U4/U5/U6 snRNP recognizes the 5' splice site in the absence of U2 snRNP

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Using an in vitro system in which a 5' splice site (5'SS) RNA oligo (A_AG GUAGAGUAAdT) is capable of inducing formation of U2/U4/U5/U6 snRNP complex we show that this oligo specifically binds to U4/U5/U6 snRNP and cross-links to U6 snRNA in the absence of U2 snRNP. Moreover, 5'SS RNA oligo bound to U4/U5/U6 snRNP is chased to U2/U4/U5/U6 snRNP complex upon addition of U2 snRNP. Recognition of the 5'SS by U4/U5/U6 snRNP correlates with the 5'SS consensus sequence. Unlike the interaction with U1 snRNP, this recognition depends largely on interactions other than RNA–RNA base pairing. Finally, the region of U6 snRNA required for this interaction with U4/U5/U6 snRNP is positioned upstream of stem I in the U4–U6 structure. We propose that the 5'SS-U4/U5/U6 snRNP complex is an intermediate in spliceosome assembly and that recognition of the 5'SS by U4/U5/U6 snRNP occurs after the 5'SS-U1 snRNA base pairing is disrupted but before the U4–U6 snRNA structure is destabilized.

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recognition of the 5'SS by U1 snRNA is effectively uncoupled from recognition by U2/U4/U5/U6 snRNP complex (Konforti et al. 1993). Here we show that the 5'SS RNA oligo binds to U4/U5/U6 snRNP and cross-links to U6 snRNA in the absence of U2 snRNP and that the region of U6 required for this interaction lies upstream of stem I in the U4–U6 snRNA structure. Mutational analysis demonstrates that 5'SS recognition by U4/U5/U6 snRNP is specific and correlates with the 5'SS consensus sequence. Although this recognition is likely to involve interactions other than RNA–RNA base pairing, recognition of the 5'SS by U1 snRNA depends largely on base pairing between the 5'SS and the 5' end of U1 snRNA. Thus, the mutational analysis strengthens the conclusion that recognition of the 5'SS by U1 snRNP and U2/U4/U5/U6 snRNP complex are distinct. Together, these data suggest that the 5'SS-U4/ U5/U6 snRNP is an intermediate in spliceosome assembly and that recognition of the 5'SS by U4/U5/U6 snRNP occurs after the 5'SS-U1 snRNA base pairing is disrupted but before the U4–U6 snRNA structure is destabilized.

Results

To study the interactions of the 5'SS RNA oligo with snRNP complexes, 5' end-labeled 5'SS RNA oligos were incubated with HeLa nuclear extracts on ice and the resulting RNA–snRNP complexes were resolved in a native gel (the binding assay) or ultraviolet light (UV)-irradiated, deproteinized, and resolved in a denaturing gel to detect the cross-linked RNA–RNA products (the cross-linking assay). Using these two assays, previous studies (Konforti et al. 1993) showed that the 5'SS RNA oligo binds to U1 snRNP [Fig. 1B, lane 3] by base pairing with the 5' end of U1 snRNA which results in a 5'SS–U1 snRNA cross-link [Fig. 1B, lane 5]. When binding of the 5'SS RNA oligo to U1 snRNP is blocked by the presence of 5'SS DNA oligo that base pairs with the 5' end of U1 snRNA, the 5'SS RNA oligo binds to U2/U4/U5/U6 snRNP complex [Fig. 1B, lane 4] which results in a cross-link between the RNA oligo and U6 snRNA [Fig. 1B, lane 6]. The identity of RNA cross-links was determined by RNase H mapping [Konforti et al. 1993]. The ability of the 5'SS RNA oligo to induce the assembly of U2/U4/U5/U6 snRNPs was determined by Northern hybridization [Hall and Konarska 1992, Fig. 1B, lanes 1,2].

Effects of single point mutations in the 5'SS on interactions with U1 snRNP and U2/U4/U5/U6 snRNP complex

To investigate the specificity of the process by which the 5'SS is recognized first by the 5' end of U1 snRNA and subsequently by a component(s) of U2/U4/U5/U6 snRNP complex, the binding and cross-linking properties of 5'SS RNA oligos containing single point mutations from positions -2 to +7 [5'SS mutant RNA oligos] were examined (see Fig. 1A for numbering of positions). 5' end labeled 5'SS mutant RNA oligos were tested for the ability to bind to U1 snRNP and cross-link to U1 snRNA under conditions which favor interaction with U1 snRNP [i.e., in the absence of 5'SS DNA oligo]. Likewise, the ability of the 5'SS mutant RNA oligos to bind to U2/U4/U5/U6 snRNP complex and cross-link to U6 snRNA under conditions which favor interaction with U2/U4/U5/U6 snRNP complex [i.e., in the presence of 5'SS DNA oligo] was examined.

