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A conformational rearrangement in the spliceosome is dependent on PRP16 and ATP hydrolysis

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PRP16 is an RNA-dependent ATPase that is required for the second catalytic step of pre-mRNA splicing. We have previously shown that PRP16 protein binds stably to spliceosomes that have completed 5′ splice site cleavage and lariat formation. PRP16 then promotes 3′ splice site cleavage and exon ligation in an ATP-dependent fashion. We now demonstrate that PRP16 can hydrolyse all nucleoside triphosphates and corresponding deoxynucleotides; complementation of the second catalytic step shows the same broad nucleotide specificity. These results link the nucleotide requirement of step 2 to PRP16. Interestingly, we find that PRP16 promotes a conformational change in the spliceosome which results in the protection of the 3′ splice site against oligo-directed RNase H cleavage. This structural rearrangement is dependent on the hydrolysis of ATP, since ATPγS, a competitive inhibitor of the PRP16 ATPase activity, does not promote the protection of the 3′ splice site and formation of mRNA.

Key words: PRP16/pre-mRNA splicing/RNA-dependent NTPase/*Saccharomyces cerevisiae*

Introduction

Splicing of nuclear pre-mRNA occurs in a large ribonucleoprotein complex, the spliceosome (Brody and Abelson, 1985; Frendewey and Keller, 1985; Grabowski *et al.*, 1985; for reviews see Green, 1986; Padgett *et al.*, 1986). The establishment of mammalian and yeast cell-free systems has greatly facilitated the analysis of the splicing process. These studies have shown that the four major small ribonucleoprotein particles (snRNPs), U1, U2, U4/6 and U5, and a number of protein factors associate with the pre-mRNA in a highly ordered pathway (for reviews see Guthrie and Patterson, 1988; Lührmann, 1988; Steitz *et al.*, 1988; Guthrie, 1991; Ruby and Abelson, 1991). After the assembly of the spliceosome is complete, splicing occurs in a two-step reaction: 5′ splice site cleavage and formation of a branched intermediate is followed by 3′ splice site (3′ss) cleavage and exon ligation (Padgett *et al.*, 1984; Ruskin *et al.*, 1984). The identification of branched intermediates strengthened the notion that nuclear splicing and splicing of organellar Group II introns occur via a fundamentally equivalent mechanism (reviewed in Cech and Bass, 1986; Sharp, 1987; Guthrie, 1989). Some Group II introns have been shown to splice autocatalytically *in vitro* in the absence of proteins (Peebles *et al.*, 1986; Van der Veen *et al.*, 1986). The reaction is known to occur via two successive transesterification reactions, with the number of phosphodiester bonds remaining conserved; no energy input in the form of ATP is necessary. Yet, in addition to the participation of many *trans*-acting factors, mRNA splicing requires ATP at many steps (Brody and Abelson, 1985; Cheng and Abelson, 1987; Lin *et al.*, 1987; Sawa *et al.*, 1988; Tazi *et al.*, 1992). The role of ATP in the splicing process is poorly understood. The energy of ATP hydrolysis may be employed to ensure accuracy in the highly ordered assembly pathway. In principle, this can be achieved by eliciting conformational changes in proteins and/or nucleic acids, as has been demonstrated in other complex processes that are coupled to nucleoside triphosphate (NTP) hydrolysis (Guthrie, 1991).

The identification and characterization of spliceosomal RNA-dependent ATPases in yeast was a major breakthrough towards understanding the role of ATP in the splicing process. These proteins show strong homology to eIF4A, a eukaryotic translation initiation factor shown to possess RNA-dependent ATPase activity (Grifo *et al.*, 1984). As such, eIF4A is the prototype of a family of so-called DEAD-box proteins (Linder *et al.*, 1989; Wassarman and Steitz, 1991; Schmid and Linder, 1992) thought to be RNA-dependent ATPases and ATP-dependent RNA helicases.

