Modulation of 5' splice site choice in pre-messenger RNA by two distinct steps

(U1 small nuclear ribonucleoprotein/Ser/Arg-rich proteins/spliceosome assembly)

WOAN-YUH TARN AND JOAN A. STEITZ*

Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, 295 Congress Avenue, New Haven, CT 06536-0812

Contributed by Joan A. Steitz, December 22, 1994

ABSTRACT Ser/Arg-rich proteins (SR proteins) are essential splicing factors that commit pre-messenger RNAs to splicing and also modulate 5' splice site choice in the presence or absence of functional U1 small nuclear ribonucleoproteins (snRNPs). Here, we perturbed the U1 snRNP in HeLa cell nuclear extract by detaching the U1-specific A protein using a 2'-O-methyl oligonucleotide (L2) complementary to its binding site in U1 RNA. In this extract, the standard adenovirus substrate is spliced normally, but excess amounts of SR proteins do not exclusively switch splicing from the normal 5' splice site to a proximal site (site 125 within the adenovirus intron), suggesting that modulation of 5' splice site choice exerted by SR proteins requires integrity of the U1 snRNP. The observation that splicing does not necessarily follow U1 binding indicates that interactions between the U1 snRNP and components assembled on the 3' splice site via SR proteins may also be critical for 5' splice site selection. Accordingly, we found that SR proteins promote the binding of the U2 snRNP to the branch site and stabilize the complex formed on a 3'-half substrate in the presence or absence of functional U1 snRNPs. A novel U2/U6/3'-half substrate crosslink was also detected and promoted by SR proteins. Our results suggest that SR proteins in collaboration with the U1 snRNP function in two distinct steps to modulate 5' splice site selection.

Removal of introns from mRNA precursors (pre-mRNA) is an important process in gene expression. In metazoans, most pre-mRNAs contain several introns, and some are alternatively spliced; therefore, splice site selection is a critical aspect of gene regulation (1). Splicing occurs in a large ribonucleoprotein particle, the spliceosome, which is composed of five small nuclear RNAs (snRNAs; U1, U2, U4, U5, and U6) and a number of protein factors (2). Functional spliceosomes are assembled stepwise by the binding of these components to the pre-mRNA accompanied by dynamic rearrangements.

The U1 small nuclear ribonucleoprotein (snRNP) and members of the Ser/Arg-rich protein (SR protein) family play vital roles in splice site selection and the early phase of spliceosome assembly (1, 2). The U1 snRNP recognizes the 5' splice site via a base-pairing interaction and is required for stable binding of the U2 snRNP to the branch site during spliceosome assembly. In yeast, base-pairing between U1 snRNA and the 5' splice site is essential for the binding of the U2 snRNP (3, 4). However, in the mammalian system, U2 binding mediated by the U1 snRNP does not depend strictly on the presence of a 5' splice site (2). Rather, interaction of the U1 snRNP with a downstream 5' splice site or with an exonic enhancer sequence may target components to the 3' splice site and stimulate the splicing reaction (5, 6).

SR proteins, which contain a Ser/Arg-rich domain, commit a pre-mRNA to splicing, apparently by prebinding and recruit-

ing the U1 snRNP to the 5' splice site (7–9). They also mediate interactions between the 5' and 3' splice site in early presplicing complexes (10, 11). This bridging function, performed by one or more SR proteins, may require collaboration with other splicing factors that also have SR domains (12). Additionally, SR proteins bind some exon sequences which act as splicing enhancers and improve the utilization of weak 3' splice sites (13–15).

Excess amounts of SR proteins can rescue splicing in nuclear extracts either depleted of U1 snRNPs (16) or pretreated with a 2'-O-methyl oligonucleotide (U1_{1–14}), which sequesters the 5' end of U1 RNA (17). Since the oligonucleotide U1_{1–14} blocks the ability of the U1 snRNP to base-pair with the 5' splice site and to promote spliceosome assembly (17, 18), we concluded that SR proteins can compensate for the loss of at least two U1–snRNP functions. In such extracts, SR proteins may bypass the initial U1 recognition step by recruiting other splicing factor(s), probably U6, to potential 5' splice sites. However, it was unclear how SR proteins overcome the defect of the debilitated U1 snRNP with respect to spliceosome assembly.

