Requirements for U2 snRNP addition to yeast pre-mRNA

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ABSTRACT

The *in vitro* spliceosome assembly pathway is conserved between yeast and mammals as U1 and U2 snRNPs associate with the pre-mRNA prior to U5 and U4/U6 snRNPs. In yeast, U1 snRNP-pre-mRNA complexes are the first splicing complexes visualized on native gels, and association with U1 snRNP apparently commits pre-mRNA to the spliceosome assembly pathway. The current study addresses U2 snRNP addition to commitment complexes. We show that commitment complex formation is relatively slow and does not require ATP, whereas U2 snRNP adds to the U1 snRNP complexes in a reaction that is relatively fast and requires ATP or hydrolyzable ATP analogs. *In vitro* spliceosome assembly was assayed in extracts derived from strains containing several U1 snRNA mutations. The results were consistent with a critical role for U1 snRNP in early complex formation. A mutation that disrupts the base-pairing between the 5' end of U1 snRNA and the 5' splice site allows some U2 snRNP addition to bypass the ATP requirement, suggesting that ATP may be used to destabilize certain U1 snRNP:pre-mRNA interactions to allow subsequent U2 snRNP addition.

INTRODUCTION

During pre-mRNA splicing *in vitro*, an ordered assembly process is observed during which snRNPs and protein factors associate with the pre-mRNA substrate to form a mature spliceosome within which the cleavage and ligation events take place (1–6). The same two regions of the substrate that are important for splicing are also important for spliceosome assembly, namely, the 5' and 3' splice site regions (e.g., 7–9). The former consists of a 6–9 nucleotide consensus sequence at the 5' end of the intron, whereas the latter contains three subregions, the branchpoint sequence, a polypyrimidine-rich sequence, and the 3' splice site PyAG (for review, see: 10).

The four splicing snRNPs contain 5 snRNAs (U1, U2, U5, and U4/U6 snRNA), which are quite conserved between mammals and yeast (10). The order of snRNP addition is also conserved: U1 and U2 snRNPs associate with the substrate prior

to addition of the U4/U5/U6 triple snRNP (11,12). We have focused on the association of U1 and U2 snRNP with pre-mRNA because an understanding of these early assembly events is likely to illuminate such biological issues as intron recognition and splice site partner assignment.

In both yeast and mammalian systems, U1 snRNP's interaction with the pre-mRNA substrate is mediated at least in part by base pairing between the highly conserved 5' end of U1 snRNA and the 5' splice site (13–15). U2 snRNP's interaction with the substrate similarly involves base pairing, in this case with the branchpoint sequence (16–18). Whereas these two interactions might suggest independent events at the two ends of the intron, recent evidence suggests that they are sequential and that, for mammals as well as for yeast, U1 snRNP is required for the ATP-dependent addition of U2 snRNP during spliceosome assembly (19,20). This requirement implicates at least an indirect interaction between U1 snRNP and the branchpoint region prior to U2 snRNP addition.

For the yeast system, several lines of direct evidence indicate that U1 snRNP forms a stable complex with pre-mRNA and interacts with both the 5' and the 3' regions independently of U2 snRNP. We originally showed that both highly conserved intron sequences, the 5' splice site and the branchpoint, are required *in cis* to compete optimally for early splicing factors and to 'commit' a pre-mRNA substrate to the *in vitro* splicing pathway. That study also indicated that this commitment step does not require added ATP nor functional U2 snRNP, and that it is necessary for the ATP-dependent binding of U2 snRNP (21). Similar conclusions were reached with affinity chromatography assays (22). Procedures were then developed for the 'genetic' depletion of functional U2 snRNP and direct visualization of the resulting U1 snRNP-pre-mRNA ('commitment') complexes by gel electrophoresis (19). The two observed commitment complex bands (CC1 and CC2; 23) were shown to be extremely stable and to be precursors of functional splicing complexes, as the addition of U2 snRNP 'chased' commitment complexes into functional spliceosomes (19).

In this communication, we examine some additional features of, and requirements for, U2 snRNP addition. The results support and extend our current view of the early events in the assembly of spliceosomes.

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MATERIAL AND METHODS

Strains

The yeast strain BS-Y20 (*MATa*, *leu2-3*, *leu2-112*, *ura3-52*, *trp1-289*, *arg4*, *ade2*, *snr19::LEU2*, p23) (19) was used as the host strain for transformation with different plasmids carrying either the wild-type U1 gene (pXL8) or mutant U1 genes (pCEN-U1-4U or pXL8 derivatives) (14,24). For the experiment in Figure 4A, the merodiploid strains expressing both the wild-type U1 gene (from p23) and the mutant U1 genes (from pXL8 derivatives) were used for extract preparations. For other experiments (except for Figure 4B), haploid strains carrying only pXL8, pXL8 derivatives or pCEN-U1-4U were obtained after selection for the loss of p23 with 5-fluoro-orotic acid (25). Unless otherwise noted, the wild-type extract was always derived from the strain XLY16, which is isogenic to BS-Y20 except with pXL8 instead of p23.

