Antisense probes containing 2-aminoadenosine allow efficient depletion of U5 snRNP from HeLa splicing extracts

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ABSTRACT

RNA duplexes containing the modified base 2-amino-adenine in place of adenine are stabilized through the formation of three hydrogen bonds in 2-amino A·U base pairs. Antisense 2'-O-alkyloligoribonucleotide probes incorporating 2-aminoadenosine are thus able to efficiently affinity select RNP particles which are otherwise inaccessible. This has allowed the efficient and specific depletion of U5 snRNP from HeLa cell nuclear splicing extracts. U5 snRNP is shown to be essential for spliceosome assembly and for both steps of pre-mRNA splicing. The absence of U5 snRNP prevents the stable association of U4/U6 but not U1 and U2 snRNPs with pre-mRNA.

INTRODUCTION

The splicing of nuclear messenger RNA precursors (pre-mRNA) occurs within a multicomponent structure termed the spliceosome. The major components of the spliceosome belong to the U-class of small nuclear ribonucleoprotein particles (U snRNPs), specifically U1, U2, U4/U6 and U5 snRNPs. These four snRNPs, together with non-snRNP protein factors, assemble along an ordered pathway to form a functional spliceosome (for recent reviews see 1–4). A combination of affinity selection methods and native gel analyses have shown that the U1, U2, U4/U6 and U5 snRNPs are present in splicing complexes (5–14). In order to study the roles of the different snRNP particles in the splicing mechanism, a variety of methods have been used to inactivate individual snRNP particles in *in vitro* splicing extracts. These include inhibition by snRNP-specific antibodies (15–17), site-specific cleavage of snRNA components by RNase H in the presence of complementary DNA oligonucleotides (17–21), and masking specific snRNA domains using antisense 2'-O-methyl oligoribonucleotides (2'-OMe RNA) (22–24). In Saccharomyces cerevisiae it has also been possible to exploit a genetic approach in which expression of a specific snRNA gene is placed under the control of the inducible β-gal promoter. This has allowed *in vivo* depletion of specific snRNP species (25–27). Using one or more of the above methods, U1, U2 and U4/U6 snRNPs have been inactivated and shown to be essential for both splicing and spliceosome formation. However, it has proved difficult to similarly demonstrate the requirement for U5 snRNP. In particular, it has not been shown that U5 snRNP is required for functional spliceosome assembly. *In vivo* depletion studies in yeast and *in vitro* reconstitution studies with HeLa splicing extracts demonstrated a strict requirement for U5 snRNP to complete the second step of the splicing reaction (27, 28). Interestingly, however, in neither of these studies was it clear whether U5 snRNP is an obligatory participant in the first step of the splicing reaction. Furthermore, it was not established to what extent U5 snRNP influences the assembly of other spliceosomal snRNPs with pre-mRNA or if U5 snRNP independently assembles into the spliceosome or joins it as part of a U4/U5/U6 triple snRNP complex. (9, 10). In Saccharomyces cerevisiae, antibodies specific for the PRP8 gene product have been shown to immunoprecipitate either U5 snRNP and/or the U4/U5/U6 triple snRNP particle (29). Immunodepletion of PRP8 from yeast splicing extracts has also been shown to inhibit splicing (30). However, although a mammalian homologue of the yeast PRP8 protein has been identified by cross reaction on protein blots with the anti-PRP8 antibody (31–33), this antibody does not efficiently immunoprecipitate U5 snRNP in HeLa splicing extracts. The lack of specific antibodies against mammalian U5 snRNP and the inaccessibility of U5 snRNA to oligonucleotide directed RNase H cleavage has made it difficult to study the role of U5 snRNP in mammalian *in vitro* splicing extracts (34). As an alternative approach we have previously shown that biotinylated 2'-O-alkyl oligoribonucleotides can be used as stable antisense probes for the affinity selection or depletion of targeted snRNP particles (23, 24, 35, 36). Using this approach, HeLa cell nuclear splicing extracts have been prepared that are efficiently depleted of either U1, U2, or U4/U6 snRNPs (35). Here we report on a development of this antisense approach that allows affinity selection of the previously inaccessible U5 snRNP particle and in principal any RNP which has only short sequences available for hybrid formation. The consequences of U5 depletion on pre-mRNA splicing and splicing complex formation are described and discussed.

