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Biochemistry

Yeast precursor mRNA processing protein PRP19 associates with the spliceosome concomitant with or just after dissociation of U4 small nuclear RNA

(ATP)

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ABSTRACT **During assembly of the spliceosome, the U4 small nuclear RNA (snRNA) interacts with the spliceosome as a preformed U4/U6–U5 triple small nuclear ribonucleoprotein (snRNP) complex. Subsequently, U4 becomes loosely associated with the spliceosome, whereas U5 and U6 remain tightly associated, suggesting unwinding of the U4/U6 duplex. We show that this step of the assembly process can be blocked by limiting the ATP concentration in the splicing reaction. We also show that the yeast precursor mRNA processing protein PRP19 becomes associated with the spliceosome during this transition. Thus, PRP19 may function in this step of spliceosome assembly.**

Splicing of precursor mRNA (pre-mRNA) occurs by a two-step transesterification mechanism: (*i*) cleavage of the 5′ splice site and formation of the lariat intermediate and (*ii*) cleavage of the 3′ splice site and ligation of the two exons. This process occurs in a large complex called the spliceosome (1–3), which is composed of U1, U2, U4/U6, and U5 small nuclear ribonucleoprotein particles (snRNPs) and a number of protein factors (4–8).

Much attention has been given to the interactions between the five small nuclear RNAs (snRNAs) and the pre-mRNA. In yeast and mammals, recognition of the 5′ splice site and the branch site are mediated, in part, through Watson–Crick base pairing with U1 and U2 snRNAs, respectively (4, 5). Recent studies suggest that U5 also interacts with the pre-mRNA, in this case with exon sequences near the two splice junctions (9, 10). U6 appears to interact with the pre-mRNA at the 5′ splice site, as shown by UV-crosslinking studies (11–13). Moreover, base-pairing between U2 and U6 snRNAs has also been shown to be important during spliceosome assembly (14).

The functions of protein factors required for splicing are relatively less well studied. Several proteins have been identified as involved in splicing of pre-mRNA in the mammalian system. These include U2 snRNP auxiliary factor (U2AF; refs. 15 and 16), splicing factor SF2/ASF (17), 35-kDa splicing component (SC-35) (18), intron-binding protein (IBP) (19, 20), polypyrimidine tract-binding protein (PTB) (21), heterogenous nuclear RNP C protein (22), and the recently identified PTB-associated splicing factor (PSF) (23). The roles these proteins play in the splicing reaction are not well understood. Identification of protein factors essential for pre-mRNA splicing has been greatly facilitated in *Saccharomyces cerevisiae* by the isolation of temperature-sensitive mutants that affect pre-mRNA splicing (24, 25). At least 30 such *PRP* (for precursor mRNA processing) genes have now been identified (24). Biochemical studies reveal that some of the PRP proteins—e.g., PRP4, PRP6, PRP8, and PRP18—are integral components of snRNPs (26–30), whereas PRP19 is not tightly associated with any of the five snRNAs required for splicing (31). PRP19, as well as PRP8 and PRP11, is also an integral component of the spliceosome (32–34). The biochemical functions for these PRP proteins are generally unknown, but PRP2 and PRP16 possess RNA-dependent NTPase activity (35–37).

Assembly of the spliceosome is a multistep process that involves sequential binding of snRNAs to the pre-mRNA in an order of U1, U2, then U4/U6 plus U5 as a preformed tri-snRNP (4–8). After all five snRNAs are associated with the pre-mRNA, U4 becomes only loosely associated with the spliceosome and does not participate in the subsequent splicing reactions (ref. 38, see Fig. 1). It is generally believed that during this step of the assembly process, base-pairing between U4 and U6 snRNAs is unwound, which reduces the affinity of U4 for the spliceosome. An RNA helicase activity is implicated in this process (24, 44). Although a number of PRP proteins, including PRP2, PRP5, PRP16, PRP22, and PRP28, contain the consensus Asp-Glu-Ala-Asp (DEAD box) or Asp-Glu-Ala-His (DEAH box) of the RNA helicase motif (24, 44), no RNA helicase activity has been demonstrated for any of these or other proteins. Furthermore, no protein has been shown to be involved in this particular step of the assembly process.