For the experiment in Figure 4B, a plasmid (pXL46, or GAL-U1WT) carrying the wild-type U1 gene under the control of GAL-UAS replaced p23 in BS-Y20 to create the strain XLY135, which was maintained in the medium containing 3% galactose and 1% sucrose and used for transformation with different plasmids carrying either the wild-type U1 gene (pXL8) or mutant U1 genes (pXL8 derivatives). The merodiploid strains were shifted from the medium containing galactose to the medium containing 4% glucose for 16 h to repress the expression of the GAL-U1WT gene before extracts were prepared. The procedure is referred to as GAL-depletion and these extracts as GAL-depleted extracts. The construction of pXL46 and that of the deletion Δ VII-VIII were described elsewhere (Stutz *et al.* submitted); the combination of Δ LII and Δ VII-VIII in the same U1 snRNA gene was achieved as described for all other U1 mutations or mutation combinations (24).

For the experiment in Figure 7, the extract containing the pseudowild-type U2 snRNP was made from the yeast strain HI70 (*MATa*, *leu2-3*, *leu2-112*, *ura3-52*, *his4-619*, *lys2*, *snr20::URA3*, YCpLYS2-U2C121U), a kind gift of M. Ares, Jr. (30).

Splicing extracts

For the experiments shown in Figures 2, 3 and 4, the glass-bead miniextract procedure was used for preparing *in vitro* splicing extracts (19); modified in (26). For all other experiments, a newly modified procedure was used. Spheroplasts were prepared according to Lin *et al.* (27) from a 500 ml culture with O.D._{600nm} = 1–3. After the cells had been resuspended in Buffer A, they were vortexed with glass beads in aliquots corresponding to 100 ml of original culture each. From that point on, the original miniextract procedure was followed. The new procedure gave extracts with significantly more activity for both complex formation and splicing.

Fractionation of extracts

Extracts were fractionated by centrifugation as in (26) except: 1) the KCl was at 50 mM (the normal concentration in the extract); 2) the centrifugation was for 4 h; and 3) Buffer D contained 0.05% Nonidet P-40 for resuspending the pellets.

In vitro assembly and gel analysis

Radioactively-labeled wild-type pre-mRNA substrate (WT- Δ 2) was synthesized as described previously (23). The *in vitro* transcription reactions for biotinylated substrate contained biotin-UTP as 15% of the UTP (28). *In vitro* assembly and splicing

reactions were as described (23) unless otherwise noted. For the experiments shown in Figure 6, endogenous ATP was depleted by preincubating the splicing extract or the pellet fraction of the same extract in the presence of splicing salts and 0.2 mM glucose at 25°C for 10 min. The ATP analogs used in Figure 6 are: cordycepin-5'-triphosphate (3' dATP), ATP- α -S, ATP- γ -S, adenylyl-(β , γ -methylene)-diphosphonate (AMP-PCP), and adenylyl-imidodiphosphate (AMP-PNP).

With the exception of the gels in Figures 3C and 4A, which did not contain glycerol, native gel analysis was as described (23) and analysis of splicing products was as in Abovich *et al.* (26).

In vitro assembly using U2-killed extract

U2-killed extract was obtained by incubating the splicing extract with the oligonucleotide RB60, which is complementary to nucleotides 29–43 of the U2 snRNA (21,29). Typically, 4 μ l of extract are incubated with 3 μ l of splicing salts and 0.2 μ g of RB60 at 25°C for 10 min followed by addition of 1 μ l each of splicing salts, ATP, and pre-mRNA and a further incubation of 20 min to form commitment complexes.

Assay for preassociation of U2 snRNP with commitment complexes

Commitment complexes were formed in large (100–200 μ l) splicing reactions, containing either wild-type or HI70 extract (HI70 contains a 'pseudowild-type' U2 snRNA gene; 30) or a 1:1 mixture of the two. After a 5-min preincubation with 0.5 mM glucose in the absence of substrate, 1 ng biotinylated or non-biotinylated pre-mRNA was added per μ l of reaction mix. After a further incubation for 30 min at 25°C, 25 μ l aliquots of the reactions were removed and treated as follows. To all aliquots containing biotinylated pre-mRNA, a 15-fold excess of non-biotinylated pre-mRNA was added. When two reactions containing non-biotinylated RNA were to be combined, one received the 15-fold excess of RNA at this point. After a brief incubation (less than 1 min), two reaction aliquots were combined into a tube either lacking (for the –ATP control) or containing ATP (to give a final concentration of 5 mM) and further incubated 5 min at 25°C. An equal volume of cold Q buffer was added (7) followed by additional KCl to a final concentration of 500 mM. 50 μ l of streptavidin-agarose (1:2 in Q buffer with 500 mM KCl) were added; the subsequent binding, washing and Proteinase K treatment steps were essentially as described in (28) except that NET-2 contained 500 mM NaCl.