Figure 1. Standard Northern, binding, and cross-linking assays using the 5'SS RNA oligo. [A] The sequence of the 5'SS RNA oligo is shown. The vertical arrow represents the cleavage site and the numbers above 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 percentage of splice sites having the consensus base for primates at positions -2 to +6 (Senapathy et al. 1990). [B] Nuclear extracts were incubated in the absence (-, lane 1) or presence (+, lane 2) of 0.25 μM 5'SS RNA oligo and 6.25 μM 5'SS DNA oligo. After incubation for 45 min at 30°C, the snRNP complexes were resolved in a 4% polyacrylamide gel, transferred to a nylon membrane, and detected by hybridization with U4 snRNA probe. Positions of U4/U5/U6 and U2/U4/U5/U6 snRNP complexes are indicated as U4/5/6 and U2/4/5/6, respectively. 5' end labeled 5'SS RNA oligo was incubated under standard conditions in the absence (-, lanes 3,5) or presence (+, lanes 4,6) of 0.25 μM 5'SS RNA oligo and 2.5 μM 5'SS DNA oligo. An aliquot of the reaction (10%) was resolved in a 4% polyacrylamide gel, transferred to a nylon membrane, and detected by hybridization with U4 snRNA probe. Positions of U4/U5/U6 and U2/U4/U5/U6 snRNP complexes are indicated as U4/5/6 and U2/4/5/6, respectively. 5' end labeled 5'SS RNA oligo was incubated under standard conditions in the absence (-, lanes 3,5) or presence (+, lanes 4,6) of 2.5 μM 5'SS DNA oligo. An aliquot of the reaction (10%) was resolved in a 4% polyacrylamide native gel (lanes 3,4). Positions of complexes formed between 5'SS RNA oligo and U1 snRNP or U2/U4/U5/U6 snRNP complex are indicated as U1 and U2/4/5/6, respectively. The remainder of the reaction was UV irradiated, deproteinized, and resolved in a 10% polyacrylamide–8 m urea gel (lanes 5,6). Positions of cross-linked products formed between 5'SS RNA oligo and U1 and U6 snRNAs are as indicated.
All 5’S S mutants cross-link to U1 snRNA but only some bind stably to U1 snRNP

All 5’S S RNA oligos with single point mutations from positions –1 to +6 reduced stable binding to U1 snRNP 10–50-fold whereas those with mutations near the ends of the RNA oligo had less of an effect on binding to U1 snRNP (Fig. 2A, closed columns). Specifically, binding of the A-2 → U 5’S S RNA oligo was comparable to that of the wild type 5’S S RNA oligo whereas binding of the A +7 → C 5’S S RNA oligo was reduced only 2–3-fold. These binding data suggest that under the conditions used here stable binding of 5’S S RNA oligo to U1 snRNP requires at least 10 bp between the 5’ end of U1 snRNA and the 5’S S RNA oligo. Any single point mutation destabilizes this interaction with the exception of those positioned near the 5’ or 3’ ends of the 5’S S RNA oligo. These findings are consistent with deletion analysis of the 5’S S RNA oligo which showed that stable binding to U1 snRNP requires at least 10 nucleotides encompassing the sequence AG ↓ GUAGUAU (Konforti et al. 1993).

To more specifically define the role of RNA–RNA base pairing in the interaction between the 5’S S RNA oligo and U1 snRNP, binding of wild-type and mutant 5’S S RNA oligos to U1 RNA oligo in the absence of nuclear extract was examined. The 11 nucleotide U1 RNA oligo [AUACUUACCUG] is complementary to the wild-type 5’S S RNA oligo [AAG ↓ GUAGUAUd(T)] in 10/11 positions and to the 5’S S mutant RNA oligos in 9/11 positions. Under native gel electrophoresis conditions a stable interaction between wild-type 5’S S RNA oligo and U1 RNA oligo was detected. In the presence of 0.5 pmole of 5’ end labeled U1 RNA oligo and a 25-fold excess of unlabeled wild-type 5’S S RNA oligo a stable duplex was observed (Fig. 2A, open columns). In this assay, 5’S S RNA oligos with single point mutations from position –1 to +6 failed to stably interact with U1 RNA oligo whereas mutant 5’S S RNA oligos A-2 → U and A +7 → C interacted with U1 RNA oligo albeit less efficiently than wild-type 5’S S RNA oligo (≈70% and ~30% of wild-type 5’S S RNA oligo, respectively). Together, these data suggest that the RNA–RNA base pairing interaction between the 5’S S RNA oligo and the 5’ end of U1 snRNA stabilizes the binding of the RNA oligo to U1 snRNP and that nucleotides at the ends of the oligo contribute less to the stability of this interaction than nucleotides at internal positions. Moreover, the similarity of the binding profiles of the 5’S S mutant RNA oligos to U1 snRNP and U1 RNA oligo suggests that the stability of the 5’S S RNA–U1 snRNP complex depends primarily on RNA–RNA base pairing interactions.

Whereas most 5’S S mutants failed to stably bind to U1 snRNP, all mutants cross-linked to U1 snRNA, albeit with varying efficiencies (Fig. 2B). Those 5’S S mutant RNA oligos which could stably bind to U1 snRNP, namely, A-2 → U and A +7 → C, cross-linked to U1 snRNA with an efficiency either comparable to or greater than that of wild-type 5’S S RNA oligo, respectively. The efficient cross-linking observed using 5’S S