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MATERIALS AND METHODS

Materials

Restriction enzymes were purchased from New England Biolabs (Beverly, MA), RNAsin from Promega (Madison, WI) and RNase H from BRL (Bethesda, MD). Streptavidin agarose beads were purchased from Sigma (St. Louis, MO), α-^{32}P rUTP and Hybond membrane were purchased from Amersham International plc (UK). T3 and T7 RNA polymerases were purchased from Stratagene (La Jolla, CA). HeLa cells used to prepare splicing extracts were purchased from the Computer Cell Culture Centre (Mons, Belgium). Autoradiography film (XAR 5) was purchased from Kodak (Rochester, NY).

Oligonucleotides

Oligonucleotides were synthesized essentially as described (37) on an Applied Biosystems synthesizer, model 380B-02 (Foster City, CA). Oligo(2'-O-allylribonucleotides) were synthesized as described for the 2'-O-methyl analogues (38) using a coupling time of 8 min. and multiple biotinylation when required was performed during the solid phase synthesis (39). The 2'-O-allyl-2-aminoadenosine monomer which is insoluble in pure acetonitrile, was dissolved in THF/acetonitrile (1:1 v/v) for synthesis. The capping reaction for oligomers containing 2'-O-allyl-2,6-diaminopurine riboside must be carried out with phenoxyacetic anhydride/N-methylimidazole instead of acetic anhydride to avoid a transamination reaction (40).

snRNP Depleted Extracts

Conditions for obtaining U5 depletion were essentially as described for the preparation of a U4/U6Δ extract (35) but with the following changes. The concentration of oligonucleotide #263 for obtaining optimum U5 depletion was titrated empirically and found to be approximately 1.2 nmol/ml of HeLa cell nuclear extract. During the two affinity selection steps, the ratio of streptavidin agarose beads to nuclear extract was 1:1 (volume refers to beads in suspension and not the packed volume). A non-depleted control extract not incubated with antisense oligonucleotide was prepared in parallel. Typical protein concentration in the depleted extract was in the range of 5−7 mg/ml.

In Vitro Splicing Assays

Splicing assays were carried out using uniformly labelled, capped pre-mRNA incubated with nuclear extracts for 3 hours at 30°C under standard *in vitro* splicing conditions (41). The total reaction volume was 30 μl. Wild type adeno pre-mRNA was transcribed from Sau3A-digested plasmid pBSAdl (10) and globin pre-mRNA was transcribed from a PvuI digested plasmid pBSGloI using T3 RNA polymerase. Splicing reconstitution assays were performed by mixing equal volumes of snRNP-depleted nuclear extracts; the total volume of nuclear extract used comprised 30% of the total reaction volume.

Gel Electrophoresis and Northern Hybridization Analyses

Splicing products were separated on 7% polyacrylamide-8M urea denaturing gel, run in 1×TBE, and splicing complexes were separated on nondenaturing agarose-polyacrylamide composite gels, as described (41). For the snRNP gel in Fig. 2B extracts were incubated under splicing conditions for 20 minutes and separated on an agarose-polyacrylamide composite gel for 2.5 hours. Gels were electroblotted onto Hybond membrane and hybridized with U5 and U1 snRNA specific riboprobes as described (35).

Pre-mRNA Affinity Selection Assays

Pre-mRNA selections were performed as described (14). The sequence (5'-3') of the biotinylated oligonucleotide targeted against the intron of adeno pre-mRNA was IACCAIAUIIACI-CIICC and carried two biotins at the 5' end.