In this paper, we have examined U1 snRNP and SR protein functions by disrupting the binding of a U1-specific protein with a 2'-O-methyl oligonucleotide rather than sequestering the 5' end of U1 snRNA. We find that an extract pretreated to detach the U1-specific A protein can splice the adenovirus pre-mRNA but exhibits different 5' splice site choice compared to a normal extract when supplemented with excess SR proteins. We also studied the relative roles of the U1 snRNP and SR proteins in promoting U2 snRNP binding to a 3'-half substrate.

MATERIALS AND METHODS

Oligonucleotides. All oligonucleotides were synthesized by John Flory at Yale University on an Applied Biosystems DNA synthesizer. The 2'-O-methyl oligonucleotides U1_{1–14}, EBER3, and the biotinylated 2'-O-methyl oligonucleotide BU1_{1–14} were described in Seiwert and Steitz (18). The 2'-O-methyl oligonucleotide L2 is complementary to nucleotides 64–75 of the U1 snRNA.

Antibodies. The anti-(U1)70k monoclonal antibody (H111) was a gift from R. Luhrmann (19). The monoclonal antibody (9A9) against the U1-specific protein A and the U2-specific protein B¹ was from W. J. van Venrooij (20).

Splicing Substrates and Splicing Reactions. The standard adenovirus splicing substrate was described in Solnick (21). For the 3'-half splicing substrate, the plasmid was constructed by insertion of a *HindIII*–*Sca I* (in exon 2) fragment of the

Abbreviations: AMT psoralen, 4'-aminomethyl-4,5',8-trimethylpsoralen; hnRNP, heterogeneous nuclear ribonucleoprotein; pre-mRNA, pre-messenger RNA; snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein; SR protein, Ser/Arg-rich protein.

*To whom reprint requests should be addressed.

adenovirus transcript between the *Hind*III and *Sma* I sites of pSP64; it was linearized with *Eco*RI for run-off transcription. The transcription reaction, splicing reactions, and analyses of splicing products and complexes were performed according to Tarn and Steitz (17); quantitation was performed on a Molecular Dynamics PhosphorImager.

Splicing Extracts and SR Proteins. Preparation of HeLa nuclear and S100 extracts and pretreatment of extracts with the 2'-*O*-methyl oligonucleotide U1₁₋₁₄ (15 μ M) or EBER3 (15 μ M) were performed according to methods described in Tarn and Steitz (17). For oligonucleotide L2 pretreatment, the high-salt nuclear extract was prepared according to Barabino *et al.* (22) and 10–20 μ M oligonucleotide was added to the high-salt dialysate in the presence of 1.5 mM ATP, 5 mM creatine phosphate, and 0.05% Nonidet P-40 (NP-40). Following a 30-min incubation at 30°C, the extract was dialyzed against buffer D containing 50 mM KCl for 2 hr. Preparation and micrococcal-nuclease treatment of SR proteins were performed according to Tarn and Steitz (17). Micrococcal nuclease-treated SR proteins were used for all experiments except the one shown in Fig. 2A.

Psoralen Crosslinking. Psoralen crosslinking was carried out as described by Tarn and Steitz (17) except that a 5% denaturing polyacrylamide gel was used to analyze products crosslinked to the 3'-half substrate. The crosslinked products were quantitated using a Molecular Dynamics PhosphorImager.