To assay for the two U2 snRNA species, oligo 23T (30) was labeled with polynucleotide kinase and used in a standard primer extension reaction containing 2.5 mM dideoxy-ATP instead of dATP. The products were then analyzed on a 15% polyacrylamide denaturing gel. The mutation in HI70 U2 snRNA is a C-to-U change at position 121; the oligo is designed to give a 32 nt product for HI70 U2 snRNA and a 35 nt product for WT U2 snRNA in the presence of ddATP.

RESULTS

U2 snRNP association with the commitment complexes is a rapid process

In our previous experiments, two novel U1 snRNP-containing commitment complexes were detected by native gel electrophoresis in the absence of U2 snRNP (19). Both complexes were undetectable or only barely detectable in a complete extract, presumably because the addition of U2 snRNP is rapid and

efficient. An assembly scheme based on these and other studies of the early events of yeast spliceosome assembly is shown in Figure 1. [In this communication, the designation 'spliceosomes' (and 'SP' in the figures) refers to U2 snRNP-containing complexes whether or not they contain U4/U6 or U5 snRNPs.] To verify this scheme and to explore other means for generating commitment complexes, we 'U2-killed' the extract with a complementary oligonucleotide and endogenous RNase H activity (21,31). The rate and extent of complex formation were compared to those of a wild-type extract.

With the U2-killed extract, there was a dramatic accumulation of commitment complexes and no spliceosome formation (Figure 2, lanes 7–12), as previously shown for extracts genetically depleted of U2 snRNP (19). Moreover, the time course and extent of commitment complex formation were similar to what was observed for spliceosome formation in the wild-type extract (lanes 1–6). The data suggest that U1 snRNP addition (commitment complex formation) is relatively slow and independent of U2 snRNP, consistent with the previous indirect assay (21), in which commitment complexes could not be seen but their formation was inferred from a subsequent chase into spliceosomes. The results also suggest that the rapid addition of U2 snRNP accounts for the failure of commitment complexes to accumulate in a complete extract.

Mutant U1 snRNP affects U2 snRNP addition to the pre-mRNA

If commitment complexes are the substrate for U2 snRNP addition, extracts derived from strains containing U1 snRNA mutations might generate mutant commitment complexes to which U2 snRNP adds poorly. To test this notion, we analyzed complex formation in extracts derived from several U1 mutant strains. The effects of these mutations had been previously characterized *in vivo*; some of them had no effect whereas others had substantial effects on splicing or growth rate (24).

One mutant strain had a deletion of the entire yeast core region of U1 snRNA (Δ YC; the deleted region is stippled in Figure 3A) and was previously shown to be temperature-sensitive for growth (24). Seven other mutant strains contained different U1 mutations; these included a deletion of the universally conserved A loop (Δ LII), a deletion of the yeast-specific helix-loop VII and VIII (Δ VII-VIII), and a C-to-U change at position 4 (U1-4U) which participates in base pairing to the 5' splice site region (Figure 3A). Also included were four multiple substitutions in helix VII (HVII-m1 to HVII-m4) (24). With the exception of the U1-4U strain, which grows slowly at 30°C and 37°C, the other six strains grow well at both 30°C and 37°C. Extracts were prepared from the nine strains (8 mutant strains and the wild-type) after growth at 30°C and assayed for spliceosome assembly *in vitro*.

Figure 3B shows a typical spliceosome assembly gel with the different extracts. Extracts from U1 mutations HVII-m1 to -m4 had not detrimental effect on complex formation; on the contrary, they generated enhanced spliceosome levels as compared to the

wild-type extract (lanes 4–7). Extracts from the strain containing the U1-4U mutation showed somewhat reduced levels of spliceosomes (lane 9 and data not shown). Extracts from the strain that carried a deletion of the universally conserved A loop (Δ LII) also gave rise to reduced levels of spliceosomes (lane 3). The smaller deletion in the yeast core region (Δ VII-VIII) gave rise to an even greater reduction in spliceosomes (lane 8). All of these strains generated parallel reductions in commitment complex formation when assayed after inactivation of U2 snRNP activity (data not shown). Most dramatic was the Δ YC extract in which there was greatly reduced spliceosome formation and a prominent accumulation of a complex that comigrated with CC2 (lane 2). The relative efficiency of *in vitro* splicing in the nine extracts paralleled the relative level of spliceosome formation (data not shown).

