Formation of the yeast splicing complex A1 and association of the splicing factor PRP19 with the pre-mRNA are independent of the 3' region of the intron

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ABSTRACT

Assembly of the spliceosome is a step-wise process and involves sequential binding of snRNAs to the pre-mRNA to form pre-splicing complex A2-1. Subsequent dissociation of U4 from the spliceosome is accompanied by formation of complex A1 (*Genes Dev.* 1, 1014–1027, 1987). We show that the 3' region of the intron sequence is not required for efficient assembly of the yeast spliceosome. Truncated precursor mRNA retaining only four or five nucleotides 3' to the TACTAAC box formed pre-splicing complex A1, kinetically the last pre-mRNA containing splicing complex identified. The subsequent cleavage–ligation reaction requires at least 23 nucleotides on the 3' side of the TACTAAC box in a sequence-independent manner. Immunoprecipitation with anti-PRP19 antibody showed that association of PRP19 with the spliceosome was also independent of the 3' region of the intron.

INTRODUCTION

The coding sequences of the majority of eukaryotic genes are interrupted by introns. Thus, the removal of intervening sequences by the process of RNA splicing is an important step in gene expression. A fundamental problem for splicing of pre-mRNA is how the splice junctions are identified and juxtaposed, despite their separation in the primary structure by as many as 100,000 nucleotides (1, 2). The development of *in vitro* systems that carry out pre-mRNA splicing has facilitated the study of the mechanism of mRNA splicing, the components required for the reaction and the pathway of assembly of the spliceosome. A two-step mechanism for the splicing reaction has been formulated. The first step is cleavage at the 5' splice site and formation of a lariat structure and the second step is cleavage at the 3' splice site and ligation of the two exons (for review, see references 3,4).

In contrast to poorly conserved metazoan introns, analysis of yeast intron sequences has revealed three highly conserved elements required for splicing. These are the 5' consensus sequence GTAPyGT, the 3' splice junction sequence PyAG and the sequence TACTAAC which lies near the 3' splice junction and functions as the branch point (5–7). Mutation at the 5' consensus sequence and the TACTAAC sequence results in

significant reduction of the splicing efficiency (8, 9), while alteration of the last nucleotide of the intron only blocks the second cleavage–ligation reaction without affecting the first (8–10). The polypyrimidine tract near the 3' splice site plays a critical role in early steps of metazoan splicing (10–12), although not important for the first cleavage–ligation reaction and early steps of spliceosome assembly in yeast (13–15). Several protein factors required early in the splicing reaction have been shown to bind to the polypyrimidine tract (16–18).

Assembly of the spliceosome is a multistep process involving sequential binding of snRNAs (small nuclear RNAs) to the pre-mRNA in an order of U1, U2 then U4/U6 plus U5 as a preformed tri-snRNP (small nuclear ribonucleoprotein particle) (19–23). 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 reaction (24). Splicing complexes representing intermediates of assembly of the spliceosome have been identified and resolved by electrophoresis on nondenaturing gels. They have previously been designated A1, A2-1, A2-2, A2-3 and B (25, 26). Splicing complex containing only U1, named as the commitment complex (CC), was identified in a different electrophoretic system (27). The order of assembly was determined to be CC→B→A2-1→A1→A2-2→A2-3 (25, 26). A summary of the assembly pathway is shown in Figure 1.

Interactions between snRNAs and the pre-mRNA have been extensively studied. In both 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 (28–29). *In vivo* analysis in the fission yeast *Schizosaccharomyces pombe* suggests that U1 also interacts with the 3' splice site of the intron through base pairing (30). Similar studies in the budding yeast *Saccharomyces cerevisiae* have demonstrated that U5 interacts with the pre-mRNA, in this case with exon sequences near the two splice junctions (31, 32). U6 appears to interact with the pre-mRNA at the 5' splice site, as shown by UV cross-linking studies (33, 34). Base-pairing between U2 and U6 has also been shown to play an important role during spliceosome assembly (35).

