SR proteins can compensate for the loss of U1 snRNP functions in vitro

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SR proteins are essential splicing factors that also influence 5' splice site choice. We show that addition of excess mixed SR proteins to a HeLa in vitro splicing system stimulates utilization of a novel 5' splice site (site 125) within the intron of the standard adenovirus pre-mRNA substrate. When U1 snRNPs are debilitated by sequestering the 5' end of U1 snRNA with a 2'-O-methyl oligoribonucleotide, excess SR proteins not only rescue splicing at the normal site and site 125 but also activate yet another 5' splice site (site 47) in the adenovirus intron. One SR protein, SC35, is sufficient to exhibit the above activities. The possibility that excess SR proteins recruit residual unblocked U1 snRNPs to participate in 5' splice site recognition has been ruled out by psoralen cross-linking studies, which demonstrate that the 2'-O-methyl oligoribonucleotide effectively blocks 5' splice site/U1 interaction. Native gel analysis reveals a nearly normal splicing complex profile in the 2'-O-methyl oligoribonucleotide pretreated, SR protein-supplemented extract. These results indicate that SR proteins can replace some functions of the U1 snRNP but underscore the contribution of U1 to the fidelity of 5' splice site selection.

[*Key Words*: SR proteins; U1 snRNPs; pre-mRNA splicing; 5' splice site selection]

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The splicing of pre-mRNA to produce functional message is a key step in gene expression. Most mammalian pre-mRNAs contain several introns, and some are alternatively spliced; therefore, the selection of splice sites must be highly accurate (for review, see Horowitz and Krainer 1993). Splicing of pre-mRNA occurs in the spliceosome, a complex ribonucleoprotein particle composed of five small nuclear RNAs (snRNAs) (U1, U2, U4, U5, and U6) and numerous protein factors (for review, see Moore et al. 1993). Splicing proceeds through two sequential transesterification reactions. In the first step, the phosphodiester bond at the 5' splice site is cleaved by nucleophilic attack of the 2'-hydroxyl group of an adenosine located near the 3' end of the intron. In the second step, the two exons are ligated with release of the intron lariat.

Complementarity between the 5' splice site and the 5' end of U1 snRNA is a major determinant in identification of the 5' splice site, but base-pairing with U1 alone is not sufficient to specify the site of nucleophilic attack (Zhuang and Weiner 1986; Seraphin et al. 1988; Siliciano and Guthrie 1988; Seraphin and Rosbash 1990). Two other small nuclear ribonucleoproteins (snRNPs), U5 and U6, are known to contribute to 5' splice site choice. Genetic experiments, initially performed in yeast, showed that mutations in the conserved first loop of U5 snRNA could activate aberrant 5' splice sites after mu-

tation of the normal 5' splice site of the pre-mRNA (Newman and Norman 1991, 1992). Similar results were obtained in analogous experiments in the mammalian system (Cortes et al. 1993). Site-specific cross-linking studies further detected dynamic interactions between the conserved loop of U5 and exon sequences adjacent to both the 5' and 3' splice sites (Wyatt et al. 1992; Sontheimer and Steitz 1993). Together, these observations suggest that the U5 snRNP might influence 5' splice site recognition by forming either Watson-Crick or noncanonical base-pairing interactions with the 5' exon. Likewise, U6 has long been suspected to serve a vital role in the catalytic reactions of the spliceosome (for review, see Guthrie 1991). Cross-linking experiments in both mammalian and yeast systems first suggested interactions between intron sequences at the 5' splice site and the invariant sequence ACAGAG in U6 snRNA (Sawa and Abelson 1992; Wassarman and Steitz 1992; Sontheimer and Steitz 1993). Genetic suppression data then showed that this U6 sequence can engage in Watson-Crick pairing with intron sequences and influence selection of the precise 5' splice site (Kandels-Lewis and Seraphin 1993; Lesser and Guthrie 1993). However, the above examples of a dominant role for U5 or U6 in determination of the 5' cleavage site represent special circumstances, occurring only when the normal 5' splice site has been mutated (Newman and Norman 1992; Kandels-Lewis and Seraphin 1993; Lesser and Guthrie 1993). Moreover, because the proposed pairings of the 5' splice site to U1 and

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U6 are mutually exclusive, these two interactions are believed to be sequential, with U6 making intron contacts only after U1 has left the 5' splice site (Konforti et al. 1993).

Yet other factors must therefore collaborate with U1 in the initial identification of the 5' splice site during the commitment step in spliceosome assembly (for review, see Rosbash and Seraphin 1991; Manley 1993). Here, a family of proteins containing arginine/serine-rich (RS) domains has been implicated (for review, see Horowitz and Krainer 1993). The so-called SR proteins include at least six related polypeptides that are conserved among metazoans (Roth et al. 1991; Zahler et al. 1992). SR proteins are essential for splicing and also influence the choice of 5' splice site in alternatively spliced pre-mRNAs (Ge and Manley 1990; Krainer et al. 1990a,b; Fu et al. 1992; Mayeda et al. 1992; Zahler et al. 1992). They commit a pre-mRNA to splicing (Fu 1993) and are required for A complex formation during spliceosome assembly (Fu and Maniatis 1990; Krainer et al. 1990a). Although the various SR proteins initially appeared to be functionally redundant, recent observations suggest differences in their ability to function as both essential and alternative splicing factors (Fu 1993; Zahler et al. 1993). With a precursor RNA containing multiple 5' splice sites, high concentrations of SR proteins favor use of the proximal (downstream) 5' splice site (Ge and Manley 1990; Krainer et al. 1990b; Fu et al. 1992; Zahler et al. 1993). The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) has been shown to antagonize the activity of SR proteins in the selection of 5' splice sites, suggesting that the relative concentrations of SR proteins and hnRNP A1 in the cell may be critical (Mayeda and Krainer 1992; Yang et al. 1994).

The mechanism by which SR proteins contribute to spliceosome assembly and 5' splice site choice is only beginning to be elucidated. One SR protein, SC35, has been shown to mediate specific interactions between U1 and U2 snRNPs bound at the 5' and 3' splice sites, respectively, in the earliest ATP-dependent splicing complex (Fu and Maniatis 1992). Using a protein-protein interaction approach, Wu and Maniatis (1993) suggested further that SC35 and other SR proteins actually function as a bridge between the U1 snRNP-specific 70K protein and the splicing factor U2AF bound to the 3' splice site. Another SR protein, SF2/ASF, has been proposed to stabilize base-pairing of U1 snRNA to the pre-mRNA (Eperon et al. 1993). Recent studies show that this protein recognizes 5' splice sites directly (Zuo and Manley 1994) and facilitates the binding of the U1 snRNP to the pre-mRNA by protein-protein interactions involving the RS domains of both ASF/SF2 and the U1-specific 70K protein (Kohtz et al. 1994). In addition, SR proteins have been shown to bind exonic purine-rich sequences and enhance the utilization of upstream 3' splice sites (Lavigne et al. 1993; Sun et al. 1993; Tian and Maniatis 1993; Amrein et al. 1994).