A number of criteria indicate that the major pre-mRNA-containing complex in the Δ YC extract is a Δ YC U1 snRNP-containing commitment complex to which stable U2 snRNP addition occurs poorly, even after prolonged incubation time (Figure 3C). Depending on gel conditions, its migration is similar to or indistinguishable from commitment complexes formed in wild-type extracts (Figures 3B and 4A). Like the wild-type commitment complexes, formation of this Δ YC complex is independent of both U2 snRNP and ATP, and they contain Δ YC U1 snRNA as shown by blotting of native gels (data not shown). Also, they chase poorly to spliceosomes upon addition of wild-type extract, indicating that these complexes, rather than soluble factors in the extracts, are deficient for U2 snRNP addition (data not shown; cf. 19,26). We conclude that the efficiency or stability of U2 snRNP addition depends on the nature of the U1 snRNP in the commitment complexes, consistent with a precursor-product relationship between commitment complexes and pre-spliceosomes.

Figure 1. Schematic of early events of spliceosome assembly. U1: U1 snRNP; U2 snRNP; CC: commitment complexes; SP: spliceosomes.

Figure 2. Radioactively-labelled pre-mRNA substrate was incubated under standard assembly conditions (see Material and Methods) with a wild-type miniextract to form spliceosomes (SP; lanes 1–6), or with U2-killed extract (see Material and Methods) to form commitment complexes (CC1 and CC2; lanes 7–12). Reactions were stopped after incubation for the various times shown above the lanes and analyzed on a native gel as described in Material and Methods.

Figure 3. (A) A few mutations of U1 snRNA are presented with the proposed secondary structure of yeast U1 snRNA. The boxed regions are deleted in Δ LII, Δ YC and Δ VII-VIII, respectively. The arrows indicate the single point mutations made in U1 snRNA. The numbering of nucleotides starts after the tri-methyl G cap. (B) Spliceosome assembly with the mutant extracts under standard conditions. The mutations of the U1 gene, shown above the lanes, have been described in (24) and/or in Figure 3A. CC1 and CC2, commitment complexes; SP, spliceosomes. (C) Pre-mRNA substrate was incubated with either a wild-type extract (lanes 1–6) or a Δ YC extract (lanes 7–12) to form spliceosomes (SP). Reactions were stopped after incubation for the various times shown above the lanes. The positions of spliceosomes and commitment complexes (CC1 and CC2) are indicated. Samples were analyzed on a native gel lacking glycerol.

A few lethal U1 mutant genes had been previously examined *in vivo* (24). Among these are mutants that contain the yeast core deletion (Δ YC) combined with the loop II deletion (Δ LII) or with point mutations in loop II (LII-m1 or LII-m3) (24). We interpreted their lethality to indicate that the double mutant U1 snRNAs could not support cell viability either because they manifested an exaggerated Δ YC phenotype, *i.e.*, they formed commitment complexes that were even less able than the Δ YC commitment complexes to progress to spliceosomes, or because they were unable to form commitment complexes. Because the strains containing the double mutant U1 snRNPs as the sole source of U1 snRNA were not viable, we initially analyzed extracts from

merodiploid strains that contained a wild-type U1 gene (carried by the plasmid p23; see Material and Methods) as well as a second U1 gene (wild-type or mutants as shown above each lane of Figure 4A).

Extracts from merodiploid strains that carry the singly mutant Δ YC U1 gene showed a semi-dominant *in vitro* phenotype as compared to their haploid 'parents'; there was a clear increase in the level of commitment complexes (compare lanes 3 and 4 with lanes 1, 2 and lane 11) characteristic of the Δ YC strain. Extracts from merodiploid strains that carried the lethal U1 genes showed no deviation from the wild-type pattern (compare lanes 5–10 with lanes 1–2), suggesting that *in vitro* activity of the

Figure 4. *In vitro* assays for phenotypes associated with the lethal U1 mutations. (A) Analyses of extracts derived from merodiploid strains expressing both the wild-type U1 gene and a mutant U1 gene. Spliceosome assembly was carried out under standard conditions and assayed on a native gel lacking glycerol. Lanes 1–10 show extracts from strains harboring p23 (wild-type U1 gene on a centromere plasmid) and a mutant U1 gene on another centromere plasmid (the latter is indicated above each lane; see 24). In the case of lanes 1 and 2, the second plasmid also carries the wild-type U1 gene. Extracts from two independent transformants of each strain were prepared and analyzed in parallel (note that the extract in lane 4 was made from half the numbers of cells as the other extracts in this gel). Lanes 11 and 12 represent extracts derived from the control haploid strains carrying only one U1 gene as indicated (either ΔYC or WT). (B) Extracts were made from merodiploid strains harboring a wild-type U1 gene under the GAL-UAS control and a mutant U1 gene as shown above the lanes (or another wild-type U1 gene for lane 1), after these strains were grown in glucose-containing medium for 16 h. Spliceosome assembly was carried out under standard conditions. SP, spliceosomes; CC1 and CC2, commitment complexes.

double mutant U1 snRNPs may be qualitatively different from that of the ΔYC U1 snRNP, consistent with the inability of the former to support detectable growth at all temperatures tested (24).