During studies of spliceosome assembly, pre-mRNA molecules deleted of various regions of the intron or exon sequence were used to examine the effect of the intron sequences on the

assembly. It is found that pre-mRNAs deleted of the 3' exon together with most of the intron sequences on the 3' side of the TACTAAC box are functional for assembly of the splicing complex A1, although with a slightly lower efficiency. Kinetically complex A1 corresponds to the last precursor mRNA containing complex before the cleavage–ligation reaction (25, 26). At least 23 nucleotides downstream of the TACTAAC sequence were required for the first cleavage–ligation reaction to take place. As further shown here that association of the yeast essential splicing factor PRP19 with the spliceosome is not affected by deletion of the 3' splice site.

MATERIALS AND METHODS

Plasmid construction

Plasmid pBS was digested with *Cla*I and *Pvu*II, and the two *Cla*I–*Pvu*II fragments were used to replace the *Cla*I–*Pvu*II fragment of pSP65Δ6 (8) to generate CS and CK.

Preparation of the splicing extracts and substrates

Yeast whole cell extracts were prepared according to Cheng *et al.* (36). Actin and CYH2^m precursor RNA were synthesized *in vitro*, using SP6 RNA polymerase according to Cheng and Abelson (25). *Eco*RI, *Cla*I or *Hpa*II digested Proteus 6-Actin DNA (37), or *Hind*III linearized pSP65Δ6 were used as templates for synthesis of actin transcripts, and *Dra*III linearized pSP64-CYH2^m DNA (38) was used for synthesis of CYH2^m transcript.

Splicing assays

In vitro splicing reactions were carried out in a buffer containing 60 mM potassium phosphate (pH 7), 1 mM spermidine, 2 mM ATP, 3% PEG, 0.4–1 nM transcript and 40% of the splicing extract, unless described otherwise. For Northern hybridization experiments, the reactions contained 4 nM transcript instead of 0.4–1 nM. In some cases, 3 mM MgCl₂ and 1 mM EDTA were included in the reaction for analysis of splicing complexes. Spliceosome formation was analyzed by electrophoresis on nondenaturing polyacrylamide gel as described in Cheng and Abelson (25).

Hybridization analysis

The small nuclear RNA content of individual splicing complexes was analyzed by Northern hybridization as described in Cheng and Abelson (25), except that the U6 probe was prepared from a DNA fragment from pT7U6 (39).

Immunoprecipitation

Immunoprecipitation with anti-PRP19 or 12CA5 antibody (Berkeley Antibody Company) as the nonspecific antibody was carried out according to Tarn *et al.* (40).

RESULTS

Actin pre-mRNA containing only four nucleotides downstream of the TACTAAC box formed the pre-splicing complex A1 but not the splicing complex A2-2

A restriction map of a portion of the yeast actin gene used for preparation of substrates for the splicing reaction is shown in Figure 2. Plasmid containing this DNA segment is usually linearized with *Eco*RI or digested with *Hpa*II in preparation of substrates for *in vitro* transcription with SP6 RNA polymerase.

This generates substrates containing a 5' exon of 88 nucleotides, an intron of 309 nucleotides and a 3' exon of either 162 nucleotides (Ac/*Eco*) or 48 nucleotides (Ac/*Hpa*) (37). Substrate RNA prepared from *Cla*I linearized DNA (Ac/*Cla*) contains only 4 nucleotides downstream of the TACTAAC box.

Figure 1. The pathway for spliceosome assembly. The scheme summarizes results from yeast and mammals. Complex CC is the commitment complex described by Séraphin and Rosbash (27). Complexes B, A2-1, A2-2 and A2-3 are described by Cheng and Abelson (25) and by Ruby and Abelson (26). Complexes CC, B, A2-1 and A1 are pre-splicing complexes containing precursor mRNA. Complexes A2-2 and A2-3 are active splicing complex containing splicing intermediates and products, respectively. The snRNA contents of each complex are indicated (25). Association of U1 with the pre-mRNA before binding of U2 is from the results of Bindereif and Green (53), Ruby and Abelson (54), and Séraphin and Rosbash (27). The absence of U1 in late complexes is the result of Wassarman and Steitz (47). Transition from complex A1 to A2-2 is blocked in heat-inactivated *prp2* mutant extracts (25). PRP19 is involved in the transition from complex A2-1 to A1 (40, 42).

