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A short 5′ splice site RNA oligo can participate in both steps of splicing in mammalian extracts

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ABSTRACT

A short 5′ splice site RNA oligonucleotide (5′SS RNA oligo) undergoes both steps of splicing when a second RNA containing the 3′ splice site region (3′SS RNA) is added in *trans*. This *trans*-splicing reaction displays the same 5′ and 3′ splice site sequence requirements as *cis*-splicing of full-length pre-mRNA. The analysis of RNA–snRNP complexes formed on each of the two splice site RNAs is consistent with the formation of partial complexes, which then associate to form the complete spliceosome. Specifically, U2 snRNP bound to the 3′SS RNA associates with U4/U5/U6 snRNP bound to the 5′SS RNA oligo. Thus, as expected, *trans*-splicing depends on the integrity of U2, U4, and U6 snRNAs. However, unlike *cis*-splicing, *trans*-splicing is enhanced when the 5′ end of U1 snRNA is blocked or removed or when the U1 snRNP is depleted. Thus, the early regulatory requirement for U1 snRNP, which is essential in *cis*-splicing, is bypassed in this *trans*-splicing system. This simplified *trans*-splicing reaction offers a unique model system in which to study the mechanistic details of pre-mRNA splicing.

Keywords: *trans*-splicing, spliceosome assembly, 5′ splice site, U1 snRNP-independent splicing

INTRODUCTION

Splicing of intervening sequences (introns) from mRNA precursors (pre-mRNAs) proceeds via a two-step transesterification reaction (for reviews see Moore et al., 1993; Madhani & Guthrie, 1994). The first step involves nucleophilic attack by the 2′ hydroxyl of the branch site adenosine residue at the 5′ splice site (5′SS). The resulting intermediates consist of a free 5′ exon and a lariat form of the intron–3′ exon. The second step involves attack by the 3′ hydroxyl of the 5′ exon at the 3′ splice site, joining the exons and releasing the lariat intron. Recognition of the correct 5′ and 3′ splice sites is necessary for accurate excision of introns from pre-mRNAs. The 5′SS consensus sequence in mammals consists of the sequence AG/GURAGU, where R = purine, Y = pyrimidine, N = any nucleotide, and / indicates a splice site. In contrast, the 3′SS region is more complex and consists of the branch site (YNYUR*A*C, where *A* is the site of branch formation), the polypyrimidine tract, and the 3′SS consensus (YAG/G).

Recognition of the splice sites takes place during assembly of the spliceosome, a multicomponent ribonucleoprotein complex containing snRNP particles (U1, U2, U4, U5, and U6 snRNPs), and a number of non-snRNP protein factors. Spliceosome formation proceeds through a stepwise assembly process. Initially, the pre-mRNA is committed to the splicing pathway by formation of an early, ATP-independent commitment complex containing U1 snRNP interacting with the 5′SS, as well as non-snRNP proteins (Legrain et al., 1988; Ruby & Abelson, 1988; Seraphin & Rosbash, 1989; Fu & Maniatis, 1992; Fu, 1993; Michaud & Reed, 1993; Wu & Maniatis, 1993; Amrein et al., 1994; Staknis & Reed, 1994). Early recognition of the 5′SS is mediated through base pairing between the 5′ end of U1 snRNA and the 5′SS. Subsequently, U2 snRNP binds to the branch site region, forming splicing complex A. The branch site region is recognized, in part, by base pairing to a sequence within U2 snRNA (5′GUAGUA3′), producing a short duplex UACUAAC:GUAGUA helix containing the branch nucleotide (Parker et al., 1987; Wu & Manley, 1989; Zhuang & Weiner, 1989; Query et al., 1994). Splicing complex A is converted to B when the U4/U5/U6 triple snRNP joins the complex. Splicing complex B formation is mediated, in part, by a base pairing interaction between the 3′ end of U6 snRNA and the 5′ end of U2 snRNA (Datta & Weiner, 1991; Wu

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& Manley, 1991) and does not appear to require base pairing between the 5′SS and U1 snRNA (Konforti et al., 1993; Crispino et al., 1994; Tarn & Steitz, 1994). Once the base pairing interaction between U1 and the 5′SS is destabilized, a new interaction between the AC of the highly conserved *A*CAGAG sequence of U6 snRNA and intron positions 6 and 5 of the 5′SS is established (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993). This rearrangement at the 5′SS almost certainly occurs before the first step of splicing (Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Wassarman & Steitz, 1993). Moreover, genetic and biochemical experiments indicate that a conserved loop of U5 snRNA interacts with exon sequences before and during both steps of splicing (Newman & Norman, 1991, 1992; Wyatt et al., 1992; Sontheimer & Steitz, 1993). The actual splicing reaction is correlated with formation of splicing complex C, which is accompanied by the apparent destabilization of U4 snRNP from the spliceosome. At this stage, the extensive U4–U6 pairing is disrupted and replaced by two (Madhani & Guthrie, 1992) or three (Sun & Manley, 1995) new intermolecular helices formed between U6 and U2 snRNAs as well as an intramolecular U6 helix. These new RNA–RNA interactions along with the U6–5′SS interaction provide a way to bring together the nucleophile of the branch site adenosine with its target phosphate at the 5′SS.

The mechanistic similarities between nuclear pre-mRNA splicing and group II intron self-splicing have led to the proposal that the snRNA components of the spliceosome actually catalyze pre-mRNA splicing (Sharp, 1985; Cech, 1986). Thus, much attention has been focused on elucidating the RNA–RNA interactions within the spliceosome. According to this model, the multistep spliceosome assembly pathway functions to build the active catalytic center composed of snRNA and pre-mRNA sequences. Although the RNA–RNA interactions are certainly important for substrate recognition and bringing the two reaction partners into proper alignment, the role of protein factors in spliceosome assembly and perhaps even catalysis, is less clear. The complexity of these RNA–RNA interactions and the dynamic nature of the spliceosome make it difficult, if not impossible, to distinguish the steps required for fidelity or regulation of splicing from those required for catalysis.

To study the mechanism of catalysis, we have developed a functional *trans*-splicing assay in mammalian nuclear extracts using a short 5′SS RNA oligonucleotide comprising the 5′SS consensus sequence (5′SS RNA oligo). We previously showed that an even shorter version of this 5′SS RNA oligo binds U1 snRNP, but more remarkably, is sufficient to induce the specific association of U2 and U4/U5/U6 snRNPs into U2/U4/U5/U6 snRNP complex, the snRNP composition of which is identical to that of splicing complex B (Hall & Konarska, 1992). Because stable 5′SS RNA–U1 snRNA base pairing prevents interaction of the 5′SS RNA with U2/U4/U5/U6 snRNP complex, we concluded that disruption of the initial base pairing between the 5′SS RNA and the 5′ end of U1 snRNA is required for subsequent spliceosome assembly (Konforti et al., 1993). Thus, in this in vitro system, recognition of the 5′SS by the 5′ end of U1 snRNA is uncoupled from recognition by U2/U4/U5/U6 snRNP complex. Moreover, we showed that the 5′SS RNA binds to U4/U5/U6 snRNP in the absence of U2 snRNP and that the specificity of 5′SS recognition by U4/U5/U6 snRNP correlates with the 5′SS consensus sequence (Konforti & Konarska, 1994). Finally, 5′SS RNA bound to U4/U5/U6 snRNP could be chased to 5′SS RNA–U2/U4/U5/U6 snRNP complex upon addition of U2 snRNP (Konforti & Konarska, 1994). Until now, these studies have focused on 5′ splice site recognition and RNA–snRNP interactions, but have been limited by the lack of a functional assay. We show here that a short 5′SS RNA oligo undergoes both steps of splicing when a second RNA containing a branch site with an adjacent polypyrimidine tract and a 3′SS (3′SS RNA) is added in *trans*. The analysis of the RNA–snRNP complexes formed on each of the two splice site RNAs and our previous binding studies are consistent with the following scenario. When the base pairing interaction between the 5′ end of U1 snRNA and the 5′SS RNA oligo is prevented, the 5′SS RNA oligo binds to U4/U5/U6 snRNP. This partial splicing complex then interacts with U2 snRNP. If the U2 snRNP is associated with the 3′SS RNA, this interaction will lead to the formation of a functional spliceosome within which the reaction partners for the two transesterification reactions are brought together.

