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Spliceosome Activation by PRP2 ATPase prior to the First Transesterification Reaction of Pre-mRNA Splicing

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In addition to small nuclear RNAs and spliceosomal proteins, ATP hydrolysis is needed for nuclear pre-mRNA splicing. A number of RNA-dependent ATPases which are involved in several distinct ATP-dependent steps in splicing have been identified in *Saccharomyces cerevisiae* and mammals. These so-called DEAD/H ATPases contain conserved RNA helicase motifs, although RNA unwinding activity has not been demonstrated in purified proteins. Here we report the role of one such DEAH protein, PRP2 of *S. cerevisiae*, in spliceosome activation. PRP2 bound to a precatalytic spliceosome prior to the first step of splicing. By blocking the activity of a novel splicing factor(s), HP, which was involved in a post-PRP2 step, we found that PRP2 hydrolyzed ATP to cause a change in the spliceosome without the occurrence of splicing. The change was quite dramatic and could account for the previously reported differences between the precatalytic, pre-mRNA-containing spliceosome and the "active," intermediate-containing spliceosome. The post-PRP2-ATP spliceosome was further isolated and could carry out the subsequent reaction apparently in the absence of PRP2 and ATP. We hypothesize that PRP2 functions as a molecular motor, similar to some DExH ATPases in transcription, in the activation of the precatalytic spliceosome for the transesterification reaction.

Several RNA-dependent ATPases which are essential for pre-mRNA splicing have been identified in *Saccharomyces cerevisiae* (reviewed in references 3, 17, 40, and 41). These proteins contain the conserved RNA helicase motifs, including the signature DEAD or DEAH sequence (reviewed in references 13, 15, 43, and 52). For example, PRP5 and PRP28 are involved in spliceosome assembly, PRP2 and PRP16 are required for the catalytic steps, and PRP22 is required for the release of mRNA from the spliceosome. Recently, a human PRP22 homolog was isolated and characterized (33, 34), and a mammalian RNA-dependent ATPase activity was found to associate with the U5 small nuclear ribonucleoprotein particle (26). This work was an attempt to address the role of one such ATPase, PRP2, in pre-mRNA splicing.

A considerable amount of work has been done on the role of PRP16 in splicing (reviewed in reference 50). PRP16 is required for an ATP-dependent reaction during the second step of splicing (44). ATP hydrolysis by PRP16 apparently can be uncoupled from the second transesterification reaction (reaction 2). This was shown by depleting splicing factors, such as PRP18 (20) or SLU7 (2, 21), which are needed after the PRP16 step from extracts. The 3′ splice site becomes resistant to RNase H after ATP hydrolysis by PRP16, suggesting that some changes to the spliceosome occur (45). Interestingly, PRP16 also plays a proofreading role in splicing (7).

Previous results from our own and other laboratories indicate that PRP2 also interacts with the spliceosome transiently (22, 24, 37). The protein binds to the pre-mRNA-containing spliceosome in the absence of ATP in vitro and is released from the spliceosome upon ATP hydrolysis and the occurrence of the first transesterification reaction (reaction 1). These results indicate that ATP hydrolysis and transesterification are tightly linked; however, whether or not reaction 1 can be uncoupled from PRP2-mediated ATP hydrolysis has not been tested. The pre-mRNA-containing spliceosome assembled in the absence of active PRP2 was called the prp2Δ spliceosome (28), the A1 complex (9), or complex I (36, 37). On native acrylamide gels, this prp2Δ splicing complex migrates more slowly than does splicing complex A2-2 (or II), which contains pre-mRNA and splicing intermediates. Kinetic studies of yeast spliceosome assembly also indicate that reaction 1 occurs during the conversion of complex A1 (complex I) to complex A2-2 (complex II) or in complex A2-2 (complex II) after the conversion (9, 36). Although ATP hydrolysis by PRP2 is required for the first step of splicing, the involvement of PRP2 in the conversion of complex A1 to A2-2 or in the transesterification reaction is less clear. It is worth noting that the difference (as revealed by native gel electrophoresis) between A1 and A2-2 is as significant as the difference between the A2-1 complex (which contains U4 small nuclear RNA) and A1 (which does not contain U4) (9, 53). Thus, it is important to investigate whether PRP2-mediated ATP hydrolysis is responsible for one of the most significant rearrangements in the spliceosome, namely A1 to A2-2, and whether the A2-2 complex is indeed the "active" spliceosome.

In this study, we identified and partially purified a protein factor(s), called HP, which was required after the PRP2 step. By blocking the HP activity with heparin, we showed that PRP2 hydrolyzed ATP and exited the spliceosome before reaction 1. The post-PRP2 spliceosome had a gradient sedimentation rate and a gel mobility indistinguishable from those of complex A2-2 (complex II). This newly identified pre-mRNA-containing spliceosome could carry out reaction 1 after it was isolated from a glycerol gradient; the reaction required the addition of HP but not ATP or PRP2. These results suggest a model in which PRP2 functions as a molecular motor to restructure the spliceosome to an active conformation so that the transesterification reaction can occur. This model has interesting similar-

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TABLE 1. Various spliceosome preparations

Spliceosome	Description	Additional factor(s) needed to complete reaction 1
SS2	Spliceosome containing pre-mRNA substrate formed in prp2Δ extracts	PRP2, ATP
hep-SS2	SS2 isolated in the presence of heparin	PRP2, HP, ATP
hs-SS2	SS2 isolated in the presence of 400 mM KCl	PRP2, HP, ATP
$SS2^{PRP2}$	SS2 with PRP2 bound	ATP
hep-$SS2^{PRP2}$	$SS2^{PRP2}$ isolated in the presence of heparin	HP, ATP
hs-$SS2^{PRP2}$	$SS2^{PRP2}$ isolated in the presence of 400 mM KCl	HP, ATP
SS3	Spliceosome containing pre-mRNA substrate formed after the PRP2 ATP step in the presence of heparin	HP
SI	Spliceosome containing splicing intermediates formed after step 1	
hep-SI	SI isolated in the presence of heparin	

ities with specific DExH ATPases involved in promoter clearance (5) and chromatin remodeling (35).

MATERIALS AND METHODS

In vitro splicing. Splicing extract isolation, ^{32}P-labeled pre-mRNA substrate preparation, and in vitro splicing reactions have been described previously (22). Wild-type actin and 3′ splice site mutant pre-mRNAs were prepared from *Eco*RI-digested SP6-actin plasmid (29) and *Hin*dIII-digested SP65-Actin-C303/C305(Δ6) plasmid (51), respectively. To assay the gradient-isolated spliceosomes for splicing activity, 0.5 μl of 20 mM ATP, 0.5 μl of 50 mM $MgCl_2$, 5 ng of purified PRP2 protein in 1 μl, and 1 μl of HP were added to 7 μl of a spliceosome-containing gradient fraction. The mixture was incubated at 23°C for 30 min, and the reaction products were extracted and analyzed on polyacrylamide-urea gels (22).

Purification of HP. HP was partially purified from a protease-deficient strain, BJ2168 (*MAT***a** *pep4-3 prb1-1122 prc1-407 leu2 trp1 ura3-52*), obtained from the Yeast Genetic Stock Center at the University of California, Berkeley. Cells from 20 liters of overnight culture in YPD medium were harvested and extracted as previously described (29). After the ultracentrifugation step, the soluble extracts (180 ml) were divided into 10-ml aliquots and heated in a boiling water bath for 5 min. The heated extracts were centrifuged in a Sorvall SS34 rotor for 30 min at 17,000 rpm. Ammonium sulfate was added to the supernatant (SUP100; 120 ml) to 72% saturation, and the mixture was centrifuged again at 17,000 rpm for 30 min. The pellet was dissolved in 5 ml of buffer A, which contained 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.4), 0.5 mM EDTA, 5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride (PMSF) (Boehringer Mannheim), 0.01% Nonidet P-40, and 10% glycerol. A 3.5-ml aliquot of the suspension was loaded on an Ultrogel AcA 34 (IBF Biotechniche) gel filtration column (2.5 by 100 cm) preequilibrated with buffer A plus 300 mM KCl. The flow rate was 9 ml/h. The gel filtration column was calibrated with gel filtration protein standards (Bio-Rad) before use. The peak fractions (40 ml) containing the HP activity were dialyzed against 2 liters of buffer A plus 50 mM KCl for 3 h and loaded on a heparin-agarose column (1.2 ml) preequilibrated with the dialysis buffer. Proteins were step eluted with 3 column volumes (each) of buffer A plus 50 to 550 mM KCl with 100 mM increments. The HP activity was eluted from the heparin-agarose column between 150 to 250 mM KCl.