The spliceosomal DEAD-box proteins PRP5 (Dalbadie-McFarland and Abelson, 1990) and PRP28 (Strauss and Guthrie, 1991) function in the assembly of the spliceosome (Ruby and Abelson, 1991; Strauss,E.J. and Guthrie,C. submitted). PRP2 (Chen and Lin, 1990), PRP16 (Burgess *et al.*, 1990) and PRP22 (Company *et al.*, 1991) show distinct variations from the DEAD-box consensus sequences and were grouped in a sub-family, the DEAH-box proteins (Company *et al.*, 1991; Wassarman and Steitz, 1991). RNA-dependent ATPase activity has been demonstrated for PRP16 (Schwer and Guthrie, 1991) and PRP2 (Kim *et al.*, 1992). PRP2 is an extrinsic factor that associates with the fully assembled spliceosome and promotes the first cleavage–ligation reaction (Lin *et al.*, 1987; King and Beggs, 1991; Kim *et al.*, 1992). The ATPase PRP16 interacts with the spliceosome after completion of the first catalytic reaction and promotes 3′ss cleavage and formation of mRNA in an ATP-dependent fashion (Schwer and Guthrie, 1991). The third member of the DEAH-box family, PRP22, is required for the release of mRNA from the spliceosome (Company *et al.*, 1991). Given the high degree of homology among these proteins it is conceivable that they act via a similar mechanism, but at distinct steps of the splicing pathway. Thus, understanding the functioning of any one of these proteins is likely to have important implications for the roles of the others.

We have previously shown that PRP16 is required for step 2 of the splicing reaction *in vitro* (Schwer and Guthrie, 1991). Studies of the mutant *prp*16-1 (Couto *et al.*, 1987; Burgess *et al.*, 1990) demonstrate that the ATPase activity of PRP16 is necessary for its function in promoting 3′ss cleavage and exon ligation (Schwer and Guthrie, 1992). We