RESULTS

A short 5′SS RNA oligo undergoes both steps of splicing

We have developed a functional *trans*-splicing assay using HeLa cell nuclear extracts in which a short 5′SS RNA oligo is used as a substrate. In most of the experiments, the 5′SS RNA contains 6 nt of exon and 11 nt of intron sequence (5′A_5G/GUAAGUAdT$dc_3$3′, where / represents the exon/intron junction). This 5′SS RNA oligo undergoes both the first and second steps of splicing when a second RNA (3′SS RNA) derived from the adenovirus major late transcription unit containing a branch site with an adjacent polypyrimidine tract and a 3′SS is added in *trans*. Typically, the 3′SS RNA was preincubated under standard splicing conditions to allow complex A formation and in the presence of 5′SS DNA oligo to block the 5′ end of U1 snRNA (see below). After the addition of the 5′SS RNA oligo, the incubation was continued for 2 h and the products were

resolved in a denaturing gel. In these experiments, the 5′SS RNA oligo was labeled by the addition of three ^{32}P-dC residues to the 3′ end (i.e., the intron was labeled) and the 3′SS RNA was unlabeled. Thus, only the branched intron intermediate and branched intron product resulting from the first and second steps of splicing, respectively, are expected to be observed. In the presence of 3′SS RNA, two labeled RNA products corresponding to the branched intermediate and the branched intron product were observed (Fig. 1A, lanes 3, 4). A time course of the *trans*-splicing reaction (Fig. 1B, lanes 1–7) shows that branched intermediates begin to appear after 30 min and accumulate with time, reaching saturation after 2 h (data not shown). Formation of these products is temperature- and ATP-dependent (Fig. 1A, lanes 5–8). When the 5′ and 3′ splice site RNAs were incubated together from the start of the reaction at 30 °C, the splicing efficiency was reduced as compared to reactions in which one of the two splice site RNAs was preincubated (Fig. 1A, compare lane 11 with lanes 9, 10). Because the splicing efficiency is optimal when the 3′SS RNA is preincubated (Fig. 1A, lane 10), most subsequent experiments include this preincubation step. At saturating concentrations of the 3′SS RNA, the amount of branched intermediate formed corresponds to a splicing efficiency of 2–10% with regard to the input 5′SS RNA oligo (data not shown).

Interestingly, in the absence of the 3′SS RNA, a specific labeled RNA product, X, is observed (Fig. 1A, lanes 1, 2). RNase H mapping of this product demonstrates that it contains U6 snRNA sequence. Characterization of this reaction suggests that it does not represent aberrant versions of either the first or second steps of splicing. Instead, the formation of X depends on the presence of a hydroxyl group at the 5′ end of the 5′SS RNA oligo and a 2′,3′-cyclic phosphate at the 3′ end of U6 snRNA. Thus, X most likely represents the addition of the 5′SS RNA oligo to the 3′ end of U6 snRNA by an RNA ligase activity in the extract (unpubl. results) and further analysis of this reaction is ongoing. This RNA ligase activity could also explain the formation of a number of labeled RNAs that occur under suboptimal splicing conditions, for example, incubation on ice (Fig. 1A, lanes 5, 6), and pretreatment with RNase H (Fig. 5A). These labeled RNAs are distinct from those generated at 30 °C and their pattern depends on the sequence of the 3′SS RNA added (data not shown). Thus, the labeled RNAs generated under suboptimal splicing conditions could represent the ligation of labeled 5′SS RNA oligo to degradation products of the 3′SS RNA substrate.

Because the 5′SS RNA oligo is complementary to the 5′ end of U1 snRNA, it readily binds to U1 snRNP in nuclear extracts. When the 5′SS RNA oligo is base paired to the 5′ end of U1 snRNA, it is unable to induce the formation of U2/U4/U5/U6 snRNP complex (Konforti et al., 1993). To determine the effect of this base pairing interaction on *trans*-splicing, the 5′ end of U1 snRNA was blocked by the binding of 5′SS DNA oligo or 2′-*O*-methyl oligo ($U1_{1\text{-}10}$), which are identical in sequence to the 5′SS RNA oligo. When the 5′SS RNA

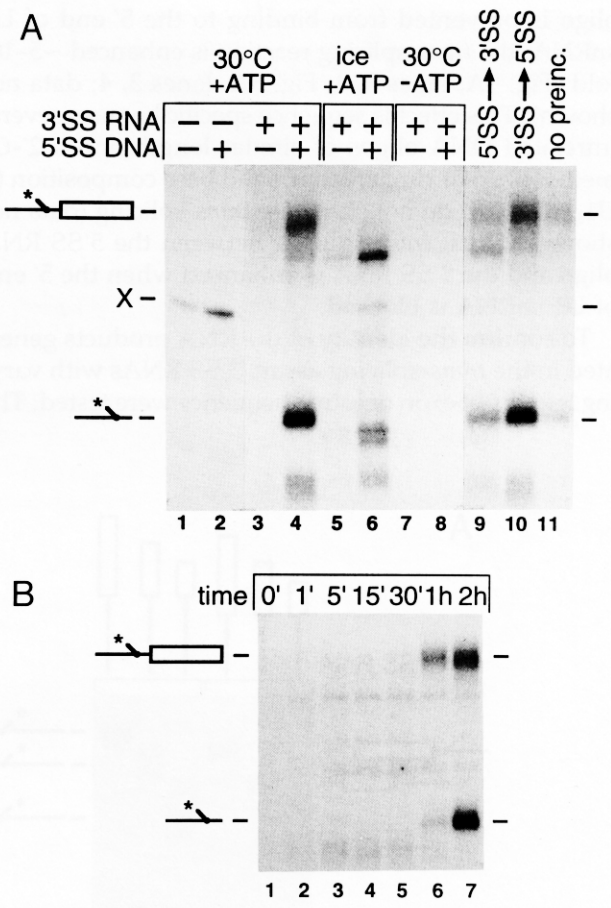

FIGURE 1. A short 5′SS RNA oligo undergoes the first and second steps of splicing when a 3′SS RNA is added in *trans*. Splicing reactions between 3′ end-labeled (*) 5′SS RNA oligo and unlabeled 3′SS RNA containing a branch site, polypyrimidine tract, 3′SS, and exon sequences. **A:** 3′SS RNA (83 nt of intron and 45 nt of exon sequence) was preincubated for 30 min at 30 °C (lanes 3, 4) or at 4 °C (lanes 5, 6) in the absence (lanes 3, 5, 7) or presence (lanes 4, 6, 8) of 5′SS DNA oligo and in the presence (lanes 1–6) or absence (lanes 7, 8) of ATP, followed by the addition of 3′ end-labeled 5′SS RNA oligo (A_5G/GUAAGUAdT*dC*dC*dC) and the incubation was continued for 2 h. Reactions lacking 3′SS RNA were conducted in the absence (lane 1) or presence (lane 2) of 5′SS DNA oligo. 3′ end-labeled 5′SS RNA oligo (lane 9) or unlabeled 3′SS RNA (lane 10) was preincubated for 30 min at 30 °C in the presence of 5′SS DNA oligo followed by the addition of unlabeled 3′SS RNA or 3′ end-labeled 5′SS RNA oligo, respectively, and the incubation was continued for 2 h. 3′SS RNA and 5′SS RNA oligo were added together at the start of the reaction (lane 11). **B:** A time course of the *trans*-splicing reaction. Unlabeled 3′SS RNA was preincubated for 30 min at 30 °C in the presence of 5′SS DNA oligo followed by the addition of 3′ end-labeled 5′SS RNA oligo and the incubation was continued for 0, 1, 15, 30, 60, and 120 min (lanes 1–7). RNA products were resolved in a 10% polyacrylamide/8 M urea gel. Because the 5′SS RNA oligo was 3′ end labeled, only the intron-containing products were detected. Positions of the branched intron–exon intermediate, branched intron product, and X are indicated.

oligo is prevented from binding to the 5′ end of U1 snRNA, the *trans*-splicing reaction is enhanced ~5–10-fold (Fig. 1A, lanes 3, 4; Fig. 5A, lanes 3, 4; data not shown). This effect is sequence specific because several unrelated DNA oligos of similar length and a 2′-*O*-methyl oligo of similar length and base composition to $U1_{1\text{-}10}$, EBER, do not stimulate *trans*-splicing (data not shown). Thus, *trans*-splicing between the 5′SS RNA oligo and the 3′SS RNA is enhanced when the 5′ end of U1 snRNA is blocked.