Proteinase K and MN digestions. To test the protease sensitivity of HP, 9 μl of the heparin-agarose fraction and 1 μl of 200-μg/ml (~10 μM) proteinase K (Boehringer Mannheim or Ambion) were mixed and incubated at 37°C for 30 min. The digestion was stopped by adding 1 μl of 100 mM PMSF, an serine protease inhibitor. To test the nuclease sensitivity, 7.5 μl of the heparin-agarose fraction was mixed with 0.5 μl of 20 mM $CaCl_2$ and 2 μl of 20-U/μl micrococcal nuclease (MN) and incubated at 30°C for 30 min. The digestion was stopped by adding 1 μl of 50 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], a calcium chelator. The preparation of MN-digested splicing extracts was performed as previously described (53).

Assembly of various spliceosomes and their isolation. The prp2Δ spliceosome (SS2) was assembled in heat-inactivated *prp2* mutant extracts in the presence of pre-mRNA substrate and ATP (22). The binding of the PRP2 protein to the spliceosome without the occurrence of splicing was accomplished by incubation with glucose to deplete ATP followed by addition of purified PRP2 protein (22). To convert $SS2^{PRP2}$ to SS3, heparin (U.S. Biochemicals) was added to the reaction mixture at 2 μg/μl and the mixture was incubated on ice for 5 min. Then ATP was added to the heparin-containing mixture at 8 mM, and the mixture was incubated at 23°C for 30 min. The spliceosome containing splicing intermediates (SI spliceosome) was formed in temperature-sensitive *prp2* (prp2 Ts) extracts supplemented with purified wild-type PRP2 protein and a 3′ splice site mutant pre-mRNA, C303/C305 (51). To isolate the spliceosomes after assembly, the mixture (50 to 65 μl) was chilled on ice, diluted to 200 μl with GT gradient buffer (40 mM potassium phosphate [pH 7.3], 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.05% Triton X-100) with or without 1 μg of heparin per μl, and sedimented in a glycerol gradient (15 to 27% of glycerol in GT gradient buffer) (53). To isolate high-salt-treated spliceosomes, KCl instead of heparin was added to the sample to a final concentration of 400 mM before sedimentation; no KCl was included in the gradient itself.

Ribonucleoprotein particle gels. Nondenaturing composite gel electrophoresis was performed essentially as previously described (46) with modifications (37a). A solution containing 0.26 g of agarose, 16 ml of 1× Tris-borate-EDTA, and 15.6 ml of water was heated to melt the agarose. Glycerol (5.2 ml of a 50% solution) and acrylamide (5.2 ml of a 30% solution with an acrylamide/bisacrylamide ratio of 60:1) were then added. After the temperature reached 38 to 40°C, 200 μl of 10% ammonium persulfate and 28 μl of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were added. The gel was cast between two glass plates (20 cm by 20 cm by 1.2 mm) at room temperature. Gradient fractions (10 μl) were mixed with 10 μl of loading dye containing 20 mM Tris-HCl, pH 8.0, and 1 mM EDTA with or without 0.8 μg of heparin per μl, and incubated on ice for 5 min before being loaded onto the gel.

Antibody production and immunoprecipitation. Anti-PRP2 antibodies were raised in rabbits against the N-terminal one-quarter of the PRP2 protein fused to *Schistosoma japonicum* glutathione *S*-transferase (48). To construct the fusion plasmid, a DNA fragment containing the N-terminal one-quarter of the PRP2 coding sequence (8) was obtained by digesting plasmid pBM-PRP2 (24) with *Bam*HI and *Hin*dIII; this 610-bp fragment was inserted into the *Bam*HI site of pGEX-3X (Pharmacia) by standard recombinant DNA techniques (42). The recombinant plasmid, pGEX-3X-PRP2N, was introduced into *Escherichia coli* HB101, and after IPTG (isopropyl-β-D-thiogalactopyranoside) induction, the glutathione *S*-transferase–PRP2N fusion protein was purified by glutathione-Sepharose CL4B (Pharmacia) affinity chromatography (16). The purified protein was used to raise antibodies in rabbits by multiple subcutaneous injections (18). The immunoglobulin G fraction was purified from the antiserum by protein A-Sepharose 6 MB (Pharmacia) affinity chromatography (18). Immunoprecipitation was performed essentially as previously described (45). Protein A-Sepharose CL4B beads (4 mg; Pharmacia) were washed and resuspended in 400 μl of IPP buffer (10 mM Tris-HCl, [pH 8.0], 0.1% Nonidet P-40) plus 500 mM NaCl. The immunoglobulin G fraction containing the anti-PRP2 antibodies (15 μg) was added, and the mixture was incubated at room temperature for 1 h on a nutator. Then the beads were washed three times and resuspended in 200 μl of IPP buffer plus 150 mM NaCl. Spliceosome-containing gradient fractions (150 μl) were incubated with these anti-PRP2–protein A beads for 1 h at 4°C on a nutator. After incubation, the beads were washed three times in IPP buffer plus 150 mM NaCl. RNA was extracted from the beads, ethanol precipitated, and analyzed on polyacrylamide-urea gels.

RESULTS

Identification of a heat-resistant splicing factor, HP. Yeast cells carrying temperature-sensitive alleles of the *PRP2* gene accumulate unspliced mRNA precursors at the restrictive temperature in vivo (38) and yield heat-sensitive splicing extracts in vitro (30). The temperature-sensitive extracts assemble an inactive yet functional spliceosome called the prp2Δ spliceosome (28), splicing complex A1 (9), or complex I (37). We call this spliceosome containing the pre-mRNA substrate the SS2 complex (Table 1). In Fig. 1, the SS2 complex, assembled in *prp2* Ts extracts with ^{32}P-labeled actin pre-mRNA, was isolated from a standard low-salt glycerol gradient (Fig. 1A). Pre-mRNA in isolated SS2 was chased to intron-exon 2 and linear exon 1 (not shown) when complemented with purified PRP2 protein and ATP (Fig. 1B, lane 3). This is consistent with our