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**Figure 2.** Effects of 5’S S RNA oligos with single point mutations from positions –2 to +7 on interactions with U1 snRNP and U2/U4/U5/U6 snRNP complex. (A) Stable binding of nonspecific, wild-type, and 5’S S mutant RNA oligos to U1 snRNP in nuclear extract (closed column). 5’ end labeled RNA oligo was incubated under standard conditions in the absence of 5’S S DNA oligo and resolved in a 4% polyacrylamide native gel. Each RNA oligo was tested 3–6 times and the error bars are as indicated. Stable binding of U1 RNA oligo to nonspecific, wild-type, and 5’S S mutant RNA oligos in the absence of nuclear extract (open column). 5’ end labeled U1 RNA oligo was incubated under standard conditions in the presence of unlabeled RNA oligo and resolved in a 20% polyacrylamide native gel. (B) Cross-linking of nonspecific, wild-type, and 5’S S mutant RNA oligos to U1 snRNA in nuclear extract (open column). 5’ end labeled RNA oligo was incubated under standard conditions in the presence of 5’S S DNA oligo, UV-irradiated, deproteinized, and resolved in a 10% polyacrylamide–8 M urea gel. Each RNA oligo was tested 3–6 times. The values for each of the RNA oligos in percent of wild type (wt) are as follows: nonspecific RNA, 3.2±1.0; wt, 100±18; A-2 → U, 102±43; G-1 → A, 99±16, G + 1 → A, 36±12; U + 2 → A, 13±7; A + 3 → U, 46±4, A + 4 → U, 60±11, G + 5 → U, 47±20, U + 6 → A, 149±53, and A + 7 → C, 392±100. (C) Stable binding of wild-type and 5’S S mutant RNA oligos to U2/U4/U5/U6 snRNP complex (closed column) and cross-linking to U6 snRNA (open column). 5’ end labeled RNA oligo was incubated under standard conditions in the presence of 2.5 μM 5’S S DNA oligo. An aliquot of the reaction (10%) was resolved in a 4% polyacrylamide native gel and the remainder of the reaction was UV-irradiated, deproteinized, and resolved in a 10% polyacrylamide–8 M urea gel. Each RNA oligo was tested 3–6 times and the error bars are as indicated. (D) Stable binding to U2/U4/U5/U6 snRNP complex (closed column) and cross-linking to U6 snRNA (open column) of RNA oligos with all possible base substitutions at position +1, +2, or +4. 5’ end labeled RNA oligo was incubated under standard conditions in the presence of 2.5 μM 5’S S DNA oligo and treated as in C.
RNA oligos cross-linked to U1 snRNA under these conditions (data not shown). The 5'SS RNA oligos, which had no significant effect on binding to U2/U4/U5/U6 snRNP complex (data not shown). Most importantly, in this extract, as in all other nuclear extracts examined to date, 5'SS RNA oligo failed to compete with U1 RNA oligo for stable binding to U1 snRNP complex, NE* behaved typically in all other respects including the ability to splice pre-mRNA (data not shown). In contrast, 5'SS RNA oligo failed to interact with U2 snRNP and U6 RNA oligo which is identical to nucleotides 33-45 of U6 RNA [GGAACGUAACAGA, data not shown] under conditions which demonstrated a stable interaction between the 5'SS RNA oligo and U1 RNA oligo (Fig. 2A, open columns). Moreover, U6 RNA oligo failed to compete with U1 RNA oligo for stable binding to 5'SS RNA oligo (data not shown). Likewise, 5'SS RNA oligo failed to cross-link to deproteinized U6 snRNA whereas wild-type and 5'SS mutant RNA oligos failed to bind to U6 snRNA under these conditions (data not shown). Thus, the ability of the 5'SS RNA oligo to stably bind to U2/U4/U5/U6 snRNP complex and cross-link to U6 snRNA depends on intact snRNPs and possibly non-snRNP associated factors suggesting the involvement of other interactions in addition to RNA–RNA base pairing.

U4/U5/U6 snRNP specifically recognizes the 5'SS

Recognition of the 5'SS by U2/U4/U5/U6 snRNP complex could occur at the level of U4/U5/U6 snRNP or it could require U2 snRNP. Several lines of evidence suggest that U4/U5/U6 snRNP recognizes the 5'SS independently of its association with U2 snRNP. Under standard reaction conditions, 5'SS RNA oligo efficiently formed a stable complex with U4/U5/U6 snRNP in one particular nuclear extract, NE* [Fig. 3, lane 4]. Except for the apparent accumulation of this 5'SS RNA–U4/U5/U6 snRNP complex, NE* behaved typically in all other respects including the ability to splice pre-mRNA (data not shown). Most importantly, in this extract, as in all other nuclear extracts examined to date, 5'SS RNA oligo induced the formation of (Fig. 3, lane 2) and bound to (Fig. 3, lane 4) U2/U4/U5/U6 snRNP complex. The 5'SS RNA oligo also bound to U4/U5/U6 snRNP in a par-
Figure 3. 5'SS RNA oligo stably binds to U4/U5/U6 snRNP and cross-links to U6 snRNA in nuclear extract and in U4/U5/U6 snRNP fraction. NE* (lanes 1-6) or glycerol gradient purified U4/U5/U6 snRNP fraction (lanes 7-9) was incubated in the absence (−, lanes 1, 7) or presence (+, lane 2) of 0.25 μM 5'SS RNA oligo and 6.25 μM 5'SS DNA oligo. After incubation for 45 min at 30°C, the snRNP complexes were resolved in a 4% polyacrylamide native gel, transferred to a nylon membrane, and detected by hybridization with U4 snRNA probe. Positions of U4/U5/U6 and U2/U4/U5/U6 snRNP complexes are indicated as U4/5/6 and U2/4/5/6, respectively. The remainder of the snRNP complexes were resolved in a 4% polyacrylamide native gel, transferred to a nylon membrane, and detected by hybridization with U4 snRNA probe. Positions of complexes formed between 5'SS RNA oligo and U6 snRNAs, respectively, as determined by Northern analysis (data not shown). Because interaction of the 5'SS RNA oligo with U2/U4/U5/U6 snRNP complex resulted in a cross-link between the oligo and U6 snRNA [Konforti et al. 1993, Fig. 1B, lane 6] the ability of the 5'SS RNA oligo to cross-link to U6 snRNA in U4/U5/U6 snRNP fraction was determined. Significantly, 5' end labeled 5'SS RNA oligo cross-linked to U6 snRNA in fractions of U4/U5/U6 snRNP [Fig. 3, lane 9]. As controls, cross-linking of the 5'SS RNA oligo in unfractionated NE* to U1 or U6 snRNAs under conditions that favor binding to U1 snRNP (in the absence of 5'SS DNA oligo) or U2/U4/U5/U6 snRNP complex (in the presence of 5'SS DNA oligo) was observed [Fig. 3, lanes 5, 6]. Together, these data demonstrate that the 5'SS RNA oligo binds to U4/U5/U6 snRNP complex and cross-links to U6 snRNA in the absence of U2 snRNP.