To confirm the identity of the RNA products generated in the *trans*-splicing assay, 3′SS RNAs with varying lengths of exon or intron sequence were tested. The mobility of the branched intron intermediate generated from the first step of splicing should depend on the length of both the exon and intron segments of the 3′SS RNA. In contrast, the mobility of the branched intron product resulting from the second step of splicing should depend only on the length of the intron sequence of the 3′SS RNA. As expected, the mobility of the branched intron intermediate decreased as the length of the exon increased (Fig. 2A, lanes 1–3 and 4–6). Although the mobility of the branched intron product was independent of the length of the exon (Fig. 2A, lanes 1–3 or 4–6), it decreased when the length of the intron was increased (Fig. 2A, lanes 1–3

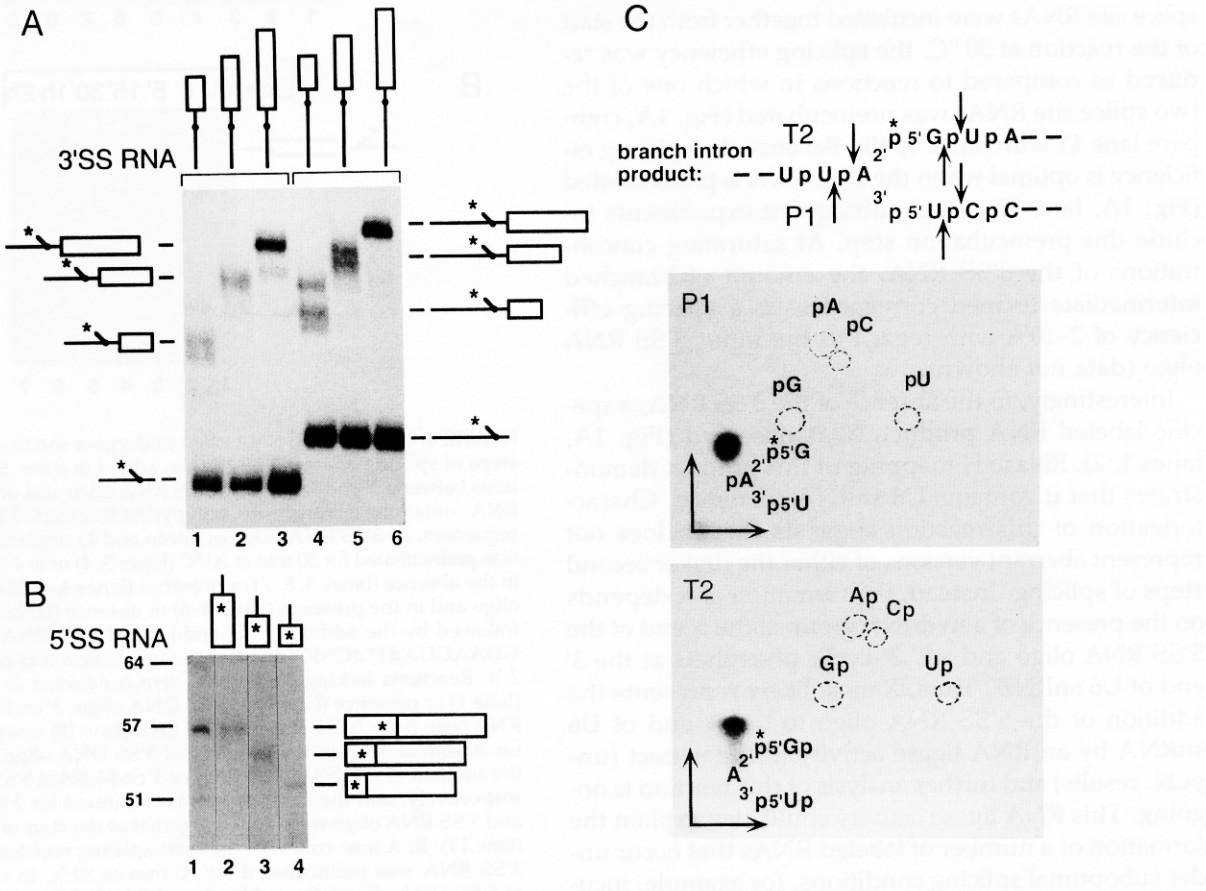

FIGURE 2. Analysis of the intermediates and products of the first and second steps of *trans*-splicing. **A:** Unlabeled 3′SS RNAs with variable intron and exon lengths were preincubated for 30 min at 30 °C in the presence of 5′SS DNA oligo followed by the addition of 3′ end-labeled (*) 5′SS RNA oligo (A_5G/GUAAGUAdT*dC*dC*dC) and the incubation was continued for 2 h. The intron length of the 3′SS RNA was 83 nt (lanes 1–3) or 90 nt (lanes 4–6), and the exon lengths were 35 (lanes 1, 4), 45 (lanes 2, 5), 62 nt (lanes 3, 6). Positions of branched intron–exon intermediates and branched intron products are indicated. **B:** Unlabeled 3′SS RNA (83 nt of intron and 45 nt of exon) was preincubated for 30 min at 30 °C in the presence of 5′SS DNA oligo followed by the addition of internally exon labeled (*) 5′SS RNA oligos of constant intron length (11 nt) and variable exon length (12, 9, and 7 nt, lanes 2–4, respectively) and the incubation was continued for 2 h. Because the exon portion of the 5′SS RNA oligo was labeled, only the ligated exons were detected. Positions of the ligated exon products, 57, 54, and 52 nt in length, and size markers are indicated. Molecular weight markers are shown in lane 1. **C:** The branch nucleotide was specifically labeled in reactions between 5′SS RNA oligo, which was internally ^{32}P-labeled (*) at the phosphate group of the exon/intron junction (5′A_6G/*GUAAGUAdT$dc_3$3′). The branched intron intermediates generated in the presence of unlabeled 3′SS RNA were isolated from a 10% polyacrylamide/8 M urea gel and digested with nuclease P1 or RNase T2. Products were resolved by two-dimensional TLC on cellulose plates in isobutyric acid/concentrated NH_4OH:H_20; 577:38:385 in the first dimension and t-butanol/concentrated HCl/H_20; 14:3:3 in the second dimension (Konarska et al., 1985a). Positions of nucleoside 5′ or 3′ monophosphate markers and the branched trinucleotide are indicated.

and 4–6). To observe the other product of the second step of splicing, the ligated exons, the exon portion of the 5′SS RNA oligo was specifically labeled. In *trans*-splicing reactions between exon labeled 5′SS RNA oligo and unlabeled 3′SS RNA, the size of the ligated exons should depend on the length of the exon segment of both the 5′SS and 3′SS RNAs. In fact, as the length of the exon of the 5′SS RNA oligo decreased, the size of the ligated exons decreased (Fig. 2B, lanes 2–4). Likewise, as the length of the exon of the 3′SS RNA increased the size of the ligated exons increased (data not shown). Thus, *trans*-splicing between labeled 5′SS RNA oligo and unlabeled 3′SS RNA generates the branched intron intermediates and products as well as the spliced exons, indicative of both steps of splicing.

The presence of the branch within the intron of the 3′SS RNA was confirmed by direct nucleotide analysis. In the expected branch structure, the phosphate group of the 2′–5′ phosphodiester bond originates from the 5′SS exon/intron junction (Padgett et al., 1984; Ruskin et al., 1984; Konarska et al., 1985a). To specifically label the branch nucleotide in the *trans*-splicing assay, $A_3^{2'p5'G}{}_{p5'U}$, the 5′SS RNA oligo was internally ^{32}P-labeled at the phosphate group of the exon/intron junction (5′A_6G/*GUAAGUAdTdc$_3$3′, where * indicates ^{32}P). This labeled 5′SS RNA oligo formed branched intron intermediates and products in the presence of unlabeled 3′SS RNA that were identical to those generated using 3′ end-labeled 5′SS RNA oligo (data not shown). Thus, branched intron intermediates and products are observed when either the 5′ or 3′ end of the intron segment of the 5′SS RNA oligo is labeled. These findings are consistent with specific cleavage at the exon/intron border in the *trans*-splicing assay. To directly test this prediction, branched intron products generated in reactions between 5′SS RNA oligo labeled at the phosphate group of the exon/intron junction and unlabeled 3′SS RNA were isolated from a denaturing gel and digested with nuclease P1 or RNase T2 and the products were separated by two-dimensional TLC. The branch trinucleotide is expected to be nuclease resistant (Padgett et al., 1984; Ruskin et al., 1984; Konarska et al., 1985a) and in both cases, the expected labeled trinucleotide was observed (Fig. 2C). Thus, like *cis*-splicing of full-length pre-mRNA, the first step of *trans*-splicing appears to involve nucleophilic attack by the 2′-OH group of the branch site adenosine on the phosphodiester bond at the exon/intron border of the 5′SS RNA oligo.