Figure 2. The restriction sites on the actin precursor RNA and the sequence 3' to the TACTAAC box of actin and CYH precursor RNAs. Open boxes are the exons. The positions of TACTAAC box and TACTAA sequence, the latter of which is deleted in the Δ6 pre-mRNA, are indicated. CK and CS are two constructs generated by replacing the *Cla*I–*Pvu*II fragment of actin pre-mRNA construct with two polylinker sequence containing *Cla*I–*Pvu*II fragments from plasmid pBS. Ac/*Cla* represents actin RNA terminated at the *Cla*I site, retaining only 4 nucleotides ATCG 3' to the TACTAAC box. Cyh/*Dra* represents pre-mRNA from the cycloheximide resistance gene terminated at the *Dra*III site, retaining only 5 nucleotides GATTT 3' to the TACTAAC box. The sequence on the 3' side of the TACTAAC box of transcripts generated from CK and CS is shown. The length of the transcripts on the 3' side of the TACTAAC box is indicated in the parentheses.

When these RNAs were used for the *in vitro* splicing reaction, RNAs Ac/Eco and Ac/Hpa spliced efficiently, whereas no splicing intermediates or products were detected with Ac/Cla RNA as shown in Figure 3A. This indicates that the intact 5' splice site and the TACTAAC box are not sufficient for the first step of the splicing reaction, consistent with that reported by Rymond *et al.* that in the 3' region of the intron, at least 29 nucleotides on the 3' side of the TACTAAC box are required for the first cleavage-ligation reaction (41). Formation of splicing complexes was also examined by electrophoresis on native polyacrylamide gel. As shown in Figure 3B, while complexes B, A1 and A2 were all seen at 10 minutes of the reaction with Ac/Hpa (lane 2), only complexes A1 and B appeared with Ac/Cla in an order of B→A1. No complex migrating at the position of A2, which may represent A2-1 or A2-2 or both, was detected. It is known that complex A2-1 is extremely short-lived and is present only in a very small amount,

usually not detected, in a regular splicing reaction with an ATP concentration above 0.2 mM (25, 42). It is likely that spliceosome assembly on Ac/Cla went through a normal pathway B→A2-1→A1, in which A2-1 was undetectable, and was blocked for the subsequent steps. Indeed, when splicing reactions were carried out in the presence of a low concentration of ATP, formation of complex A1 was blocked, and complex A2-1 accumulated in both Ac/Eco and Ac/Cla (data not shown, see reference 42). Formation of complex A1 but no complex A2-2 on Ac/Cla indicates that with most sequences downstream of the TACTAAC box deleted, pre-mRNA molecules still undergo spliceosome assembly through a regular pathway, but was blocked for the subsequent cleavage-ligation reactions.

To prove that the complexes seen at positions of A1 and B truly represent splicing complexes, snRNA contents of these complexes were examined by Northern blot analysis. The splicing reactions were carried out with unlabeled RNAs Ac/Eco and Ac/Cla for 2, 5 and 10 minutes, and the splicing complexes were separated by electrophoresis on native polyacrylamide gel. The reaction mixtures contained 1 mM EDTA and 3 mM MgCl₂ for more efficient accumulation of complex A1 with untruncated pre-mRNA (25). These complexes were then electroblotted to a GeneScreen membrane and hybridized with probes for actin, U2, U5 and U6 as shown in Figure 4. On the Northern blot, the free U2 snRNP can be detected as a faster migrating complex than complex B (25). The fast migrating complex with Ac/Eco on the actin blot is a non-specific complex, which was not seen with Ac/Cla due to the small size of the RNA. As in the case for Ac/Eco, Ac/Cla formed complex B contained U2 RNA, and complex A1 contained U2, U5 and U6 RNAs. This indicates that complexes formed on Ac/Cla RNA were specific splicing complexes, containing specific snRNAs as those formed with untruncated RNA. However, the amounts of complexes formed with Ac/Cla RNA appeared to be approximately 3-fold lower for both complexes B and A1 than those with Ac/Eco RNA (Figure 3). This suggests that the 3'-region deleted pre-mRNA undergoes spliceosome assembly through a normal pathway to form pre-splicing complex A1, but with a slightly lower efficiency even at early steps of the assembly process.