We set out to ask whether SR proteins can promote the interaction between a 5' splice site and the U6 snRNP, allowing U6 to replace U1 in interacting with the 5'

splice site during the conversion of early splicing complexes to the active spliceosome. SR proteins from HeLa cells were therefore added to a pre-mRNA splicing reaction in the presence or in the absence of functional U1 snRNPs. We discovered that excess amounts of SR proteins can activate a novel 5' splice site within the intron of the standard splicing substrate derived from the adenovirus major late transcript (Solnick 1985). Furthermore, when the U1 snRNP was debilitated by stably sequestering its 5' end with a 2'-O-methyl oligoribonucleotide (Seiwert and Steitz 1993), excess SR proteins rescued the splicing activity of the extract, activating yet another novel 5' splice site within the intron. We conclude that SR proteins can compensate for the requirement for the 5' end of U1 snRNA in the splicing of pre-mRNA but do not distinguish true from alternative 5' splice sites. We discuss the relative roles of SR proteins, U1 and U6 snRNPs in 5' splice site selection, and spliceosome assembly.

Results

Excess SR proteins induce use of a novel 5' splice site within the intron of the adenovirus pre-mRNA substrate

Isolation of SR proteins from HeLa nuclear extracts was carried out by a two-step salt precipitation as described by Zahler et al. (1992). The SR protein preparation contained six primary polypeptides (data not shown), as reported (Zahler et al. 1992). The SR proteins were assayed for their ability to complement a HeLa S100 extract using two standard splicing substrates, derived from either the adenovirus major late or the human β -globin transcript. As shown in Figure 1A, the unsupplemented S100 extract was unable to splice the pre-mRNA (lanes 2,6). However, S100 extract became splicing competent by complementation with mixed SR proteins (at approximately twofold the level in nuclear extract), exhibiting the same splicing pattern as the nuclear extract (lane 4, products marked ● and lane 8). Addition of excess SR proteins (~20-fold the endogenous level) altered the pattern of splicing products from the adenovirus substrate substantially (lane 3, products marked *), suggesting the activation of an alternative splice site. Titration of SR proteins while holding the amount of S100 extract constant showed that the ratio of utilization of the alternative site relative to the normal site increased with increasing amounts of SR proteins (data not shown). Likewise, when nuclear extract was supplemented with additional SR proteins, the ratio of alternative to normal splicing of the adenovirus transcript increased in a concentration-dependent manner (data not shown). In contrast, the β -globin transcript showed stimulation, but an unchanged pattern of splicing products, in the presence of high concentrations of SR proteins (Fig. 1A, lane 7).

To determine the position of the novel adenovirus splice site activated by excess SR proteins, individual RNAs were gel purified from the splicing reaction and then incubated with the S100 extract, which contains

Figure 1. Alternative splicing of the adenovirus substrate induced by mixed SR proteins. (A) Splicing was carried out in HeLa nuclear extract (NE), or S100 extract (S100), or S100 extract complemented with different amounts of SR proteins using a pre-mRNA derived from the adenovirus major late transcript (Ade) or the β -globin pre-mRNA (β -globin). Symbols at *left* indicate the adenovirus precursor, as well as the splicing intermediates and products using the normal 5' splice site (●) or an alternative 5' splice site at position +125 within the intron (*). Symbols at *right* indicate the β -globin precursor RNA, splicing intermediates, and products. Marker shows 32 P-labeled pBR322 DNA digested with *Msp*I, with the fragment lengths in nucleotides given at *left*. The smear in lane 1 migrating below the excised intron lariat is polyadenylated substrate (data not shown). (B) The sequence at the splice junction of the alternatively spliced product (excised from a gel as in A, lane 3) was determined by dideoxy sequencing. The sequencing reaction, using oligonucleotide AdEII as primer, was carried out in the absence of dideoxynucleotide (lane 1) or in the presence of ddA (lane 2), ddT (lane 3), ddG (lane 4), or ddC (lane 5). The arrow denotes the splice junction. (C) Sequences at the novel 5' splice site (+125) within the intron of the adenovirus transcript and the 3' splice site are shown with uppercase and lowercase letters representing exon and intron regions, respectively. The size of the normal intron is 241 nucleotides, whereas the intron removed by splicing at site +125 is 117 nucleotides.

debranching activity. Two molecules containing a branch (the lariat and lariat intermediate indicated by * in Fig. 1A, lane 3) were identified by their altered mobility on a denaturing gel (data not shown), and their 5' ends were determined by primer extension. These analyses established the position of the novel 5' splice site 125 nucleotides downstream of the normal site (data not shown). The sequences adjacent to the cleavage–ligation site were then determined by dideoxy sequencing of the spliced product (Fig. 1B) and revealed that the novel 5' splice site within the intron becomes joined to the normal adenovirus 3' splice site (Fig. 1C). It should be noted that the first intron of the adenovirus major late transcript has been shortened in our pre-mRNA construct (Solnick 1985); in an *in vivo* adenovirus transcript, a much longer sequence (~800 nucleotides) intervenes between site 125 and the branch site.

SR proteins can activate yet another 5' splice site (site 47) in the absence of functional U1 snRNA

To ask whether SR proteins act autonomously in selecting the novel 5' splice site at position 125 of the adenovirus intron, the splicing extract was pretreated with an antisense 2'-O-methyl oligoribonucleotide (U1₁₋₁₄) complementary to the sequence at the 5' end of U1 snRNA. The U1₁₋₁₄ oligonucleotide had been shown previously to effectively abolish splicing not only of the standard

adenovirus pre-mRNA but also of spliced leader (SL) RNA-containing chimeric substrates; in contrast to shorter anti-U1 oligonucleotides, which do not block splicing of such chimeric transcripts, U1₁₋₁₄ interferes with an additional function of the U1 snRNP and prevents formation of stable complex A (Seiwert and Steitz 1993). Two other 2'-O-methyl oligoribonucleotides, U2b and U7₃₋₂₀, were used as controls. U2b is complementary to a region of U2 that recognizes the branch site region of the intron and has been shown to block A complex formation completely (Lamond et al. 1989); U7₃₋₂₀ is complementary to a nonspliceosomal RNA.

Consistent with previous results, preincubation of nuclear extract with either the U1₁₋₁₄ or U2b oligonucleotide significantly blocked splicing activity (Fig. 2A, lanes 5,6), whereas the U7₃₋₂₀ oligonucleotide had no effect except for nonspecific increases in RNA stability and debranching activity (lane 7). Addition of a high level of mixed SR proteins to S100, mock-treated or U7₃₋₂₀-pretreated nuclear extract (lanes 3,8,11) induced selection of the alternative 5' splice site, 125 nucleotides downstream of the normal site, as expected. Surprisingly, the extract pretreated with the U1₁₋₁₄ oligonucleotide also became splicing competent after supplementation with the same high amount of SR proteins (lane 9), suggesting that SR proteins can somehow substitute for the U1 snRNP functions blocked by this oligonucleotide. The same extract pretreated with the U2b oligo-