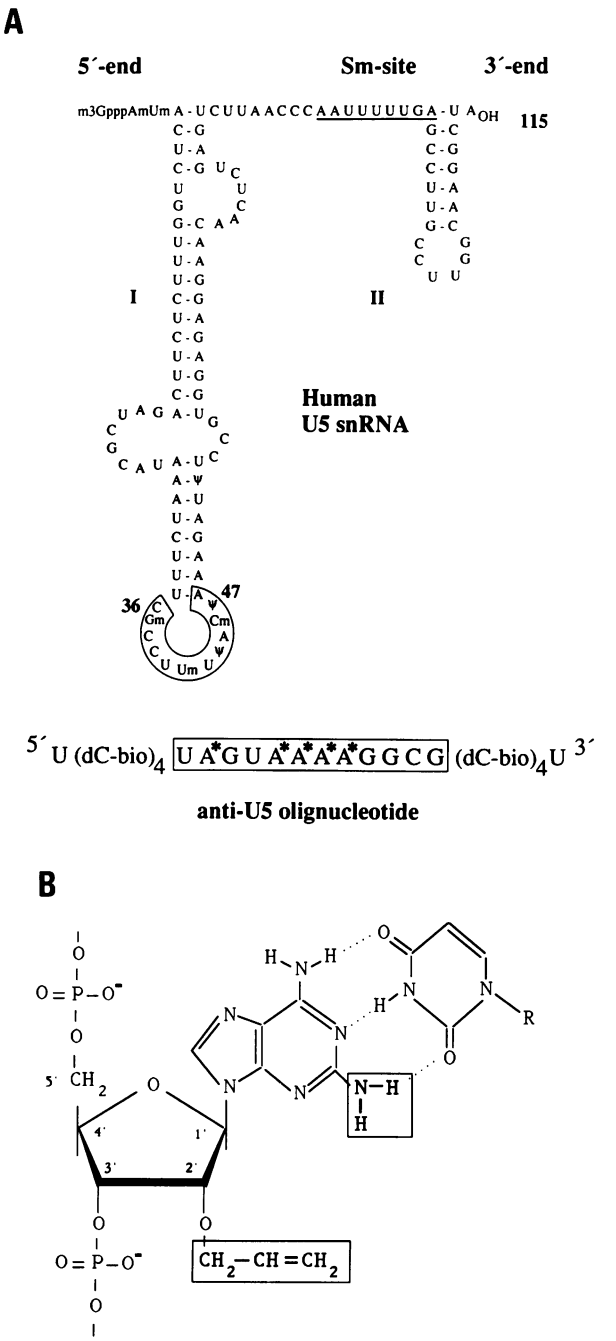

Figure 1. Antisense Targeting of U5 snRNP. **(A)** Secondary structure model of human U5 snRNA drawn according to the phylogenetic consensus structure of Krol et al. (47). U5 nucleotides complementary to the anti U5 oligonucleotide (oligonucleotide #263) are shown boxed (nts. 36−47). The sequence of oligonucleotide #263 is shown below the U5 structure. 5' and 3' terminal biotin residues (indicated as dC-bio) are attached to a modified deoxycytidine nucleotide by a long carbon spacer arm (39). Positions of the five 2'-O-allyl 2-aminoadenosine residues are indicated by asterisks. **(B)** Structure of 2'-O-allyl 2-aminoadenosine (37) shown forming three hydrogen bonds with uracil. Position of the additional NH_2 group on the adenine base is shown highlighted in a square box. Structure of the 2'-O-allyl group is shown highlighted in a rectangular box.

RNase H Assays

RNase H protection assays were performed essentially as described (35). Sequences of adeno pre-mRNA complementary DNA oligonucleotides used were (5′ to 3′):

# 142 (5′ss):	ACTCACCCCAAC
# 151 (I):	TACTGCGCCCTA
# 153 (PPy):	GGAAAAAAAAGG
# 152 (BP):	ACAGGATAAGTA
# 154 (3′ss):	CGCGAGCTGTGG