Recognition of the 5′ and 3′ splice sites is sequence specific

The GU of the 5′ splice site (intron positions 1 and 2) is the most highly conserved element within the 5′SS consensus sequence (Senapathy et al., 1990) and single point mutations at either of these two positions were found to be important in recognition of the 5′SS by U4/U5/U6 snRNP using the binding assay (Konforti & Konarska, 1994). Although the GU of the 5′SS is required for the formation of spliced products, it is not critical for the first step of splicing. Instead, *cis*-splicing of full-length pre-mRNAs with G1 → A or U2 → A mutations forms lariat intermediates at reduced levels, but effects a total block at the second step (Aebi et al., 1987; Lamond et al., 1987; Siliciano & Guthrie, 1988). To determine the 5′SS sequence specificity of the *trans*-splicing reaction, 5′SS RNA oligos with single point mutations at intron positions 1, 2, or 4 were examined. The G1 → A 5′SS RNA oligo formed branched intron intermediate with an efficiency comparable to that of wild-type 5′SS RNA oligo (Fig. 3, lane 4), whereas the U2 → A 5′SS RNA oligo formed branched intron intermediate with ~5% the efficiency of wild-type 5′SS

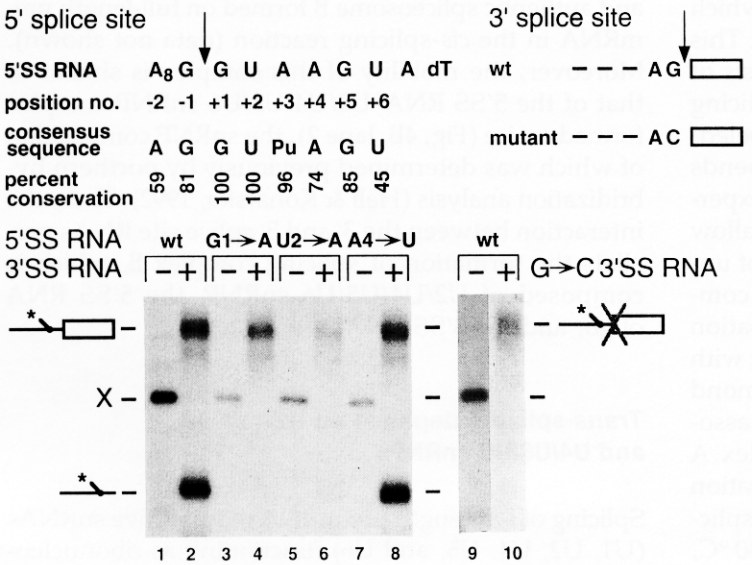

FIGURE 3. Recognition of the 5′ and 3′ splice sites is sequence specific. The 5′SS RNA oligo is shown. The vertical arrow represents the cleavage site and the numbers below the 5′SS RNA oligo sequence indicate exon (−) and intron (+) positions relative to the cleavage site. The consensus sequence is shown and the numbers below indicate the frequency of splice sites in primates that match the consensus base at positions −2 to +6 (Senapathy et al., 1990). Wild-type (lanes 1, 2) and mutant (lanes 3–8) 5′SS oligos with a single point mutation at position +1, +2, or +4 were tested in the *trans*-splicing assay in the absence or presence of wild-type 3′SS RNA. 3′SS RNA with a single point mutation in the last nucleotide of the intron (G → C) was tested in the *trans*-splicing assay (lanes 9, 10). In all reactions, wild-type or mutant 3′SS RNAs were preincubated for 30 min at 30 °C in the presence of 5′SS DNA oligo, followed by the addition of 3′ end-labeled 5′SS RNA oligo, and the incubation was continued for 2 h. RNA products were resolved in a 10% polyacrylamide/8 M urea gel. Positions of the branched intron–exon intermediate, branched intron product, and X are indicated.

RNA oligo (Fig. 3, lane 6). Both mutations dramatically reduced (~1% of wild-type 5′SS RNA oligo) the second step of splicing. In contrast, 5′SS RNA oligo with a mutation at intron position 4 (A4 → U), had little or no effect on the efficiency of the first or second steps of splicing (Fig. 3, lane 8). This position is an A in 74% of introns (Senapathy et al., 1990) and is not specifically recognized by U4/U5/U6 snRNP in the binding assay (Konforti & Konarska, 1994). Thus, the sequence specificity of the 5′SS in the *trans*-splicing reaction is comparable to that observed in *cis*-splicing of full-length pre-mRNA.

In the context of full-length pre-mRNAs, mutations of the A or G (the second last and last nucleotides of the intron) of the 3′SS severely inhibited the first step and completely blocked the second step of *cis*-splicing (Aebi et al., 1987; Lamond et al., 1987; Siliciano & Guthrie, 1988). To determine the 3′SS sequence specificity of the *trans*-splicing reaction, 3′SS RNA with a single point mutation at the last G of the intron was examined. As in full-length pre-mRNA *cis*-splicing, an AG → AC mutation at the 3′SS resulted in a ~fivefold reduction in the formation of the branched intron intermediate and a complete block (<1%) in the second step of splicing in comparison to that of wild-type 3′SS RNA (Fig. 3, lane 10). Together these data demonstrate that *trans*-splicing of a short 5′SS RNA oligo to a 3′SS RNA displays the same 5′ and 3′ splice site sequence specificity as *cis*-splicing of full-length pre-mRNA.

Interaction between the 5′ and 3′ splice sites

Although *cis*-splicing of full-length pre-mRNA involves the stepwise assembly of splicing complexes, which brings the 5′ and 3′ splice sites into juxtaposition, the details of the specific RNA–snRNP interactions that occur at each of the two splice sites can be more readily studied using the *trans*-splicing assay. The experiments that follow are consistent with the formation of partial splicing complexes on the 5′SS and 3′ SS RNAs, which then associate to form the complete spliceosome. This process was assayed by native gel electrophoresis of the RNA–snRNP complexes formed in *trans*-splicing reactions in which one of the two RNAs was labeled.

The conversion of splicing complex A to B depends on the presence of the 5′SS RNA oligo. In these experiments, labeled 3′SS RNA was preincubated to allow complex A formation, followed by the addition of unlabeled 5′SS RNA oligo, and the RNA–snRNP complexes that formed after various times of incubation were resolved in a nondenaturing gel. Consistent with previous studies (Konarska & Sharp, 1986; Lamond et al., 1987), the 3′SS RNA efficiently and stably associated with U2 snRNP to form splicing complex A (Fig. 4A, lane 2), which was dependent on incubation at 30 °C (Fig. 4A, lane 1). Complex B formed in splicing reactions that were incubated for 10 min at 30 °C, followed by the addition of 5′SS RNA oligo and subsequent incubation on ice (Fig. 4A, lane 4). Neither the 5′SS DNA oligo, which is identical in sequence to the 5′SS RNA oligo, the U1 RNA oligo, which is identical in sequence to the 5′ end of U1 snRNA, nor the RNA oligo $A_{12\text{-}18}$ induced complex B formation (Fig. 4A, lanes 5–7). Moreover, complex B formation did not require base pairing between the 5′SS RNA oligo and U1 snRNA because DNA oligo-directed RNase H cleavage of the 5′ end of U1 snRNA did not inhibit this process (Fig. 4A, lane 10). Together, these data suggest that U2 snRNP, which is stably associated with the 3′SS RNA, interacts with U4/U5/U6 snRNP, which is bound to the 5′SS RNA, at least transiently (see below), to generate complex B.

Binding of the 5′SS RNA oligo to U2/U4/U5/U6 snRNP complex, the snRNP composition of which is identical to splicing complex B, is enhanced in the presence of the 3′SS RNA. When labeled 5′SS RNA oligo was incubated at 30 °C in the absence of the 3′SS RNA, binding of the 5′SS RNA oligo to U4/U5/U6 and U2/U4/U5/U6 snRNP was observed (Fig. 4B, lane 3). Binding of the labeled 5′SS RNA oligo to U2/U4/U5/U6 snRNP complex was greatly enhanced in the presence of 3′SS RNA (Fig. 4B, lane 4). These RNA–snRNP complexes form rapidly (<30 s) and are stable on ice (Konforti & Konarska, 1994), but are less stable at 30 °C, under chase conditions (data not shown). Thus, it is likely that the 3′SS RNA stabilizes the interaction between the 5′SS RNA oligo and U2/U4/U5/U6 snRNP complex. Enhanced binding of the 5′SS RNA oligo to U2/U4/U5/U6 snRNP complex in the presence of the 3′SS RNA was also observed in splicing reactions in which unlabeled 3′SS RNA was preincubated for 10 min at 30 °C followed by the addition of labeled 5′SS RNA oligo and continued incubation (Fig. 4B, lane 5). The identity of this complex as U2/U4/U5/U6 snRNP is based, in part, on its comigration with splicing complex B formed in the *trans*-splicing assay (Fig. 4B, lane 7) and authentic spliceosome B formed on full-length pre-mRNA in the *cis*-splicing reaction (data not shown). Moreover, the mobility of this complex is similar to that of the 5′SS RNA–U2/U4/U5/U6 snRNP complex formed on ice (Fig. 4B, lane 2), the snRNP composition of which was determined previously by northern hybridization analysis (Hall & Konarska, 1992). Thus, the interaction between the 3′ and 5′ splice site RNAs parallels the formation of splicing complex B, which is composed of U2/U4/U5/U6 snRNP, the 5′SS RNA oligo, and the 3′SS RNA.