Previously, we have shown that PRP19 is not a component of snRNPs but is associated with the spliceosome during the splicing reaction (31, 32). To determine in which step of the assembly process PRP19 is involved, we took advantage of the sequential binding of snRNPs to the pre-mRNA and blocked spliceosome assembly at various steps by inactivating specific snRNAs. Splicing complexes formed in extracts depleted of U2 or U6 were examined for their association with PRP19 by immunoprecipitation with anti-PRP19 antibody. The results show that PRP19 is not associated with the spliceosome formed in extracts in which U2 or U6 has been inactivated by oligonucleotide-directed RNase H cleavage, indicating that PRP19 is associated with the spliceosome only after or concomitant with binding of U6 to the pre-mRNA (31). However, PRP19 is associated with the functional splicing complex formed without splicing intermediates or products in heat-inactivated *prp2* mutant extracts (43). In other words, PRP19 is associated with complex A1 (see Fig. 1). Therefore, PRP19 appears to enter the spliceosome after binding of U2 to the pre-mRNA at the earliest but before formation of the functional spliceosome—i.e., after formation of complex B but before formation of complex A1 as shown in Fig. 1. We were unable to determine whether PRP19

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Abbreviations: snRNA, small nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; PRP, precursor mRNA processing; anti-TMG, anti-trimethyl guanidine.

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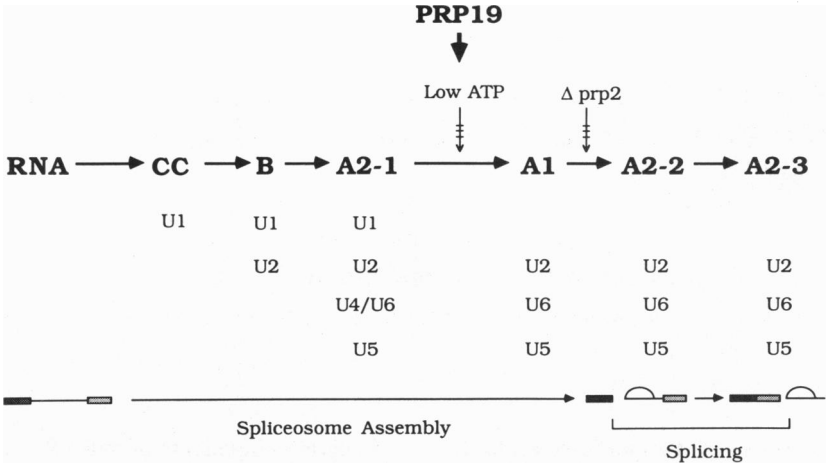

FIG. 1. Scheme for spliceosome assembly, summarizing results from yeast and mammals. Complex CC is the commitment complex described by Séraphin and Rosbash (39). Complexes B, A2-1, A1, A2-2, and A2-3 are described by Cheng and Abelson (40) and by Ruby and Abelson (24). Complexes A2-2, and A2-3 are active splicing complexes and contain splicing intermediates and products, respectively. The snRNA contents of each complex are indicated below each complex (40). Association of U1 with the pre-mRNA early in the splicing reaction is from the results of Ruby and Abelson (41), Bindereif and Green (42), and Séraphin and Rosbash (39). The absence of U1 in complexes A1, A2-2, and A2-3 is from the results of Wassarman and Steitz (11) that U1 is absent from the spliceosome concomitant with U4 dissociation. Formation of complex A2-2 can be blocked by using heat-inactivated *prp2* mutant extracts (43). Formation of complex A1 can be blocked by limiting ATP concentration in the splicing reaction, as described in text. The involvement of PRP19 in the transition from complex A2-1 to complex A1 is also reported in text.

is involved in formation of complex A2-1 or complex A1 because so far we know of no factor that can be depleted to block the formation of complex A1. In this report, we show that formation of A1 can be blocked by limiting the concentration of ATP in the splicing reaction. We further show that PRP19 is involved in the transition from complex A2-1 to A1, the step U4 dissociates from the spliceosome.