Figure 3. Pre-mRNA deleted of the 3' region of the intron does not splice, but forms splicing complex A1. **A**, the splicing reactions of actin pre-mRNAs Ac/Eco, Ac/Hpa and Ac/Cla. **B**, splicing complex formation of actin pre-mRNAs, Ac/Hpa and Ac/Cla. Ac/Eco, Ac/Hpa and Ac/Cla represent actin pre-mRNA terminated at the *Eco*RI site, *Hpa*II site and *Cla*I site, respectively.

Figure 4. Northern blot analysis of the splicing complexes. The splicing reactions were carried out under standard condition except in the presence of 3 mM MgCl₂ and 1 mM EDTA, and in the presence or absence of 4 nM of extremely low specific activity transcript of Ac/Eco and Ac/Cla, and the reaction mixtures were fractionated by electrophoresis on nondenaturing polyacrylamide gel. RNA was then transferred to GeneScreen membrane and subjected to hybridization analyses with probes of actin, U2, U5 and U6.

Figure 5. Spliceosome formation of Ac/Eco, Ac/Cla, $\Delta 6$ /Cla and Cyh/Dra. The splicing reactions were carried out under standard condition with Ac/Eco, Ac/Cla, $\Delta 6$ /Cla and Cyh/Dra, and analyzed by electrophoresis on nondenaturing polyacrylamide gel.

Figure 6. Immunoprecipitation of splicing complexes by anti-PRP19 antibody. The splicing reactions were carried out under standard condition with Ac/Eco, Ac/Cla, $\Delta 6$ /Cla and Cyh/Dra, and the reaction mixtures were subjected to immunoprecipitation with anti-PRP19 (α -PRP19) or a nonspecific (NS) antibody. RNA from the precipitates and from 1/10 volume of the reaction mixtures (RXN) was extracted and analyzed by electrophoresis on nondenaturing polyacrylamide gel.

Formation of complex A1 with 3' region truncated pre-mRNAs is independent of the cryptic TACTAC sequence in the actin gene

It is known that the TACTAAC sequence plays an important role in early steps of the spliceosome assembly process in yeast. U2 recognition of the pre-mRNA is mediated through base-pairing between the 5' end of the U2 RNA and the TACTAAC sequence (29). A single point mutation in the TACTAAC sequence could block formation of all splicing complexes (25) except complex

CC (43). The actin intron contains the sequence TACTAAG seven nucleotides upstream of the TACTAAC box. It has been reported that this sequence can function as a branch point in the absence of the authentic TACTAAC box (12). It is not clear whether formation of complex A1 on Ac/Cla was due to utilization of the authentic TACTAAC box, or the cryptic TACTAA sequence for recognition by U2, since there are only 4 nucleotides 3' to the TACTAAC box. Splicing complex formation was then examined with RNA $\Delta 6$ /Cla, in which the cryptic TACTAA sequence is deleted. Figure 5 shows that the $\Delta 6$ /Cla RNA also formed splicing complexes (lanes 5–7) in a fashion similar to Ac/Cla, but with slightly lower efficiency (lanes 2–4). This suggests that assembly of the spliceosome on Ac/Cla may be in part, but not entirely, due to utilization of the cryptic TACTAA sequence, and this sequence only 4 nucleotides on the 3' side of the TACTAAC box was sufficient for the assembly.