To show more directly that the U1 snRNPs expressed from the lethal U1 genes were not functional, several additional merodiploid strains were constructed and examined. These contained a wild-type U1 gene under control of the GAL-UAS (provided by pXL46, see Material and Methods) as well as another U1 gene (wild-type or mutant) with a normal U1 promoter. To extinguish expression of the wild-type U1 snRNA, the carbon source was shifted from the galactose to glucose. Even with a lethal U1 mutation, the cells continue growing normally for 10–16 h (utilizing the previously synthesized, stable wild-type U1 snRNPs for splicing), during which time they dilute substantially the wild-type snRNPs and continue to synthesize the mutant snRNPs. After 16 h, the wild-type U1 snRNA expressed from the GAL-U1WT gene was essentially depleted by this treatment (19,24), and these extracts contain a single population (>95%) of U1 snRNP expressed from the other U1 gene with the conventional U1 promoter. This provides an *in vivo* method to assemble even lethal U1 snRNA mutants into U1 snRNPs that can then be assayed *in vitro* in the absence of the wild-type U1 snRNP.

As predicted, the phenotypes of the GAL-depleted extracts from strains that carried viable U1 mutants were the same as those obtained from haploid strains that contained the corresponding mutant U1 genes as the sole source of U1 RNA (Figure 4B). The wild-type (WT) pattern (lane 1) was similar to that from a wild-type strain (e.g., Figure 3B, lane 1). The pattern from the viable double mutant combination $\Delta YC + LII26A$ [the ΔYC deletion and the LII26A point mutation; LII26A was formerly LII-27A in (24)] resembled that of a ΔYC extract (compare Figure 4B, lane 4 with Figure 3B, lane 2) as well as that from a haploid strain that carried the same double mutant combination as the sole source of U1 snRNA (data not shown). The assembly pattern from another GAL-depleted extract that contained a different viable U1 gene, $\Delta LII + \Delta VII-VIII$, gave rise to only a small amount of complex formation (Figure 4B, lane 5), indistinguishable from what was observed with the corresponding, viable haploid strain (data not shown). The extracts containing the lethal combination of the yeast core deletion and the loop II deletion ($\Delta YC + \Delta LII$) formed neither spliceosomes nor commitment complexes (Figure 4B, lane 2). Another lethal combination, $\Delta YC + LII29A$ [see Figure 3A; LII29A was formerly LII-30A in (24)], almost completely eliminated the spliceosome signals; there was a faint commitment complex signal reminiscent of the ΔYC extract phenotype. We conclude that the GAL-depletion approach can address the *in vitro* phenotype of lethal U1 mutations and that the lethal combination of $\Delta YC + \Delta LII$ is unable to form splicing complexes *in vitro*.

Commitment complex formation is an ATP-independent step

Previous observations indicated that commitment complex formation does not require added ATP, in contrast to U2 snRNP addition (19,21). To show that this is likely to be a qualitative rather than a quantitative difference, *i.e.*, that commitment complex accumulation does not depend on the presence of endogenous ATP in the extract, we adopted a strategy to deplete ATP from the extract. The protocol exploits the hydrolysis of ATP by hexokinase, which converts glucose to glucose 6-phosphate (32). After first preincubating splicing extracts with hexokinase as well as glucose, we found that the addition of only

Figure 5. Pre-mRNA substrate was incubated under standard conditions in a wild-type spheroplast extract (see Material and Methods) with various amounts of glucose in the absence (lanes 1–3) or the presence (lanes 4–6) of 2 mM ATP. (Note that lane numbers correspond to identical samples between panels A and B; lane 2 is omitted from panel A.) Splicing intermediates (L, lariet intermediate; I, intron lariet) and unspliced pre-mRNA (P) were assayed on a 15% polyacrylamide denaturing gel (panel A), while commitment complexes (CC1 and CC2) and spliceosomes (SP) were assayed on a native gel (panel B).

glucose had identical effects (data not shown and see below), due presumably to the presence of substantial hexokinase in the splicing extracts.

Addition of 1 mM glucose to the extracts completely inhibited splicing, even in the presence of 2 mM ATP, which would normally support substantial activity (Figure 5A, lane 6). Similar results were observed for spliceosome formation, as the addition of glucose inhibited U2 snRNP addition and led to the accumulation of commitment complexes (Figure 5B, lanes 1–3). As expected, the addition of a sufficient excess of ATP overcame the inhibitory effect of glucose (Figure 5B, lane 5 vs. lane 2). The results show that the small amount of spliceosome formation observed without ATP addition (Figure 5B, lane 1) is due to residual ATP in the extract, that commitment complex formation does not require ATP, and that spliceosome formation requires ATP.