Trans-splicing depends on U2 and U4/U5/U6 snRNPs

Splicing of full-length pre-mRNA requires five snRNAs (U1, U2, U4, U5, and U6) functioning as ribonucleo-

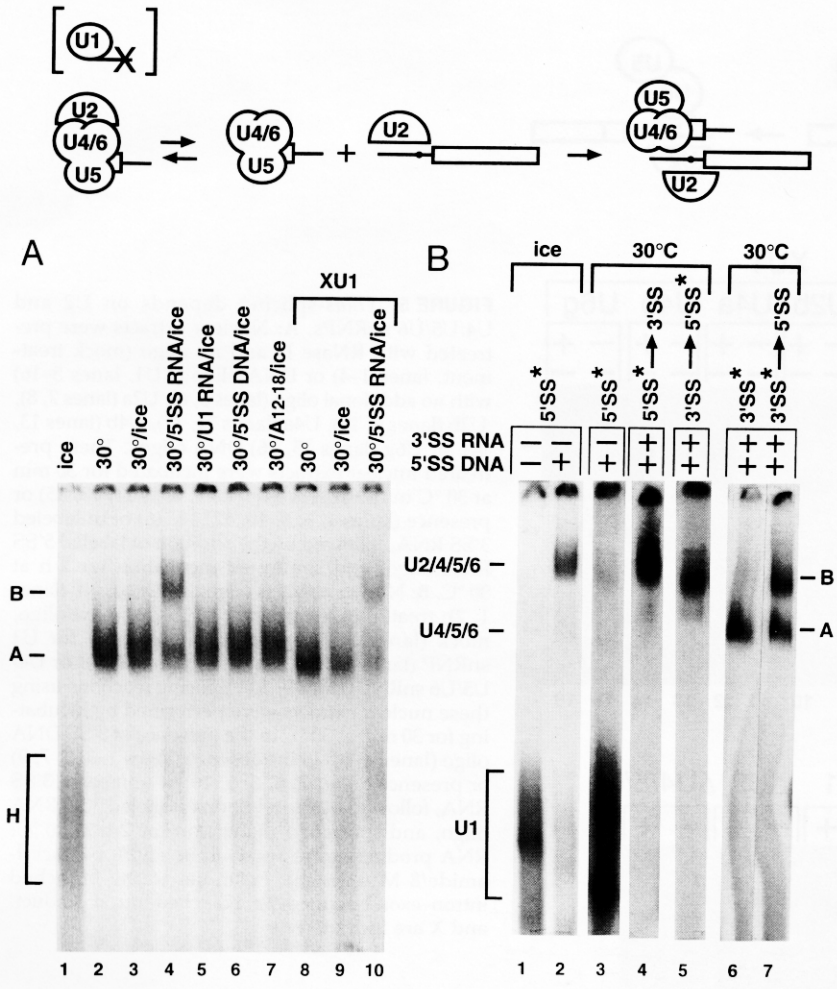

FIGURE 4. Formation of partial splicing complexes on 5′ and 3′SS RNAs. **A:** Splicing reactions containing labeled 3′SS RNA were incubated for 30 min at 4 °C (lane 1), 30 min at 30 °C (lane 2), or 10 min at 30 °C followed by a 15-min incubation on ice in the absence (lane 3) or presence of 5′SS RNA oligo, U1 RNA oligo, 5′SS DNA oligo, or oligo $A_{12\text{-}18}$ (0.02 μg/μL, lanes 4–7). Nuclear extracts used in lanes 8–10 were subjected to oligo-directed RNase H cleavage of U1 snRNA. Reactions were incubated for 30 min at 30 °C (lane 8), or 10 min at 30 °C followed by a 15-min incubation on ice in the absence (lane 9) or presence (lane 10) of 5′SS RNA oligo. **B:** Splicing reactions containing 3′ end-labeled 5′SS RNA oligo were incubated for 5 min at 4 °C in the absence (lane 1) or presence (lane 2) of 5′SS DNA oligo, 5 min at 30 °C in the presence of 5′SS DNA oligo (lane 3), or 5 min at 30 °C in the presence of 5′SS DNA oligo, followed by the addition of unlabeled 3′SS RNA, and the incubation was continued at 30 °C 15 min (lane 4). In lane 5, the unlabeled 3′SS RNA was incubated for 15 min at 30 °C in the presence of 5′SS DNA oligo, followed by the addition of labeled 5′SS RNA oligo, and the incubation was continued at 30 °C for 15 min. Splicing reactions containing labeled 3′SS RNA were incubated for 15 min at 30 °C in the presence of 5′SS DNA oligo followed by a 15-min incubation at 30 °C in the absence (lane 6) or presence of 5′SS RNA oligo (lane 7). Three-microliter aliquots were resolved by electrophoresis in a 4% nondenaturing polyacrylamide gel. Positions of splicing complexes A, B, and H (heterogeneous) and 5′SS RNA oligo-U1, -U4/U5/U6, and -U2/U4/U5/U6 snRNP complexes are indicated.

proteins. To determine which snRNPs are required for splicing in *trans*, nuclear extracts in which specific regions of U1, U2, U4, or U6 snRNAs were removed by DNA oligo-directed RNase H cleavage (Kramer et al., 1984; Black et al., 1985) were tested. The effectiveness of this treatment was monitored by denaturing gel electrophoresis followed by northern hybridization analysis (data not shown). Removal of the 5′ end of U1 snRNA (nt 1–11) enhanced the efficiency of *trans*-splicing ~10-fold (Fig. 5A, lane 6) in comparison to the mock reaction (Fig. 5A, lane 4). This effect is comparable to the enhancement of *trans*-splicing observed in the presence of 5′SS DNA or 2′-*O*-methyl oligos, which are complementary to the 5′ end of U1 snRNA, and thus, prevent base pairing of the 5′SS RNA oligo (Fig. 1A, lanes 3, 4; Fig. 5A, lanes 3, 4; data not shown). Although *trans*-splicing was inhibited when specific segments of U2, U4, or U6 snRNAs were individually removed (data not shown), the relative effect of this treatment was more dramatic in nuclear extracts lacking the 5′ end of U1 snRNA (U1 knock-out extracts; Fig. 5A, lanes 7–16). Removal of the first 15 nt of U2 snRNA resulted in a ~10-fold reduction in the efficiency of splicing (Fig. 5A, lane 8). This region of U2 base pairs with the 3′ terminal segment of U6 snRNA (Datta & Weiner, 1991; Wu & Manley, 1991). When the region of U2 snRNA that is known to base pair with the branch site (nt 28–42) is removed, no *trans*-splicing (<1% of U1 knock-out extract) was detected (Fig. 5A, lane 10). Removal of nt 1–15 or 58–76 of U4 snRNA, which decreases the potential base pairing between U4 and U6 snRNAs (Hashimoto & Steitz, 1984; Rinke et al., 1985; Brow & Guthrie, 1988), substantially diminished the levels of U4/U5/U6 triple snRNP in the extract, as evidenced by native gel analysis followed by northern hybridization (data not shown), and resulted in a ~4-fold reduction in *trans*-splicing (Fig. 5A, lanes 12, 14). Finally, removing nt 33–45 of U6 snRNA abolishes (<1% of U1 knock-out extract) *trans*-splicing (Fig. 5A, lane 16). This region of U6 snRNA includes the ACAGA of the phylogenetically invariant ACAGAG sequence (reviewed by Guthrie, 1991). Together, these data demonstrate that *trans*-splicing requires the regions of U2 snRNA that base pair with the branch site or U6 snRNA and the region of U6 snRNA that base pairs with the 5′SS.