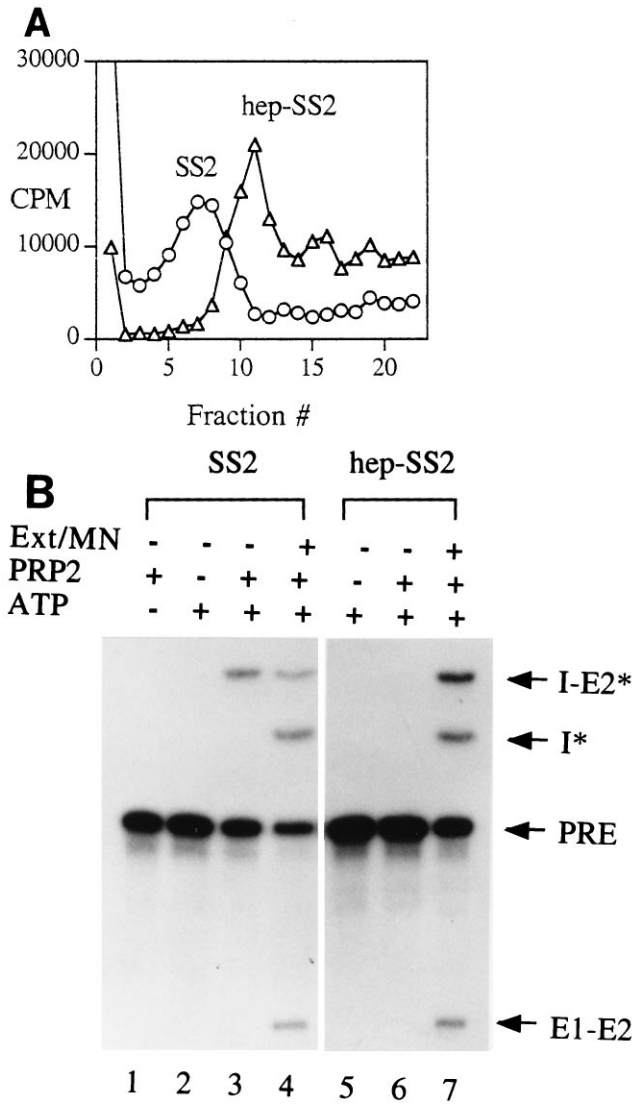


FIG. 1. Heparin treatment of the SS2 spliceosome. (A) Gradient profile. The SS2 spliceosome was assembled with ^{32}P-labeled actin pre-mRNA in *prp2* Ts extracts and sedimented in standard low-salt gradients with (hep-SS2) or without (SS2) heparin treatment. Fractions near the bottom of each gradient are plotted closer to the left. (B) Chase gel. The SS2 (lanes 1 through 4) and hep-SS2 (lanes 5 through 7) spliceosome fractions were incubated with various combinations of MN-digested *prp2* Ts extracts (Ext/MN), the purified PRP2 protein, and ATP. RNA was extracted and separated on polyacrylamide-urea gels. I-E2*, lariat intron-exon 2; I*, lariat intron; PRE, pre-mRNA; E1-E2, ligated exons; +, present; −, absent.

previous results (22). Complete splicing occurred when MN-treated prp2 extracts were supplied to the reaction in addition to PRP2 and ATP (Fig. 1B, lane 4). However, when the SS2 complex in the temperature-sensitive extract was incubated with heparin before gradient isolation, the sedimentation rate and complementing factor requirement of the spliceosome changed (Fig. 1). These differences were also observed when we analyzed the small nuclear RNAs in isolated spliceosomes (54). The heparin-treated SS2 complex, hep-SS2 (Table 1), sedimented about three or four fractions less densely than did SS2 (Fig. 1A). No transesterification reaction occurred in isolated hep-SS2 when only PRP2 and ATP were added (Fig. 1B, lane 6). Nonetheless, hep-SS2 was still functional since complete splicing occurred when MN-treated prp2 extracts were included during the chase (Fig. 1B, lane 7). This result suggested that protein factors (collectively called HP for heat stable and heparin binding [see below]) besides PRP2 were needed to complement the heparin-treated spliceosome.

We then proceeded to purify HP with the hope that this factor(s) could be a tool to further dissect the PRP2/reaction 1 step. The chase reaction using gradient-isolated hep-SS2 was used to monitor the HP activity throughout purification. Interestingly, we coincidentally found out that HP was heat resistant; the activity was retained in the supernatant after extracts were incubated at 100°C for 5 min (Fig. 2, lane 2). This unusual property of HP provided a powerful first step in its purification. The supernatant from boiled extracts (Fig. 2, lane 2) was further fractionated by ammonium sulfate precipitation, gel filtration, and heparin-agarose chromatography. This purification procedure resulted in a ~500-fold increase in specific activity of HP with a 3% yield. The final heparin fraction contains more than 10 protein bands as revealed by denaturing sodium dodecyl sulfate protein gels (data not shown). Thus, it is not yet possible to determine whether HP is a single factor or a collection of several splicing factors. Nonetheless, the partially purified HP after the heparin-agarose step was used for the biochemical characterization of HP (Fig. 2) and for later in vitro splicing experiments.

First, we tested whether the partially purified HP factor remained heat resistant as it did in the crude extract. The

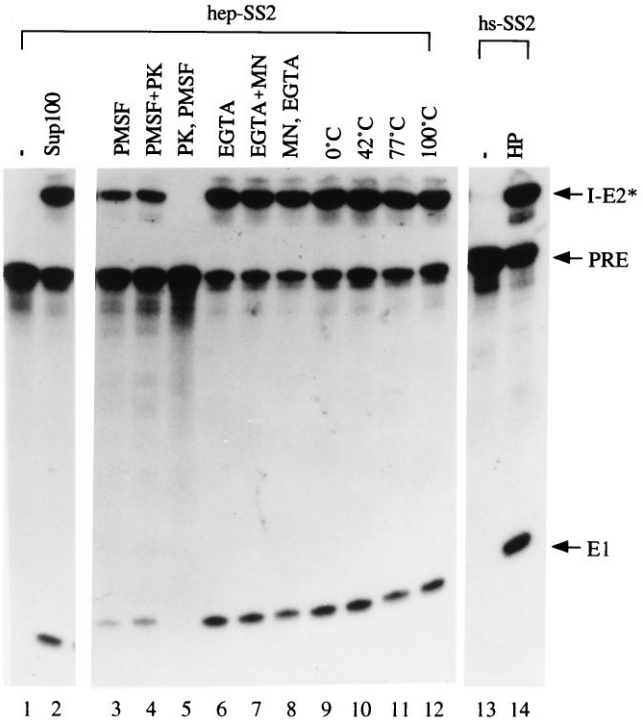


FIG. 2. Spliceosome-complementing activity of HP. The heparin-treated hep-SS2 (lanes 1 through 12) and 400 mM KCl-treated hs-SS2 (lanes 13 and 14) spliceosomes were assembled and isolated from glycerol gradients. The spliceosome fractions were incubated with partially purified HP (boiled supernatant Sup100 [lane 2] and heparin-agarose fraction [lanes 3 through 12 and 14), and RNA was analyzed on polyacrylamide-urea gels. Lane 4, PMSF was added to HP before incubation with proteinase K (PK); lane 5, PMSF was added after the proteinase K digestion of HP; lane 7, EGTA was added to HP before MN; lane 8, EGTA was added after MN digestion; lanes 9 through 12, HP was incubated at the specified temperature for 5 min. I-E2*, lariat intron-exon 2; PRE, pre-mRNA; E1, linear exon 1.

heparin HP fraction was incubated at various temperatures before being assayed for its ability to complement hep-SS2 in conjunction with PRP2 and ATP (Fig. 2, lanes 9 through 12). Nearly full activity was recovered even after an incubation of HP at 100°C for 5 min (Fig. 2, lane 12). Second, to show that HP contains a protein moiety, the HP fraction was digested with proteinase K. Then the protease inhibitor PMSF was added, and the digested HP was used to complement the hep-SS2 spliceosome. The HP activity was completely eliminated after proteinase K digestion (Fig. 2, lane 5), while PMSF itself has only a little effect on the chasing reaction (lane 3). In contrast, the chasing activity was retained when the inhibitor was added to HP before being incubated with proteinase K (Fig. 2, lane 4). This experiment indicated that HP has an essential protein moiety. Third, to test whether HP contained nucleic acid, the HP fraction was digested with or without MN, quenched by inhibitor EGTA, and then added to the spliceosome (Fig. 2, lanes 8 and 6, respectively). As a control, EGTA was added to the HP fraction before being incubated with MN (Fig. 2, lane 7). All three samples retained the HP activity, indicating that HP is resistant to MN digestion (Fig. 2, lanes 6 through 8). Although we could not exclude the possibility that HP contains a tightly bound nucleic acid molecule, we refer to HP as a heat-stable protein factor(s) here.