Figure 2. SR proteins rescue splicing and activate a second novel 5' splice site (+47) within the intron of the adenovirus substrate when the 5' end of U1 snRNA is sequestered by an antisense 2'-O-methyl oligoribonucleotide. (A) Lanes 1–3 show 10-μl splicing reactions carried out with HeLa nuclear extract (NE), S100 extract (S100), or S100 extract supplemented with 1.5 μg of mixed SR proteins. For lanes 4–11, nuclear extracts were preincubated under splicing conditions for 10 min in the absence of oligonucleotide (lanes 4,8) or in the presence of 2'-O-methyl oligoribonucleotide U1_{1–14} (lanes 5,9), U2b (lanes 6,10) or U7_{3–20} (lanes 7,11). Then, labeled adenovirus substrate, along with 5% glycerol-containing buffer D (lanes 4–7) or micrococcal nuclease-treated SR proteins (1.5 μg) (lanes 8–11), were added and incubation continued for 90 min. Symbols at left indicate the precursor RNA, as well as the splicing intermediates and products using the normal 5' splice site (●), using site 125 (*) or using site 47 (Δ). Size markers are as in Fig. 1A. Note that the spliced product of site 125 reproducibly migrates slightly fast in lane 8 but behaves normally in both lanes 9 and 11. When lane 8 was examined at earlier times, this band migrated normally after 30 min of incubation and smeared slightly at 60 min; when the extract was supplemented with SR proteins that were not treated with micrococcal nuclease, its mobility was normal. Thus, it appears that some micrococcal nuclease activity remains in mock-treated extracts, which can be competed by oligoribonucleotides and prevents digestion of the RNA in lanes 9 and 11. The comigration of the debranched lariat from site 47 with excised exon from site 125 was established by primer extension analysis. (B) The sequence at the splice junction of the site 47 spliced product (excised from a gel as in A, lane 9) was determined by dideoxy sequencing. The sequencing reaction, using oligonucleotide AdEII as primer, was carried out in the absence of dideoxynucleotide (lane 1), or in the presence of ddA (lane 2), ddT (lane 3), ddG (lane 4), or ddC (lane 5). The arrow denotes the splice junction. (C) Sequences at site 47 within the intron of the adenovirus transcript and at the 3' splice site are presented, with uppercase and lowercase letters representing exon and intron regions, respectively. The size of the intron removed by splicing at site +47 is 195 nucleotides. (D) Lane 1 shows a 10-μl splicing reaction performed with normal nuclear extract. For lanes 2–6, the nuclear extract was pretreated with the 2'-O-methyl oligoribonucleotide U1_{1–14} as in A, lanes 5 and 9. Then, labeled adenovirus substrate, along with 5% glycerol-containing buffer D (lane 2) or increasing amounts of micrococcal nuclease-treated SR proteins, was added (lanes 3–6), and incubation continued for 90 min. Gel fractionation and symbols are as in A.

nucleotide was unable to be activated by SR proteins (lane 10). Analogous experiments performed in S100 extracts (not shown) yielded the same results.

Because SR proteins prepared by salt precipitation contain trace amounts of U1 snRNA (~14–20 fmoles of U1 snRNA per microgram of SR proteins; data not shown), we pretreated the preparation extensively with micrococcal nuclease. In Northern blot analyses, the amount of U1 was reduced to undetectable levels (data not shown). Yet, the ability of the treated SR protein preparation to activate splicing even when the 5' end of U1 was blocked was no different from untreated SR proteins

(data not shown). Therefore, micrococcal nuclease-treated SR protein preparations were used in Figure 2 and in further experiments. The possible involvement of residual U1 snRNPs in complemented splicing reactions is addressed below.

When the U1_{1–14} oligonucleotide-pretreated extract was supplemented with excess SR proteins, not only did splicing occur at both the normal and +125 sites, but at least one more splice site was activated (Fig. 2A, lane 9). As described above, each RNA species was excised from the gel and subjected to the debranching assay. In addition to those bands characterized in Fig. 1 (corresponding

to use of the +125 site), two new prominent species were sensitive to the debranching activity (data not shown). The 5' ends of these RNAs were determined by primer extension, revealing yet another 5' splice site located 47 nucleotides downstream of the normal site (data not shown). Again, the exon sequences flanking the splice junction were confirmed by dideoxy sequencing of the spliced product (Fig. 2B), demonstrating that site 47 also becomes joined to the normal adenovirus 3' splice site (Fig. 2C). The intermediates and products associated with splicing at position 47 are indicated (Δ) in Figure 2A.

We showed above that titration of SR proteins into S100 (Fig. 1) or nuclear extracts (both of which have functional U1 snRNPs) causes a concentration-dependent switch from use of the normal to the alternative (site 125) 5' splice site. When the U1₁₋₁₄ oligonucleotide-pretreated extract was likewise supplemented with various amounts of SR proteins, optimal splicing (more than severalfold the level in normal extracts) was obtained at 10-fold the endogenous level of SR proteins (Fig. 2D, lane 5). At this SR protein concentration, use of the normal (+1) 5' splice site began to approach that in normal extracts (cf. ● in lane 5 with lane 1). Increasing the amount of SR proteins further (to 20-fold) resulted in a decrease in splicing at the normal 5' splice site relative to the alternative site 125, but little change was found in use of site 47, which predominates (cf. lanes 5 and 6). Quantitation of the efficiency of splicing to each of the three 5' splice sites (for details, see Fig. 6 legend) under such conditions is presented in Figure 6.

Finally, we used an alternative method to inactivate the U1 snRNP and assessed the effect of excess SR proteins. Previous reports established that the splicing of the adenovirus substrate is almost completely abolished when the 5' end of U1 snRNA is destroyed by RNase H digestion in the presence of the U1₁₋₁₂ deoxyoligonucleotide (see Seiwert and Steitz 1993 and references therein). After such pretreatment of the extract, we observed that excess SR proteins were able to restore the splicing efficiency as well as activate three 5' splice sites concomitantly (data not shown).

In summary, our results indicate that excess mixed SR proteins stimulate utilization of a novel 5' splice site at position 125 of the adenovirus intron in S100 and nuclear extracts, whereas the additional presence of the U1₁₋₁₄ oligonucleotide leads to concomitant use of three 5' splice sites. Site 47 is silent in the presence of functional U1 snRNA but is selected predominantly when the 5' end of U1 snRNA is sequestered. Moreover, when a titration of the U1₁₋₁₄ oligonucleotide (1.5–15 mM) was performed, we found that the efficiency of splicing at site 47 increased with increasing amounts of the oligonucleotide in the SR protein-supplemented extract, confirming a direct correlation between activation of site 47 and inactivation of U1 snRNA (data not shown). Because excess SR proteins can efficiently (but with reduced fidelity) rescue the splicing defect of an extract in which the 5' end of U1 snRNA is masked by an antisense oligonucleotide, it appears that SR proteins can substitute at least partially for functions of the U1 snRNP.

SC35 alone can rescue splicing when the 5' end of U1 snRNA is sequestered

Different SR proteins have distinguishable characteristics (Fu 1993; Sun et al. 1993; Zahler et al. 1993), raising the question of which is contributing to the splicing we observe in the presence of debilitated U1 snRNPs. SC35 purified from the baculovirus expression system (the kind gift of M. Tian and T. Maniatis, Harvard University, Cambridge, MA) was added to a U1₁₋₁₄ oligonucleotide-pretreated extract. Figure 3, lane 6, shows that SC35 alone can rescue the splicing activity of such an extract and mediate utilization of the same three 5' splice sites as a comparable amount of mixed SR proteins. Because only limited amounts of purified SC35 were available, we altered the assay conditions in Figure 3 to use 40% rather than 60% extract (see Materials and methods). Although the same amount of SR proteins as in Figure 2A was supplied, splicing to site 125 was preferred over site 47 (by ~1.5-fold) instead of the reverse (cf. Fig. 3, lane 4, with Fig. 2A, lane 9). This suggests that some extract factor that exhibits a preference for site 47 may collaborate with SR proteins in the selection of 5' splice sites in the absence of U1 snRNPs. Again, the splicing activity of the U2b oligonucleotide-pretreated extract

Figure 3. SC35 can rescue splicing and activate site 47 in the absence of functional U1 snRNA. Nuclear extracts were pretreated in bulk with 2'-O-methyl oligoribonucleotide U1₁₋₁₄ (lanes 2,4,6,8) or U2b (lanes 3,5,7,9) under splicing conditions for 10 min. After addition of ³²P-labeled adenovirus substrate along with 5% glycerol-containing buffer D (lanes 2,3), 1.5 μg of mixed SR proteins (lanes 4,5), 1.6 μg of purified SC35 (lanes 6,7), or 1.6 μg of BSA (lanes 8,9), incubation was continued for 90 min. Lane 1 shows splicing in an untreated nuclear extract. Size markers and symbols are as in Fig. 2A.

was not able to be rescued either by mixed SR proteins or by SC35 (Fig. 3, lanes 5,7). Likewise, BSA did not restore the splicing activity of oligonucleotide-pretreated extracts (lanes 8,9).