MATERIALS AND METHODS

Antibodies. Anti-PRP19 antibody was prepared as described in Cheng *et al.* (32). Anti-trimethyl guanidine (anti-TMG) and anti-PRP4 antibodies were from Reinhard Lührmann (Max-Planck-Institut) and Josette Banroques (Centre National de la Recherche Scientifique), respectively. The 12CA5 monoclonal antibody was obtained from Berkeley Antibody (Richmond, CA).

Preparation of Splicing Extracts and Substrates. Yeast whole-cell extracts were prepared according to Cheng *et al.* (45). Actin precursors were synthesized *in vitro*, using SP6 RNA polymerase according to Cheng and Abelson (40).

Splicing Assays and Immunoprecipitation. Splicing assays were done according to Cheng and Abelson (40) in the presence or absence of 3 mM $MgCl_2$ and 1 mM EDTA and with various concentrations of ATP according to the particular experiment. Immunoprecipitation was done as described in Tarn *et al.* (31) for anti-PRP19 and 12CA5 antibodies. For anti-PRP4 antibody, 10 μl of the immune or preimmune serum was used per 10 μl of protein A-Sepharose.

PAGE and RNA Blot Analysis. Fractionation of splicing complexes by electrophoresis on nondenaturing polyacrylamide gels and Northern blot analysis were done according to Cheng and Abelson (40).

RESULTS

Formation of Complex A1 Was Blocked by Limiting the Concentration of ATP in the Splicing Reaction. Conversion of complex A2-1 to complex A1 involves a major conformational change in which U4 snRNP becomes loosely associated with the spliceosome (40). It has recently been shown that this dissociation of U4 is accompanied by changes in modes of interaction between the pre-mRNA and all snRNAs (11). Therefore, we reasoned that ATP might be important in causing the major conformational change; thus, reduced ATP concentration might provide a way to block the formation of complex A1. Consequently we examined the ATP dependence of complex A1 formation. The splicing reaction was done in the presence of 0, 0.06, 0.2, 0.6, or 2 mM ATP and analyzed for formation of the splicing intermediates and products and for formation of splicing complexes by electrophoresis on nondenaturing gels (40). Fig. 2*A* shows that no splicing intermediates or products were detected at 0 or 0.06 mM ATP. At 0.2 mM or higher concentrations of ATP, splicing was very efficient, as large amounts of intermediates appear. Examination of splicing complexes formed at low ATP concentrations (0 or 0.06 mM) revealed the formation of complex B and a complex migrating at the position of A2, which may represent complexes A2-1 and/or A2-2 and A2-3 (Fig. 2*B*). Since formation of complex B requires ATP (40), the small amounts of complexes formed without ATP addition must have been due to residual amounts of ATP in the whole-cell extract (40). Greater amounts of complexes B and A2 accumulated at 0.06 mM ATP than when ATP was absent. Complex A2-1 is a pre-splicing complex and contains only precursor mRNA without splicing intermediates or products, whereas complexes A2-2 and A2-3 are active splicing complexes capable of generating splicing intermediates and products, respectively (24, 40). Since no splicing intermediates or products were formed at 0 or 0.06 mM ATP, the complex migrating at the position of A2 should theoretically represent only complex A2-1. At higher ATP concentrations (0.2 mM or higher), complex A1 was also detected, as well as complexes B and A2. Complex A2 should consist of A2-1, A2-2, and A2-3 under these conditions. Thus, formation of complex A1 was blocked by limiting the ATP concentration in the splicing reaction.

Accumulation of More Complex A2-1 by Limiting ATP Concentration. Complex A2-1 is extremely short-lived in the assembly process under normal splicing conditions (in the presence of 2 mM ATP). It is present only in small amounts throughout the reaction, as indicated by the amount of U4 in the complex (40). U4 is not detected in any other complexes in the assembly pathway when assayed by electrophoresis on nondenaturing polyacrylamide gels (40). To further show that the A2-1 complex accumulated at 0.06 mM ATP, the snRNA