RNA Cyh/Dra was transcribed from a portion of the yeast *CYH* gene and contained portions of the 5' exon and the intron with only 5 nucleotides on the 3' side of the TACTAAC box as shown in Figure 2. No cryptic TACTAAC sequence is present in this RNA. As shown in Figure 5, Cyh/Dra also formed splicing complexes in a fashion similar to Ac/Cla and $\Delta 6$ /Cla (lanes 8–10). This further confirms that the 3' splice site was dispensable for assembly of splicing complex A1. Only 4 or 5 nucleotides 3' to the branch site sequence are thus sufficient for assembly of complex A1, although the efficiency of complex formation is somewhat reduced. Furthermore, this phenomena is not only specific for the actin RNA.

PRP19 is associated with the complex A1 formed with 3'-region truncated pre-mRNAs

We have previously shown that the yeast PRP19 protein is associated with the spliceosome during spliceosome assembly (44). PRP19 becomes associated with the spliceosome during transition from complex A2-1 to A1, and remains associated throughout the cleavage–ligation reactions (40, 42). The fact that complex A1 formed in the absence of the 3' splice site suggests that binding of PRP19 to the spliceosome may be independent of the 3' portion of the intron sequence. This was examined by immunoprecipitation with the anti-PRP19 antibody. The splicing reactions were carried out using substrate RNAs Ac/Eco, Ac/Cla, $\Delta 6$ /Cla or Cyh/Dra, and the reaction mixtures were subjected to immunoprecipitation with anti-PRP19 antibody. As shown in Figure 6, precipitation of the splicing reaction mixture with Ac/Eco precipitated approximately 10% of the splicing intermediates, excised IVS and the precursor, but not the mature message. Precipitation of the splicing intermediates and precursor RNA but not the mature message is diagnostic for precipitation of the spliceosome (44, 45). With Ac/Cla, $\Delta 6$ /Cla or Cyh/Dra, approximately 5–10% of the RNA were precipitated. This indicates that PRP19 is tightly associated with the splicing complexes formed on Ac/Cla and Cyh/Dra. Association of PRP19 with the spliceosome formed on precursor mRNAs deleted of the 3' portion of the intron and the 3' exon was nearly as efficient as with that formed on the untruncated pre-mRNA.

Transition from complex A1 to A2-2 requires at least 23 nucleotides downstream of the TACTAAC sequence in a sequence-independent manner

The sequence requirement for transition from complex A1 to A2-2 was examined. To synthesize pre-mRNAs containing

different lengths and sequences of RNA 3' to the TACTAAC box, two plasmids were constructed, in which the *Clal*–*PvuII* fragment of the plasmid pSP65Δ6 was replaced with two *Clal*–*PvuII* fragments from plasmid pBS, each containing one half of the polylinker region. As shown in Figure 2, CK linearized with *XhoI*, *ApaI* or *KpnI* generates transcripts containing 20, 25 and 31 nucleotides, respectively, downstream of the TACTAAC box. Transcripts containing 11, 15, 23, 33, 41 and 53 nucleotides downstream of the TACTAAC box can be obtained by digestion of the CS construct with *HindIII*, *EcoRV*, *EcoRI*, *SmaI*, *BamHI* and *XbaI*, respectively.

These transcripts were examined for their abilities to form complex A2-2 during spliceosome assembly. As formation of complex A2-2 is accompanied by the occurrence of the first cleavage–ligation reaction, formation of the lariat structure was used to monitor formation of complex A2-2. As shown in Figure 7, no lariat structure was detected with CS/Hd, which contains 11 nucleotides downstream of the TACTAAC box. A transcript containing 18 nucleotides on the 3' side of the TACTAAC box also generated no lariat RNA (data not shown). A very small amount of the lariat RNA formed with CS/Eco and CK/Apa, which contain 23 and 25 nucleotides, respectively, downstream of the TACTAAC box. Increasing amounts of the lariat RNA formed with increasing length of RNA 3' to the TACTAAC box. Two species of lariat RNA molecules were detected when the length 3' to the TACTAAC box was more than 33 nucleotides (CS/Sma and CS/Bam), indicating possible occurrence of the second cleavage–ligation reaction. Since CS/Eco and CK/Apa contain different sequences, we conclude that formation of complex A2-2, or the first cleavage–ligation reaction, requires at least 23 nucleotides downstream of the TACTAAC box in a sequence-independent manner.