In addition to the standard adenovirus pre-mRNA, we examined a chimeric substrate (C/Ad) (Bruzik and Steitz 1990), which contains an SL RNA 5' splice site and lacks adenovirus sites 47 and 125, to ask whether the ability of mixed SR proteins or SC35 to compensate for functions of the U1 snRNP is general. Splicing of the C/Ad construct was shown previously to be blocked completely by preincubation with the U1₁₋₁₄ oligonucleotide (Seiwert and Steitz 1993). We observed that this block can be rescued fully by supplementation with either mixed SR proteins or SC35 alone (data not shown). Thus, mixed SR proteins or SC35 alone can commit even a variant 5' splice site to splicing in the presence of debilitated U1 snRNPs. Moreover, the splicing of C/Ad occurred only at the authentic 5' splice site in the absence of competitive sites, indicating that reconstitution by SR proteins is not limited to certain special downstream sites.

Psoralen cross-linking of U1 snRNA to 5' splice sites is effectively blocked by the U1₁₋₁₄ oligonucleotide in SR protein-supplemented extract

There are several possible explanations for the observation that SR proteins can rescue the splicing activity of an extract in which the 5' end of U1 RNA is blocked by an antisense 2'-O-methyl oligoribonucleotide. It could be that an RNA unwinding activity induced by addition of SR proteins disrupts the U1 snRNA-antisense oligonucleotide duplex, reactivating U1 to perform its normal role in splicing. Alternatively, a tiny fraction of the U1 snRNPs in the extract could remain free and be actively recruited to potential 5' splice sites by excess SR proteins; in our standard splicing assay, there is an ~1000-fold excess of U1 snRNP over substrate. Finally, SR proteins may be capable of substituting for those U1 snRNP functions blocked by the oligonucleotide. We employed psoralen cross-linking (Wassarman and Steitz 1992; Seiwert and Steitz 1993) to differentiate among these possibilities.

Because splicing in the presence of the U1₁₋₁₄ oligonucleotide and excess SR proteins is highly efficient (Figs. 2A and 3), cross-linking to the pre-mRNA should not be diminished if U1 snRNPs are being either reactivated or recruited from a small residual pool. The adenovirus substrate was incubated under splicing conditions for 10 min and then irradiated on ice with 365-nm light in the presence of AMT psoralen. No specific cross-linked products were observed in the absence of nuclear extract (Fig. 4A, lane 2), whereas U1 cross-linking to the normal 5' splice site (U1×1; see below) was seen in both the nuclear extract and the SR protein-supplemented extract (lanes 3,5). At high concentrations of SR proteins, formation of the U1×1 cross-linked product was enhanced to more than three-fold that in the unsupplemented extract (lane 5), and two new closely migrating bands corresponding to U1 cross-linking to site 125

(U1×125; see below) appeared. The species labeled U1×1 and U1×125 were determined to contain U1 snRNA by oligonucleotide-directed RNase H cleavage; both were cleaved with an oligonucleotide complementary to U1 snRNA, U1₆₄₋₇₅ (lanes 4,6). These data demonstrate that both 5' splice sites are occupied by U1 snRNPs even though high concentrations of SR proteins lead to preferred use of the downstream site (site 125). Note that because the efficiency of cross-linking depends on the geometry (sequence) of the reactive sites, relative cross-linking is not a measure of the relative occupancy of the two sites by the U1 snRNP.

In the nuclear extract pretreated with the 2'-O-methyl oligonucleotide U1₁₋₁₄ (Fig. 4A, lanes 7,8), the U1-specific cross-linked products disappeared, as expected. Titrations of the oligonucleotide moreover revealed a direct correlation between splicing and cross-linking at sites +1 and +125 (data not shown). Significantly, when splicing was restored to the U1₁₋₁₄ oligonucleotide-pretreated extract by complementing with SR proteins, the cross-linked products containing U1 snRNA did not reappear (lanes 9,10), although cross-linked products with U2 were formed (see below). Likewise, in the absence of ATP, conditions favoring only formation of the earliest pre-splicing complex (E complex) (Michaud and Reed 1991), no cross-linked products were detected in the U1₁₋₁₄-pretreated and SR protein-supplemented extract, whereas in a normal extract lacking ATP, U1 cross-linking to site +1 was enhanced (data not shown). These results argue that the 5' end of U1 snRNA does not participate in the splicing events activated by excess SR proteins in the presence of the U1₁₋₁₄ oligonucleotide. We can rule out the possibility that excess SR proteins block the access of psoralen because U1-substrate cross-linking is stimulated by SR proteins in the presence of functional U1 snRNPs (lane 5 vs. lane 3).

To locate the sites of cross-linking precisely on the adenovirus substrate and U1 RNA, we performed primer extension analyses on gel-purified cross-linked RNAs. Reverse transcription from a primer complementary to a region 3' of a cross-linked site will terminate at the residue preceding a psoralen cross-linked nucleotide (Piette and Hearst 1983). Analysis of cross-linked RNA U1×1 using an antisense adenovirus sequence as primer mapped blockage primarily to nucleotide U⁺⁹ (the ninth nucleotide of the intron) but also to C⁺⁸ (Fig. 4B). Previously, the second nucleotide of U1 snRNA (U²) had been identified as cross-linking to nucleotides C⁺⁸ and U⁺⁹ of the adenovirus intron under normal splicing conditions (Wassarman and Steitz 1992). The two U1×125 cross-linked bands were analyzed separately; both showed stops prior to nucleotide U⁺¹²³ and nucleotide U⁺¹⁴⁷ of the intron (Fig. 4C; data not shown). It is unclear why the U1×125 band is a doublet; however, we determined that the stop at nucleotide U⁺¹⁴⁷ probably results from intramolecular cross-linking or a psoralen monoadduct because primer extension on precursor RNA cross-linked in the absence of extract yielded this product (data not shown). To determine which nucleotide of U1 snRNA becomes cross-linked to residue