Western Blot Analysis. To deplete the U1 snRNP from extracts by affinity selection, 20 μ l of mock-treated or oligonucleotide L2-pretreated extracts was incubated in a 25- μ l reaction mixture containing 10 μ M biotinylated 2'-*O*-methyl oligonucleotide BU1₁₋₁₄, 0.5 mM ATP, 20 mM creatine phosphate, 2.4 mM MgCl₂, and 0.05% NP-40 for 30 min at 30°C. The mixtures were then incubated with 10 μ l of preblocked streptavidin agarose beads (18) for 1.5 hr at 4°C. After a 10-sec spin in the microcentrifuge, 6- μ l portions of supernatant were fractionated by 10% SDS/PAGE followed by Western blot analysis using the enhanced chemiluminescence detection system (Amersham). Both hybridoma cell supernatants (H111 and 9A9) were diluted 1:100 for detection.

RESULTS

Modulation of 5' Splice Site Utilization Depends on Integrity of the U1 snRNP. We devised a method to detach the U1-specific A protein from U1 snRNP particles utilizing a 2'-*O*-methyl oligonucleotide, L2, which is complementary to the binding site of the U1 A protein, loop 2 of U1 snRNA (23, 24). We introduced the L2 oligonucleotide into a high-salt nuclear extract as described by Barabino *et al.* (22) in the hope of competitively inhibiting protein binding. To assess whether the U1 A protein was effectively displaced, supernatants of extracts were examined by Western blot analyses after depletion of U1 snRNP particles by affinity selection with a biotinylated oligonucleotide (BU1₁₋₁₄). As shown in Fig. 1A, removal of U1 snRNPs reduced the levels of U1-specific proteins A and 70k (to about 20%) in the mock-treated extract (lane 2). In contrast, significant amounts of the U1 A protein, but not 70k, remained in the supernatant when the extract had been pretreated with the L2 oligonucleotide (lane 4). While most of U1 A became dislodged from the U1 snRNP particle, the level of the U2-specific protein B', which cross-reacts with the monoclonal antibody 9A9, was not different in the L2-treated vs. mock-treated extract (lanes 1–4). The affinity-selected fractions were also subjected to Western blot analyses (data not shown); only a small fraction ($\approx$ 10%) of the U1 A protein remained associated with the U1 particle in the L2 oligonucleotide-pretreated extract. In addition, interaction of U1 loop 2 with the 2'-*O*-methyl oligonucleotide was confirmed by the observation that U1 snRNA extracted from the affinity-selected U1 snRNPs became

FIG. 1. Splicing at the normal 5' splice site in the presence of excess SR proteins and A protein-deficient U1 snRNPs. (A) Immunoblotting of mock-treated or 2'-*O*-methyl oligonucleotide L2-pretreated nuclear extracts (NE) after affinity-depletion of U1 snRNPs in the absence (–) or presence (+) of the biotinylated 2'-*O*-methyl oligonucleotide BU1₁₋₁₄. (B) Splicing reactions were carried out for 1.5 hr in mock-treated, 2'-*O*-methyl oligonucleotide EBER3-, U1₁₋₁₄-, or L2-pretreated extracts in the absence (–) or presence (+) of SR proteins (at 0.12 μ g/ μ l). Symbols on the left indicate the adenovirus precursor, as well as its splicing intermediates and products resulting from use of the normal 5' splice site (●), site 47 (Δ), or site 125 (*). Relative splicing efficiencies shown at the bottom for the three 5' splice sites in each reaction were determined by quantitating the excised exons (marked on the gel) and normalizing for the length of each band. The appearance of a degraded version of the spliced product from site 125 has been discussed previously (17). Unidentified fragments occasionally appeared in some batches of nuclear extract but not in S100 extract. The lengths in nucleotides of DNA markers are shown on the left. (C) Splicing was performed for 10 min in mock-treated or L2-pretreated extract in the absence (–) or presence (+) of additional SR proteins. 4'-Aminomethyl-4,5',8-trimethylpsoralen (AMT psoralen) was added to the reaction mixtures and irradiation with 365-nm light was carried out. All crosslinked species, labeled at the left, were identified previously (17). After prolonged exposure, a U2/substrate crosslink could be seen in the unsupplemented, L2-pretreated extract. Lanes 1 and 2 show precursor RNA that was not or was irradiated with 365-nm light in the presence of AMT psoralen. Below is reported the fold stimulation of U1-crosslinks at the normal and +125 sites induced by excess SR proteins, determined by quantitating the crosslink yield in the presence vs. absence of added SR proteins.