DISCUSSION

The results reported herein demonstrate that truncated pre-mRNAs, retaining only 4 or 5 nucleotides on the 3' side of the TACTAAC box, undergo efficient formation of splicing complex A1. This is based on two lines of evidence. First, analysis of spliceosome formation by electrophoresis on nondenaturing polyacrylamide gel revealed that complex A1 formed on truncated pre-mRNA which retained only 4 or 5 nucleotides on the 3' side of the TACTAAC box. The complex A1, thus formed, contained snRNAs U2, U5 and U6 comparable to that formed with the untruncated pre-mRNA, suggesting it is specific splicing complex. Second, anti-PRP19 antibody precipitated splicing complexes formed on the 3' region-truncated pre-mRNA. It has previously been demonstrated that during the splicing reaction, PRP19 becomes associated with the spliceosome concomitant with or just after dissociation of U4 from the spliceosome (42), and remained associated throughout the reaction (40, 44). In other words, PRP19 is not associated with complexes CC, B or A2-1, but is associated with complexes A1, A2-2 and A2-3, among which A1 is the only complex containing unspliced pre-mRNA (see Figure 1 and reference 25). Precipitation of the truncated pre-mRNA by the anti-PRP19 antibody, therefore, indicates formation of complex A1 with the truncated pre-mRNA. We cannot exclude the possibility that the complex A1 formed on the 3'-region truncated pre-mRNA is a premature form of the A1 complex in lack of some other component(s), although this complex shows similar electrophoretic mobility as complex A1 and contains U4, U5, U6 and PRP19.

Figure 7. Length- and sequence-dependency in the 3'-region of the intron for the splicing reaction. RNAs Δ6/Hd, CS/Hd, CK/Apa, CK/Kpn, CS/Sma, CS/Bam and CS/Eco were assayed for splicing under standard conditions, and analyzed on 8% polyacrylamide-8 M urea gels. The length of the RNA downstream of the TACTAAC box of individual pre-mRNAs is indicated in the parentheses.

Rymond *et al.* investigated the importance of sequences between the yeast branch point and 3' splice site on the pre-mRNA splicing reaction (41). Their results indicate that at least 29 nucleotides of RNA on the 3' side of the TACTAAC box are required for 5' cleavage and lariat formation. Truncated pre-mRNAs retaining only a few nucleotides on the 3' side of the TACTAAC box, although incapable of forming lariat intermediate, form splicing complexes I and III, with accumulation of complex I upon prolonged incubation (41). Formation of complex II, which contains splicing intermediates and products, is inhibited (41). In their electrophoretic system for the analysis of splicing complexes, complexes I, II and III correspond to our complexes A2-1, A2-2/A2-3 and B, respectively (25, 46). Complex A1 is not detected in their electrophoretic system. Thus, it was not clear whether truncation of the 3' region inhibits the formation of complex A1 or the formation of complex A2-2. Our result is consistent with their report that formation of splicing complexes containing processed mRNA was blocked with the 3' region-truncated pre-mRNAs. Furthermore, this result shows that these 3' region-truncated pre-mRNAs are capable of forming the identified complex A1, which is kinetically the last precursor mRNA containing splicing complex.

By addition of homoribopolymer tails to the 3' side of the TACTAAC box, Rymond *et al.* also show that the 3' region requirement for 5' cleavage and lariat formation is length-dependent but sequence-independent (41). Here, we utilize a different method to generate transcripts of various lengths and sequences on the 3' side of the TACTAAC box, using constructs CS and CK, to examine the sequence dependency for transition from complex A1 to A2-2. We reach a similar conclusion that at least 23 nucleotides behind the TACTAAC box are required in a sequence-independent manner.