RESULTS

Targeting of U5 snRNP

Two short regions of U5 snRNA have been shown to be accessible to chemical modification within the intact U5 snRNP particle (34) and these were focussed on to identify target sites for binding antisense probes. A set of overlapping oligonucleotide probes identified a region of twelve nucleotides in U5 snRNA (nt. 36−47) as being the most accessible (Fig. 1A). For optimum efficiency of affinity depletion it was essential to stabilize the hybrid between the antisense probe and the accessible, short, U-rich region of U5 snRNA. Replacement of adenosine with 2-aminoadenosine (Fig. 1B) allows the formation of a third hydrogen bond in 2-aminoA:U base pairs, resulting in an increased Tm of hybrid formation (42). This is particularly effective in RNA duplexes, which form A type helices. In B type helices formed by DNA duplexes, stabilization resulting from the extra H-bond in 2-aminoA:T base pairs is counteracted by a destabilization effect resulting from disruption of the spine of hydration (42). The use of 2-aminoadenosine is therefore well suited to the antisense approach based on 2′-O-alkyl RNA (22, 23, 24, 35, 36), rather than DNA oligonucleotides.

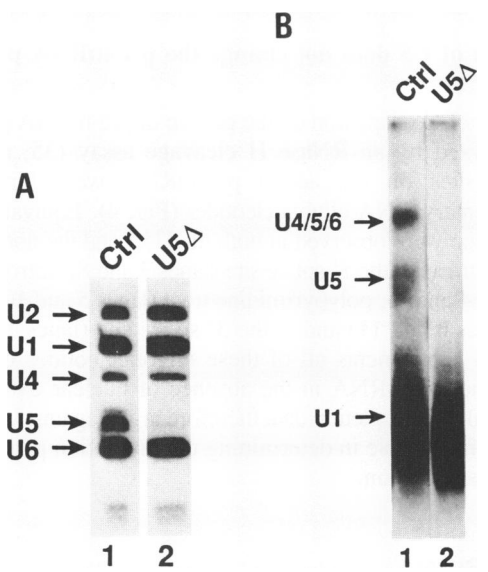

Figure 2. Antisense Affinity depletion of U5 snRNP. **(A)** RNA recovered from snRNP depleted HeLa cell nuclear extracts is shown separated on a 10% polyacrylamide-8M urea denaturing gel, detected by Northern hybridization with U snRNA specific riboprobes. Lane 1 shows a non-depleted control nuclear extract. Lane 2 shows an extract depleted with the oligonucleotide #263 of U5 snRNA. **(B)** Characterization of U5 snRNP-depleted (U5Δ) extract by non-denaturing gel electrophoresis. Lane 1 shows a non-depleted control nuclear extract and lane 2 a U5Δ nuclear extract.

Efficient depletion of U5 snRNP

The most efficient affinity depletion was obtained using a probe in which all five adenosine residues were replaced with 2-aminoadenosine and which carried four biotin moieties (39) at both the 5′ and 3′ termini (cf. Fig. 1A). This probe was used to remove U5 snRNP from a HeLa cell nuclear splicing extract according to the antisense affinity depletion procedure previously described (35) (for details see Fig. 2 methods). Figure 2A shows the level of spliceosomal snRNAs present in the non-depleted control (lane 1) and in the U5-depleted (U5Δ) nuclear extract (lane 2). Figure 2B shows the level of U5 snRNP in a non-depleted control extract (lane 1) and in an extract depleted of U5 snRNP (lane 2), analyzed by non-denaturing gel electrophoresis. Depletion levels of both U5 snRNA (Fig 2A) and snRNP (Fig 2B) were above 95%, as determined by quantitative Northern hybridization analyses. Little change in the level of the non-targeted RNA species was observed (Fig 2A and other data not shown). In Fig 2B, the removal of U5 snRNP also resulted in a loss of the U4/U5/U6 triple snRNP band with little or no change in the level of U1 snRNP which was probed in parallel as an internal control. From these data we conclude that the use of 2-aminoadenosine has allowed us to prepare an extract efficiently and specifically depleted of U5 snRNP.