To determine the specificity of 5'SS recognition by U4/U5/U6 snRNP, the binding properties of the previously described 5'SS mutant RNA oligos were examined in NE*. Using the standard binding assay, we found that single point mutations affected binding of the RNA oligo to U4/U5/U6 snRNP and to U2/U4/U5/U6 snRNP complex to a similar extent [Fig. 4A and B]. This, together with the ability of U4/U5/U6 snRNP to recognize the 5'SS in the absence of U2 snRNP [Fig. 3] indicates that the presence of U2 snRNP does not appear to contribute to the specificity of 5'SS recognition by U4/U5/U6 snRNP.

Finally, binding of the 5'SS RNA oligo to U4/U5/U6 snRNP and U2/U4/U5/U6 snRNP complex is abolished in the presence of DNA oligos complementary to one of two highly conserved regions of U6 snRNA [Fabrizio and Abelson 1990; Madhani et al. 1990]. NE* was preincubated in the presence of 5'SS DNA oligo and DNA oligos complementary to segments of U2, U4, U5, or U6 snRNA for 15 min, followed by the addition of 5' end labeled 5'SS RNA oligo and the incubation was continued for 45 min. The resulting 5'SS RNA oligo–snRNP complexes were resolved in a native gel [Fig. 5A]. Preincubation with DNA oligos complementary to positions 29–42, 39–50, or 18–31 of U2 snRNA [Fig. 5A, lanes 3–5], 1–15, or 58–76 of U4 snRNA [lanes 6, 7] or 35–47 of U5 snRNA [lanes 8] had no effect on binding of 5'SS RNA oligo to U4/U5/U6 snRNP or U2/U4/U5/U6 snRNP complex. In contrast, DNA oligos complementary to positions 33–48 (U6f) or 33–45 (U6g) of U6 snRNA [lanes 9, 10] abolished binding of 5'SS RNA oligo to U4/U5/U6 snRNP and U2/U4/U5/U6 snRNP complex. The effect of the U6f and U6g DNA oligos was specific because DNA oligos complementary to positions 1–20, 29–40, 59–76, or 94–106 of U6 snRNA had no effect on binding of 5'SS RNA oligo to U4/U5/U6 snRNP or U2/U4/U5/U6 snRNP complex [data not shown]. To rule out the possibility that U6f and U6g DNA oligos prevent U2/U4/U5/U6 snRNP complex formation by disrupting U4/U5/U6 snRNP, the stability of snRNP associations was analyzed in the presence of these oligos. The effect of adding increasing concentrations of U6g DNA
U4/U5/U6 snRNP recognizes the 5' splice site

5'SS RNA oligo bound to U4/U5/U6 snRNP is chased to U2/U4/U5/U6 snRNP complex

The experiments presented thus far show that the 5'SS RNA oligo binds stably to U4/U5/U6 snRNP in the ab-

Figure 4. U4/U5/U6 snRNP specifically recognizes the 5'SS RNA oligo. (A) Stable binding of wild-type 5'SS RNA oligo [wt] and 5'SS RNA oligos with single point mutations from positions -2 to +7 to U4/U5/U6 snRNP and U2/U4/U5/U6 snRNP complex in NE*. 5' end labeled RNA oligo was incubated under standard conditions in the presence of 2.5 μM 5'SS DNA oligo and resolved in a 4% polyacrylamide native gel. Positions of complexes formed between 5'SS RNA oligo and U4/U5/U6 snRNP or U2/U4/U5/U6 snRNP complex are indicated as U4/5/6 and U2/4/5/6, respectively. (B) The percent of wild-type 5'SS RNA oligo [wt] bound to U2/U4/U5/U6 snRNP complex [closed column] and U4/U5/U6 snRNP [open column] observed in A as a function of the single point mutation in the 5'SS RNA oligo.