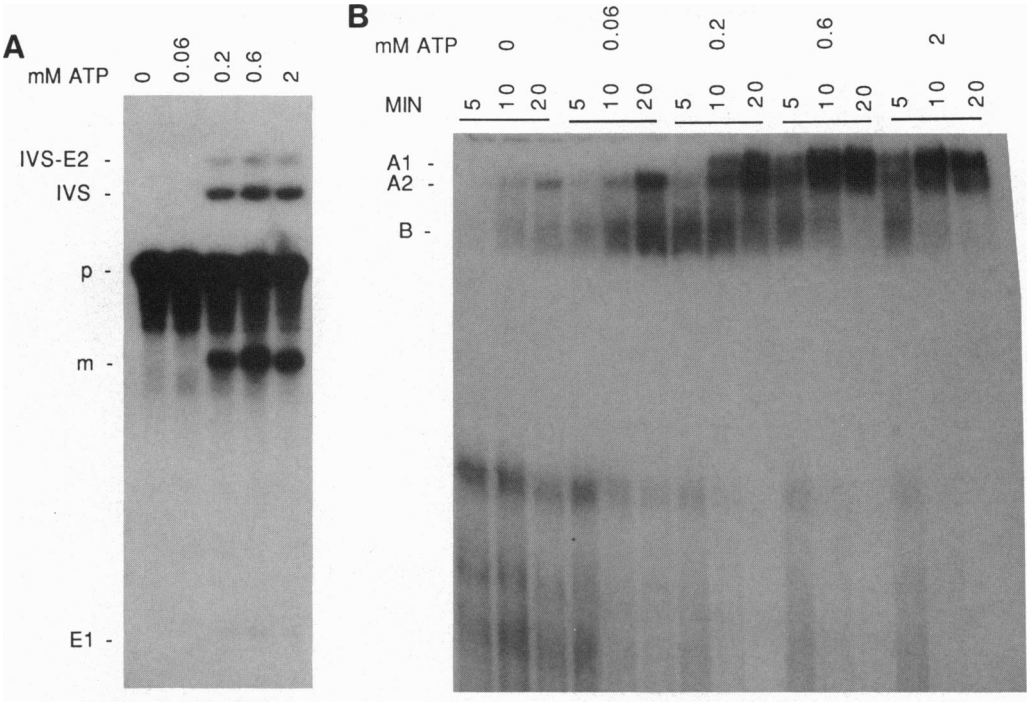

FIG. 2. Splicing reactions and splicing complex formation at various ATP concentrations. (*B*) The splicing reaction was done in the presence of 3 mM $MgCl_2$/1 mM EDTA, and 0, 0.06, 0.2, 0.6, or 2 mM of ATP. At 5, 10, and 20 min of incubation, aliquots were removed from each reaction and electrophoresed on a 4% nondenaturing polyacrylamide gel. (*A*) An aliquot of the reaction mixture after 20 min was also extracted to isolate RNA for analysis on an 8% polyacrylamide–8 M urea gel. IVS-E2, intervening sequence-exon 2; IVS, intervening sequence; p, precursor mRNA; m, mature mRNA.

content of each splicing complex formed at different ATP concentrations was investigated. Splicing complexes separated on nondenaturing polyacrylamide gels were electroblotted to GeneScreen membrane followed by hybridization with probes for actin and each of the relevant snRNAs (40). Fig. 3 shows that the amount of U4 in complex A2 increased with increased ATP concentrations up to 0.06 mM and then decreased at higher ATP concentrations, concomitant with the appearance of complex A1. Conversely, the amounts of U2, U5, and U6 in complex A2 increased as ATP concentration was raised above 0.06 mM. This result indicates that the complex A2 that accumulated at 0.06 mM ATP was, indeed, complex A2-1. Conversion of complex A2-1 to complex A1, which involves loosening of U4 association, was blocked at low ATP concentrations.

The accumulation of complex A2-1 at low concentrations of ATP can be further shown by immunoprecipitation of the complex with an antibody against a protein component of the U4 snRNP, PRP4. The protein has been found tightly associated with U4/U6 in the splicing extract (27, 28) and is associated with the 5′ portion of U4 snRNA (46). Previous attempts to immunoprecipitate the spliceosome with anti-PRP4 antibody have been unsuccessful and have only detected a very small amount of precursor mRNA in the precipitate (J. Banroques, personal communication). This result is consistent with the fact that the only splicing complex with which U4 is tightly associated, complex A2-1, is present at a very low level when ATP is abundant and contains only precursor mRNA. We repeated the immunoprecipitation experiments with splicing reactions at 2 mM and