more resistant to RNase H digestion directed by deoxyoligonucleotide U1₆₄₋₇₅ in the L2-pretreated extract (data not shown).

Fig. 1B shows the splicing activity of extracts containing U1 snRNPs debilitated in two different ways. In contrast to the

U1₁₋₁₄ oligonucleotide-pretreated extract (lane 5), the L2 oligonucleotide-pretreated extract did splice the adenovirus pre-mRNA substrate (lane 7). Addition of excess amounts (≈ 20 -fold the endogenous level) of SR proteins to mock-treated or EBER3 (nonspecific) oligonucleotide-pretreated extracts caused complete switching from the normal site ($\bullet$) to the +125 site within the intron of the adenovirus transcript (*, lanes 2 and 4), as previously observed (17). In contrast, the L2-pretreated extract continued to utilize the normal site (+1) efficiently and switched splicing only slightly (15%) to site 125 even in the presence of high concentrations of SR proteins (compare all products labeled by $\bullet$ in lane 8 to those in lanes 2 and 4). Another novel 5' splice site (site 47) was activated concomitantly when the extract was treated with the U1₁₋₁₄ oligonucleotide, which sequesters the 5' end of U1 snRNA (lane 6); we previously concluded (17) that activation of site 47 depends on inactivation of the U1 snRNA.

Psoralen crosslinking was previously utilized to detect U1 snRNA interaction with 5' splice sites in the adenovirus pre-mRNA (17). We therefore examined U1 binding to the normal and +125 sites in mock-treated and L2-pretreated extracts by this method. In the absence of additional SR proteins, U1 crosslinking to the normal site (U1 $\times$ 1), but not to the +125 site, occurred in both extracts (Fig. 1C, lanes 3 and 5). When either extract was supplemented with high concentrations of SR proteins, formation of the U1 $\times$ 1 crosslinked product increased 3-fold over that in the unsupplemented extract (lanes 4 and 6). Yet, splicing in the mock-treated extract did not occur at the normal site (Fig. 1B, lane 2), while in the L2-pretreated extract, excess SR proteins stimulated splicing at the normal site about 2.5-fold (Fig. 1B, lanes 7 and 8), consistent with the relative levels of U1 binding. These results suggest that modulation of 5' splice site utilization exerted by SR proteins depends on integrity of the U1 snRNP. In the presence of excess SR proteins, the U1 $\times$ 125 crosslink appeared at similar levels whether or not the U1-A protein had been detached (Fig. 1C, lanes 4 and 6). Thus, in the L2-pretreated extract, splicing induced by SR proteins at sites +1 and +125 does quite closely follow U1 snRNP binding.

SR Proteins Are Required for the Stable Binding of U2 snRNP to the Pre-mRNA. Our finding that splicing at the distal (+1) 5' splice site in normal extract supplemented with excess SR proteins does not correlate with binding of the U1 snRNP is consistent with previous reports (17, 25, 26). Therefore, 5' splice site choice induced by excess SR proteins may reflect a specific role in promoting interactions between the U1 snRNP and components assembling at the 3' splice site. Splicing substrates that lack 5' splice sites can form stable complexes containing U2 snRNPs in nuclear extract (22, 27, 28). We therefore investigated the contribution of SR proteins to this partial reaction.