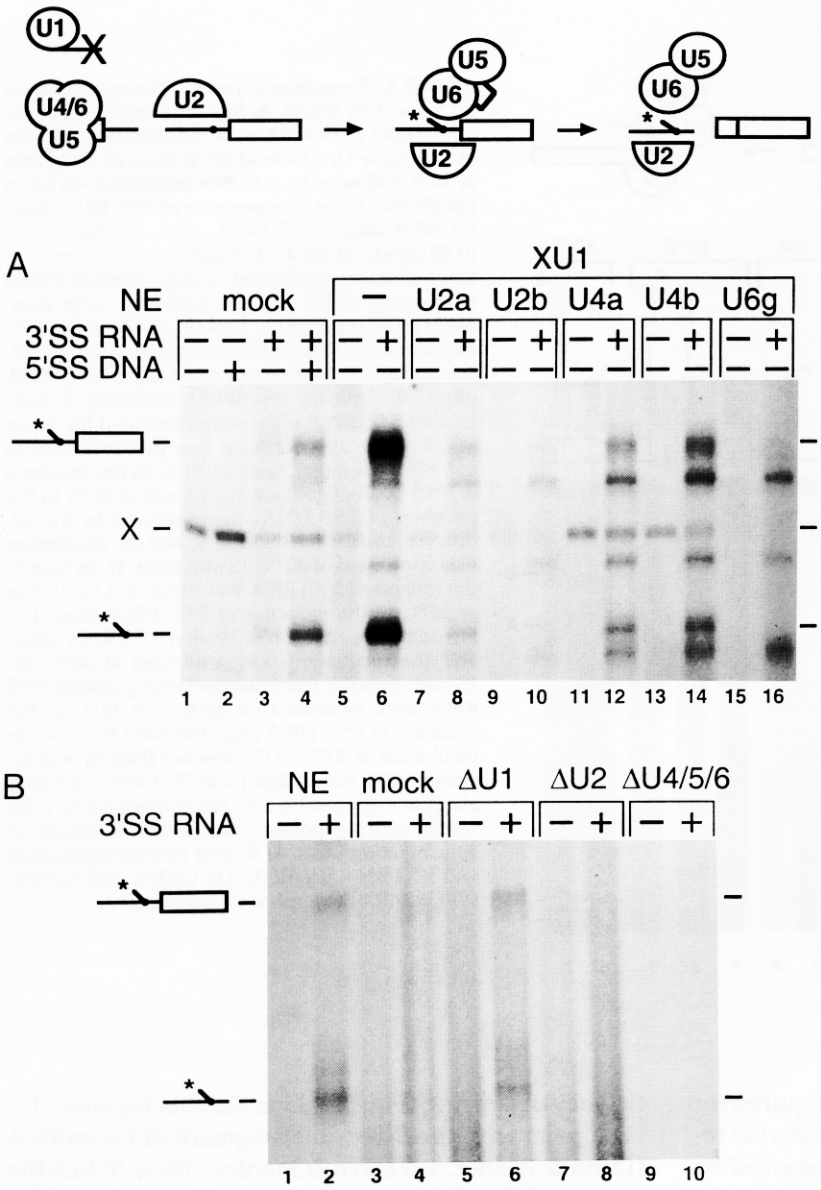

FIGURE 5. *Trans*-splicing depends on U2 and U4/U5/U6 snRNPs. **A:** Nuclear extracts were pretreated with RNase H and no oligo (mock treatment, lanes 1–4) or DNA oligo (XU1, lanes 5–16) with no additional oligo (lanes 5, 6), U2a (lanes 7, 8), U2b (lanes 9, 10), U4a (lanes 11, 12), U4b (lanes 13, 14), or U6g (lanes 15, 16) DNA oligos. These pretreated nuclear extracts were incubated for 30 min at 30 °C in the absence (lanes 5, 7, 9, 11, 13, 15) or presence (lanes 4, 6, 8, 10, 12, 14, 16) of unlabeled 3′SS RNA, followed by the addition of labeled 5′SS RNA oligo, and continued incubation for 2 h at 30 °C. **B:** Nuclear extracts were untreated, NE (lanes 1, 2); treated in the absence of 2′-*O*-methyl oligo, mock (lanes 3, 4); specifically depleted for U1 snRNP (lanes 5, 6); U2 snRNP (lanes 7, 8); or U4/U5/U6 snRNP (lanes 9, 10). Splicing reactions using these nuclear extracts were performed by incubating for 30 min at 30 °C in the presence of 5′SS DNA oligo (lanes 1–10) in the absence (lanes 1, 3, 5, 7, 9) or presence (lanes 2, 4, 6, 8, 10) of unlabeled 3′SS RNA, followed by the addition of labeled 5′SS RNA oligo, and continued incubation for 2 h at 30 °C. RNA products were resolved in a 10% polyacrylamide/8 M urea gel. Positions of the branched intron–exon intermediate, branched intron product, and X are indicated.

Splicing of the short 5′SS RNA oligo to a 3′SS RNA added in *trans* is enhanced by blocking (Fig. 1A, lane 4; Fig. 5A, lane 4) or removing (Fig. 5A, lane 6) the 5′ end of U1 snRNA. To determine whether the U1 snRNP particle is required for *trans*-splicing, nuclear extracts specifically depleted of U1, U2, or U4/U5/U6 snRNPs by 2′-*O*-methyl oligonucleotide affinity selection (Blencowe & Barabino, 1994) (a generous gift of J.D. Crispino & P.A. Sharp) were examined. The U1-depleted extracts supported *trans*-splicing, whereas extracts depleted of U2 snRNP or U4/U5/U6 snRNP did not (Fig. 5B, lanes 6, 8, 9). Moreover, the efficiency of the reaction in the U1-depleted extract was enhanced in comparison to the mock-depleted extract (Fig. 5B, compare lanes 6, 4). Thus, the *trans*-splicing reaction does not depend on the U1 snRNP and is, in fact, enhanced in its absence.

DISCUSSION

The ordered and sequential nature of the spliceosome assembly pathway that functions to build the active site depends, at least in part, on an extensive and ordered series of dynamic interactions involving pre-mRNA, snRNP particles, and protein factors. The multistep spliceosome assembly pathway has two important consequences. First, it allows for multiple recognition of splice sites, which could increase the fidelity of splicing. Second, it provides the opportunity for regulation at each step in the assembly pathway, which could affect splice site choice as well as the efficiency of splicing. Although these multiple regulatory steps are essential for accurate splicing of full-length pre-mRNA, they provide little insight into the actual mechanism of

catalysis. Thus, to consider in detail how the active catalytic center of the spliceosome promotes the chemical events in splicing, a simplified splicing system was developed in which at least some of these regulatory steps have been bypassed.

Similarities between *cis*- and *trans*-splicing

In this simplified splicing system, a short RNA oligo comprising the 5′SS consensus sequence (5′SS RNA oligo) is capable of forming branched intermediates and products as well as ligated exons, indicative of both steps of splicing, when a second RNA containing a branch site with an adjacent polypyrimidine tract and a 3′SS (3′SS RNA) is added in *trans*. The identity of these intermediates and products was confirmed by varying the length of the exon or intron sequences of one of the two RNAs. As expected, the size of the branched intermediate depends on the length of both the exon and intron of the 3′SS RNA, whereas the size of the branched intron product depends only on the length of the intron (Fig. 2A). The size of the ligated exons depends on the exon length of both the 5′SS and 3′SS RNAs (Fig. 2B; and data not shown). Furthermore, the branched intermediates contain the predicted branch structure as shown by direct nucleotide analysis (Fig. 2C). Thus, the mechanism of the *trans*-splicing reaction appears indistinguishable from that characterized for the *cis*-splicing reaction of full-length pre-mRNA.

Although the first two bases of the intron (GU) comprise the most highly conserved element within the 5′SS consensus sequence, cleavage at the 5′SS does not absolutely depend on the identity of this GU dinucleotide in *cis*-splicing of full-length pre-mRNA (Aebi et al., 1987; Lamond et al., 1987; Siliciano & Guthrie, 1988). Likewise, in the *trans*-splicing reaction, 5′SS RNA oligos with a G1 → A or a U2 → A mutation proceed through the first step of splicing with the same or a reduced efficiency, respectively (Fig. 3). In *cis*-splicing, cleavage at the 5′SS can depend on the identity of the 3′SS (Reed, 1989; Reich et al., 1992), however, this requirement is not universal (Seraphin & Kandels-Lewis, 1993) and, in fact, the first step of splicing does not strictly depend on the G of the AG dinucleotide at the intron/exon boundary of the 3′SS in the *trans*-splicing system (Fig. 3).

In contrast to the sequence flexibility of the first step of splicing, the second step is more stringent with regard to base sequence requirements. In the context of full-length pre-mRNAs, mutational analysis has revealed that the second step depends on the identity of the first two (GU) and last two (AG) nucleotides of the intron, and the branch site (Vijayraghavan et al., 1986; Aebi et al., 1987; Lamond et al., 1987). Likewise, 5′SS RNA oligos with a G1 → A or a U2 → A mutation proceed through the second step of splicing at a reduced efficiency when compared to the wild type. Furthermore, a AG → AC mutation at the 3′SS (the last nucleotide of the intron of the 3′SS RNA) completely blocks the second step of *trans*-splicing (Fig. 3). Thus, *trans*-splicing of a short 5′SS RNA oligo to a second RNA containing the 3′SS region displays the same 5′ and 3′ splice site sequence specificity as does *cis*-splicing of full-length pre-mRNA.