To further test whether the requirement for HP during the chase was unique to the spliceosome isolated in the presence of heparin, we decided to isolate the SS2 complex treated with a high concentration of KCl (4, 10) instead of heparin. After the assembly of SS2 in *prp2* Ts extracts, KCl was added to a final concentration of 400 mM and the mixture was sedimented through a standard low-salt gradient. As shown in Fig. 2, this high-salt-treated spliceosome, hs-SS2 complex (Table 1), could not carry out the transesterification reaction without HP (lane 13). In addition to PRP2 and ATP, HP was essential and sufficient to complete the complementation (Fig. 2, lane 14). Thus, additional HP was not necessary to chase standard SS2 preparations but it was required to chase spliceosomes isolated after heparin or high-salt treatment.

HP is required for complementing the PRP2-bound spliceosome. Before we could use the HP requirement in an attempt to uncouple the ATP hydrolysis by PRP2 from the actual transesterification reaction, it was necessary to test whether HP was still needed after PRP2 bound to the spliceosome. We have shown previously that a PRP2-bound spliceosome, $SS2^{PRP2}$ (Table 1), isolated from a standard low-salt gradient without heparin or high-salt treatment, can perform the first step of splicing when incubated with ATP without adding any protein factors (22). In the following experiments, *prp2* Ts extracts were incubated with ^{32}P-labeled pre-mRNA to assemble SS2. After ATP was depleted by incubating with glucose and endogenous hexokinases, PRP2 protein was added to bind to the SS2 complex. The mixture was then incubated with heparin or 400 mM KCl prior to the isolation of the spliceosome by standard low-salt glycerol gradients. The PRP2 protein remained bound to the spliceosome under these conditions (data not shown) (see below). Note that ATP was added to all of the following spliceosome chase reaction mixtures. The $SS2^{PRP2}$ complex isolated after heparin or high-salt treatment (hep-$SS2^{PRP2}$ or hs-$SS2^{PRP2}$, respectively [Table 1]) could not carry out the transesterification reaction when incubated with only ATP (Fig. 3, lanes 1 and 5). The addition of PRP2 did not help (Fig. 3, lane 2). Apparently, what was needed for these spliceosome preparations was HP since adding HP and ATP was sufficient to trigger reaction 1 in both hep-$SS2^{PRP2}$ (Fig. 3, lane 3) and hs-$SS2^{PRP2}$ (lane 6). Adding extra PRP2 did not increase the amounts of reaction 1 products (Fig. 3, lane 4), indicating the presence of PRP2 in these spliceosomes. This was confirmed later, as the pre-mRNA in these spliceosomes was immunoprecipitated by anti-PRP2 antibodies (see Fig. 7). We concluded that HP was required for the transesterification reaction even when PRP2 was already bound to the spliceosome. The next step was to determine whether PRP2 could hydrolyze ATP in the spliceosome without the HP activity and to monitor the consequence of the ATP hydrolysis event if it did occur.

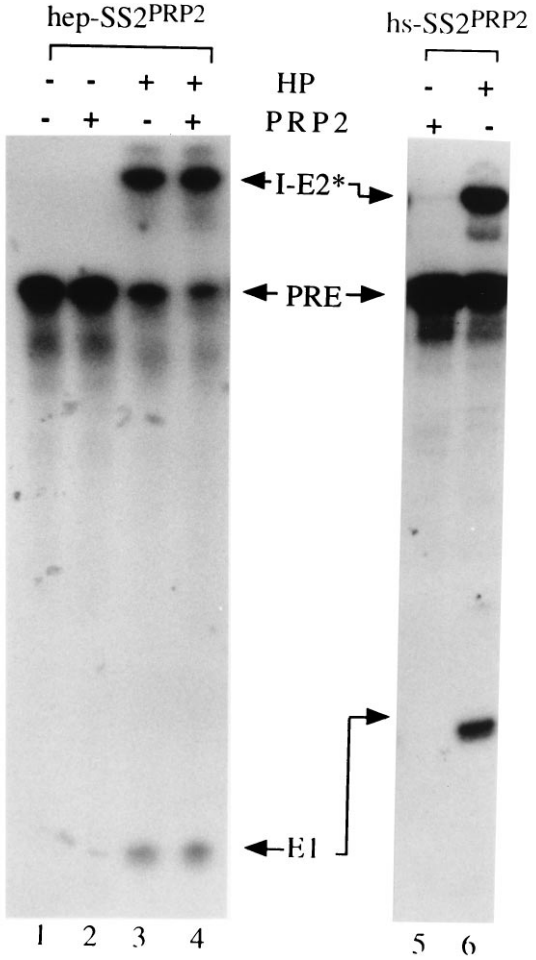


FIG. 3. Splicing in PRP2-bound spliceosomes. The PRP2 protein was allowed to bind in the absence of ATP to the SS2 spliceosome assembled in *prp2* Ts extracts. After the addition of heparin (hep-$SS2^{PRP2}$; lanes 1 through 4) or 400 mM KCl (hs-$SS2^{PRP2}$; lanes 5 and 6), the spliceosomes were isolated from glycerol gradients. The spliceosome fractions were incubated with (+) or without (−) HP and PRP2, as indicated, in the presence of ATP, and the RNA was analyzed on polyacrylamide-urea gels. I-E2*, lariat intron-exon 2; PRE, pre-mRNA; E1, linear exon 1.

A novel pre-mRNA-containing spliceosome, SS3. The identification and partial purification of HP provided an opportunity to test whether ATP hydrolysis and the first transesterification reaction could be uncoupled. After the assembly of SS2 in *prp2* Ts extracts followed by depletion of ATP, wild-type PRP2 protein was added to the mixture to bind to the spliceosome. Heparin was then added to the mixture to inactivate or sequester HP in the extract. Up to this point, no transesterification reaction occurred and PRP2 remained bound to the spliceosome (hep-$SS2^{PRP2}$ [Table 1]). Finally, ATP was added back and the mixture was incubated before being subjected to

glycerol gradient sedimentation. Without adding back ATP, splicing could not occur and the hep-SS2^{PRP2} spliceosome sedimented to a position around fraction 10 (Fig. 4A, gradient I). In contrast, by adding back ATP during the last step, a new spliceosome peak was detected around fractions 12 and 13 (Fig. 4A, gradient II). The formation of this new spliceosome was PRP2 dependent since this new peak was not detected when the wild-type PRP2 protein was not added to the reaction mixture prior to the heparin addition step (data not shown). To verify whether the transesterification reaction had occurred in these spliceosomes, RNA was extracted from gradient fractions (fraction 10 of gradient I and fractions 10, 12, and 14 of gradient II) and analyzed on a polyacrylamide-urea gel (Fig. 4B, lanes 1 through 4). Only pre-mRNA was detected in these gradient fractions, suggesting that the transesterification reactions did not occur in these PRP2-bound spliceosomes even when ATP was added back after the heparin treatment (Fig. 4B, lanes 2 through 4). The results indicated that the spliceosome shifted, in a PRP2- and ATP-dependent fashion, its sedimentation position (from fraction 10 to 12) without the occurrence of reaction 1. Thus, a novel pre-mRNA-containing spliceosome was identified in this experiment (Fig. 4A, gradient II), and this newly identified SS3 spliceosome was formed by incubating the PRP2-bound spliceosome with heparin and ATP (Table 1).

