- and *cis*-splicing rescue in *A. lumbricoides* SR protein-depleted whole-cell extract, large differences in the activity of mammalian and nematode SR proteins were observed only in *trans*-splicing reactions. We do not yet know if this reflects a *trans*-splicing specific SR protein or the overall balance of activities among SR proteins from these organisms. If SR proteins exist in trypanosomatids, which exclusively *trans*-splice their pre-mRNAs, it would be interesting to determine their activity in *trans*- and *cis*-splicing assays. In addition, we have recently observed that *A. lumbricoides* and HeLa SR proteins can exhibit marked differences in alternative splicing assays on multiple substrates (J.R. Sanford, S. Nesich, & J.P. Bruzik, unpubl. observations). Since both *trans*- and *cis*-splicing coexist in the same cell and can also occur on the same pre-mRNA transcript, potential interactions between SR proteins and U1 snRNP or SL RNP associated factors as well as components of the complex at the 3' splice site might play a role in the discrimination between *trans*- and *cis*-splicing.

MATERIALS AND METHODS

Splicing extracts, pre-mRNA transcripts, and splicing reactions

A. lumbricoides whole-cell and SR protein-depleted extracts were prepared as previously described (Hannon et al., 1990),

with the following modification for the latter. The supernatant following centrifugation at 15,000 rpm for 10 min in a JA-20 rotor (Beckman) was made 10 mM MgCl_2 by the addition of 0.01 vol of 1 M MgCl_2 , mixed gently, and incubated on ice 5 min. The supernatant was then centrifuged at 35,000 rpm for 20 min in a ti50 rotor (Beckman). The supernatant was then carefully removed, so as not to disturb the pellet (from this point SR protein-depleted extract preparation is identical to the previously described procedure). The pellet was resuspended in 250 μL BC100 (100 mM KCl, 50 mM Tris HCl, pH 7.5, 0.1 mM EDTA, and 20% glycerol) and centrifuged 1 min in a microfuge. The supernatant was collected, frozen on dry ice and stored at -80°C . The concentration of the cleared, resuspended pellet was determined by Bradford assay (BioRad).

Body-labeled pre-mRNA splicing substrates were prepared in vitro as previously described (Hannon et al., 1990). Templates for in vitro transcription were previously described (Hannon et al., 1990, 1991). All in vitro splicing assays were performed as previously described (Hannon et al., 1990). Micrococcal nuclease (Worthington Biochemical Corp.) treatment of *A. lumbricoides* whole-cell extract, resuspended 10 mM MgCl_2 pellet, and SR proteins was performed under the following conditions. *A. lumbricoides* whole-cell extract was incubated with MN (7.7 U/ μL final) or ddH₂O for mock treatment, under splicing conditions (minus substrate), with 1 mM CaCl_2 at 30°C for 30 min. The MN was then inactivated by the addition of EGTA to 4 mM and tRNA (0.06 $\mu\text{g}/\mu\text{L}$ final). Pre-mRNA substrate was added to initiate the in vitro splicing reactions. Resuspended 10 mM MgCl_2 pellet or SR proteins (~6 μg) were also treated with MN under identical conditions, prior to their addition to in vitro *trans*- and *cis*-splicing assays. Approximately 1.5 μg of SR proteins and resuspended 10 mM MgCl_2 pellet were added back to in vitro splicing reactions in SR protein-depleted whole-cell extract. Spliced products were resolved on denaturing 5% polyacrylamide gels followed by autoradiography.

Western blot analysis was used to determine the amount of SR proteins present in both the *A. lumbricoides* whole-cell and SR protein-depleted extracts. Briefly, 3.3 mg (200 μL) of each extract were fractionated by adding saturated ammonium sulfate to 65%. The insoluble material was pelleted by microcentrifugation for 15 min and the supernatant was dialyzed against 100 volumes of dialysis buffer (Hannon et al., 1990). Equivalent amounts of starting material were resolved by 12% SDS-PAGE and transferred in Immobilon NC (Millipore). The membrane was then probed with the SR protein-specific monoclonal antibody MAb104 (Roth et al., 1990). Following incubation with the secondary antibody (Immuno-ure Goat Anti-Mouse IgG + IgM, (H+L), Peroxidase Conjugated, Pierce) the immune complexes were detected with SuperSignal Ultra Chemiluminescent Substrate (Pierce) and exposed against film.

Protein purification and analysis

SR proteins were purified from 5.5×10^9 HeLa cells and from 100 g of synchronous 32-cell *A. lumbricoides* embryos by ammonium sulfate/magnesium precipitation as previously described (Zahler et al., 1992). GST-tagged SRPK1 in the pAcG2T Baculovirus Transfer Vector (Pharmlingen, provided by X.-D. Fu) was used to construct a recombinant baculo-

virus. The fusion protein was expressed in SF9 cells. GST-SRPK1 was affinity purified using Glutathione Sepharose 4B following the manufacturer's instructions (Pharmacia). The unit definition was determined as previously described (Gui et al., 1994b).

SR proteins purified from HeLa cells and ~32-cell *A. lumbricoides* embryos (6 μg) were dephosphorylated by alkaline phosphatase (1 U, Promega) in BC100, for 30 min at 37°C . Phosphatase-treated or untreated SR proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose (Immobilon NC, Millipore) for Western blot analysis with MAb104 (as described above) or stained with Coomassie Blue (Sigma).

SR protein phosphorylation/dephosphorylation

A. lumbricoides SR proteins (7.5 μg) were dephosphorylated with protein phosphatase 1 (PP1, Gibco-BRL) at a final concentration of 0.064 U/ μL , or ddH₂O for control, in modification buffer (50 mM Tris HCl, pH 7.5, 1.5 mM MgCl_2 , and 1 mM DTT) for 60 min at 37°C . The dephosphorylation reactions were then divided into three aliquots, for in vitro *trans*- and *cis*-splicing assays (as described above, 1.5 μg per reaction) and for SRPK1 kinase assay. PP1 was heat inactivated prior to rephosphorylation reactions with SRPK1 and $\gamma^{32}\text{P}$ -ATP. In vitro SRPK1 kinase assay of mock treated or PP1 treated SR proteins was performed as previously described (Gui et al., 1994a). Radiolabeled SR proteins were resolved by 12% SDS-PAGE and visualized by autoradiography.

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