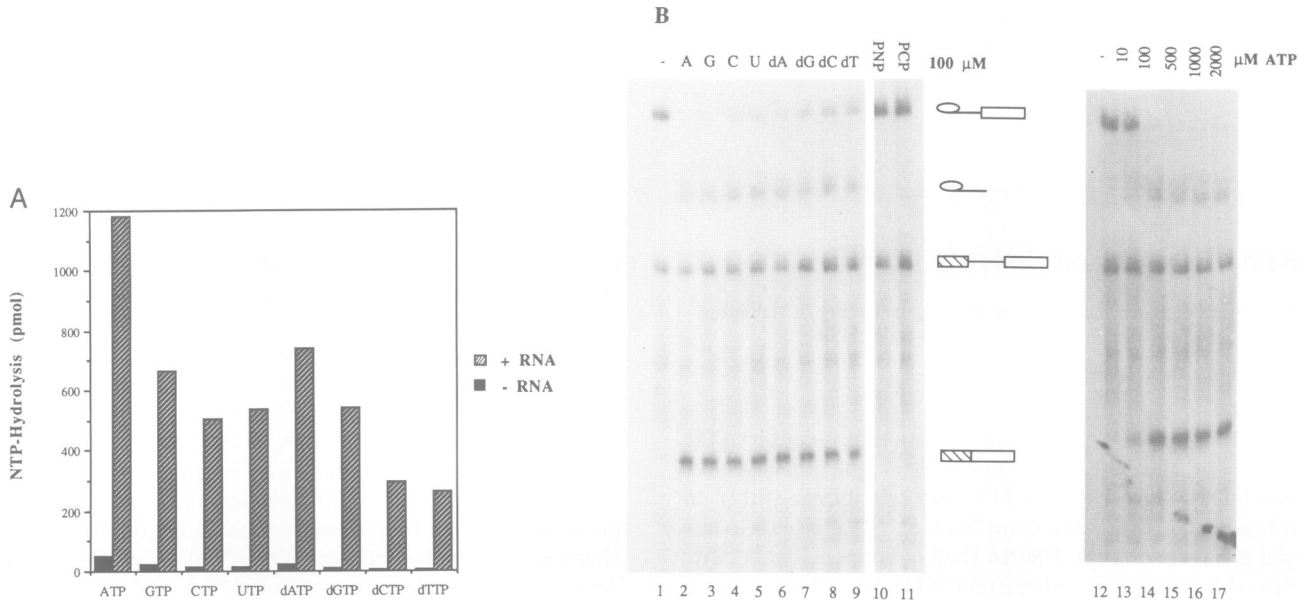

Fig. 1. PRP16 has broad nucleotide specificity. (**A**) Reaction mixtures containing 1.5 pmol of purified PRP16 protein were incubated with 1 mM NTP/dNTP in the presence (hatched bars) or absence (black bars) of poly(U) RNA. NTP hydrolysis in 15 min at 24°C was measured as formation of [^{32}P]NDP (in pmol) by polyethyleneimine chromatography followed by Phosphorimaging. The variation between duplicate reactions was <5%, the background (−enzyme) has been deducted from each value. (**B**) Splicing was performed for 25 min at 24°C in extracts immunodepleted of PRP16 (ΔPRP16). ATP was then removed by gel filtration and purified PRP16 added. Aliquots were further incubated without additional NTP (lanes 1 and 12), with increasing amounts of ATP as indicated (lanes 13−17) and with 100 μM of each NTP/dNTP (lanes 2−9) or two non-hydrolysable analogues, AMP-PNP (lane 10) and AMP-PCP (lane 11). The symbols depict the precursor and products of the splicing reaction; exon 1 is represented by a hatched box, exon 2 by an empty box and intron by a line.

now show that the wild-type protein can hydrolyse all four nucleoside triphosphates and the corresponding deoxynucleoside triphosphates. The usage of these nucleotides in the NTPase assay correlates with their effectiveness in complementing the second catalytic step of splicing, suggesting that PRP16 is the only NTPase that is required for the second catalytic reaction. In this report we present evidence that PRP16 uses ATP hydrolysis to promote a conformational change in the spliceosome. This structural rearrangement results in the protection of the 3′ss against oligo-directed RNase H degradation.

Results

PRP16 can hydrolyse all NTPs/dNTPs

We have previously shown that PRP16 exhibits RNA-dependent ATPase activity (Schwer and Guthrie, 1991). In order to test the nucleotide specificity of PRP16, we assayed the purified enzyme for its ability to hydrolyse all nucleoside triphosphates (NTP) and the corresponding deoxynucleoside triphosphates (dNTP). Figure 1A shows that PRP16 catalyzes the hydrolysis of all NTPs in the following order of efficiency: ATP (100%) > dATP (62.4%) > GTP (56.4%) > dGTP (45.6%) > UTP (45.3%) > CTP (42.6%) > dCTP (25%) > dTTP (23.6%). The products of the reactions were determined to be nucleoside diphosphate and inorganic phosphate (P_i). We sought to determine whether this broad NTP specificity parallels the NTP usage in the splicing reaction (Figure 1B). Intermediates were formed in extracts immunodepleted of PRP16 (ΔPRP16), then ATP was removed by gel filtration. PRP16 was added and aliquots of this reaction were further incubated with 100 μM of the respective NTP/dNTP and with two non-hydrolysable analogues, adenosine β-γ-imido (AMP-PNP) and β-γ-methylene (AMP-PCP) triphosphate and assayed for mRNA formation (Figure 1B, lanes 2−11). We chose 100 μM to exclude the possibility that complementation was due to a minor (<10%) contamination of the tested NTP with ATP (although we could not detect any ATP by polyethyleneimine−cellulose chromatography with a detection level of ~5%). As shown in the ATP titration experiment (Figure 1B, lanes 12−17), 100 μM ATP (lane 14) was sufficient for the maximum formation of mRNA in 10 min; however, decreasing the ATP concentration to 10 μM (lane 13) resulted in a large reduction in the mRNA formed during 10 min of incubation. We observed complementation with all eight NTPs/dNTPs but not with the non-hydrolysable ATP analogues AMP-PNP and AMP-PCP (lanes 10 and 11). The finding that PRP16 can hydrolyse all NTPs/dNTPs and that these nucleotides can also support the second step of the splicing reaction, links the nucleotide requirement in our complementation assay to PRP16. However, we cannot exclude the possibility that another ATPase acting after PRP16 would have the same broad nucleotide specificity. Furthermore, since we cannot rule out undetectable amounts of ATP, an ATPase with a very high affinity for ATP might act after PRP16.