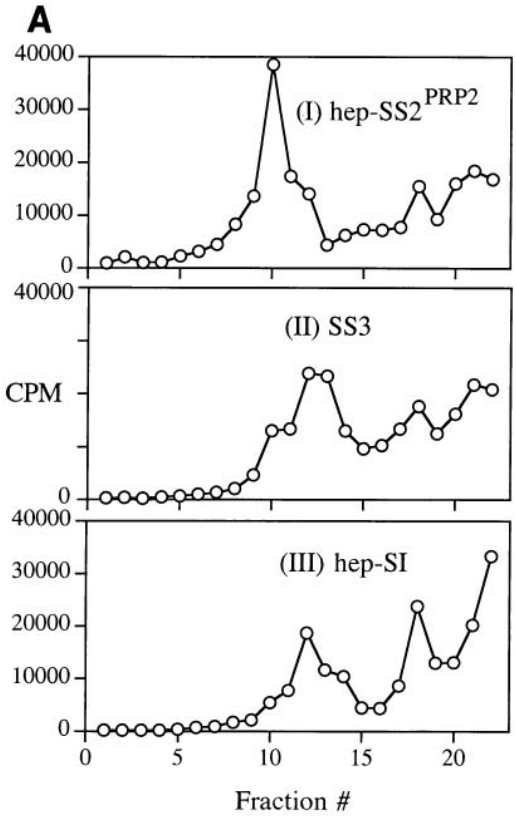
Next, we showed that the SS3 complex had a gradient sedimentation rate similar to that of the SI spliceosome which contained the splicing intermediates formed after reaction 1. The SI spliceosome was accumulated by using mutant pre-mRNA substrate C303/C305, with a C instead of a G at the 3′ end of the actin intron, to block the second catalytic step (51). A splicing reaction containing the *prp2* Ts extract, the wild-type PRP2 protein, and C303/C305 pre-mRNA was carried out in the presence of ATP. After the addition of heparin, the mixture was sedimented through a standard gradient (Fig. 4A, gradient III). The hep-SI complex (Table 1) sedimented around fraction 12, similar to SS3; and as expected, RNA extracted from fractions 10, 12, and 14 of gradient III contained pre-mRNA and the products of reaction 1, lariat intron-exon 2 and excised exon 1 (Fig. 4B, lanes 5 through 7).

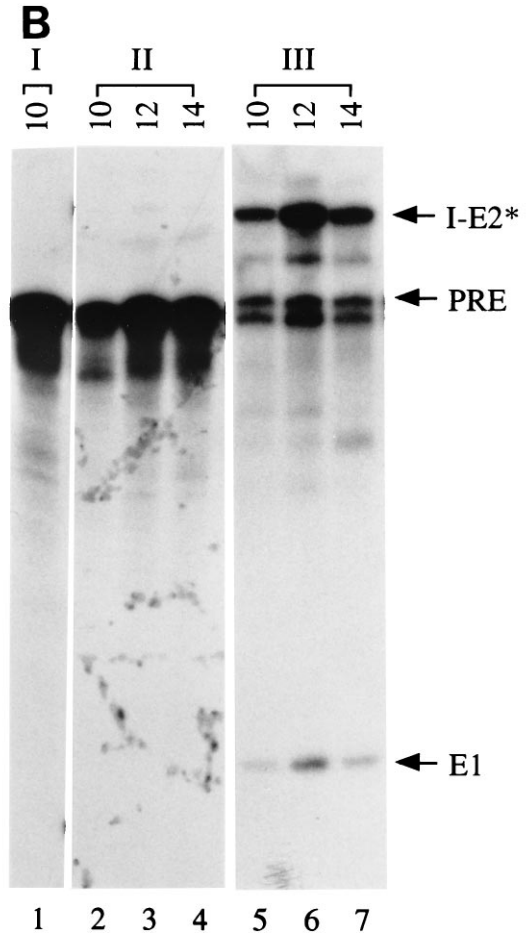
To further detect differences between these spliceosomes, fractions from the gradients shown in Fig. 4A were also analyzed on a nondenaturing polyacrylamide-agarose composite gel (Fig. 4C). A complex migrating to the position marked P1 was detected in fraction 10 of gradient I (Fig. 4C, lane 1). This complex, representing the hep-SS2^{PRP2} spliceosome, had a mobility similar to that of the precatalytic spliceosome previously described as complex A1 (9) or complex I (36). A small amount of an SS2-like complex migrating to position P1 was also detected in fraction 10 of gradient II (Fig. 4C, lane 2). This SS2-like complex probably represented unreacted SS2 or SS2^{PRP2} spliceosomes. In addition to a minute amount of an SS2-like complex at P1, a new complex migrating to position P2 and another migrating to position X were detected in fraction 12 of gradient II (Fig. 4C, lane 3). The P2 complex was most likely the SS3 complex since its appearance coincided with the appearance and activity of SS3 (data not shown) (see below). A small amount of SS3 and an increased amount of complex X were detected in fraction 14 of gradient II (Fig. 4C, lane 4). The nature of complex X was not further investigated. The mobility of the complex corresponding to SS3 was similar to the previously reported "active" spliceosome called complex A2-2 (9) or complex II (36). In agreement with that observation, a complex representing the SI spliceosome was detected in fraction 12 of gradient III at position P2 (Fig. 4C, lane 6). Again, complex X was detected in fraction 14 of gradient III (Fig. 4C, lane 7). Since SS3 migrates like SI not SS2, this gel analysis result suggests that the change of the spliceosome from the precatalytic stage (A1 or I) to the active stage (A2 or II) has largely taken place during the SS2-to-SS3 step. Moreover, it is worth noting that transesterification does not occur during this PRP2- and ATP-dependent step (Fig. 4B).

Spliceosomes representing SS2 (without heparin treatment), hep-SS2, hep-SS2^{PRP2}, and SS3 (with heparin treatment) (Table 1) were isolated from another set of gradients to further compare their gel mobilities. The spliceosomal peak fractions were mixed with Tris-EDTA loading dye identical to that used in Fig. 4C and analyzed on a native gel (Fig. 5). The SS2, hep-SS2, and hep-SS2^{PRP2} spliceosomes migrated nearly indistinguishably to the P1 position (Fig. 5, lanes 1 through 3), while the SS3 spliceosome migrated to the faster P2 position (lane 4). The SS3 complex was clearly different from the P1 complexes since both P1 and P2 bands could be distinctly detected (Fig. 5, lane 5) when the hep-SS2^{PRP2} gradient fraction (lane 3) was mixed 1 to 1 with the SS3 fraction (lane 4) before gel electrophoresis. It is interesting that the SS2 sample (Fig. 5, lane 1) without ever being exposed to heparin migrated almost like the heparin-treated SS2 (lane 2), yet these two complexes have different sedimentation rates and HP requirements (Fig. 1). To further distinguish SS3 from SS2, the spliceosomal fractions were mixed with a Tris-EDTA dye containing heparin prior to gel electrophoresis. Interestingly, the heparin in the gel loading dye has little effect on the mobility of SS2, hep-SS2, and hep-SS2^{PRP2} (Fig. 5, lanes 6 through 8). However, the addition of heparin to the dye apparently caused the disassembly of the SS3 spliceosome, perhaps to free pre-mRNA, during gel electrophoresis (Fig. 5, lane 9, band D). The instability of gradient-isolated SS3 during gel electrophoresis was likely an intrinsic property of SS3 since the additional heparin caused disassembly of only SS3, not hep-SS2^{PRP2}, in a 1:1 mixture of the two spliceosomal fractions (Fig. 5, lane 10). Taken together, the SS3 spliceosome is clearly different from other precatalytic spliceosomes in its gradient sedimentation rate, gel mobility, and heparin sensitivity. Thus, we conclude that SS3 is a novel spliceosomal intermediate.

PRP2 hydrolyzes ATP and leaves SS3. As described above, both PRP2 and ATP were needed to convert SS2 to SS3. To further test whether ATP hydrolysis was involved in the conversion, splicing reaction mixtures containing SS2^{PRP2} and heparin were incubated with ATP, a nonhydrolyzable ATP analog (AMPPNP), or water prior to the gradient sedimentation. Only the sample incubated with ATP produced the shifted SS3 peak, while the samples with AMPPNP or no ATP produced the typical hep-SS2^{PRP2} peak (data not shown). The peak spliceosomal fractions from these gradients were then analyzed on a native gel with or without additional heparin in the gel loading dye. As shown in Fig. 6, only hep-SS2^{PRP2} was detected in samples incubated with water or AMPPNP (lanes 1 and 2), while SS3 was detected in the sample with ATP (lane 3). Furthermore, incubation with the nonhydrolyzable analog did not produce a heparin-hypersensitive spliceosome during gel electrophoresis (Fig. 6, lane 5), while ATP did as previously shown (lane 6). Thus, this result suggests that ATP hydrolysis is involved in the production of SS3. This conclusion is supported by the work of Jean Beggs' group, who studied a dominant-negative prp2 mutant with a mutation in the conserved SAT motif within the ATPase domain (37, 49). The mutant protein has a lower ATPase activity and cannot convert splicing complex I (equivalent to SS2) to complex II (equivalent to SS3 and SI) even in the presence of hydrolyzable ATP.