As shown in Fig. 2A, formation of the 3'-half complex occurred in nuclear extract but not in splicing-deficient S100 extract (lanes 1 and 2). Addition of ≈ 4 -fold the endogenous level of SR proteins to S100 extract restored complex formation (lane 3). Since U2 auxiliary factor (U2AF) is essential for the binding of U2 snRNPs to the pre-mRNA (29), one might argue that magnesium-precipitated SR proteins are contaminated with U2AF, which also contains the SR motif. However, neither subunit of U2AF could be detected in our mixed SR protein preparation by Western blot analysis (data not shown). Indeed, purified SC35, but not U2AF, was sufficient to rescue 3'-half complex formation in S100 extract (data not shown).

Psoralen crosslinking was then used to establish whether SR proteins simply increase the stability of the 3'-half complex or also facilitate the initial binding of U2 snRNA to the branch site. Fig. 2B (lane 1) shows the formation in the nuclear extract of several crosslinked products whose snRNA content was determined by oligonucleotide-mediated RNase H digestion (data not shown). In contrast, no specific crosslinked products

FIG. 2. SR proteins recruit the U2 snRNP to the branch site and stabilize complexes on the 3'-half splicing substrate. (A) Reactions were carried out under splicing conditions for 30 min in HeLa nuclear extract, S100 extract, or S100 extract supplemented with SR proteins (at 30 ng/ μ l). Complex 3'CP and H represent the specific and the nonspecific complexes assembled on the 3'-half substrate, respectively. A, B, and C indicate the migration of splicing complexes assembled on the intact pre-mRNA (not shown in this figure). (B) Psoralen crosslinking was performed on the reactions shown in A. Crosslinked products were identified by deoxyoligonucleotide-mediated RNase H digestion. The nonspecific crosslink appearing near the top does not contain U1 or U2 snRNA. X represents an intramolecular crosslink.

were observed in the unsupplemented S100 extract (lane 2). Addition of SR proteins to S100 extract rescued the U2/3'-half substrate and U2/U6/3'-half substrate crosslinks, as well as lower amounts of U1/3'-half substrate crosslinks (lane 3). Appearance of a novel U2/U6/3'-half substrate crosslink indicates that a complex containing multiple snRNPs does form under these conditions but is too unstable to have been previously observed in nondenaturing gels (22, 27, 28). We believe that base-pairing of U1 snRNA to the 3'-half substrate is not essential for subsequent steps in spliceosome assembly, because the U1/3' crosslinks were barely detected in SR protein-supplemented S100 extract (Fig. 2B), where the 3'-half complex can form (Fig. 2A), and because the crosslinked site is located in an intron region (data not shown) rather than in an exon (5).

SR Proteins Can Rescue the Binding of U2 to the Pre-mRNA when U1 snRNPs Are Debilitated by the U1₁₋₁₄ 2'-O-Methyl Oligonucleotide. Our results with the 3'-half substrate suggest that SR proteins may be directly involved in the stable binding of the U2 snRNP to the pre-mRNA. Since U2 association with

the branch site was previously shown to require the U1 snRNP (22, 28), we asked whether excess SR proteins can compensate for debilitated U1 snRNPs in promoting U2 snRNP binding. When the 5' end of U1 snRNA was sequestered by the 2'-*O*-methyl oligonucleotide U1₁₋₁₄ (17, 18), no 3'-half substrate complex assembled in the nuclear extract (Fig. 3*A*, lane 2), whereas increasing concentrations of mixed SR proteins (lanes 3–6) or purified SC35 (data not shown) reversed the assembly blockage. This result is consistent with previous observations that splicing of a pre-mRNA can be rescued by supplementing a U1-debilitated extract with excess SR proteins or SC35 (17). Likewise, in extracts containing A protein-deficient U1 snRNPs, stable 3'-half complexes did not form (even though splicing of the intact adenovirus substrate could occur; Fig. 1*B* and data not shown); excess SR proteins rescued complex formation.