U5 snRNP is essential for splicing

The U5Δ extract was assayed for its ability to splice exogenously added pre-mRNA (Fig. 3A) and to assemble spliceosomes (Fig. 3B). No splicing of a rabbit β-globin pre-mRNA substrate was observed in a U5Δ extract (Fig. 3A, lane 2), although the same substrate was spliced in a non-depleted control extract (Fig. 3A, lane 1). Complementation experiments showed that splicing activity can be restored by mixing the U5Δ extract with extracts specifically depleted of either U1 or U4/U6 snRNP (Fig 3A, lanes 5 & 6). The U4/U6 and U1 depleted extracts alone are unable to splice pre-mRNA (ref. 35 and Fig. 3A, lanes 3 & 4). Splicing could also be restored by complementing the U5Δ extract with an extract depleted uniquely of U2 snRNP (data not shown) and identical results were obtained using an adeno pre-mRNA substrate (data not shown). These complementation studies demonstrate that the lack of splicing activity in the U5Δ extract can be attributed to the absence of U5 snRNP and is not due to nonspecific inhibitory effects resulting from the depletion strategy.

The U5Δ extract was also tested for spliceosome formation on the adeno pre-mRNA substrate using a nondenaturing gel mobility retardation assay (Fig. 3B). No spliceosome formation was observed in the U5Δ extract (Fig. 3B, lane 2). Instead, accumulation of a complex which approximately comigrated with the presplicing complex 'A' was detected. A similar effect was observed in a U4/U6Δ extract (ref 35 and Fig. 3B, lane 3). Parallel restoration of splicing activity and spliceosome formation was observed upon mixing U5Δ and U4/U6Δ extracts (Fig. 3 lane 5), U5Δ and U2Δ extracts (Fig. 3 lane 6), U4/U6Δ and U2Δ extracts (Fig.3 lane 7) and U5Δ and U1Δ extracts (data not shown). We conclude that U5 snRNP is essential for spliceosome formation.

U5 affects snRNP binding to pre-mRNA

The ability of snRNPs present in the U5Δ extract to bind to splicing substrates was assayed by selecting the pre-mRNA with a biotinylated 2′-OMe oligoribonucleotide (14). In a non-depleted control extract, U1, U2, U4, U5 and U6 snRNAs were detected