Since previous studies indicated that PRP2 transiently associates with the spliceosome and exits from the spliceosome





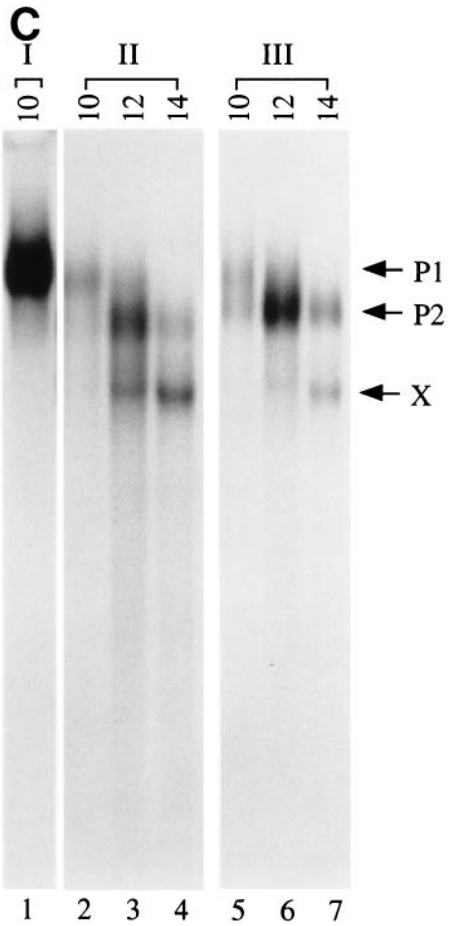


FIG. 4. Formation of a new spliceosome, SS3, after the addition of ATP. (A) Gradient profile. The SS2^{PRP2} spliceosome was assembled in the absence of ATP, with the subsequent addition of heparin. The mixture was incubated without (gradient I) or with (gradient II) ATP before gradient sedimentation. For gradient III, the intermediate-containing spliceosome (SI) was assembled in *prp2* Ts extracts with PRP2 and C303/C305 mutant pre-mRNA in the presence of ATP. Heparin was then added before sedimenting in the gradient. (B) Denaturing gel analysis of RNA. RNA was extracted from fraction 10 of gradient I (lane 1), fractions 10 through 14 of gradient II (lanes 2 through 4), and fractions 10 through 14 of gradient III (lanes 5 through 7). The RNA was separated on polyacrylamide-urea gels. I-E2*, lariat intron-exon 2; PRE, pre-mRNA; E1, linear exon 1. (C) Native gel analysis of ribonucleoprotein particles. The gradient fractions from panel B were incubated with gel loading dye without heparin and analyzed on native agarose-acrylamide composite gels.

after ATP hydrolysis (22, 24, 37), it was logical to suspect that PRP2 might have dissociated from SS3. The presence or absence of PRP2 in the spliceosome can be verified by immunoprecipitating the spliceosome-bound pre-mRNA with anti-PRP2 antibodies. If PRP2 leaves the spliceosome after the conversion of SS2 to SS3, pre-mRNA in SS3 should not be precipitated by the antibody. To test this, gradient fractions containing hep-SS2, hep-SS2^{PRP2}, or SS3 were incubated with anti-PRP2 antibodies immobilized on protein A beads. Then RNA was extracted from the pellet beads and analyzed on a denaturing gel (Fig. 7). The ^{32}P-labeled pre-mRNA was precipitated from SS2^{PRP2} by the antibody (Fig. 7, lane 3) but was not precipitated by preimmune serum (lane 2) as expected. Also, a spliceosome lacking PRP2 was not precipitated by the antibody (Fig. 7, lane 1). As suspected, the anti-PRP2 antibody failed to bring down the pre-mRNA in SS3 (Fig. 7, lane 4). To show that equal amounts of spliceosomes were used for each

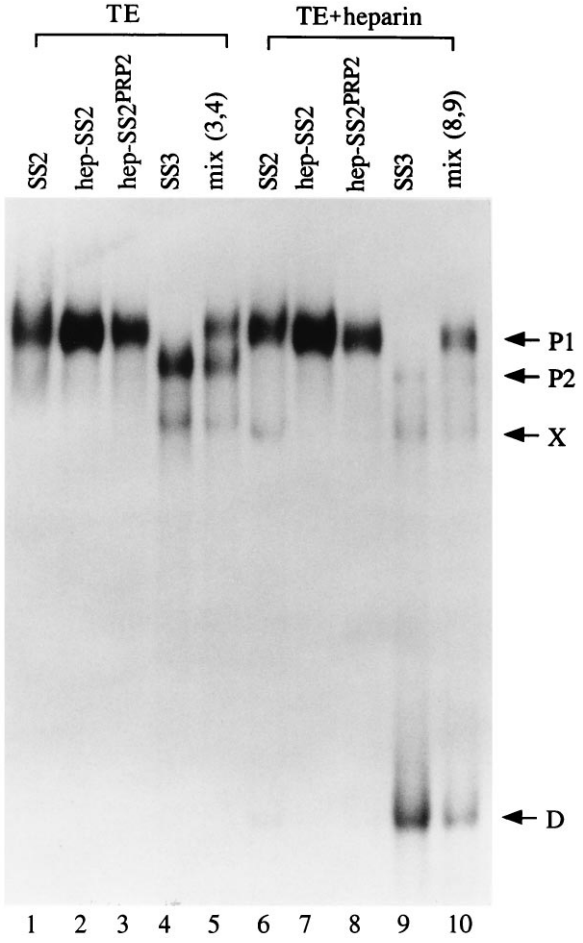


FIG. 5. Heparin sensitivity of purified spliceosomes. Spliceosomes (Table 1) were assembled and isolated from glycerol gradients. The main spliceosome fractions were incubated with gel loading dye without (TE; lanes 1 through 5) or with (TE+heparin; lanes 6 through 10) heparin. The samples were separated on a native agarose-acrylamide gel. Lane 5, a 1:1 mixture of the samples in lanes 3 and 4; lane 10, a 1:1 mixture of the samples in lanes 8 and 9.

immunoprecipitation, total RNA before immunoprecipitation was extracted and displayed (Fig. 7, lanes 5 through 7). Furthermore, only pre-mRNA was present in these gradient fractions, indicating that no splicing had occurred in any of these samples (Fig. 7, lanes 5 through 7). Thus, before the transesterification reaction, PRP2 most likely hydrolyzed ATP and left the spliceosome during or after the conversion of the SS2 spliceosome to SS3. These results also suggest that the PRP2-mediated ATP hydrolysis event can be uncoupled from the actual transesterification reaction when SS3 remains splicing competent (see below).