Figure 5. 5'SS RNA oligo interacts with a region of U6 snRNA upstream of U4/U6 helix I. (A) Stable binding of 5'SS RNA oligo to U4/U5/U6 snRNP and U2/U4/U5/U6 snRNP complex is abolished in the presence of U6f or U6g DNA oligos. NE* was preincubated in the presence of 5'SS DNA oligo [2.5 μM] and U2e, U2f, U2g, U4a, U4b, U5c, U6f, or U6g DNA oligos [2.5 μM] for 15 min at 4°C (lanes 3–10). As controls, NE* was preincubated in the absence of any DNA oligos [lane 1] or in the presence of 2.5 μM 5'SS DNA oligo [lane 2]. 5' end labeled 5'SS RNA oligo was then added [10 nM] and the incubation continued for 45 min at 4°C. Binding of 5'SS RNA oligo to U2/U4/U5/U6 snRNP complex, U4/U5/U6 snRNP, or U1 snRNP was measured by resolving the reaction in a 4% polyacrylamide native gel. (B) U6g DNA oligo inhibits formation of U2/U4/U5/U6 snRNP complex without affecting U4/U5/U6 snRNP. NE* was preincubated in the presence of 0.25 μM 5'SS RNA oligo and 6.25 μM 5'SS DNA oligo and increasing concentrations of U6g DNA oligo. After incubation for 45 min at 30°C, the snRNP complexes were resolved in a 4% polyacrylamide native gel, transferred to a nylon membrane, and detected by hybridization with U4 snRNA probe. The amount of U4/U5/U6 snRNP [closed diamonds] and U2/U4/U5/U6 snRNP complex [open squares] in arbitrary units as a function of the concentration of U6g DNA oligo is shown.
The precursor-product relationship exists between U4/U5/U6 snRNP fraction. The resulting 5'SS RNA oligo-snRNP complexes were resolved in a native gel (Fig. 6A, lanes 1-5). As the concentration of U2 snRNP increased, binding of the 5'SS RNA oligo to U2/U4/U5/U6 snRNP complex increased and saturated at ~2.0 μl of U2 snRNP fraction, which is equivalent to ~1.3 μl of complete nuclear extract with regard to U6 snRNA (data not shown), and increasing amounts of the U2 snRNP fraction. The resulting 5'SS RNA oligo-snRNP complexes were resolved in a native gel (Fig. 6A, lanes 1-5). The ability of the 5'SS RNA oligo to induce the formation of U2/U4/U5/U6 snRNP complex in reactions containing U4/U5/U6 and U2 snRNP fractions was confirmed by Northern analysis (Fig. 6A, lanes 6,7). In the presence of 5'SS RNA oligo and 5'SS DNA oligo, a majority of U4/U5/U6 snRNP was found in U2/U4/U5/U6 snRNP complex (Fig. 6A, lane 7). 5'SS DNA oligo was included in these reactions because the U2 snRNP fraction was contaminated with U1 snRNP (data not shown). Thus, 5'SS RNA oligo induced the assembly of and bound to U2/U4/U5/U6 snRNP complex in reactions containing U4/U5/U6 and U2 snRNP fractions.

Figure 6. Binding of 5'SS RNA oligo to U4/U5/U6 snRNP can act as an intermediate along the pathway to U2/U4/U5/U6 snRNP complex formation. (A) 5'SS RNA oligo induces the formation of and binds to U2/U4/U5/U6 snRNP complex in the presence of glycerol gradient purified U2 and U4/U5/U6 snRNP fractions. U4/U5/U6 snRNP fraction (1 μl) was incubated in the presence of 5' end labeled 5'SS RNA oligo and increasing amounts of U2 snRNP fraction (0, 1.0, 1.5, 2.0, or 2.5 μl) under standard conditions in the presence of 2.5 μM 5'SS DNA oligo and resolved in a 4% polyacrylamide native gel (lanes 1-5). Positions of complexes formed between 5'SS RNA oligo and U4/U5/U6 snRNP or U2/U4/U5/U6 snRNP complex are indicated as U4/U5/U6 or U2/U4/U5/U6, respectively. U4/U5/U6 (1 μl) and U2 snRNP (2 μl) fractions were incubated in the absence (−, lane 6) or presence of 0.25 μM 5'SS RNA oligo and 6.25 μM 5'SS DNA oligo (+, lane 7). After incubation for 45 min at 30°C, the snRNP complexes were resolved in a 4% polyacrylamide native gel, transferred to a nylon membrane, and detected by hybridization with U4 snRNA probe. Positions of U4/U5/U6 and U2/U4/U5/U6 snRNP complexes are indicated as U4/5/6 and U2/4/5/6, respectively. 5'SS DNA oligo was included in these reactions because the U2 snRNP fraction was contaminated with U1 snRNP (data not shown). (B) 5'SS RNA oligo bound to U4/U5/U6 snRNP is chased to U2/U4/U5/U6 snRNP complex in reactions containing U4/U5/U6 and U2 snRNP fractions. Reactions containing 1 μl U5/U6 snRNP fraction were preincubated with 5' end labeled 5'SS RNA oligo (10 nm) in the absence of 5'SS DNA oligo (−) under standard conditions for 30 min at 4°C (lanes 1-4). A 500-fold excess of unlabeled 5'SS RNA oligo was added [lanes 2-4] and the incubation was continued for 30 min at 4°C in the absence [lane 2] or presence of 1 μl of nuclear extract [lane 3] or 2 μl of U2 snRNP fraction [lane 4]. BSA [0.1 mg/ml] was included as a nonspecific protein in binding reactions containing U4/U5/U6 snRNP fraction [lanes 1-4]. Note that the mobility of U4/U5/U6 snRNP is affected by differences in salt and/or protein concentrations in the binding reactions [cf. lanes 1,2 and 3,4]. Nuclear extracts were preincubated with 5' end labeled 5'SS RNA oligo (10 nm) for 30 min at 4°C in the absence [−, lanes 5,7,9] or presence [+, lane 6,8,10] of 2.5 μM 5'SS DNA oligo. A 500-fold excess of unlabeled 5'SS RNA oligo was added at t = 0 min [lanes 7,8] or at t = 30 min [lanes 9,10] and the incubation was continued for a total of 60 min at 4°C. The RNA-snRNP complexes were resolved in a 4% polyacrylamide native gel. Positions of complexes formed between 5'SS RNA oligo and U1 snRNP or U4/U5/U6 snRNP or U2/U4/U5/U6 snRNP complex are indicated as U1, U4/5/6 or U2/4/5/6, respectively.
Discussion