PRP16 affects the 3′ss protection pattern

In order for the second catalytic reaction to occur, the 3′ss has to be recognized and the two splice sites need to be brought into close proximity. It has previously been described that the 3′ss is dispensible for the first catalytic reaction in the yeast *in vitro* splicing reaction (Rymond and Rosbash, 1985); thus a conformational change involving the 3′ss of the pre-mRNA presumably occurs prior to (or concomitant with) the second catalytic step. We sought to detect an effect of PRP16 in the spliceosome by probing the

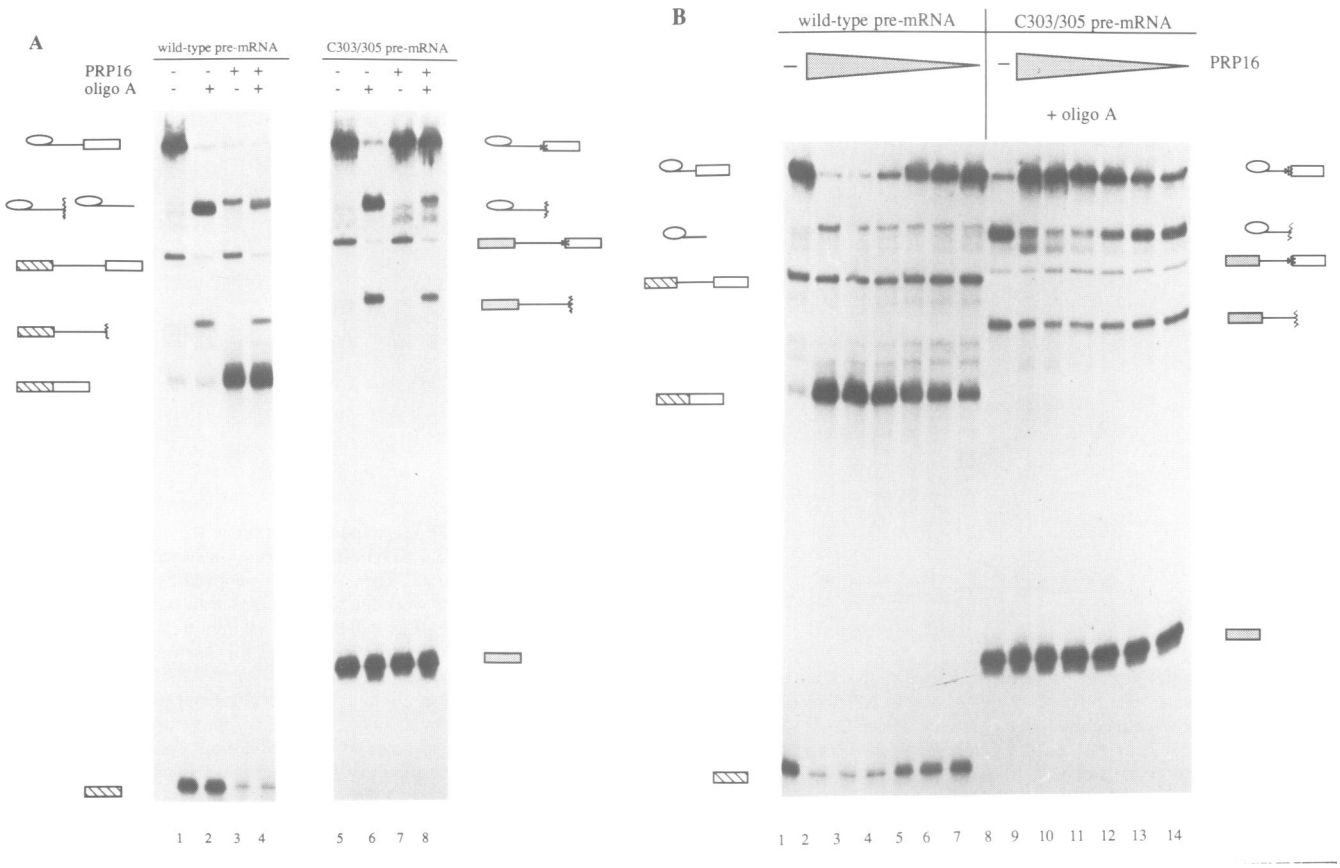

Fig. 2. PRP16 promotes a conformational change involving the 3′ss. Splicing was performed in ΔPRP16 extracts using wild-type (A, lanes 1−4; B, lanes 1−7) or mutant C303/305 (A, lanes 5−8; B, lanes 8−14) pre-mRNA. (**A**) Aliquots from these reactions were further incubated for 10 min at 24°C without (lanes 1, 2, 5 and 6) or with (lanes 3, 4, 7 and 8) PRP16. Then oligo A, which is complementary to the 3′ss, was added as indicated (lanes 2, 4, 6 and 8). After 10 min, the reactions were stopped and the RNA analysed by denaturing PAGE. (**B**) Intermediates formed in the absence of PRP16 were incubated for 15 min at 24°C in the absence (lanes 1 and 8) or presence (lanes 2−7 and 9−14) of decreasing amounts (from left to right: 1500, 150, 15, 3, 1.5 and 0.75 fmol) of PRP16 protein. The reactions were stopped (lanes 1−7) or further incubated for 10 min upon addition of oligo A and analysed by denaturing PAGE. The symbols show the precursor and products of the reactions; exon 1 is represented by a hatched (wild-type) or shaded (C303/305) box, exon 2 by an empty box and intron by a line. Specific cleavage by RNase H is shown as a wavy line, and the 3′ss mutation by an asterisk at the intron−exon 2 junction.