Figure 4. Evidence that the 5' end of U1 snRNA is not involved in 5' splice site recognition in the U1₁₋₁₄ oligonucleotide-pretreated and SR protein-supplemented extract. (A) Splicing reactions were performed for 10 min in mock-treated nuclear extract (lanes 3,4), mock-treated and SR protein-supplemented extract (lanes 5,6), U1₁₋₁₄ oligonucleotide-pretreated extract (lanes 7,8), or U1₁₋₁₄ oligonucleotide-pretreated and SR protein-supplemented extract (lanes 9,10). AMT psoralen was added to the reaction mixtures and irradiation with 365-nm light was carried out. RNAs recovered from each reaction were aliquoted into two fractions, followed by addition of RNase H alone (lanes 3,5,7,9) or RNase H plus 1 μ g of U1₆₄₋₇₅ (lanes 4,6,8,10). The identities of the cross-linked species, as determined below, are indicated at left. (Lanes 1,2) Precursor RNA that was not or was irradiated with 365-nm light in the presence of AMT psoralen, respectively. Quantitation indicated that $\sim$ 15% of the U1 $\times$ 1 bands remained undigested by RNase H in both lanes 4 and 6; $<$ 1.5% residual U1 $\times$ 125 and U1 $\times$ 1 cross-linking was detected in the U1-blocked extracts (cf. lane 9 to lane 5). (B) The cross-linked U1 $\times$ 1 RNA was gel-purified from a reaction containing excess SR proteins, as in lane 5 of A, and the cross-linked site mapped on the precursor RNA by primer extension using oligonucleotide Ad1a as primer (lane 6). Dideoxy sequencing reactions using the same primer included no dideoxynucleotide (lane 1), ddA (lane 2), ddT (lane 3), ddG (lane 4), or ddC (lane 5). Although stops at C⁺¹⁰ and U⁺⁹ are clearest, the faint stop at A⁺⁷ may indicate weak cross-linking to U⁺⁶, as well. (C) The cross-linked site of the U1 $\times$ 125 species was mapped similarly on the precursor RNA by primer extension using oligonucleotide Ad1b as primer (lane 6). (D) The cross-linked site of U1 $\times$ 125 was mapped on U1 snRNA, using oligonucleotide U1₆₄₋₇₅ as primer (lane 6) and gel-purified U1 snRNA as the template in lanes 1–5. (E) Complementarity between the adenovirus 5' splice sites (+1 or +125) and U1 snRNA is indicated by thick vertical lines for Watson–Crick base pairs or dots for G–U. The cross-linked nucleotides identified here are indicated in bold, with that determined previously by Wassarman and Steitz (1992) in outline. The cross-linked nucleotides are indicated by a lightning bolt. The 5' splice site is marked by an arrow. (F) Cross-linked RNA was isolated from a reaction as in lanes 9 and 10 of A. Oligonucleotide-directed RNase H cleavage was performed in the absence of oligonucleotide (lane 1) or in the presence of U1₆₄₋₇₅ (lane 2) or U2₇₉₋₉₈ (lane 3). Note that the higher yield of U2–U6–substrate cross-link in F compared with A is attributable to a different extract.

U⁺¹²³, we also performed primer extension analysis on the U1 $\times$ 125 bands with an oligonucleotide complementary to U1 snRNA. As illustrated in Figure 4D, the cross-link mapped to nucleotide U¹⁰ of U1 snRNA, show-

ing that base-pairing with U1 snRNA occurs when usage of site 125 is induced by excess SR proteins (and the 5' end of U1 is not blocked). Primer extension analysis performed on U1 $\times$ 1- or U1 $\times$ 125-comigrating RNA isolated

from a cross-linking reaction in the absence of the splicing substrate showed no specific stops (data not shown). Deduced base-pairing interactions adjacent to cross-linked nucleotides in U1 snRNA and the normal 5' splice site, as well as in the downstream site (site 125), are shown in Figure 4E.

Finally, we identified other snRNAs that became cross-linked to the pre-mRNA in reactions performed in the presence of the U1₁₋₁₄ oligonucleotide and excess SR proteins. RNase H cleavage experiments were conducted using oligonucleotides complementary to U1 or U2 (Fig. 4F) and to U4, U5, or U6 (data not shown). The bands targeted in the presence of the U2 oligonucleotide correspond to U2-substrate and U2-U6-substrate cross-linked products, both identified previously (Wassarman and Steitz 1992). None of the cross-linked species obtained at this early time in the splicing reaction (10 min) were found to contain U4 or U5 snRNAs. These results suggest that the U2 snRNP contributes to splicing in the U1-blocked, SR protein-supplemented extract as in a normal reaction.

Splicing complexes in the U1₁₋₁₄ oligonucleotide-pretreated extract rescued by SR proteins

Seiwert and Steitz (1993) showed that formation of pre-splicing complex A is slowed significantly in extracts pretreated with the U1₁₋₁₄ 2'-O-methyl oligonucleotide, whereas the appearance of later complexes B and C is almost completely abolished. We therefore examined the assembly of splicing complexes when SR proteins rescue splicing in extracts blocked with the U1₁₋₁₄ oligonucleotide. Consistent with previous results (Seiwert and Steitz 1993), only a trace amount of complex A was formed in the U1₁₋₁₄ oligonucleotide-pretreated extract (Fig. 5, lanes 7–9). In contrast, a profile showing splicing complexes A, B (which migrates slightly faster than normal), and C was restored by addition of excess SR proteins (lanes 10–12). However, constant levels of complex A and a novel complex (marked *) were observed at all time points, whereas both complexes A and B peak early and then diminish in the mock-treated and SR protein-supplemented extract (lanes 4–6) or in a U7₃₋₂₀-pretreated and SR protein-supplemented extract (data not shown). Preliminary studies of the accumulated complex A and the new complex showed that they both contain U2, but not U6, and that their assembly requires ATP and 30°C incubation. The buildup of complex A may be explained if association of U6 with the 5' splice site in the absence of functional U1 becomes rate-limiting, causing the accumulation of a previous splicing complex. The kinetics of splicing (data not shown) and the formation and snRNP constituents of complex C do not differ significantly in mock-treated extract or in SR protein-supplemented extracts with or without U1₁₋₁₄ (Fig. 5, lanes 1–6, 10–12; data not shown). These results suggest that SR proteins facilitate the formation of early splicing complexes, whether or not the U1 snRNP is fully functional. Even excess SR proteins do not appear

Figure 5. Native gel electrophoresis of splicing complexes formed in SR protein-rescued extracts. Splicing reactions were carried out in mock-treated extract (lanes 1–3), mock-treated, SR protein-supplemented extract (at 0.15 $\mu\text{g}/\mu\text{l}$) (lanes 4–6), U1₁₋₁₄ oligonucleotide-pretreated extract (lanes 7–9) or U1₁₋₁₄ oligonucleotide-pretreated, SR protein-supplemented (at 0.15 $\mu\text{g}/\mu\text{l}$) extract (lanes 10–12) for 5, 15, or 30 min. Complexes H, A, B, and C are labeled according to Konarska (1990); a distinct complex (*) appeared only in the U1₁₋₁₄ oligonucleotide-pretreated and SR protein-supplemented extract. The SR proteins used in this experiment were not pretreated with micrococcal nuclease because, for unknown reasons, splicing complexes (except complex C) change their mobility in native gels after exposure to EGTA-inactivated micrococcal nuclease (data not shown). Yet, untreated SR proteins behave identically to micrococcal nuclease-treated preparations in the reconstitution of splicing, as assayed by denaturing gels.

to enhance whatever the rate-limiting step prior to catalysis might be.

Discussion

In this paper we report several novel functions of SR proteins. First, a silent 5' splice site within the intron of the adenovirus pre-mRNA substrate, which is not usually thought of as an alternatively spliced RNA, can be activated by high concentrations of SR proteins. Second, excess SR proteins can compensate for at least some functions of the U1 snRNP. Third, SR proteins can activate yet another novel 5' splice site within the adenovirus intron when the 5' end of U1 snRNA is sequestered. These results suggest that SR proteins may contribute to more than the earliest steps of spliceosome assembly and give us new insights into the mechanism of 5' splice site selection and functions and evolution of the U1 snRNP.