To define more specifically the interactions of the spliceosome with the 5'SS, we have used an in vitro system in which a short RNA oligonucleotide comprising the 5'SS consensus sequence (5'SS RNA oligo) is sufficient to bind U1 snRNP or to induce the association of U2 snRNP and U4/U5/U6 snRNP (Hall and Konarska 1992, Konforti et al. 1993). The 5'SS RNA oligo binds to U1 snRNP via base pairing with the 5' end of U1 snRNA. When this pairing is blocked, for example, by the addition of a DNA oligo which is complementary to the 5' end of U1 snRNA, the 5' SS RNA oligo induces the assembly of U2/U4/U5/U6 snRNP complex. In this in vitro system, recognition of the 5'SS by U1 snRNP is effectively uncoupled from subsequent recognition by the spliceosome.

Specificity of 5' SS recognition by U4/U5/U6 snRNP correlates with the 5' SS consensus sequence

Mutational analysis of the 5' SS demonstrates that recognition of the 5' SS by U4/U5/U6 snRNP complex correlates with the 5' SS consensus sequence derived from a compilation of all naturally occurring 5' splice sites (Senapathy et al. 1990). This correlation is also true for U2/U4/U5/U6 snRNP complex because the specificity of 5' SS recognition by U4/U5/U6 snRNP is indistinguishable from that displayed by U2/U4/U5/U6 snRNP complex (Fig. 4). Specifically, the binding and cross-linking properties of 5' SS mutant RNA oligos to U2/U4/U5/U6 snRNP complex and U6 snRNA, respectively, [Fig. 2C] are consistent with the degree to which positions −1 to +5 are conserved among natural 5' SS [see Fig. 1A for numbering of positions] and with in vivo experiments using full length pre-mRNAs [for review, see Green 1991; Guthrie 1991]. For example, the first two positions of the intron, G +1 and U +2 are invariant and 5' SS RNA oligos with all possible base substitutions at these positions, with the exception of U +2 → G, most dramatically reduced binding to U2/U4/U5/U6 snRNP complex and cross-linking to U6 snRNA [Fig. 2D]. These data are consistent with in vivo experiments using full-length pre-mRNAs which showed that all possible single point mutations at positions G +1 or U +2 reduced the efficiency of 5' SS cleavage [Aebi et al. 1987]. In addition, the requirement for a G at position +1 is consistent with recent genetic experiments in yeast which proposed a direct, non-Watson-Crick interaction between the gua-
nosines at the 5' (G1) and 3' (G303) splice sites (Parker and Silicicano 1993). Likewise, a G at positions −1 and +5 occurs in 81 and 85 percent of mammalian 5'SS, respectively [Senapathy et al. 1990] and mutations at these positions also significantly reduced binding to U2/U4/U5/U6 snRNP and cross-linking to U6 snRNA (Fig. 2C). Consistently, both in vitro and in vivo experiments showed that pre-mRNAs with a mutation at the +5 position significantly reduced 5'SS cleavage (Treisman et al. 1983; Krainer et al. 1984). Intron position 3 is a purine in 96% of mammalian introns. Thus, as expected, the A+3 → U mutation severely affected 5'SS recognition in 96% of mammalian introns. Thus, as expected, the recognition by U2/U4/U5/U6 snRNP complex does not appear to require specific interactions at this position.

Although recognition of the 5'SS by U1 snRNP is a major determinant of 5' splice site selection [Aebi et al. 1987; Nelson and Green 1990] and spliceosome assembly [Ruby and Abelson 1988; Rosbash and Seraphin 1991], only 4% of natural 5'SS are perfectly complementary to the first 9 nucleotides of U1 snRNA [Mount 1982, Ohshima and Gotoh 1987, Shapiro and Senapathy 1987]. Thus, as expected, all single point mutations in the 5'SS interacted with U1 snRNA as evidenced by cross-linking to U1 snRNA (Fig. 2A). Moreover, mutational analysis of the 5'SS demonstrates that the stability of the interaction between the 5'SS RNA oligo and U1 snRNP correlates with the stability of the RNA–RNA base pairing interactions between the 5'SS and the 5' end of U1 snRNA. This conclusion is based on binding experiments which showed that all single point mutants, with the exception of those positioned at the ends of the RNA duplex, failed to stably bind to U1 snRNP or U1 RNA oligo (Fig. 2A). Likewise, the 5'SS mutant RNA oligos cross-linked to U1 snRNA in the complete [Fig. 2B] and deproteinized [data not shown] nuclear extract indicating that the ability of 5' SS RNA oligos to interact with U1 snRNP and cross-link to U1 snRNA can be explained by RNA–RNA base pairing interactions. However, during pre-mRNA splicing this interaction is stabilized by additional factors, such as U1-specific proteins and multiple non-snRNP factors [Kohtz et al. 1994 and references therein].