In *cis*-splicing of full-length pre-mRNA, DNA oligo-directed RNase H cleavage of specific segments of individual snRNAs (Kramer et al., 1984; Black et al., 1985) was an important tool in determining the snRNA sequence requirements. This same approach showed that *trans*-splicing also depends on the integrity of U2, U6, and to a lesser extent, U4 snRNAs (Fig. 5A). When the region of U2 snRNA that base pairs with the branch site was removed, *trans*-splicing was blocked. Likewise, removal of the region of U2 snRNA that base pairs with U6 snRNA to form part of the catalytic center of the spliceosome (Madhani & Guthrie, 1992; Sun & Manley, 1995) dramatically reduced the efficiency of *trans*-splicing. When the region of U6 snRNA that interacts with the 5′SS (Sawa & Abelson, 1992; Kandels-Lewis & Seraphin, 1993; Lesser & Guthrie, 1993; Wassarman & Steitz, 1993) was removed, *trans*-splicing was completely abolished. This region of U6 snRNA contains the phylogenetically invariant ACAGAG sequence that is thought to be at or near the catalytic center of the spliceosome (reviewed by Guthrie, 1991). By analogy to yeast, the first two nucleotides of the ACAGAG sequence should base pair with intron positions 6 and 5 of the 5′SS RNA oligo, respectively. However, in this *trans*-splicing system, the complementarity between the 5′SS RNA oligo and this region of U6 snRNA is even more extensive, such that five base pairs could potentially form. The importance of this interaction between the 5′SS and U6 snRNA is supported by recent experiments, which showed that, when this base pairing is improved, the efficiency of *cis*-splicing of full-length pre-mRNA increases (Crispino & Sharp, 1995). Thus, *trans*-splicing requires the regions of U2 snRNA which base pair with the branch site or with U6 snRNA, and the region of U6 snRNA which base pairs with the 5′SS. By comparison, the role of U4 snRNA in the *trans*-splicing reaction appears to be less important. Removal of regions of U4 snRNA that base pair with U6 snRNA (Hashimoto & Steitz, 1984; Rinke et al., 1985; Brow & Guthrie, 1988) had less of an effect on the efficiency of *trans*-splicing (Fig. 5A). This finding could be explained if a low level of interaction between U5 and U6 snRNPs persists and is capable of participating in splicing. This interpretation is consistent with the fact that before or concomitant with the first step of splicing, the extensive U4/U6 base pairing is disrupted (Cheng & Abelson, 1987; Konarska & Sharp, 1987; Lamond et al., 1988; Blencowe et al., 1989; Yean & Lin, 1991). Thereafter, U4 snRNP remains only

loosely associated with the spliceosome (Blencowe et al., 1989) and, in fact, does not participate further in the splicing reaction (Yean & Lin, 1991).

Regulatory role of U1 snRNP is bypassed in *trans*-splicing

The early requirement for U1 snRNP in pre-mRNA splicing is, at least in part, due to its role in exon/intron definition (Robberson et al., 1990; Talerico & Berget, 1990). According to this model, U1 and U2 snRNPs, along with non-snRNP proteins, select pairs of splice sites, thus commiting them to the splicing pathway (Legrain et al., 1988; Ruby & Abelson, 1988; Seraphin & Rosbash, 1989; Fu & Maniatis, 1992; Fu, 1993; Michaud & Reed, 1993; Wu & Maniatis, 1993; Amrein et al., 1994; Staknis & Reed, 1994). Specifically, U1 snRNP interacts with the 5′SS via base pairing, and the U1-70K protein directly interacts with SC35 or other SR proteins, a family of general splicing factors (Zahler et al., 1992; Horowitz & Krainer, 1994). SC35 then interacts with $U2AF^{35}$, which in turn interacts with $U2AF^{65}$ bound to the polypyrimidine tract near the 3′SS. In this way, the two splice sites interact. Unlike *cis*-splicing, *trans*-splicing between the 5′SS RNA oligo and the 3′SS RNA does not depend on U1 snRNP. In fact, *trans*-splicing is enhanced when the 5′ end of U1 snRNA is blocked or removed or when the U1 snRNP is depleted from the nuclear extract (Figs. 1A, 5A,B). Thus, the SR proteins that mediate the interaction between the two splice sites in *cis*-splicing of full-length pre-mRNA would not be expected to play a role in this simplified splicing system. The finding that U1 snRNP actually inhibits splicing in this *trans*-splicing system is consistent with several lines of evidence. First, previous in vitro studies of 5′SS RNA oligo-snRNP interactions using the native gel binding assay showed that, when 5′SS RNA–U1 snRNA base pairing is prevented, formation of U2/U4/U5/U6 snRNP complex is enhanced (Konforti et al., 1993). Thus, we concluded that disruption of this initial base pairing interaction is required for subsequent spliceosome assembly. Second, binding studies of 5′SS RNA mutant oligos showed that specific recognition of the 5′SS occurs in the absence of the 5′ end of U1 snRNA. In fact, the specificity resides within U4/U5/U6 snRNP and correlates with the 5′SS consensus sequence (Konforti & Konarska, 1994). Moreover, the effect of mutations at the 5′ and 3′ splice sites is nearly identical in the *cis*- and *trans*-splicing systems, despite the fact that, in the *trans*-splicing reaction, the 5′ end of U1 snRNA was removed. These findings suggest that the splice site sequence specificity of spliceosome assembly and catalysis does not depend on the 5′ end of U1 snRNA. Third, when U1 snRNP is depleted from nuclear extracts (Crispino et al., 1994), or when the 5′ end of U1 snRNA is blocked by the presence of a complementary 2′-*O*-methyl oligo (Tarn & Steitz, 1994), splicing of full-length pre-mRNA can be restored by high concentrations of SR proteins. Thus, the 5′ end of U1 snRNA is not absolutely required for *cis*-splicing. Together, these observations suggest that U1 snRNP plays an early role, along with other commitment factors, which include members of the SR protein family, to identify the substrate before transferring it to the catalytic center. In this scenario, the base pairing interaction between the 5′SS and U1 snRNP is maintained at early stages of spliceosome assembly when U1 snRNP interacts with U2 snRNP. Later, when the U4/U5/U6 snRNP joins the spliceosome, this interaction is disrupted and U6 snRNA replaces the 5′ end of U1 snRNA to base pair with the 5′SS. Thus, U1 snRNP does not appear to play any subsequent role in catalysis and the role of U1 snRNP can be bypassed when the 5′SS is allowed to interact more directly with U6 snRNA.

The demonstration of low levels of *trans*-splicing of normally *cis*-spliced RNAs in mammalian extracts (Konarska et al., 1985b; Solnick, 1985) suggested that the two splice site RNAs physically interact, at least transiently. In fact, the efficiency of *trans*-splicing was increased if the ends of the two intron sequences were complementary (Konarska et al., 1985b). More recently, *trans*-splicing in mammalian extracts was demonstrated in reactions that were dependent on splicing enhancers (Chiara & Reed, 1995) and stimulated by SR proteins (Bruzik & Maniatis, 1995). In contrast, *trans*-splicing between a short 5′SS RNA oligo and a second RNA containing the 3′SS region occurs in the absence of complementarity between the two RNAs and does not depend on splicing enhancers, high concentrations of SR proteins, or U1 snRNP. In fact, when 3′SS RNA substrates that included a naturally occurring downstream 5′SS (derived from the second intron of the tripartite leader of adenovirus) were tested in this *trans*-splicing system, no stimulation of splicing was observed. Moreover, mutation of this downstream 5′SS had no effect on *trans*-splicing (data not shown). Thus, in this simplified *trans*-splicing reaction, which does not depend on the interactions required for proper splice site selection, namely those mediated by U1 and U2 snRNPs and SR proteins, formation of an active spliceosome must require other, more direct, interactions between the two splice sites.