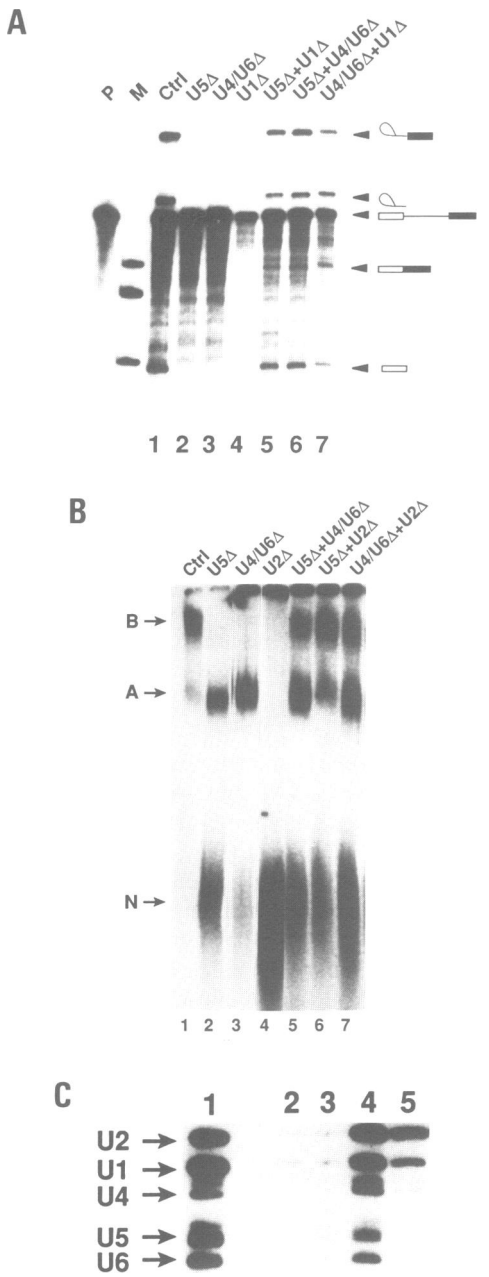

Figure 3. Pre-mRNA Splicing, Splicing Complex Formation and Affinity Selected Splicing Complexes in a U5Δ Nuclear Extract. **(A)** β-Globin pre-mRNA splicing products were separated on a 7% polyacrylamide-8M urea denaturing gel. Lane 1 shows a splicing reaction with a non-depleted control nuclear extract. Lanes 2,3 and 4 show splicing reactions with U5Δ, U4/U6Δ and U1Δ nuclear extracts, respectively. Lanes 5–7 show splicing reactions with combinations of two different snRNP-depleted extracts: U5Δ+U1Δ (lane 5), U5Δ+U4/U6Δ (lane 6) and U4/U6Δ+U1Δ (lane 7). Markers are end-labelled Msp1-digested pBR322 fragments (M) and full length pre-mRNA (P). **(B)** Splicing complexes assembled on adeno pre-mRNA were separated on an agarose-polyacrylamide composite gel. Lane 2, 3 and 4 show splicing reactions in a U5Δ, U4/U6Δ and U2 snRNP-depleted nuclear extract, respectively. Lanes 5–7 show reactions incubated with pairwise combinations of different snRNP-depleted nuclear extracts: U5Δ+U4/U6Δ (lane 5) U5Δ+U2Δ (lane 6), and U4/U6Δ+U2Δ (lane 7). Lane 1 shows a splicing reaction with a non-depleted control nuclear extract. **(C)** Affinity selected snRNAs using adeno pre-mRNA and an anti-intron probe under splicing conditions were separated on a 10% polyacrylamide-8M urea denaturing gel. Lane 4 shows snRNAs selected in a non-depleted control nuclear extract. Lane 5 shows snRNAs selected in a U5Δ nuclear extract. Controls include incubation of pre-mRNA without anti-intron probe (lane 2) and *vice versa* (Lane 3). Markers (Lane 1) correspond to total HeLa nuclear RNA.

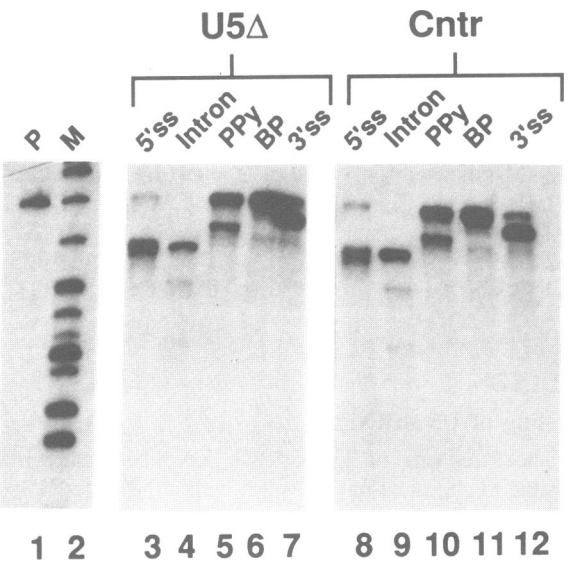

Figure 4. RNase H Protection Assay. The pre-mRNA protection pattern at regions containing the 5′ splice site (lanes 3 and 8), intron (lanes 4 and 9), polypyrimidine tract (lanes 5 and 10), branch site (lanes 6 and 11), and 3′ splice site (lanes 7 and 12) are shown in a U5 snRNP-depleted and a non-depleted control nuclear extract. Size markers are end-labelled Msp1-digested pBR322 fragments (lane 2) and full-length adenovirus pre-mRNA (lane 1).

binding to pre-mRNA (Fig. 3C, lane 4). In the U5Δ extract only U1 and U2 and not U4 or U6 snRNAs were detected (lane 5). Control experiments, including incubation of pre-mRNA without oligonucleotide (Fig. 3C, lane 2), and incubation of oligonucleotide without pre-mRNA (Fig. 3C, lane 3) showed no snRNA selection. We conclude that U4/U6 snRNP cannot stably assemble with pre-mRNA in the absence of U5 snRNP. This is consistent with the U4/U5/U6 triple snRNP particle being a functional intermediate during spliceosome assembly (9, 10, 29).