Hydrolyzable ATP analogs promote U2 snRNP addition

We tested the ability of a dozen nucleotides (including 5 ATP analogs) to promote spliceosome assembly and splicing after preincubation of the extract in 0.2 mM glucose to deplete endogenous ATP (see Material and Methods). With the exception of the two non-hydrolyzable ATP analogs AMP-PCP and AMP-PNP (Figure 6A, lanes 13 and 14), all of the nucleotides tested were able to effect spliceosome formation (Figure 6A, lanes 2–12). To test whether this was due to the direct utilization of these molecules by splicing factors or to phosphate transfer enzymes, we separated the whole cell extract into a snRNP and a non-snRNP fraction by ultracentrifugation (26; see Material and Methods). The ability of the nucleotides to promote spliceosome assembly in the snRNP fraction was then examined (Figure 6B). In this case, we also observed spliceosome formation with ATP and hydrolyzable ATP analogs (dATP and 3' dATP)

Figure 6. The wild-type extract (Figure 5A) and the pellet fraction of the same extract (Figure 5B) (see Material and Methods) were preincubated in the presence of splicing salts and 0.2 mM glucose to deplete the endogenous ATP. Spliceosome assembly was assayed with the subsequent addition of pre-mRNA and 2 mM NTP, dNTP, or ATP analog as shown above each lane (see Material and Methods for abbreviations). Commitment complexes (CC1 and CC2) and spliceosomes (SP) were assayed on native gels.

and with two poorly hydrolyzable ATP analogs (ATP-α-S, and ATP-γ-S) (Figure 6B, lanes 2, 6, 10, 11, and 12, respectively). However, in contrast to the results obtained with the whole extract, all other ribonucleoside- and deoxyribonucleoside-5'-triphosphates were unable to promote spliceosome assembly in the pellet fraction, suggesting that some nucleoside diphosphate kinase-like activity did not fractionate with the active snRNPs and was responsible for the activity of these triphosphates in whole extract.

U2 snRNP is not stably associated with the commitment complexes in the absence of ATP

The ATP requirement for pre-spliceosome formation cannot exclude the possibility that a U2 snRNP-containing splicing complex is formed without ATP but is unstable during the subsequent assay, for example, during gel electrophoresis. By this hypothesis, U2 snRNP addition would be ATP-independent and ATP would be necessary to stabilize a loose interaction between U2 snRNP and other components of this pre-spliceosome complex (cf. 33–35).

To address this possibility, we devised an experiment that employed two distinguishable U2 snRNAs, encoded by a wild-

Figure 7. Analysis of U2 snRNA species coprecipitated with biotinylated pre-mRNA by streptavidin-agarose. Assembly reactions (lacking added ATP) were carried out in the presence of glucose in various extracts ['Extract 1', as shown above lanes 1–7; wild-type (WT), HI70 or a mixture (WT + HI70)]. Biotinylated pre-mRNA was the substrate for all reactions (except that in lane 6, for which non-biotinylated pre-mRNA was used). After 30 min, a 15-fold excess of non-biotinylated pre-mRNA was added, followed (after 1 min) by the simultaneous addition of ATP and another assembly reaction done with non-biotinylated pre-mRNA in another extract ('Extract 2', as shown above lanes 1–7). After 5 min, the reactions were quenched and the complexes purified with streptavidin-agarose. For lanes 3 and 4, the reactions were mixed after incubation with ATP and quenching; for lane 7, no ATP was added (see Material and Methods for further details). U2 snRNAs in the complexes bound to streptavidin were purified and assayed by primer extension with oligo 23T and ddATP; the products were analyzed on a 15% polyacrylamide denaturing gel. The positions of the products corresponding to the two U2 snRNAs are indicated on the left; their sizes are 35 nt (WT U2) and 32 nt (HI70 U2), respectively. Lane 8 shows the wild-type U2 product in 8 μ g yeast RNA.

type gene and a pseudowild-type gene (WT and HI70, respectively; 30). The basic protocol included a two-step incubation of biotinylated pre-mRNA substrate, first in the presence of glucose and one type of U2 snRNP and then (after addition of excess non-biotinylated pre-mRNA) in the presence of ATP and the second U2 snRNP (which had been similarly incubated but with a non-biotinylated pre-mRNA). Complexes formed during the first incubation should be chased into stable U2 snRNP-containing pre-spliceosomes during the second incubation with ATP. If U2 snRNP is irreversibly, albeit loosely, associated with the pre-mRNA prior to the addition of ATP and the second extract, only the U2 snRNA from the first extract should be present in the complexes that bind to streptavidin-agarose. On the other hand, if U2 snRNP is not associated with the pre-mRNA in the absence of ATP (or exchanges rapidly), both types of U2 snRNA molecules should be equally present in the spliceosomes subsequently bound to streptavidin-agarose.