precursor RNA for changes in its accessibility to oligo-directed RNase H cleavage, an assay developed previously by Ruskin and Green (1985a). We probed lariat intermediates formed in the absence of PRP16 for 3′ss protection (Figure 2A). Intermediates were allowed to form in ΔPRP16 extracts for 25 min (lane 1) and then an oligo complementary to 16 nucleotides upstream of the 3′ss (oligo A, see Materials and methods) was added and the reaction incubated for 10 min at 24°C. Yeast whole cell extract contains RNase H activity, thus no additional RNase H needed to be added. A positive control for the functioning of the RNase H and the oligo is given by the precursor RNA in the reaction mix, which is specifically cleaved in the presence of oligo A (Figure 2A, lanes 2, 4, 6 and 8). In the absence of PRP16, lariat intermediates were converted into a product that migrates at the size of excised lariat, reflecting specific cleavage at the 3′ss by RNase H when oligo A was present (Figure 2A, lane 2). The products of the RNase H cleavage are indicated by the symbols; the 3′ half molecule appears to be susceptible to exonuclease activity in the extract as has been described before (Rymond and Rosbash, 1985), the 5′ half molecule is protected by the cap (GpppG) structure incorporated in the *in vitro* transcription reaction. Upon the addition of PRP16 and incubation for 10 min (Figure 2A, lanes 3 and 4), mRNA is formed readily, so the status of the 3′ss region could not be determined. In order to circumvent the rapid conversion of lariat intermediates to mRNA by PRP16, we made use of a precursor that contains a 3′ss mutation (CAG/AG to CAC/AC, named C303/305 precursor) (Figure 2A, lanes 5−8). This mutant precursor proceeds through 5′ss cleavage and lariat formation; however, it does not allow 3′ss cleavage and exon ligation (Vijayraghavan *et al.*, 1986). When we assayed intermediates formed with mutant precursor in extracts lacking PRP16, we found that the 3′ss was accessible (Figure 2A, lane 6) as expected from the results obtained with wild-type pre-mRNA (lane 2). Upon incubation of the pre-formed lariat intermediates with PRP16, however, the subsequently added oligo A did not target the 3′ss for cleavage by RNase H (~80% of the intermediates were protected, lane 8). These data argue that PRP16 promotes a rearrangement in the spliceosome that renders the 3′ss inaccessible to oligo-directed RNase H cleavage.