Assembly of the spliceosome involves sequential binding of snRNAs to the pre-mRNA to form splicing complex A2-1 (19–23). Subsequently, U4 is dissociated from the spliceosome to form complex A1 (25). A large conformational rearrangement occurs during this transition since new base-pairings between snRNAs, and between snRNAs and the pre-mRNA form (47). It is interesting that RNA 3' to the TACTAAC box is not required for all of these steps but is required for a later step for formation of complex A2-2. Transition from complex A1 to A2-2 requires at least two trans-acting factors, b2 (PRP2) and bn (48). While factor bn remains unidentified, PRP2 is known to belong to the family of 'DEAD box' proteins, which share a number of highly conserved amino acid motifs characteristic of ATP-dependent RNA-helicase (26), and has been demonstrated RNA-dependent ATPase activity (49). Although the PRP2 protein has been shown to interact with the spliceosome transiently during the splicing reaction (47, 50), the function of PRP2 in transition from complex A1 to A2-2 is not known. It is possible that interactions of PRP2 and/or bn with the spliceosome requires extended sequences 3' to the TACTAAC box to induce a conformational change so that the cleavage–ligation reaction can take place. Alternatively, such interactions may not require sequence in the 3' region of the intron, but the continuity of the RNA chain 3' to the TACTAAC box may be required for the conformational rearrangement. A dominant negative allele of *PRP2* has been isolated. The mutant PRP2 protein is capable of binding to the spliceosome and remains tightly associated with the spliceosome, preventing the subsequent cleavage–ligation reaction (51). It will be interesting to see whether the mutant PRP2 protein can interact with the spliceosome formed with the 3' splice site truncated pre-mRNA.

Genetic analysis in the budding yeast *Saccharomyces cerevisiae* demonstrates that U5 snRNA interacts with the 5' exon and the first two bases of the 3' exon (32). Similar analysis in the fission yeast *Schizosaccharomyces pombe* shows that U1 also interacts with the 3' splice sites (30) in addition to base-pairing with the 5' splice site of the intron. Nevertheless, the stages of the spliceosome assembly process such interactions occur are not demonstrated. It has been shown that U5 becomes associated with the spliceosome in a form of U4/U6–U5 tri-snRNP particle upon formation of complex A2-1, and remains tightly associated with the spliceosome throughout the splicing reaction (25, 46). By crosslinking studies, base-pairing between U5 and the 5' splice site is detected upon dissociation of U4 in the mammalian spliceosome (47). This step corresponds to formation of complex A1 in yeast (see Figure 1). Since our results show that the 3' region of the pre-mRNA, including the complete 3' exon sequence, is dispensable for formation of complex A1, at least in *Saccharomyces cerevisiae*, interaction of U5 with the 3' exon possibly occurs or is important only after formation of complex A1. Consistently, in a recent report, crosslink between U5 and exon 2 is detected on the lariat IVS-E2 intermediate but not on the pre-mRNA in mammalian spliceosome, suggesting that U5 may interact with exon 2 only after the first cleavage–ligation reaction (55).

Base-pairing between U1 and the 5' splice site has been well established in both yeast and mammals (28, 52). During spliceosome assembly, U1 is the first snRNA to bind to the pre-mRNA early in the assembly (53, 54), presumably through base-pairing with the 5' splice site. Interaction of U1 with the 3' splice site, demonstrated in the fission yeast, may occur in a later step of the assembly process. Such interaction, if occurs, does not require base pairing between U1 and the 3' splice site (56). The

result shown here that the 3' region of the intron is dispensable for spliceosome assembly suggests that in the budding yeast such interaction is also not essential for the assembly prior to the cleavage–ligation reaction.

Previously, it was shown that PRP19 is associated with the spliceosome during spliceosome assembly (44). It becomes associated when complex A1 forms, but is not associated with complexes formed before formation of complex A1 in the assembly pathway (40, 42). It is shown here that formation of complex A1 does not require the 3' region of the intron. Additionally, the 3' region of the intron is also dispensable for binding of PRP19 to the spliceosome. Thus, association of PRP19 with the spliceosome and complex A1 formation occur simultaneously not only with a regular pre-mRNA, but also with a pre-mRNA truncated of the 3' region. This implies that PRP19 may play an important role in mediating formation of complex A1.

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