How do SR proteins compensate for functions of the U1 snRNP?

U1 is the spliceosomal snRNP that was first hypothe-

sized and confirmed to be involved in the splicing of pre-mRNA (for review, see Sharp 1994). The critical role of the 5' end of U1 snRNA is recognition of the 5' splice site by Watson–Crick base-pairing, leading to commitment of a pre-mRNA to the splicing pathway (Michaud and Reed 1991; for review, see Rosbash and Seraphin 1991). Here, we have employed a 2'-O-methyl oligonucleotide U1_{1–14} to sequester elements near the 5' end of U1 snRNA. As described by Seiwert and Steitz (1993), in addition to the 5' splice site pairing function, some other function of U1 snRNA that is essential for complex A formation is blocked (Fig. 5). Even under these conditions (as well as when only the 5' end of U1 snRNA is removed), excess SR proteins can rescue splicing of pre-mRNA substrates (Figs. 2 and 3).

Several results argue that in our oligonucleotide-pretreated extracts, excess SR proteins are not acting simply by reversing the block or recruiting a tiny fraction of residual U1 snRNPs. (1) Psoralen cross-linking between U1 and the pre-mRNA is undetectable (Fig. 4). (2) Purified recombinant SC35, which does not contain detectable amounts of snRNPs, can rescue splicing (Fig. 3). (3) The rescued extracts exhibit an altered 5' splice site preference: In addition to the normal and the +125 sites, a novel 5' splice site (site 47), not used in the presence of unblocked U1 snRNPs, is activated dominantly (Figs. 2 and 3). (4) Crispino et al. (1994) have demonstrated that extracts depleted of U1 snRNPs by affinity selection using a tethered 2'-O-methyl oligonucleotide can likewise be rescued for splicing by SR proteins. Although we cannot rule out the possibility that excess SR proteins stimulate residual U1 snRNPs to act so quickly that their interactions cannot be captured by cross-linking, the normal kinetics of splicing (data not shown) and complex assembly (Fig. 5) and the increased cross-linking of U1 snRNA in unblocked extracts (Fig. 4) argue strongly against this explanation.

The recent finding that SR proteins can directly recognize 5' splice sites (Zuo and Manley 1994) is probably key to their ability to rescue splicing in the absence of active U1 snRNPs. (It should, however, be noted that site 47 is not as purine-rich as most 5' splice sites.) Whereas in normal extracts binding of the U1 snRNP is enhanced (Eperon et al. 1993), apparently other splicing factors can also be recruited to potential 5' splice sites in the pre-mRNA by SR proteins. The U6 snRNP, a splicing factor, which (like U1) can engage in 5' splice site pairing (Sawa and Abelson 1992; Wassarman and Steitz 1992; Kandels-Lewis and Seraphin 1993; Konforti et al. 1993; Lesser and Guthrie 1993), is a likely candidate (see below).

In addition to its role in recognizing the 5' splice site, the U1 snRNP assists formation of pre-splicing complex A by promoting binding of the U2 snRNP (Barabino et al. 1990), probably in collaboration with SR proteins (Fu and Maniatis 1992; W.-Y. Tarn and J. Steitz, unpubl.). Cloned SC35 behaved almost identically to mixed SR proteins in our reconstitution experiments, except that its efficiency in rescuing splicing was lower (Fig. 3). This protein has been proposed to bridge the U1 70K protein and U2AF via homodimeric or heterodimeric interactions

(Wu and Maniatis 1993). We cannot exclude the possibility that SC35 collaborates with other members of the SR protein family to achieve optimal communication between the 5' and 3' splice sites. Likewise, the remainder of the U1 snRNP particle may continue to participate in splicing in our U1-debilitated system. By immunoprecipitation with anti-(U1)70K antibodies (Kastner et al. 1992), we found that the 70K protein was still associated with the pre-mRNA and splicing intermediates in the U1 snRNA-blocked and SR protein-supplemented extract, whereas no precipitation of RNAs was observed without excess SR proteins (W.-Y. Tarn and J. Steitz, unpubl.). To determine whether free 70K protein exists in cells and extracts or the debilitated U1 snRNP provides this bridging function will require further investigation.

Potential role of U6 in 5' splice site selection

We argue above that SR proteins can bypass the normal role of the U1 snRNP by directly recruiting other splicing factors to potential 5' splice sites. What then might be responsible for selecting site 47, which is never seen to act as a 5' splice site in the presence of functional U1 snRNPs? The observation that lowering the amount of extract in the reaction decreases the efficiency of splicing to site 47 (Fig. 3) suggested that some extract factor(s), rather than only components of the SR protein preparation, participates. Potential base-pairing between U6 snRNA and the three 5' splice sites utilized in SR protein-supplemented and U1-blocked extracts is shown in Figure 6; interestingly, predicted pairing with site 47 is strongest. We therefore tested the hypothesis that U6 might play a determining role in selection of site 47 by mutating one nucleotide (position +52) of the intron to weaken the potential base-pairing interaction between U6 snRNA and site 47. The substrate was assayed in the U1_{1–14} oligonucleotide-pretreated extract following supplementation with excess amounts of SR proteins, and the efficiency of splicing to the mutated site 47 was reduced to 30% of the wild type (W.-Y. Tarn and J. Steitz, unpubl.). These results are consistent with our suspicion that U6 may be involved in 5' splice site choice in the absence of functional U1 snRNPs. Because compensatory mutations in the U6 snRNA are not yet available, we cannot exclude alternative possibilities.

Kandels-Lewis and Seraphin (1993) and Lesser and Guthrie (1993) suggested previously that U6 snRNA provides a "proofreading function," specifying the precise bond at the 5' splice site for nucleophilic attack. In their genetic suppression studies in yeast, the aberrant cleavage sites activated by U6 were adjacent to an authentic 5' splice site that had been mutated, implicating the participation of U1 as well. However, using a different approach, Konforti and co-workers have recently shown that an oligoribonucleotide mimicking the 5' splice site can associate with snRNP complexes containing U6 when the 5' end of U1 is blocked (Konforti et al. 1993; Konforti and Konarska 1994). Although the system they used represents only a partial reaction of splicing, their result constitutes the first evidence that U6 per se can

Figure 6. Alternative splicing of the adenovirus pre-mRNA substrate induced by SR proteins. (A) The precursor RNA derived from the adenovirus major late transcript contains 67 nucleotides of exon 1 (E1), 241 nucleotides of intron, and 99 nucleotides of exon 2 (E2). The 5' splice sites characterized here are +1 (the normal site), +47, and +125. (B) The spliced products and potential base-pairing of U1 and U6 to the various 5' splice sites are shown. Limiting amount of mixed SR proteins means 1- to 2-fold the endogenous level, whereas excess amounts means ~20-fold the endogenous level. The relative splicing efficiency of site 125 and site 47 was determined by quantitating the spliced products and normalizing for the length of each band. The usage of site 125 is slightly overestimated because the spliced product comigrates with a much lower amount (5–10%) of cognate lariat intermediate. Because the normal spliced product was barely detected in the U1-blocked extract complemented with 20-fold SR proteins, the relative splicing efficiency of the normal site vs. site 47 was determined by quantitating the excised exons 1 and normalizing for the length of each band. Values given are the average of three independent experiments.

recognize and bind a 5' splice site sequence. Here, we provide a novel approach to explore further the hypothesis that U6 can function dominantly in the determination of the 5' splice site during active splicing *in vitro*.