Although genetic experiments in yeast demonstrate that base pairing interactions between the 5'SS and U6 snRNA are important for the specificity of 5'SS cleavage [Kandels-Lewis and Seraphin 1993; Lesser and Guthrie 1993], several lines of evidence suggest that other factors must be involved in recognition of the 5'SS. For example, the A+7 → C mutation which is predicted to decrease base pairing between the 5'SS and U6 snRNA according to the model proposed in mammals [Wassarman and Steitz 1992] had no effect on the ability of the 5'SS to interact with U2/U4/U5/U6 snRNP complex (Fig. 2C). Moreover, the 5'SS RNA oligo failed to cross-link to U6 snRNA in deproteinized nuclear extract [data not shown]. Finally, recognition by U2/U4/U5/U6 snRNP complex also appears to involve identification of the ribose backbone [Konforti et al. 1993]. Thus, RNA–RNA base pairing is not the only factor that influences the interaction between the 5'SS and U2/U4/U5/U6 snRNP complex.

**Temporal sequence of RNA–RNA interactions**

A summary of our findings with regard to the temporal order of interactions involving the 5'SS is schematically depicted in Figure 7A and the early steps in spliceosome assembly which these partial in vitro reactions are likely to mimic is shown in Figure 7B. Initially, the 5' end of U1 snRNA base pairs with the 5'SS to form a stable U1 snRNP–5'SS complex in vitro. Within the context of the full length pre-mRNA, this interaction recruits the RNA into commitment complexes [Ruby and Abelson 1988; Seraphin and Rosbash 1989; Michaud and Reed 1991; Jamison and Garcia-Blanco 1992] which are additionally stabilized by protein factors [Kohtz et al. 1994 and references therein]. This, together with an estimated 10-fold excess of U1 over U4/U5/U6 snRNPs, ensures that the 5'SS is initially recognized by U1 snRNP. Subsequently, base pairing between the 5' end of U1 snRNA and the 5'SS is disrupted [Konforti et al. 1993], which allows the 5'SS to interact with U4/U5/U6 snRNP. At this stage, the 5'SS interacts with a sequence in U6 snRNA upstream of stem I in the U4-U6 structure. In vitro the interaction between the 5'SS RNA oligo and U4/U5/U6 snRNP is detected in the absence of U2 snRNP and the resulting complex is converted to U2/U4/U5/U6 snRNP complex upon addition of U2 snRNP. Because the 5’S5–U4/U5/U6 snRNP intermediate does not accumulate in reactions with standard nuclear extracts and the 5'SS RNA oligo or the full-length pre-mRNA, the addition of U2 snRNP does not appear to be rate limiting in those cases. In fact, during pre-mRNA spliceosome assembly, early interaction between U1 and U2 snRNPs in complex A [Barabino et al. 1990] positions U2 snRNP near the 5'SS, promoting its association with U4/U5/U6 snRNP in this hypothetical intermediate complex and thus formation of splicing complex B (Fig. 7B). Before or concomitant with the first step of splicing, the U4–U6 snRNA interaction is disrupted [Pikielny et al. 1986; Cheng and Abelson 1987; Konarska and Sharp 1987; La- mond et al. 1988; Yean and Lin 1991] and replaced by a U2–U6 snRNA interaction [Madhani and Guthrie 1992]. Because the pairing between U6 and U2 snRNAs is mutually exclusive with pairing between U4 and U6 snRNAs within which U6 snRNA is proposed to be inactive [Guthrie and Patterson 1988], this rearrangement could represent the catalytic activation of the spliceosome [Madhani and Guthrie 1992]. Thus, spliceosome
assembly involves several conformational rearrangements which are apparently regulated with respect to timing.

**Materials and methods**

**Oligonucleotides and snRNA probes**

RNA oligonucleotides were synthesized on an Applied Biosystems 390 synthesizer using Milligen/Biosearch (Burlington, MA) and Glen Research (Sterling, VA) nucleoside phosphoramidites and purified as recommended by Applied Biosystems. The wild-type 5'SS RNA oligo, 5'-AAG/GUAAGUAdT-3', was the oligo to which all RNA oligos with single point mutations were compared. The nonspecific RNA oligo was a 10-mer of the sequence 5'-AUACUUACCUG-3', was complementary to wild-type 5'SS RNA oligo but lacked consensus sequence and committed the pre-mRNA to the splicing pathway. U1 snRNP recognizes the 5'SS RNA oligo and U4/U5/U6 snRNP interacts with the 5'SS in the absence of U2 snRNP (dashed bracket). Subsequent interaction between U4/U5/U6 and U2 snRNPs results in the formation of complex B. Although the intermediate in the dashed bracket has not been detected in vitro, the commitment complex, complex A and B are macromolecular complexes which have been distinguished biochemically and/or genetically. The relative positions of the snRNPs with respect to each other and the 5'SS RNA oligo or pre-mRNA are arbitrary. Other, non-snRNP factors which are required for spliceosome assembly are not shown.