A simplified spliceosome assembly pathway in *trans*-splicing

Because the 5′SS RNA oligo is known to specifically interact with U4/U5/U6 snRNP (Konforti & Konarska, 1994) and the 3′SS RNA forms complex A upon U2 snRNP binding, the functional interaction between the 5′ and 3′ splice sites could occur by the association of these partial splicing complexes. This scenario is con-

sistent with the analysis of RNA–snRNP complexes formed on each of the two splice site RNAs. When the 3′SS RNA is preincubated in the absence of the 5′SS RNA oligo, U2 snRNP and a number of protein factors bind to the 3′SS RNA to form splicing complex A. Upon addition of the 5′SS RNA oligo, complex A is converted to splicing complex B (Fig. 4A). When the 5′SS RNA oligo is incubated in the absence of the 3′SS RNA, it rapidly (<30 s) binds to U4/U5/U6 and U2/U4/U5/U6 snRNP complexes (Fig. 4B). Although these RNA–snRNP complexes are stable on ice (Konforti & Konarska, 1994), they are less stable at 30 °C (data not shown). Thus, at 30 °C, rapid dissociation and reassociation of these RNA–snRNP complexes is likely to occur. However, addition of the 3′SS RNA enhances binding of the 5′SS RNA oligo to U2/U4/U5/U6 snRNP complex (Fig. 4B), most likely by stabilizing this interaction. Likewise, upon addition of the 5′SS RNA oligo, preformed complex A is very rapidly (<30 s) converted to splicing complex B, which represents the addition of U4/U5/U6 snRNP complex and the 5′SS RNA oligo (Fig. 4A,B; and data not shown). Finally, the functional interaction between U4/U5/U6 snRNP bound to the 5′SS and U2 snRNP bound to the 3′SS is also consistent with the fact that, at high 5′SS RNA oligo concentrations, a majority of U4/U5/U6 snRNP is involved in formation of U2/U4/U5/U6 snRNP complex (Hall & Konarska, 1992). Likewise, under splicing conditions, high concentrations of the 5′SS RNA oligo would drive formation of U2/U4/U5/U6 snRNP complex and any 3′SS RNA associated with U2 snRNP would be passively introduced into this complex. Thus, the 5′SS RNA oligo-induced U2/U4/U5/U6 snRNP complex B appears to be functionally active.

By using a short 5′SS RNA oligo as a substrate, the early regulatory role of U1 snRNP, which is essential in *cis*-splicing of full-length pre-mRNA, has been bypassed. By analogy, minimizing the size of the 3′SS RNA could further simplify the system at least with respect to the substrates and perhaps also with regard to the non-snRNP factors involved in catalysis. More importantly, chemical synthesis of short RNA oligos allows easy incorporation of modified nucleotides at specific positions that can be used directly as *trans*-splicing substrates. The combination of several different assays, which follows spliceosome assembly (binding assay), RNA–RNA and RNA–protein interactions (cross-linking assay), and splicing activity (*trans*-splicing assay), offers a unique system in which to study the mechanistic details of splicing.

MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides were synthesized using an Applied Biosystems 390 synthesizer. Phosphoramidites were from Glen Research (Sterling, Massachusetts).

5′SS RNAs

3′ end labeling of oligonucleotides was carried out by annealing the RNA oligo to a complementary DNA oligo, such that the 5′ end of the DNA oligo overhangs the 3′ end of the RNA oligo. The RNA of this heteroduplex was extended with the Klenow fragment of *Escherichia coli* DNA polymerase (Boehringer Mannheim) in the presence of α^{32}P dCTP (NEN) to incorporate three dC residues at the 3′ end (Hausner et al., 1990). These RNAs were resolved in a 20% polyacrylamide/8 M urea gel to separate the DNA oligo and unincorporated nucleotide from the RNA oligo. RNAs containing three labeled dC residues were isolated from the gel and the concentration of the 5′SS RNA oligo was determined based on the known specific activity of the label. The 3′ end-labeled 5′SS RNA oligo used in the experiments described in Figures 1A,B, 2A, 4B, and 5 has the sequence A_5G/GUAAGUA dT*dC_3. 5′ end labeling of oligos was carried out using T4 polynucleotide kinase (New England Biolabs) and γ^{32}P ATP (NEN). To allow for accurate measurement of the RNA oligo concentration, a twofold molar excess of γ^{32}P ATP was used to ensure quantitative labeling. The 5′SS RNA oligo that was specifically labeled at the phosphate of the exon/intron junction (A_6G/*GUAAGUAdTdC_3) used in the experiments described in Figure 2C was prepared by 5′ end labeling of /GUAAGUAdTdCdCdC oligo and subsequent ligation to A_6G using T4 RNA ligase. The RNA oligos used in the experiments described in Figure 3 were prepared by ligation of 3′ end-labeled wild type, AAG/GUAAGUAdT*dC_3; G1 → A, AAG/*A*UAAGUAdT*dC_3; U2 → A, AAG/G*A*AAGUAdT*dC_3; or A4 → U, AAG/GUA*U*GUAdT*dC_3 to A_6 using T4 RNA ligase (Boehringer Mannheim). The exon-labeled RNA oligos used in the experiments described in Figure 2B were prepared by 5′ end labeling A_5G/GUAAGUAdTdC_3, A_2G/GUAAGUAdTdC_3, or G/GUAAGUAdTdC_3 oligos and subsequently ligating to A_6 using T4 RNA ligase. The following 2′-*O*-methyl oligonucleotides, a gift of S.D. Seiwert and J.A. Steitz, were used: $U1_{1\text{-}10}$, AGGUAAGUAU; and EBER3, GCAAACCUCUAGG (Seiwert & Steitz, 1993).

3′SS RNAs

In most experiments, the 3′SS RNA contained 83 nt of intron and 45 nt of exon sequence and was prepared by transcription with T7 RNA polymerase from a pBSAd13 plasmid template cut with *Sau* 3A1 (Konarska, 1989). The 3′SS RNAs used in the experiments described in Figure 2A were prepared by transcription with T7 or T3 RNA polymerase from pBSAd13 or pBSAd7 plasmid template cut with *Sac* I, *Sau* 3A1, or *Hae* III to generate transcripts with 83 or 90 nt of intron and 35, 45, or 62 nt of exon sequence, respectively. pBSAd7 was constructed by inserting a 180-nt *Hin*d III–*Hin*c II fragment of pBSAd1 (Konarska & Sharp, 1987) between the *Hin*d III and *Hin*c II sites of pBS$^-$ vector. 3′SS RNA with a single point mutation at the 3′SS, AG → AC, was generated by PCR-directed oligonucleotide mutagenesis and sequenced to confirm the presence of the mutation. Labeled 3′SS RNAs were generated as above in the presence of α^{32}P GTP. The concentration of unlabeled 3′SS RNA was estimated by preparing an identical transcription reaction in the presence of a known trace amount of α^{32}P GTP. Based on the number of Gs in the transcript and the specific activity of the transcrip-

tion reaction, the RNA concentration was determined and confirmed by denaturing gel electrophoresis and ethidium bromide staining. Typically, the transcription reactions yielded ~1 μg of RNA per μL of reaction.

Trans-splicing assays

Splicing reactions (15 μL) contained 40% nuclear extract (Dignam et al., 1983), 1 mM ATP, 2 mM Mg acetate, 5 mM creatine-phosphate, ±0.25 μg (4 μM) 5′SS DNA oligo (5′CAGGTAAGTAT3′), 5′SS RNA oligo (~10^5 cpm, 0.7 nM), and 3′SS RNA (150 nM). Typically, splicing reactions containing all components except the 5′SS RNA oligo were preincubated for 30 min at 30 °C followed by the addition of the labeled 5′SS RNA oligo and the incubation was continued for 2 h at 30 °C. Splicing reactions lacking ATP contained only nuclear extract and Mg acetate and were preincubated for 15 min at 30 °C to deplete the ATP in the nuclear extract prior to the addition of the 3′SS RNA. RNA products were phenol extracted, ethanol precipitated, and resolved in a 10% polyacrylamide/8 M urea gel such that the free, unreacted 5′SS RNA oligo remained in the bottom of the gel and a PhosphorImager (Molecular Dynamics) was used for quantitation. The efficiency of the first step of splicing was calculated as a fraction of the total (unreacted 5′SS RNA + branch intron intermediate + branch intron product) recovered in the branch intron intermediate + branch intron product, multiplied by the total concentration of the 5′SS RNA oligo. The efficiency of the second step of splicing was calculated as a fraction of the total recovered in the branch intron product, multiplied by the total concentration of the 5′SS RNA oligo.

Binding assay

Splicing complexes were resolved in a 4% polyacrylamide/50 mM Tris-glycine nondenaturing gel at ~20 V/cm at 4 °C for ~3 h, transferred to 3MM Whatman paper, dried, and autoradiographed as previously described (Konforti & Konarska, 1994).

Oligonucleotide-directed cleavage of snRNAs and snRNP-depleted nuclear extracts

RNase H (Boehringer Mannheim) digestions were carried out as previously described (Konarska & Sharp, 1987) using DNA oligonucleotides complementary to the following regions in corresponding snRNA sequences: U1, 1–15; U2a, 1–15; U2b, 28–42; U4a, 1–15; U4b, 58–76; U6g, 33–45. snRNAs of nuclear extracts treated in this way were phenol extracted and resolved in a 10% polyacrylamide/8 M urea gel, transferred, and hybridized with a mixture of RNA probes complementary to U1, U2, U4, and U6 snRNAs as described above. A PhosphorImager (Molecular Dynamics) was used for quantitation. Nuclear extracts depleted of U1, U2, or U4/U5/U6 snRNPs, and mock-depleted extracts prepared as described (Blencowe & Barabino, 1994), were a generous gift of J.D. Crispino and P.A. Sharp.

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