Finally, an alternative possibility, that the U5 snRNP is dominant in 5' splice site choice when functions of the U1 snRNP are blocked and splicing is activated by excess SR proteins, must be considered. Under normal circumstances, the U5 loop is believed to function by interacting with the terminus of the exon throughout the splicing reaction, rather than by defining the 5' splice site (Wyatt et al. 1992; Sontheimer and Steitz 1993; for review, see Madhani and Guthrie 1994). When a conserved intron sequence has been mutated, Watson-Crick interactions can apparently occur between the U5 loop and the mutated pre-mRNA, allowing splicing at cryptic sites (Newman and Norman 1992; Cortes et al. 1993). In our rescue experiments, the exon sequences at the novel sites (+47 and +125) exhibit weaker complementarity to the invariant sequence of the U5 loop than the normal site (+1). Therefore, the U5 snRNP seems unlikely to be a major determinant in selection of 5' splice sites in the

presence of debilitated U1 snRNPs and excess SR proteins.

The mechanism of 5' splice site selection

Two aspects of our results extend our understanding of how the splicing machinery discriminates among candidate 5' splice sites. Previously, it was demonstrated that SR proteins favor the utilization of the proximal site in pre-mRNAs that contain alternative 5' splice sites (for review, see Horowitz and Krainer 1993). Here, we have discovered that excess SR proteins can activate (Fig. 1) and recruit U1 snRNPs (Fig. 4) to a 5' splice site (site 125) that is normally silent within the intron of the adenovirus substrate. These observations suggest that as the concentration of SR proteins builds beyond its normal level, additional sites on a pre-mRNA can become occupied and the site that is most proximal is then selected for splicing. On the other hand, splicing does not necessarily follow U1 binding, as has been discussed (Reed and Maniatis 1986; Nelson and Green 1988; Eperon et al. 1993). Our psoralen cross-linking data confirm this no-

tion by showing that SR proteins enhance binding of U1 to the normal 5' splice site (Fig. 4), even though this site is utilized very inefficiently for splicing (Fig. 2A). A. Zahler and M. Roth (in prep.) have also observed by cross-linking that U1 snRNPs occupy sites that are not used. More investigation is needed to understand the interactions that effectively commit a 5' splice site to the splicing pathway.

Our other finding is the activation of a second silent 5' splice site in the presence of excess SR proteins and debilitated U1 snRNPs. Site 47 was never potentiated by SR proteins in the presence of functional U1 snRNPs; moreover, a cross-link between the 5' end of U1 and site 47 (even though it is pyrimidine-rich and should be an excellent cross-linker) was not observed under any conditions. These results argue that U1 plays an influential role in 5' splice site utilization even at high concentrations of SR proteins. On the other hand, the use of three 5' splice sites in the U1₁₋₁₄ oligonucleotide-pretreated extract indicates that SR proteins can circumvent the participation of U1 and may facilitate association of U6. Because increasing the concentration of SR proteins in blocked extracts increased the utilization of site 125 (the most proximal site) (Fig. 2D), we also conclude that SR proteins can promote proximal 5' splice site selection, regardless of whether U1 or (presumably) U6 is functioning as the initial base-pairing partner.

Functions and evolution of the U1 snRNA

Our observations add yet another entry to a growing list of conditions under which splicing has been observed to be "U1 independent." Previously, splicing of the SV40 large T intron and SL RNA-containing adenovirus constructs was shown to be less sensitive to RNase H-mediated destruction of the 5' end of U1 (Black and Steitz 1986; Black 1987; Bruzik and Steitz 1990), and injection of antibodies against the U1 snRNP into *Xenopus* oocytes did not prevent utilization of some 5' splice sites (Fradin et al. 1984). Also, Eperon et al. (1993) have argued that splicing can be independent of the 5' end of U1 when 5' splice sites are in close proximity. These initially puzzling findings have been clarified recently by accumulating evidence that U1 serves only an early function in the spliceosome and is dispensable for subsequent catalysis (S.-L. Yean and R.-J. Lin, pers. comm.; for review, see Newman 1994). Later, U6 appears to take over and proofread the 5' splice site following destabilization of U1 (Wassarman and Steitz 1992; Kandels-Lewis and Seraphin 1993; Konforti et al. 1993; Lesser and Guthrie 1993; Konforti and Konarska 1994). Our findings now suggest that U6 may cooperate with SR proteins to replace the early function of U1 in recognition of the 5' splice site. Why then does the splicing of nuclear pre-mRNAs require the U1 snRNP at all?

Spliceosomal snRNAs have been viewed as fragments of an RNA sequence that might originally have functioned as a single self-splicing molecule (for review, see Guthrie 1991; Sharp 1991) and then evolved through simpler multicomponent splicing systems. In this con-

text, progress in understanding the mechanisms of both group II splicing and *trans*-splicing should provide insights into the functions of the spliceosomal snRNAs, including U1, in *cis*-splicing. For the group II intron, whose excision is mechanistically similar to the splicing of nuclear pre-mRNA, no analog to the U1–5' splice site pairing has been identified; the conserved ϵ - ϵ' pairing in group II introns appears instead to mimic the 5' splice site–U6 interaction (for review, see Wise 1993; Newman 1994). Likewise, no U1 homolog has been identified in trypanosomatids, in which all mRNAs are believed to arise via *trans*-splicing (for review, see Nilsen 1992). The recent analysis of a new *trans*-spliceosomal U6 RNA gene (Xu et al. 1994) has revealed compensatory base changes within the conserved hexanucleotide sequence of U6 and the 5' splice sites of SL RNAs, suggesting that the 5' splice site–U6 snRNA interaction is universal among splicing systems. In contrast, functions attributed to U1 snRNA appear to be dispensable for group II intron removal and for *trans*-splicing.

Our results suggest that U1 snRNPs may function in the first step of a double proofreading scheme in the spliceosome. Although the role of U1 in 5' splice site recognition can be substituted by excess SR proteins and perhaps also by U6, inappropriate 5' splice sites are activated. Thus, it seems that the U1 snRNP is critical for faithful selection of the correct 5' splice site from many potential sites bound by SR proteins; extensive base-pairing of its 5' end with the true sequence is probably an important determinant (Fig. 6). After destabilization of U1 from the 5' splice site, U6 proofreads the cleavage site by limited base-pairing (Fig. 6) and allows conversion of the spliceosome to a catalytically active state. We conclude that sequential recognition of the 5' splice site by SR proteins, U1, and U6 is vital to the accuracy of splicing of complicated pre-mRNAs. Therefore, only in a system that has evolved the versatility to splice a multitude of structurally heterogeneous introns is U1 an essential splicing component.