Psoralen crosslinking experiments confirmed that interactions of the 3'-half substrate with U1 snRNA were completely blocked when the U1 snRNP was inactivated by the U1₁₋₁₄ oligonucleotide (Fig. 3*B*, lane 2). Titration of SR proteins into this extract generated an increase in specific crosslinked products (lanes 3–6) that correlates with complex formation

(Fig. 3*A*). This result also agrees with the previous observation that excess SR proteins can rescue U2/substrate interactions and even a U2/U6/substrate crosslink in the absence of the functional U1 snRNPs (17). We conclude that excess amounts of SR proteins can substitute for the U1 snRNP's role in mediating U2 snRNP binding and stabilizing the complex assembled on a 3'-half substrate.

DISCUSSION

It is not understood how the splicing machinery selects true 5' splice sites from many potential sequences with high fidelity. Prior studies have suggested a double proofreading mechanism for 5' splice site recognition: SR proteins initially recruit the U1 snRNP to the 5' splice site and U6 proofreads the cleavage site prior to the first catalytic reaction (ref. 17 and references therein). However, the scenario becomes more complicated in the presence of excess SR proteins, which may recruit U1 snRNPs to other potential sites. By exploiting psoralen crosslinking to examine the binding of U1 to 5' splice sites, we showed previously that high levels of SR proteins not only recruit U1 snRNPs to a proximal site (site 125 in the adenovirus intron) but also increase U1 interaction with the normal (+1) site (17). Yet, splicing occurred exclusively at site 125, indicating that the binding of a U1 snRNP to a 5' splice site is not sufficient to specify nucleophilic attack by the branchpoint nucleotide, especially in the presence of excess SR proteins. A similar result with the alternatively spliced simian virus 40 transcript was obtained recently by Zahler and Roth (26). These findings are consistent with the involvement of another factor(s) in 5' splice site selection.

Here, we have devised conditions under which a distal 5' splice site is spliced following U1 snRNP binding in the presence of excess SR proteins. In contrast to a normal extract, splicing in an extract pretreated with the 2'-*O*-methyl oligonucleotide, L2, which detaches the A protein from U1 snRNA, did not switch completely from the normal site to site 125 even at high concentrations of SR proteins (Fig. 1). We have therefore succeeded in uncoupling two steps executed by SR proteins in conjunction with U1 snRNPs that influence 5' splice site utilization. First, SR proteins direct the U1 snRNP to the normal 5' splice site; then, as the concentration of SR proteins builds beyond its normal level, other potential sequences on a pre-mRNA become occupied by U1 snRNPs. At this stage, the U1 snRNP lacking the A protein is able to bind to the normal 5' splice site (M. A. Garcia-Blanco, personal communication) and be recruited by excess SR proteins to site 125 (Fig. 1*C*). In a second step, SR proteins collaborate with the U1 snRNP to actively pair the 5' and 3' splice sites: If the U1 snRNP is intact, excess SR proteins contribute to a mechanism whereby splicing is inhibited at the normal (distal) site and occurs instead at the proximal site. In the oligonucleotide L2-pretreated extract, U1 snRNP interactions with SR proteins appear to be perturbed because the A protein is displaced; consequently, U1 snRNA–5' splice site base-pairing interactions take over to facilitate presplicing complex formation and allow splicing to occur at all sites bound by the U1 snRNP.

Using a 3'-half splicing substrate to examine other roles of SR proteins in spliceosome formation, we found that SR proteins can directly or indirectly recruit U2 to the branch site and stabilize the complex assembled on the 3' splice site in the presence of functional U1 snRNPs (Fig. 2). Such a complex can subsequently communicate with a 5' splice site bound by a U1 snRNP. Indeed, J. Bruzik and T. Maniatis (personal communication) observed that SR proteins promote formation of a complex on a substrate containing the 3' splice site and an enhancer and that this complex is able to engage a 5' splice site in trans. This result rules out the possibility that aberrant complexes assemble on 3'-half substrates and supports the idea