Depletion of U5 does not change the pre-mRNA protection pattern

The effect of U5 depletion on the pattern of pre-mRNA protection was analyzed by an RNase H cleavage assay (35, 43). Five separate sites on the adeno pre-mRNA were targeted by complementary DNA oligonucleotides (Fig. 4). Equivalent levels of protection were observed in both the U5Δ and the non-depleted control extracts at the 5′ splice site (lanes 3 and 8), intron internal site (lanes 4 and 9), polypyrimidine tract (lanes 5 and 10), branch point (lanes 6 and 11) and at the 3′ splice site (lanes 7 and 12). In control experiments all of these oligonucleotides efficiently cleaved the pre-mRNA in the absence of nuclear extract (data not shown). The depletion data therefore argue against U5 snRNP playing a major role in determining the protection pattern at the 3′ splice site region.

DISCUSSION

We have shown that the incorporation of 2-aminoadenosine into an antisense probe made of 2′-O-allyl RNA allows efficient affinity depletion of U5 snRNP from a HeLa cell nuclear splicing extract. As the depletion of U5 snRNA is not accompanied by significant depletion of U4 or U6 snRNAs it is unlikely that we are observing direct selection of the U4/U5/U6 triple snRNP

particle. Instead, it is likely that the association of U5 with the U4/U6 snRNP is not stable under the high salt conditions used for the affinity depletion procedure. Alternatively, the presence of the antisense probe may affect the level of triple snRNP in the extract.

The sole region in U5 snRNA that we have found accessible to an antisense probe (nts. 36–47) is also highly conserved phylogenetically. Interestingly, a recent study in Saccharomyces cerevisiae showed that point mutations in this same region of U5 snRNA can suppress 5′ splice site mutations of a yeast CYH2 pre-mRNA *in vivo* by affecting the position of 5′ splice site cleavage (44). The accessibility of this region to an antisense oligonucleotide could indicate that it is not the binding site for a U5 snRNP protein, but may rather interact with other components of the splicing apparatus or even with the pre-mRNA directly.

Two earlier studies have also addressed the requirement for U5 snRNP for pre-mRNA splicing (27, 28). It was shown in yeast that *in vivo* depletion of U5 snRNA results in the accumulation of unspliced pre-mRNAs (27). However, in this case it was unclear whether U5 was absolutely required for the first step of the splicing reaction since with some precursors *in vivo* accumulation of the lariat-3′ exon intermediate was observed (27). A subsequent, *in vitro* study using snRNP-enriched HeLa cell nuclear fractions demonstrated that a large reduction in the U5 snRNP concentration did not completely inhibit the first step of splicing but did absolutely block the second step (28). Our results extend these studies in several respects. First, we show here more clearly that U5 snRNP is absolutely required for both steps of the splicing reaction. A likely explanation for this is the high efficiency of U5 depletion achieved by the antisense affinity depletion method. A depletion level of over 95% is required for both steps of the splicing reaction to be blocked (our unpublished observations). Second, we establish that U5 snRNP is required not only for splicing, but also for spliceosome assembly.

Characterization of the U5 snRNP-depleted extract has demonstrated that U5 removal prevents spliceosome assembly on several different pre-mRNAs. Removal of U5 prevents stable association of the U4/U6 snRNP with pre-mRNA. This result, together with our previous observation (35) that removal of U4/U6 snRNP prevents stable binding of U5 to pre-mRNA, strongly supports the model that U4/U6 and U5 join the spliceosome as a pre-formed U4/U5/U6 triple snRNP (9, 10, 29). The present data also demonstrate that U5 snRNP is not required to promote stable binding of U1 and U2 snRNPs to pre-mRNA.

We note that recent studies in yeast support our results and come to similar conclusions. Séraphin et al. (45) prepared U5 snRNP-depleted yeast extracts and Brown and Beggs (46) used *in vivo* depletion of the yeast U5-specific protein PRP8 to show that U5 snRNP is required for both steps of splicing and for spliceosome assembly.

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