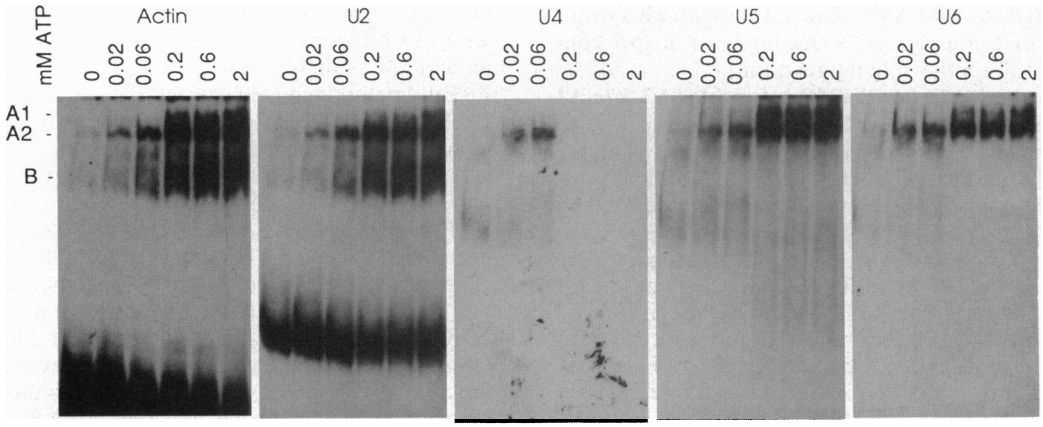

FIG. 3. RNA blot analysis of the splicing complexes formed at various ATP concentrations. Splicing reactions were done in the presence of 3 mM $MgCl_2$/1 mM EDTA, and 0, 0.02, 0.06, 0.2, 0.06, or 2 mM ATP for 20 min, with extremely low specific activity transcript. The complexes were separated by electrophoresis on a nondenaturing polyacrylamide gel. RNA was then transferred electrophoretically to GeneScreen membrane and subjected to hybridization analyses with probes of actin, U2, U4, U5, and U6.

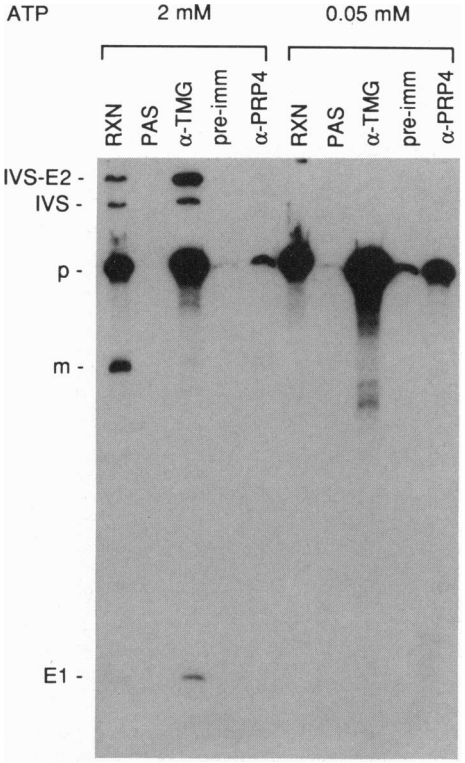

FIG. 4. Immunoprecipitation of splicing reaction mixtures. The splicing reaction was done in the presence of 2 mM or 0.05 mM ATP without $MgCl_2$ or EDTA, and the reaction mixtures were immunoprecipitated with protein A-Sepharose (PAS) alone, or protein A-Sepharose-coupled anti-TMG (α-TMG) or anti-PRP4 antibody (α-PRP4), or preimmune serum to anti-PRP4 antibody (pre-imm). RNA from the precipitates and from 2 μl of the reaction mixture was extracted and analyzed on an 8% polyacrylamide/8 M urea gel. RXN, reaction; IVS-E2, intervening sequence-exon 2; IVS, intervening sequence; p, precursor mRNA; m, mature mRNA.