FIG. 3. SR proteins rescue U2 snRNP binding to the branch site and stabilize 3'-half complexes in the presence of the 2'-*O*-methyl oligonucleotide U1₁₋₁₄-debilitated U1 snRNP. (A) Reaction mixtures (10 μ l) were incubated for 30 min and contained mock-treated or U1₁₋₁₄ oligonucleotide-pretreated extract in the absence or presence of increasing amounts of SR proteins. The disappearance of complex H in lanes 3–6 was caused by exposure to EGTA-inactivated micrococcal nuclease. (B) Psoralen crosslinking was performed on the reactions shown in A. Crosslinked species are described in the legend to Fig. 2*B*.

that selection of 5' splice sites can be directed by 3' splice site complexes. Even when U1 snRNP functions were blocked by sequestering the 5' end of U1 or removing the A protein, we observed that excess amounts of SR proteins can recruit the U2 snRNP and stabilize the 3'-half complex (Fig. 3 and data not shown). Therefore, cross-talk between this stabilized complex and a 5' splice site specified by U6 is also a possible explanation for the formation of an active splicing complex involving 5' and 3' splice sites.

Our results imply that SR proteins, like heterogeneous nuclear ribonucleoproteins (hnRNPs) (32), may function as RNA matchmakers or chaperones during pre-mRNA splicing. hnRNP proteins and SR proteins possess RNA recognition motifs. Those in SR proteins exhibit specificity for purine-rich enhancer sequences (11, 13, 14) and are linked to SR domains, which may mediate protein-protein interactions (12). One SR protein, SF2/ASF, can promote the annealing of complementary RNAs (33) and directly recruit U1 snRNPs to a 5' splice site (8, 9). We showed previously that SR proteins can bypass the normal role of U1 snRNPs, presumably by directing other splicing factors, probably U6, to potential 5' splice sites (17). The data presented here indicate that SR proteins can also promote U2 binding to the branch site and stimulate U2-U6 interactions even in the absence of functional U1 snRNPs. Although the binding of U2 to the branch site may trigger a conformational change in the U2 snRNP that allows the 5' end of U2 snRNA to become accessible to U6 (refs. 34 and 35; unpublished data), we have also observed that SR proteins, or SC35 alone, stimulate formation of U2-U6 helix II (30, 31) in the absence of an exogenous splicing substrate (unpublished data). These observations suggest that SR proteins play multiple roles in the intricate rearrangements involving snRNAs and the pre-mRNA during the splicing reaction.

Note Added in Proof. We recently learned that R. Roscigno and M. Garcia-Blanco (personal communication) have demonstrated that SR proteins are involved in the joining of the U4/U6-U5 tri-snRNP to the assembling spliceosome. This finding explains our observation of a U2/U6/3'-half substrate crosslink (Fig. 3) and supports our conclusion that SR proteins facilitate all snRNP-substrate interactions in the spliceosome.

We are most grateful to R. Luhrmann for providing the monoclonal antibody anti-(U1)70k, W. van Venrooij for the monoclonal antibody 9A9, M. Tian and T. Maniatis for purified SC35, and J. Valcarcel and M. Green for purified U2AF and antibodies against U2AF subunits. We are indebted to M. Garcia-Blanco for communicating unpublished results. We thank the C. Guthrie laboratory, J. Bruzik, L. Scharl, and C. Smith for discussions and critical reading of the manuscript. This work was supported by Grant GM26154 from the National Institutes of Health.