Materials and methods

Oligonucleotides

Both deoxyoligonucleotides and 2'-O-methyl oligoribonucleotides were synthesized by John Flory at Yale University on an Applied Biosystems DNA synthesizer. The following 2'-O-methyl oligoribonucleotides were used for pretreatment of nuclear extracts: U1₁₋₁₄ (5'-UGCCAGGUAAGUAU-3'), complementary to nucleotides 1–14 of U1 snRNA (Seiwert and Steitz 1993); U2b, complementary to nucleotides 27–49 of U2 snRNA (Lamond et al. 1989); and U7₃₋₂₀, complementary to nucleotides 3–20 of U7 snRNA (Scharl and Steitz 1994). Deoxyoligonucleotides used for primer extension were AdEII (5'-GGAGGC-CGACGGGTTTCCGATCC-3'), complementary to nucleotides 45–67 of the 3' exon, AdIa (5'-CACCTCAAAGGCAT-CACCG-3'), complementary to nucleotides 91–110 of the intron, AdIb (5'-ATCATCAAGGAAACCCTGGA-3'), complementary to nucleotides 189–208 of the intron, and the antisense U1 oligonucleotide, U1₆₄₋₇₅. Antisense deoxyoligonucleotides used for RNase H cleavage were U1₆₄₋₇₅ and U2₇₉₋₉₈.

Splicing extracts and substrates

HeLa nuclear and S100 extracts were prepared according to Dignam et al. (1983) except that buffer D contained 50 mM KCl. Transcription reactions were carried out in 10- μ l reaction mixtures containing 1 μ g of linearized plasmid, 20 μ Ci of [α -³²P]UTP, 0.5 mM ATP and CTP, 0.1 mM GTP and UTP, 1 mM diguanosine triphosphate, 10 mM dithiothreitol, 40 mM Tris-HCl (pH 8.0), 6 mM MgCl₂, 2 mM spermidine, 4 units/ μ l of RNase inhibitor, and 4.5 U/ μ l of SP6 RNA polymerase (Pharmacia). For psoralen cross-linking experiments, the splicing substrate was labeled to a higher specific activity by doubling the amount of [α -³²P]UTP to 40 μ Ci and lowering the amount of cold UTP to 40 μ M. All transcripts were gel purified.

In vitro splicing assay and pretreatment of nuclear extracts with 2'-O-methyl oligoribonucleotides

Splicing reactions were carried out in 10 μ l containing 60% nuclear extract or S100, 0.5 mM ATP, 20 mM creatine phosphate, 2.4 mM (for nuclear extracts) or 2 mM (for S100 extracts) MgCl₂, 1 U/ μ l of RNasin and 1 $\times$ 10⁴ cpm of radiolabeled precursor RNA (~3 fmoles) for 90 min at 30°C. For 2'-O-methyl oligoribonucleotide-pretreated extracts, 0.1 mM cordycepin (3'-dATP) was included to prevent nonspecific polyadenylation of the precursor RNA. For SR protein-supplemented experiments, 2 μ l of SR proteins (0.15–1.5 μ g) with or without micrococcal nuclease treatment were added into a 10 μ l reaction mixture containing S100 extract or extract pretreated with a 2'-O-methyl oligoribonucleotide (see below). For Figure 3 only, splicing was performed in 25- μ l reaction mixtures containing 40% nuclear extract, 1 mM ATP, 20 mM creatine phosphate, 3 mM MgCl₂, 0.8 U/ μ l RNasin, 0.1 mM cordycepin, and 1 $\times$ 10⁴ cpm of pre-mRNA (~3 fmoles) for 2 hr at 30°C. RNA was isolated from the splicing reaction and fractionated on an 8% denaturing polyacrylamide gel. Spliced products were quantitated using a Molecular Dynamics PhosphorImager.

Pretreatment of the nuclear extract with 2'-O-methyl oligoribonucleotides was performed by incubating with 15 μ M U1_{1–14}, 4 μ M U2b, or 10 μ M U7_{3–20} under splicing conditions for 10 min before adding the splicing substrate and SR proteins. The concentration of the U1_{1–14} oligonucleotide used for inactivation of splicing is ~30-fold higher than that of the U1 snRNA.

Preparation of SR proteins

Mixed SR proteins were prepared from HeLa nuclei (Cell Culture Center) according to methods described by Krainer et al. (1990a) and Zahler et al. (1992), with modifications. Frozen HeLa nuclei (from a 20-liter culture) were thawed at room temperature, washed with buffer A (Dignam et al. 1983), and centrifuged at 14,000 rpm in an SS34 rotor for 20 min. The nuclei were resuspended in an equal volume of buffer C [20 mM HEPES (pH 7.9), 0.42 M NaCl, 25% (vol/vol) glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF] by 12 strokes of a tight pestle in a glass Dounce homogenizer. The nuclear suspension was then sonicated in four 10-sec bursts at 1-min intervals. The homogenate was nutated for 30 min at 4°C and then centrifuged at 16,500 rpm in an SS34 rotor for 30 min. The pellet was resuspended in an equal volume of buffer C, except that the salt was reduced to 0.21 M. The supernatants were pooled and dialyzed for 3 hr against buffer D omitting glycerol. The dialysate was centrifuged at 16,500 rpm for 30 min, and ammonium sulfate was added to the supernatant to 60% saturation. After 2 hr of stirring, the suspension was centrifuged at 17,000 rpm for 20 min. The supernatant was collected, and ammonium sulfate

was increased to 90% saturation, stirred for 2 hr and centrifuged as above. The 60–90% pellet was resuspended in 1.2 ml of SR dialysis buffer (Zahler et al. 1992), dialyzed against the same buffer for 4 hr, and centrifuged at 17,000 rpm for 20 min to remove insoluble materials. MgCl₂ was added into the dialysate to a final concentration of 20 mM. After 1 hr of incubation on ice, the suspension was centrifuged at 13,000g for 30 min in a microcentrifuge. The pellet containing SR proteins was resuspended in 160 μ l of buffer D containing 5% glycerol and dialyzed against the same buffer for 2 hr.

For complementation of the U1_{1–14} oligonucleotide-pretreated extracts, each microgram of SR proteins was pretreated with 4 units of micrococcal nuclease (Worthington Biochem.) in the presence of 1 mM CaCl₂ for 10 min at 30°C. Digestion was stopped by addition of 2 mM EGTA.

Psoralen cross-linking and RNase H digestion

Splicing reactions were performed as described above except that 1 $\times$ 10⁵ cpm of radiolabeled substrate with high specific activity was used. After incubation at 30°C for 10 min, the reaction mixtures were irradiated with 365-nm light for 10 min on ice in the presence of 20 μ g/ml of AMT (4'-aminomethyl-4,5',8-trimethyl) psoralen (HRI Associates). RNase H digestions of naked RNA were carried out in 10 μ l containing 40 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 1 μ g of oligonucleotide, 2 μ g of carrier RNA (yeast tRNA) and 2 units of RNase H (Boehringer-Mannheim) for 60 min at 37°C. Gel analysis of cross-linked products was as described by Wasserman and Steitz (1992); quantitation was performed on a Molecular Dynamics PhosphorImager.

Primer extension

Splicing reactions were performed as described above except that the volume of reaction was enlarged to 50–100 μ l and 0.5 ng/ μ l of unlabeled precursor RNA substrate was included. Bands were excised from the gel, eluted, and recovered by ethanol precipitation. Primer extension was performed as described by Parker and Steitz (1987), with minor modifications.

Native gels

Splicing reactions were carried out as described above and stopped after the indicated incubation time by addition of 1 mg/ml of heparin. Reactions were then incubated on ice for 10 min and loaded onto a 4% polyacrylamide gel (Konarska and Sharp 1987). Electrophoresis was performed at 300 V for 30 min and then at 250 V for 3 hr and 30 min.

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