No U2 snRNA copurified with the biotinylated pre-mRNA when the extracts were incubated without ATP (Figure 7, lane 7). The key part of the experiment monitored the U2 species purified after mixing the extracts and adding ATP simultaneously (Figure 7, lanes 1 and 2); the data show that both types of U2 snRNA were found in spliceosomes, irrespective of which U2 snRNP-containing extract was incubated with biotinylated substrate during the first incubation without ATP. Mixing the extracts after addition of ATP and quenching restricted the substrate to the U2 species present during the initial incubation (Figure 7, lanes 3 and 4). Consistent with prior gel results, the data indicate that U2 snRNP is not detectably associated with the U1 snRNP-pre-mRNA complex in the absence of ATP.

U1-4U extract forms pre-spliceosomes in the absence of ATP

Although ATP appears strictly required for stable U2 snRNP addition, we noticed that extracts derived from a strain containing a U1 snRNA mutation, U1-4U (14, also see Figure 3A),

Figure 8. Analysis of complexes formed in a U1-4U extract without ATP. Standard assembly reactions with pre-mRNA substrate were carried out with two independent U1-4U extracts in the presence of 2 mM glucose, which is about 10-fold higher than necessary to deplete a wild-type extract of endogenous ATP (see Figure 5B). The positions of spliceosomes (SP) and commitment complexes (CC1 and CC2) are indicated. A wild-type extract made and assayed in parallel did not give any spliceosome signal, even at a lower glucose concentration (lane 1). Lanes 4–6 are lanes 1–3 subjected to longer exposure.

consistently produced some spliceosome-like complexes in the absence of ATP, *i.e.*, in the presence of glucose (Figure 8). The U1-4U spliceosome-like complex observed in the absence of ATP not only comigrates with wild-type spliceosomes but fails to form if functional U2 snRNP is eliminated from the U1-4U extract by U2-killing (data not shown), suggesting that it represents bona fide spliceosomes whose formation bypassed the ATP requirement.

DISCUSSION

The experiments presented in this report were designed to address the step of U2 snRNP addition to the commitment complexes in the yeast spliceosome assembly pathway. There is substantial evidence in both yeast and mammalian systems that U2 snRNP addition precedes and does not require the U4/U5/U6 triple snRNP (11,12), and the experiments presented here were directed at steps occurring before the addition of this triple snRNP.

We addressed the issue of pre-spliceosome formation, in order to confirm both that the substrate for U2 snRNP addition is the commitment complex and that the formation of commitment complex is ATP-independent. This view is based on our previous native gel electrophoresis assays, which showed that commitment complexes accumulated in extracts 'genetically' depleted of U2 snRNP (19). Those complexes were biologically active because they could be chased into pre-spliceosomes and functional splicing complexes by the addition of U2 snRNP (19). Similar results were reported for the mutant strain *prp9*, which accumulates commitment complexes at the expense of spliceosomes (26). The experiments described in the present report employed three additional procedures that prevent U2 snRNP addition and give

rise to commitment complexes: i) digestion of U2 snRNA by oligonucleotide-directed RNase H, ii) the use of an extract that contains a mutant U1 snRNA (Δ YC), and iii) the elimination of ATP with glucose and endogenous hexokinase. In mammalian extracts, a similar commitment complex (E complex) also accumulates only in the absence of ATP (34). Taken together with our previous observations and with affinity chromatography assays indicating that U2 snRNP addition is dependent on functional U1 snRNP (22), these experiments suggest strongly that the U1 snRNP-containing complexes are indeed intermediates in spliceosome formation.

The GAL-depletion procedure provides an strategy for the *in vivo* assembly and *in vitro* assay of U1 snRNPs that even contain lethal mutations. Because the GAL-depleted extracts containing U1 snRNP that carried viable U1 snRNA mutations had the same phenotypes as extracts from haploid strains containing the corresponding U1 genes, it is likely that the GAL-depleted extracts faithfully reflect the *in vitro* phenotypes of the mutant U1 snRNPs. The most severe 'lethal' U1 snRNA-containing extracts gave rise to little or no spliceosomes and little or no commitment complexes upon U2-killing (Figure 4B and data not shown), suggesting that the mutations affect the formation or stability of the U1 snRNP complexes. Yet mutant U1 snRNPs were present in these extracts, as shown by hybridization of snRNP blots with U1 snRNA probes (data not shown), indicating that the absence of complex formation cannot be accounted for by the absence of mutant U1-containing snRNP. The results suggest that the mutant U1 transcripts are able to assemble into U1 snRNPs but that these mutant U1 snRNPs function poorly.