It was important to determine whether the 3′ss protection, assayed with C303/305 precursor, correlates with the formation of mRNA using wild-type precursor. To this aim, we compared the effects of various amounts of PRP16 protein in the RNase H protection and the complementation assays. The results are shown in Figure 2B. About 2 fmol of precursor (lanes 1−7, wt and 8−14 C303/305) were in-

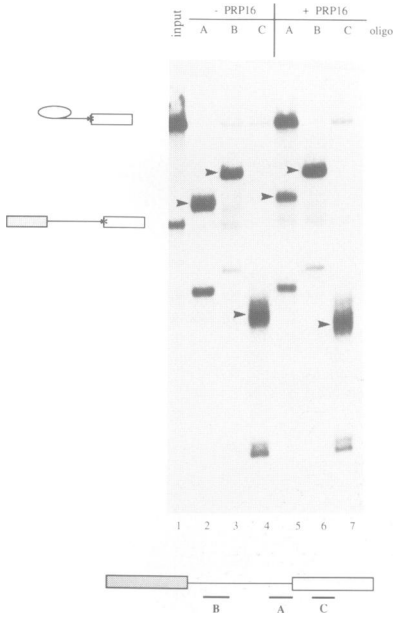

Fig. 3. Change in protection pattern is restricted to the 3′ss. Using C303/305 precursor, intermediates were formed in ΔPRP16 extract (input). Aliquots were incubated in the absence (lanes 2–4) or presence (lanes 5–7) of PRP16 (0.5 pmol each) for 10 min at 24°C. Then 50 ng of oligo A, B or C (complementary to regions indicated in the schematic drawing) were added. After 10 min the reactions were stopped and the products analysed by denaturing PAGE. The arrowheads mark the branched products that result from specific RNase H cleavage of lariat intermediates.

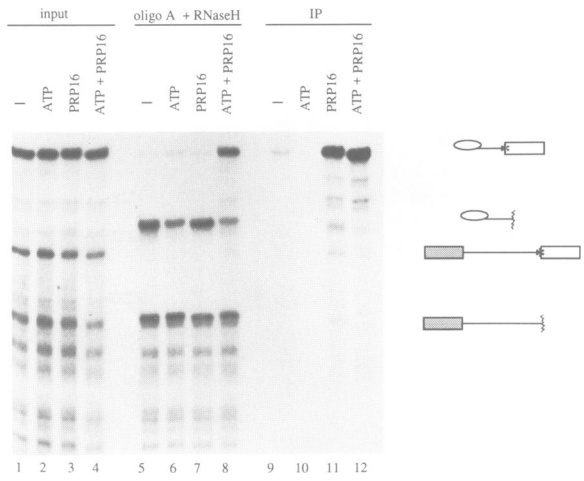

Fig. 4. PRP16 plus ATP are required to promote the conformational change. Using the mutant C303/305 precursor, intermediates were formed in ΔPRP16 extract. Then ATP was removed by gel filtration. Aliquots of this mixture were further incubated with buffer (–), ATP, PRP16 or ATP + PRP16 as indicated for 10 min at 24°C (input). Aliquots of each reaction were then assayed for 3′ss protection (lanes 5–8) and immunoprecipitation (in IPP_{150} without NP40) with α-PRP16 antiserum (lanes 9–12). The precursor and products of the reactions are indicated by symbols; exon 1 is represented by a shaded box, exon 2 by an empty box and intron by a line. Specific cleavage by RNase H is indicated by wavy lines, and the 3′ss mutation by an asterisk at the intron–exon 2 junction.