1. Horowitz, D. S. & Krainer, A. R. (1993) *Trends Genet.* **10**, 100-106.
2. Moore, J. M., Query, C. C. & Sharp, P. A. (1993) in *The RNA World*, eds. Gesteland, R. F. & Atkins, J. F. (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 303-358.
3. Ruby, S. W. & Abelson, J. (1988) *Science* **242**, 1028-1035.
4. Seraphin, B., Kretzner, L. & Rosbash, M. (1988) *EMBO J.* **7**, 2533-2538.
5. Watakabe, A., Tanaka, K. & Shimura, Y. (1993) *Genes Dev.* **7**, 407-418.
6. Hoffman, B. E. & Grabowski, P. J. (1992) *Genes Dev.* **6**, 2554-2568.
7. Fu, X.-D. (1993) *Nature (London)* **365**, 82-85.
8. Zuo, P. & Manley, J. L. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 3363-3367.
9. Kohtz, J. D., Jamison, S. F., Will, C. L., Zuo, P., Luhrmann, R., Garcia-Blanco, M. A. & Manley, J. L. (1994) *Nature (London)* **368**, 119-124.
10. Fu, X.-D. & Maniatis, T. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1725-1729.
11. Staknis, D. & Reed, R. (1994) *Mol. Cell. Biol.* **14**, 7670-7682.
12. Wu, J. Y. & Maniatis, T. (1993) *Cell* **75**, 1061-1070.
13. Lavigne, A., La Branche, H., Kornblihtt, A. R. & Chabot, B. (1993) *Genes Dev.* **7**, 2405-2417.
14. Sun, Q., Mayeda, A., Hampson, R. K., Krainer, A. R. & Rottman, F. M. (1993) *Genes Dev.* **7**, 2598-2608.
15. Tian, M. & Maniatis, T. (1993) *Cell* **74**, 105-114.
16. Crispino, J. D., Blencowe, B. J. & Sharp, P. A. (1994) *Science* **265**, 1866-1869.
17. Tarn, W.-Y. & Steitz, J. A. (1994) *Genes Dev.* **8**, 2704-2717.
18. Seiwert, S. D. & Steitz, J. A. (1993) *Mol. Cell. Biol.* **13**, 3135-3145.
19. Kastner, B., Kornstadt, U., Bach, M. & Luhrmann, R. (1992) *J. Cell Biol.* **116**, 839-849.
20. Habets, W. J., Hoet, M. H., De Jong, B. A. W., Van der Kemp, A. & van Venrooij, W. J. (1989) *J. Immunol.* **143**, 2560-2566.
21. Solnick, D. (1985) *Cell* **42**, 157-164.
22. Barabino, S. M. L., Blencowe, B. J., Ryder, U., Sproat, B. S. & Lamond, A. I. (1990) *Cell* **63**, 293-302.
23. Scherly, D., Boelens, W., van Venrooij, W. J., Nathan, N. A. & Mattaj, I. W. (1989) *EMBO J.* **8**, 4163-4170.
24. Lutz-Freyermuth, C. & Keene, J. D. (1989) *Mol. Cell. Biol.* **9**, 2975-2982.
25. Eperon, I. C., Ireland, D. C., Smith, R. A., Mayeda, A. & Krainer, A. R. (1993) *EMBO J.* **12**, 3607-3617.
26. Zahler, A. M. & Roth, M. B. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 2642-2646.
27. Konarska, M. M. & Sharp, P. A. (1986) *Cell* **46**, 845-855.
28. Robberson, B. L., Cote, G. J. & Berget, S. M. (1990) *Mol. Cell. Biol.* **10**, 84-94.
29. Zamore, P. D. & Green, M. R. (1991) *EMBO J.* **10**, 207-214.
30. Datta, B. & Weiner, A. M. (1991) *Nature (London)* **352**, 818-824.
31. Wu, J. & Manley, J. L. (1991) *Nature (London)* **352**, 818-821.
32. Portman, D. S. & Dreyfuss, G. (1994) *EMBO J.* **13**, 213-221.
33. Krainer, A. R., Conway, G. C. & Kozak, D. (1990) *Genes Dev.* **4**, 1158-1171.
34. Lamond, A. I., Sproat, B., Ryder, U. & Hamm, J. (1989) *Cell* **58**, 383-390.
35. Wassarman, D. A. & Steitz, J. A. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 7139-7143.