Of the U1 mutants examined, the Δ YC phenotype is unique in that high levels of commitment complex are formed and U2 snRNP addition is inhibited. The mobilities of the Δ YC U1 snRNP complexes (both Δ YC-CC1 and Δ YC-CC2) were altered compared to the wild-type CC1 and CC2 when assayed on a native gel system lacking glycerol (e.g., Figures 3C and 4A), probably as a consequence of the substantial U1 snRNA deletion. In the presence of glycerol, however, only CC2 was observed, and its migration was indistinguishable from that of wild-type CC2 (Figure 3B). Since CC2 has been defined as the commitment complex subspecies containing a branchpoint sequence-recognizing factor X (23), the increased ratio of CC2:CC1 in the Δ YC extract suggests a tighter association of X with the other components of the Δ YC commitment complex. Attempts to challenge these complexes with non-specific competitor RNA support this view (H.V.Colot, unpublished data). A tighter association with the substrate branchpoint sequence may be related to, if not responsible for, the inefficient addition of U2 snRNP, *i.e.*, an intimate interaction between U2 snRNP and the branchpoint sequence may be required for U2 snRNP addition, and this interaction may be inhibited by a too tight interaction between the Δ YC U1 snRNP and the same branchpoint region.

A requirement for ATP or hydrolyzable ATP analogs, although not apparent from assays in whole cell extracts, is indicated by the nucleotide requirements for U2 snRNP addition in an enriched snRNP fraction (Figure 6). Presumably, a phosphate transfer activity fractionates away from the snRNPs, so that the true nucleotide requirement is revealed. The requirement for ATP or hydrolyzable ATP analogs for U2 snRNP addition in yeast extracts is identical to what has been reported for mammalian extracts (36,37). Although this ATP requirement provides an operational distinction between stable U2 snRNP addition and commitment complex formation, it remains somewhat enigmatic

and presumably reflects a requirement for a helicase or for some other protein that effects a substantial conformational change. The yeast splicing factor PRP5 is a candidate for such a factor, as it has been reported to be required for U2 snRNP addition and its sequence indicates that it is a member of a family of proteins that include known RNA helicases (38). Consistent with this suggestion, the RNA helicase activity of the founding member of this family, eIF-4A, has identical nucleotide requirements to those reported here for U2 snRNP addition (39).

Because spliceosome formation and commitment complex formation have the same kinetics (Figure 2), commitment complex formation appears to be rate-limiting for spliceosome formation. Previous experiments of this kind were done with extracts made from cells genetically depleted of U2 snRNP. Because the cells start to grow poorly after 8 h of U2 depletion (19), those extracts made after 16 h of U2 depletion could not be compared quantitatively to wild-type extracts. The results shown here suggest that U2 snRNP is added rapidly after commitment complexes are formed. We cannot address the biological significance of slow commitment complex formation and a subsequent rapid U2 snRNP addition step, because the relative activities of splicing factors *in vitro* may not reflect their relative activities *in vivo*. We note, however, that the formation of the mammalian commitment complex (E complex) also appears to be the rate-determining step in spliceosome formation (34).

The subsequent rapid addition of yeast U2 snRNP might suggest an undetected ATP-independent association of U2 snRNP with commitment complex, but the experiment shown in Figure 7 either argues against this possibility or indicates that such an association is not stable. This conclusion is also consistent with our interpretation of results from an early indirect assay, which indicated that, in contrast to substrate commitment, U2 snRNP addition was rapid and required ATP (21). Although all of the available evidence suggests that there is a strict ATP requirement for U2 snRNP addition in the yeast system, we cannot rule out an ATP-independent weak interaction that might be revealed by more sensitive assays like those used to demonstrate an ATP-independent association of U2 snRNP with the mammalian E complex (34).

The only exception we have observed to this ATP requirement provides a hint as to its possible functional significance. We consistently observed a small amount of ATP-independent U2 snRNP addition in extracts derived from the U1-4U mutant strain. We cannot exclude the possibility that this property reflects a bizarre characteristic of the mutant strain that is an indirect consequence of the U1 snRNP mutation, but the most straightforward interpretation is that commitment complexes that contain the mutant U1 snRNP have a reduced requirement for the helicase or other protein(s) that hydrolyzes ATP. An alteration in U1 snRNP structure, resulting from the U1-4U mutation, might permit an as yet undefined conformational change that normally requires ATP. Alternatively, the ATP-independent U2 snRNP addition could be related to the altered base pairing between the 5' splice site and the 5' end of U1 snRNA. Position 4 of U1 snRNA normally contains a C and forms a G:C base pair with position 5 of the 5' splice site. The G:U base-pair between the 5' splice site and the 5' end of U1-4U snRNA is expected to be weaker than the wild-type G:C pair. Thus, the putative ATP-dependent protein could normally function to destabilize the canonical 5' splice site:U1 base pairing. We have previously proposed that the 5' splice site sequence interacts with other splicing factors after it undergoes pairing with U1 snRNP (14),

and this putative destabilization might serve to make this sequence available for a subsequent interaction, for example, with U5 snRNP (40,41).

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