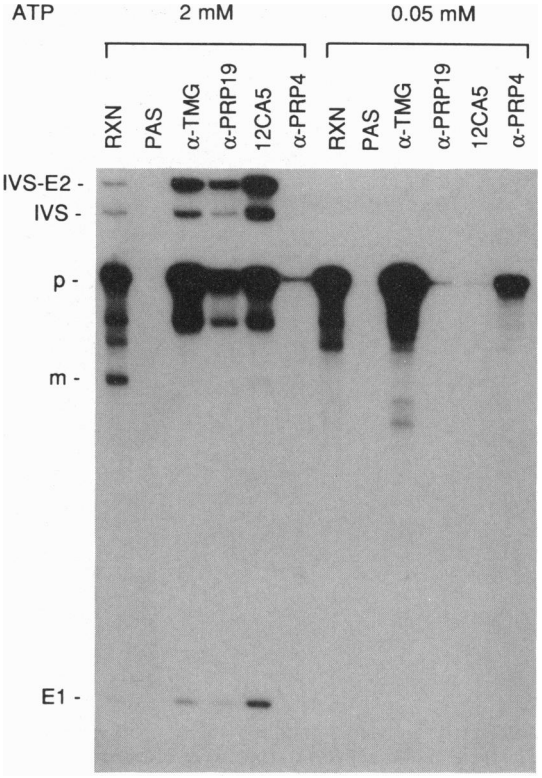

FIG. 5. Immunoprecipitation of splicing reaction mixtures. The splicing reaction was done as in Fig. 4, and the reaction mixtures were immunoprecipitated with protein A-Sepharose (PAS) alone or with protein A-Sepharose-coupled anti-TMG (α-TMG), anti-PRP4 (α-PRP4), anti-PRP19 (α-PRP19), or 12CA5 antibody. RNA was extracted from the precipitates and from 2 μl of the reaction mixture for analysis as for Fig. 4 (see Fig. 4 legend).

0.05 mM ATP, respectively. Fig. 4 shows that at 2 mM ATP anti-TMG antibody precipitated splicing intermediates and the precursor but did not precipitate the mature message, indicative of precipitation of the spliceosome, whereas anti-PRP4 antibody precipitated only a very small amount of the precursor, consistent with previous observations. At 0.05 mM ATP, the amount of precursor precipitated by anti-PRP4 antibody was much higher than that at 2 mM ATP. Prolonged incubation further increased the amount of precursor RNA precipitated at 0.05 mM ATP but decreased the amount of RNA precipitated at 2 mM ATP (data not shown). This result further shows that complex A2-1 accumulates at low concentrations of ATP in the splicing reaction.

PRP19 Is Involved in Transition from Complex A2-1 to A1 During Spliceosome Assembly. Having established a way to block formation of complex A1 and accumulate complex A2-1, we then examined whether PRP19 is specifically associated with complex A2-1. In this experiment, we used an extract prepared from a strain of *S. cerevisiae* in which the PRP19 protein was tagged with the epitope recognized by monoclonal antibody 12CA5 at its C terminus, as described in Tarn *et al.* (31), so that PRP19 could be monitored by monoclonal antibody 12CA5 as well as by anti-PRP19 antibody. The splicing reaction was done with 0.05 mM or 2 mM of ATP, and the reaction mixtures were immunoprecipitated with anti-TMG, anti-PRP4, anti-PRP19, or 12CA5 antibody. Fig. 5 shows that at 2 mM ATP, anti-TMG, anti-PRP19, and 12CA5 antibodies precipitated splicing intermediates and the precursor mRNA but did not precipitate the mature message, as reported (32, 47). In contrast, anti-PRP4 antibody precipitated only a very small amount of the precursor mRNA. At 0.05 mM ATP, a significant amount of the precursor RNA was precipitated by anti-PRP4 antibody. Precipitation with anti-PRP19 or 12CA5 antibody did not significantly precipitate any RNA. This finding indicates that PRP19 is not associated with the complexes formed at 0.05 mM ATP; these include complexes B and A2-1 and perhaps also commitment complex CC (see Figs. 1 and 2). Since we have previously shown that PRP19 is not associated with complex B, this result indicates that PRP19 is also not associated with complex A2-1. As the protein appears in complex A1 (31), PRP19 must become associated with the spliceosome during the transition from complex A2-1 to A1. This result is consistent with our previous observation that complex B and a small amount of complex A2-1 but not of A1 accumulated in PRP19-immunodepleted extract (data not shown). Therefore, PRP19 is likely to play a role in transition from complex A2-1 to A1 during spliceosome assembly.