Reaction 1 occurs in SS3 in the presence of HP. If SS3 is an intermediate spliceosome, splicing should resume when gradient-purified SS3 is incubated with the missing factors. Moreover, if PRP2 indeed hydrolyzed ATP and left SS3, we would predict that ATP and PRP2 was not required for SS3 spliceosomes to complete reaction 1. To test this, the hep-SS2^{PRP2} and SS3 spliceosomes were isolated from separate gradients. ATP, PRP2, and HP in various combinations were added to the peak spliceosomal fractions, and the samples were incubated. RNA was extracted and analyzed by denaturing gels (Fig. 8). As shown previously, reaction 1 occurred in hep-SS2^{PRP2} when both ATP and HP were supplemented to chase the spliceosome-bound pre-mRNA to intron-exon 2 and exon 1 (Fig. 8, lane 2). The reaction on hep-SS2^{PRP2} required ATP (Fig. 8, lane 1), while additional PRP2 was not necessary since PRP2 was already present in the spliceosome (lane 3). In the SS3 spliceosome, both intron-exon 2 and exon 1 were produced from the pre-mRNA whenever HP was supplemented (Fig. 8, lanes 5, 6, 8, and 9). The reaction in SS3 did not require ATP (Fig. 8, lanes 5 and 6) or PRP2 (lanes 5 and 8) as predicted. In fact, HP was the only missing macromolecule(s) that needed to be supplemented (Fig. 8, lane 5). Thus, SS3 is a splicing-competent intermediate. Although the gradient-purified SS3 spliceosome might be contaminated with other pre-mRNA-containing complexes as revealed by native gels (Fig. 4C, 5, and 6), the level of contamination was too low to account for the amounts of the products generated in the SS3 samples (Fig. 8). Furthermore, any contaminating hep-SS2 or hep-SS2^{PRP2} spliceosomes in the SS3 gradient preparation would not be active without ATP. Lastly, this ATP- and PRP2-independent splicing activity, representing the SS3 spliceosome, copurified in the gradient with the complex that migrated to position P2 on native gels (data not shown). Thus, SS3 is indeed a functional intermediate spliceosome formed after the PRP2 step that can further carry out the first step of splicing in the absence of ATP. Although the purification scheme we developed could not rule out the possible presence of tightly bound ATP in the SS3 spliceosome, there was no evidence to suggest that the

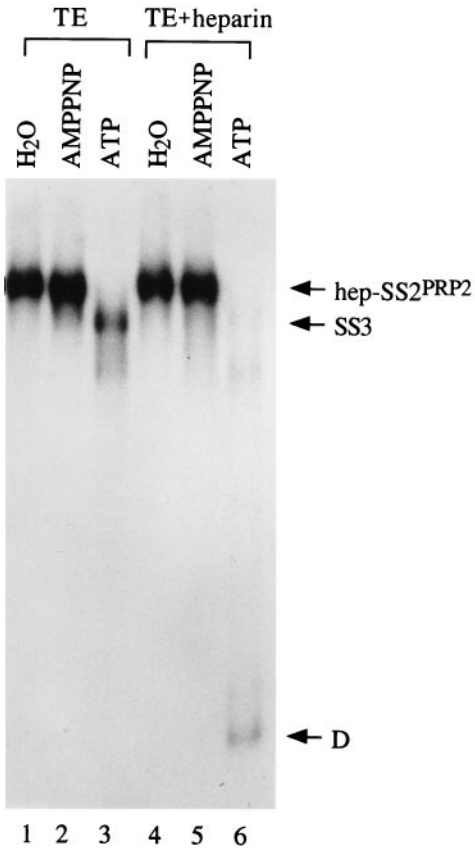


FIG. 6. Involvement of ATP hydrolysis in converting SS2 to SS3. The hep-SS2^{PRP2} spliceosome was assembled. After the addition of heparin, the samples were incubated with water, AMPPNP, or ATP before sedimenting in glycerol gradients. The spliceosome fractions were incubated with TE dye (lanes 1 through 3) or TE dye plus heparin (lanes 4 through 6) and separated on a native agarose-acrylamide gel.

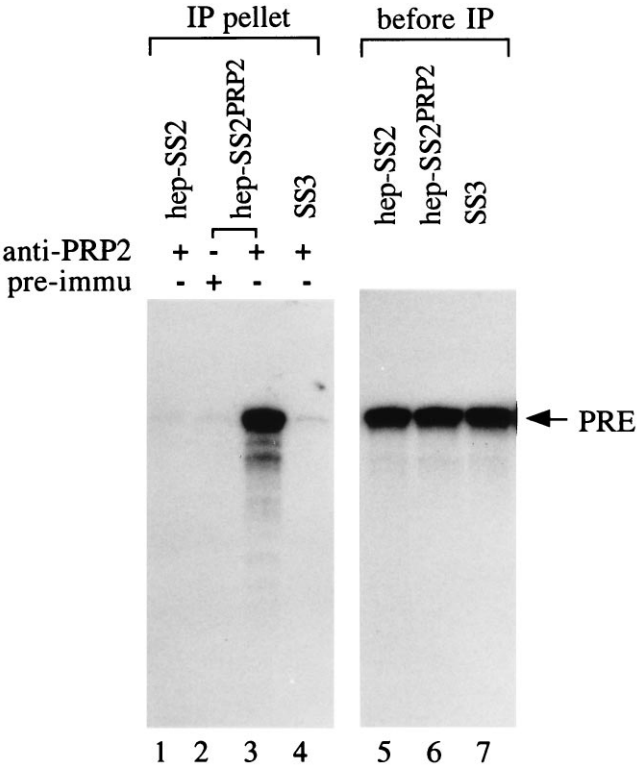


FIG. 7. Dissociation of PRP2 from the SS3 spliceosome. The hep-SS2, hep-SS2^{PRP2}, and SS3 spliceosomes were isolated from glycerol gradients. Gradient fractions containing the spliceosome (150 μl) were incubated with protein A beads coupled with (+) or without (−) anti-PRP2 antibodies (lanes 1, 3, and 4) or preimmune serum (pre-immu; lane 2). Then RNA was extracted from the pelleted beads (IP pellet; lanes 1 through 4) and separated on a polyacrylamide-urea gel. Total RNA was also extracted from the corresponding spliceosome fractions (10 μl) without immunoprecipitation (before IP; lanes 5 through 7) and was displayed on the same gel. PRE, pre-mRNA.

occurrence of reaction 1 in this post-PRP2 spliceosome required ATP. Conversely, in order for SS3 to continue through the second step of splicing, ATP and other splicing factors, including the PRP16 ATPase, were needed in addition to HP (22a).

Taking these results together, we conclude that (i) ATP hydrolysis by PRP2 and the transesterification reaction can be uncoupled; (ii) ATP and PRP2 are involved in converting SS2 to a functional intermediate spliceosome, SS3; (iii) PRP2 leaves the spliceosome before reaction 1; (iv) HP is needed to allow splicing to occur in purified SS3; and (v) ATP is apparently not required for reaction 1 in SS3. Thus, our data are consistent with a model in which the role of the PRP2 ATPase is to activate the spliceosome by hydrolyzing ATP and the newly identified SS3 represents the post-PRP2 active spliceosome.

DISCUSSION

By the identification and partial purification of a novel splicing factor(s), HP, we have shown that ATP hydrolysis by PRP2 and the first transesterification reaction could be uncoupled in the spliceosome. The requirement for PRP2 in splicing was apparently to cause the spliceosome to undergo the structural changes needed for the first catalytic step in a process we have called spliceosome activation. Although the nature of these changes was not understood, the changes seemed quite dramatic and could account for the previously reported differences between the precatalytic, pre-mRNA-containing spliceosome and the active, intermediate-containing spliceosome (9, 36). Moreover, we isolated from glycerol gradients the post-PRP2-ATP spliceosome and have shown that it could carry out the subsequent splicing reaction upon incubation with HP.