A typical reaction mixture containing 5'-end labeled 5'SS RNA oligo was incubated at 4°C for 45 min. Note that the concentration of 5'SS RNA oligo in the absence of U2 snRNP [dashed bracket] was 0.4 μg/ml in our previous paper (Konforti et al. 1993). The resulting RNA oligo–snRNP complexes were resolved in a 12% polyacrylamide/60 mM Tris-glycine native gel at 4°C at ~20 V/cm for 3 hr, transferred to 3M Whatman paper, dried, and autoradiographed. For quantitation, RNA oligo–snRNP complexes were resolved in the same system at 12°C for 2.5 hr. Under these conditions, the input oligo remained in the gel and a PhosphorImager (Molecular Dynamics) was used for quantitation.

In all preparations of nuclear extract 5'SS RNA oligo induced the formation of and bound to U2/U4/U5/U6 snRNP complex. However, in one particular nuclear extract, NE*, 5'SS RNA oligo efficiently formed a stable complex with U4/U5/U6 snRNP. One possible explanation for this unusual property is the age of NE* (5 yr old). However, several more recently prepared nuclear extracts also show formation of a 5'SS RNA–U4/U5/U6 snRNP complex, although at a reduced efficiency in comparison to NE* (data not shown). Except for the apparent accumulation of 5'SS RNA–U4/U5/U6 snRNP complex, NE* behaved typically in all other respects including the ability to splice pre-mRNA (data not shown).

**Cross-linking assays**

A typical reaction mixture containing 5'-end labeled 5'SS RNA oligo was incubated for 45 min at 4°C. The reactions were UV-irradiated (wavelength 254 nm; Spectrolight ENF-260C) 3× 3 min in a microtiter plate on ice at a distance of 1 cm from the
Konforti and Konarska

lamp (—200 mW/cm²). The irradiated samples were deproteinized with protease K [Boehringer Mannheim] at 37°C for 30 min, followed by phenol extraction and ethanol precipitation, and resolved by electrophoresis in a 10% polyacrylamide–8 M urea gel [Bromophenol blue, 3 cm from the bottom of the gel]. The gel was directly autoradiographed and a PhosphorImager (Molecular Dynamics) was used for quantitation.

RNA–RNA duplex stability assay

A standard reaction (10 μl) contained 2 mM Mg acetate, 1 mM ATP, 5 mM creatine phosphate, 6 mM HEPES (pH 7.6), 30 mM KCl, 0.06 mM EDTA, and 6% (vol/vol) glycerol. A typical reaction mixture contained 0.5 pmole 5’ end labeled U1 RNA (or U6 RNA) and unlabeled 5’SS RNA oligo was titrated up to 50 pmole. The reactions were incubated at 4°C for 2 hr. The resulting RNA–RNA duplexes were resolved in a 20% polyacrylamide–50 mM Tris-glycine native gel at 4°C at 24 V/cm for 2 hr, transferred to 3 MM Whatman paper, dried, and autoradiographed. A PhosphorImager (Molecular Dynamics) was used for quantitation.

Northern hybridization analysis

A standard reaction (10 μl) contained 1–2 μl of a HeLa nuclear extract [Dignam et al. 1983], 2 mM Mg acetate, 1 mM ATP, 5 mM creatine phosphate, 6 mM HEPES (pH 7.6), 30 mM KCl, 0.06 mM EDTA, and 6% (vol/vol) glycerol. A typical reaction mixture containing 0.25 mM 5’SS RNA oligo and 6.25 mM 5’SS DNA oligo was incubated at 30°C for 45 min. The resulting RNA oligo–snRNP complexes were resolved in a 4% polyacrylamide–50 mM Tris-glycine native gel at 4°C at ~20 V/cm for 3 hr. RNA was transferred to Hybond-N (Amersham) by electroblotting at 4°C for 16 hr in TAE buffer. RNAs were cross-linked to the membrane using a UV Stratalinker 2400 (Stratagene) and hybridization was carried out as described (Konarska and Sharp 1987). A PhosphorImager (Molecular Dynamics) was used for quantitation.

Gradient sedimentation

For the gradient sedimentation analysis, 110 μl of nuclear extract was sedimented through a 15–35% glycerol–50 mM Tris-glycine gradient in an SW 41 rotor at 40K rpm for 17 hr at 4°C. Gradients were divided into 80 fractions (150 μl each). Typically, 25-μl aliquots of fractions 10–60 were analyzed by native gel electrophoresis followed by Northern hybridization with U1, U2, or U4 snRNA probes. Fractions containing U2 or U4/5’U6 snRNPs were pooled and concentrated by sedimentation at 60K rpm for 12 hr at 4°C in a TLA100.3 rotor in a TL100 Ultracentrifuge [Beckman]. snRNP pellets were resuspended in snRNP-free supernatant of nuclear extract on ice for several hours and aliquots were frozen at ~70°C. These partially purified U2 and U4/5’U6 fractions were analyzed in native and denaturing gels followed by hybridization with U1, U2, U4, U5, and U6 snRNA probes. The U2 snRNP fraction (1 μl) was equivalent to ~0.65 μl, ~0.3 μl, and ~0.14 μl of complete nuclear extract with regard to U2, U1, and U6 snRNAs, respectively, as determined by denaturing gel electrophoresis followed by quantitative Northern hybridization (data not shown). Likewise, 1 μl of the U4/5’U6 snRNP fraction was equivalent to ~0.65 μl, ~0.01 μl, and ~0.01 μl of complete nuclear extract with regard to U6, U2, and U1 snRNAs (data not shown).

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B B Konforti and M M Konarska

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