DISCUSSION

ATP is required for assembly of the spliceosome in essentially every step except for binding of U1 to the pre-mRNA (41). With the identification of a group of splicing factors containing the "DEAD box" motif for RNA helicase and demonstration of RNA-dependent NTPase activities for PRP2 and PRP16 (35–37), it is speculated that a subset of protein factors might function as ATPases using ATP to drive conformational rearrangements of the spliceosome and snRNPs during spliceosome assembly (44). Such rearrangement is best revealed by changes in base-pairing between pre-mRNA and snRNAs during the course of spliceosome assembly, as shown by crosslinking studies (11). An ATP-dependent conformational

change in the spliceosome catalyzed by PRP16 has also been detected (37). In light of this, the ability to dissect the assembly pathway and to block the assembly at various steps so that the intermediate complexes can be isolated is important. We have previously shown (40) that formation of complex A2-2 can be blocked by EDTA in the splicing reaction or by using heat-inactivated *prp2* mutant extracts. We show here that the step at which U4 is dissociated from the spliceosome—i.e., transition from complex A2-1 to A1—can be blocked by limiting the ATP concentration in the splicing reaction. The nature of such blockage is unclear. Perhaps this transition requires a higher amount of ATP to accomplish a large conformational rearrangement. It is also possible that the factor(s) responsive to ATP at this step requires a higher ATP concentration for its function. In our experiments, the amount of complex B accumulated in proportion to that of complex A2-1 when ATP was limited (see Figs. 2 and 3), which suggests that the ATP requirements for the formation of complex B and complex A2-1 are in parallel but are lower than that for formation of complex A1.

The conversion of complex A2-1 to A1 involves unwinding of the U4/U6 duplex. An RNA helicase activity has been implicated in this step, but no identified protein factor has been shown to possess RNA helicase activity. Concomitant with U4 dissociation, the modes of interaction between the pre-mRNA and snRNAs also change (11). New base-pairings between U2 and U6, and U6 and the 5′-splice site region of the pre-mRNA are detected (11). These events presumably occur after disruption of U4/U6 base-pairings. The factors that mediate such rearrangements also remain to be identified. We had previously tried to determine which step of the spliceosome assembly process requires PRP19 by examining complex formation in PRP19-immunodepleted extracts or heat-inactivated *prp19* mutant extracts. Although we observed the accumulation of complex B and a small amount of complex A2-1 but not of complex A1, we could not conclude that PRP19 was required for transition from complex A2-1 to complex A1 due to difficulty in judging whether the PRP19 protein was completely immunodepleted or heat-inactivated in the mutant extract. We therefore examined which splicing complex PRP19 was associated with during spliceosome assembly and showed that PRP19 was associated with complex A1 but not with complex B, suggesting that PRP19 is involved in the step for the formation of complex A2-1 or complex A1 (31). Here, we demonstrate that PRP19 is not associated with complex A2-1. Although we cannot exclude the possibility that the protein is associated with the complex but is not accessible to either anti-PRP19 or 12CA5 antibody, this result is consistent with our observation that complex A1 did not form in the PRP19-immunodepleted extract. Moreover, while no significant amount of the endogenous spliceosomes accumulated in the wild-type extract, accumulation of splicing complexes migrating at the position of complexes B and A2, presumably representing A2-1, was observed in the *prp19* mutant extract (data not shown). Therefore, PRP19 must become associated with the complex during the transition from complex A2-1 to A1 and is likely to play a role in this transition.

The role of PRP19 in transition from complex A2-1 to A1 is not known. Because this process involves a major conformational rearrangement, one would expect involvement of ATPase, RNA helicase, RNA hybridizing activities, or ATP-dependent chaperone-like molecules in this process. It is likely that other protein factors, in addition to PRP19, are involved in this process. Although its precise function is still unknown, PRP19 is now positively identified as a protein factor that associates with the spliceosome at this step in the assembly pathway.

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