What is the role of HP in splicing? When precatalytic spliceosomes like SS2 and SS2^{PRP2} were isolated from standard low-salt gradients, they did not require additional HP for activity (22) (this study); however, HP needed to be supplemented when the spliceosomes were isolated after heparin or high-salt treatment (54) (this study). One possibility is that HP is a constituent of the spliceosome under low-salt conditions but that it dissociates from the spliceosome in the presence of heparin or high salt. In this scenario, HP may be involved in the transesterification reaction itself, for instance, as a component of the catalytic center. Alternatively, it may be required for yet another precatalytic substep situated between PRP2-mediated ATP hydrolysis and reaction 1. A small protein called SPP2 interacts with PRP2 genetically and biochemically (39); however, SPP2 is required for the binding of PRP2 to the spliceosome and appears not to be the same as HP. The involvement of additional protein factors in a post-ATPase step during the second transesterification reaction has been reported. In the second step of yeast splicing, the PRP16 ATPase is involved (44). At least two protein factors, PRP18 (20) and SLU7 (2, 21), are required for the post-PRP16 step. Thus, HP

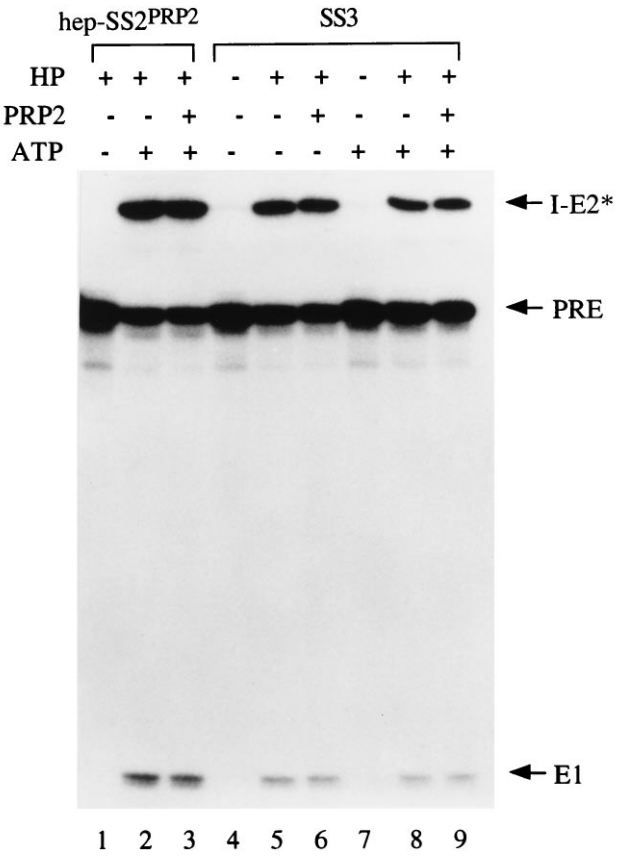


FIG. 8. Occurrence of reaction 1 in SS3. The hep-SS2^{PRP2} and SS3 spliceosomes were isolated from glycerol gradients. The spliceosome fractions were incubated with various combinations of HP, PRP2, and ATP, as indicated. RNA was extracted and separated on a polyacrylamide-urea gel. +, present; −, absent. I-E2*, lariat intron-exon 2; PRE, pre-mRNA; E1, linear exon 1.

may be the counterpart of PRP18 or SLU7 for the first step of splicing.

The second scenario for the role of HP in splicing is that perhaps HP is a molecular chaperone required for maintaining the active conformation of the spliceosome. For example, under certain conditions, such as low-salt conditions, the spliceosome can proceed to the catalytic step right after the PRP2-ATP activation step. Reaction 1 and ATP hydrolysis by PRP2 are almost linked. The reports that anti-PRP2 antibodies can immunoprecipitate splicing intermediates in addition to the pre-mRNA (24) and that reaction 1 readily occurs after incubation of a gradient-purified PRP2-bound spliceosome with ATP (22) support a tight linkage between the two events. However, in the presence of heparin or high salt, the spliceosome may be in a different or inactive conformation. For example, it has been shown that spliceosomes isolated from a 100 mM KCl gradient have a tighter structure than do spliceosomes from a 400 mM KCl gradient (10). In our experiments, PRP2 was always allowed to bind to the spliceosome under low-salt conditions before adjusting to high-salt conditions or adding heparin. Conceivably, once bound, PRP2 continued to interact properly with the spliceosome, and when ATP was added, PRP2 hydrolyzed ATP to induce the structural change. However, under these conditions, the spliceosome could not carry out splicing since it was in an inactive conformation. The incubation with HP might facilitate the conversion of the spliceosome back to the active conformation. Thus, the role of HP may be to fold or maintain the spliceosome in a conformation that can undergo splicing once activated by the ATPase. This possibility suggests that HP is an RNA chaperone (19) that folds or maintains correct RNA structures within the spliceosome. We are currently purifying HP in order to investigate its function.

What can account for the changes in gel mobility and sedimentation rate of the spliceosome induced by the PRP2 activation step? A similar gel mobility change has been reported to occur when the spliceosome undergoes a rearrangement of its small nuclear RNAs (9, 25, 27, 36). The spliceosome moves from position P2 to position P1 on a native gel (Fig. 5) when U4-U6 interactions are replaced by U2-U6 interactions (reviewed in references 31, 32, and 47). Since PRP2 contains RNA helicase motifs, it is reasonable to suspect that some RNA structures in the spliceosome may be altered after ATP hydrolysis by PRP2. We have shown that purified PRP2 does not unwind artificial RNA duplexes (23). In fact, none of the DEAD/H splicing proteins tested so far have an RNA unwinding activity by themselves. It is possible that these splicing proteins need auxiliary protein factors and only unwind authentic RNA substrates. Now we are in the position to answer these questions since the PRP2 action is defined as the conversion of SS2 to SS3. The authentic RNA substrates and any auxiliary factors needed for the PRP2 helicase reaction should all be present in the SS2 spliceosome preparation. Any differences in RNA structure between SS2 and SS3 are potentially the effect of PRP2. Interestingly, it has been reported that PRP2 can be cross-linked to pre-mRNA in the spliceosome (49). By probing with dimethyl sulfate, we have detected changes in RNA structure in the spliceosome by PRP2 (55). Although the dimethyl sulfate results do not demonstrate that PRP2 is indeed a helicase, they do show that some RNA interactions which are potentially important for catalysis are altered. Schwer and Guthrie have also shown that some conformational rearrangement occurs as a result of the PRP16-ATP step (45). The 3′ splice site region of the pre-mRNA becomes resistant to an oligonucleotide-directed RNase H attack after ATP hydrolysis by PRP16 but apparently before the second transesterification reaction. However, a gross change in gel mobility or sedimentation rate during the PRP16 step has not been reported.

The release of proteins from the spliceosome as a consequence of ATP hydrolysis by PRP2 has also been reported. In addition to the release of PRP2 itself (22, 37) (this study), the SPP2 protein is also released from the spliceosome after ATP hydrolysis by PRP2 (39). Considering the molecular masses of these two proteins (100 kDa for PRP2 and 23 kDa for SPP2), it is unlikely that the loss of these two proteins contributes significantly to the change in gel mobility or sedimentation rate of the spliceosome. Moreover, the spliceosome migrates to almost the same position (P1) on a native gel regardless of the presence or absence of SPP2 and/or PRP2. We are investigating whether there are other proteins that exit with PRP2 from the spliceosome after ATP hydrolysis.

The vast change in the spliceosome upon ATP hydrolysis by PRP2 does not address whether PRP2 has a proofreading role at step 1 similar to the proofreading role of PRP16 at step 2 (7); however, it does suggest that PRP2 functions like an ATPase motor (1). It is tempting to draw analogies between PRP2 and some other DExH proteins (15) that also function as motors in multicomponent nucleoprotein complexes. For example, in transcription, TFIIH is required to convert the preinitiation complex to an elongation complex in a step called promoter clearance (5). TFIIH contains DExH DNA helicases, ERCC2/RAD3 and ERCC3/RAD25/SSL2 (12). ATP hydrolysis is involved, and both TFIIH and another factor, TFIIE, are released from the preinitiation complex after the promoter clearance step (6, 14). Another example is chromatin remodeling; SWI/SNF is required for changing the nucleosome structure during the activation of certain genes (35). Again, SWI/SNF contains a DExH DNA-dependent ATPase, SWI2/SNF2. Both ATP hydrolysis and the release of histone proteins are involved in the process (11). Although highly speculative, this view suggests that one common function of DExH proteins is to utilize ATP hydrolysis to restructure a nucleoprotein complex so that the complex becomes activated for the subsequent step. In this case, the role of PRP2 is to activate the spliceosome for the first transesterification reaction in pre-mRNA splicing.

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