cubated in ΔPRP16 extract for 25 min at 24°C. Then decreasing amounts of PRP16 (1.5 pmol to 0.75 fmol) were added and the reaction stopped after 15 min (lanes 1–7), or oligo A was added and further incubated for 10 min (lanes 8–14). The amount of 3′ss protection roughly parallels the formation of mRNA and the amount of PRP16 added: an ~7-fold excess of PRP16 protein over precursor is sufficient to promote full protection (lane 11) and formation of mRNA lane 4) in 15 min. The formation of mRNA depends linearly on amounts of PRP16 protein added and the incubation time; when the amount of PRP16 was reduced, the incubation time had to be increased to give maximal mRNA formation (data not shown). These results demonstrate that the function of PRP16 in promoting step 2 correlates with a change in the spliceosome that renders the 3′ss protected against oligo-directed RNase H cleavage.

Only the 3′ss protection pattern is affected by PRP16

Is the structural rearrangement locally restricted to the 3′ss region? In order to rule out global conformational changes in the spliceosome, we probed lariat intermediates formed in the absence of PRP16 using oligos complementary to intron sequences (oligo B) and exon 2 sequences (oligo C) (Figure 3). Using the mutant C303/305 precursor, lariat intermediates were formed in the absence of PRP16 (Figure 3, input). Then 50 ng of the oligos were added before (lanes 2–4) or after (lanes 5–7) incubation of lariat intermediates with purified PRP16 protein. The addition of PRP16 resulted in protection (~80%) of the 3′ss (probed with oligo A) against targeted RNase H cleavage (Figure 3, compare lanes 2 and 5), while the pattern of cleavage did not change for oligos B and C (lanes 3 and 6, and lanes 4 and 7, respectively); that is, the sites in the RNA remained accessible to the complementary oligo and hence specific cleavage. The branched nature of the products of targeted RNase H cleavage , indicated by arrowheads, was confirmed by incubation with HeLa S100 extract containing debranching activity (Ruskin and Green, 1985b) (data not shown). These data show that the conformational change that is promoted by PRP16 appears to affect specifically the 3′ss region. Experiments are under way to determine the exact region of protection and also to probe the whole pre-mRNA molecule for changes in response to PRP16 addition.

Both ATP and PRP16 are required for the change in the 3′ss protection pattern

The formation of mRNA has previously been shown to require the PRP16 protein and ATP (Schwer and Guthrie, 1991). Using the C303/305 precursor, we could now determine whether PRP16 alone, or together with ATP, was responsible for the conformational change that renders the 3′ss protected (Figure 4). Intermediates were formed in ΔPRP16 extracts using the mutant C303/305 precursor. After ATP had been removed by gel filtration, either buffer, ATP, PRP16 or both were added and the reaction was allowed to continue for 10 min at room temperature. One-fifth of each reaction was used to analyse the input of each reaction (Figure 4, lanes 1–4), another one-fifth was probed for RNase H cleavage (lanes 5–8) and the remainder was assayed by immunoprecipitation (lanes 9–12). In the RNase H assay (lanes 5–8), oligo A (25 ng) and 1 U of RNase H were added for 10 min; the addition of RNase H was required since the gel filtration step removes the RNase H that is present in crude yeast extract. The addition of PRP16 plus ATP renders the 3′ss inaccessible to the hybridization of oligo A and hence insensitive to RNase H cleavage (Figure 4, lane 8). Thus, PRP16 can promote the

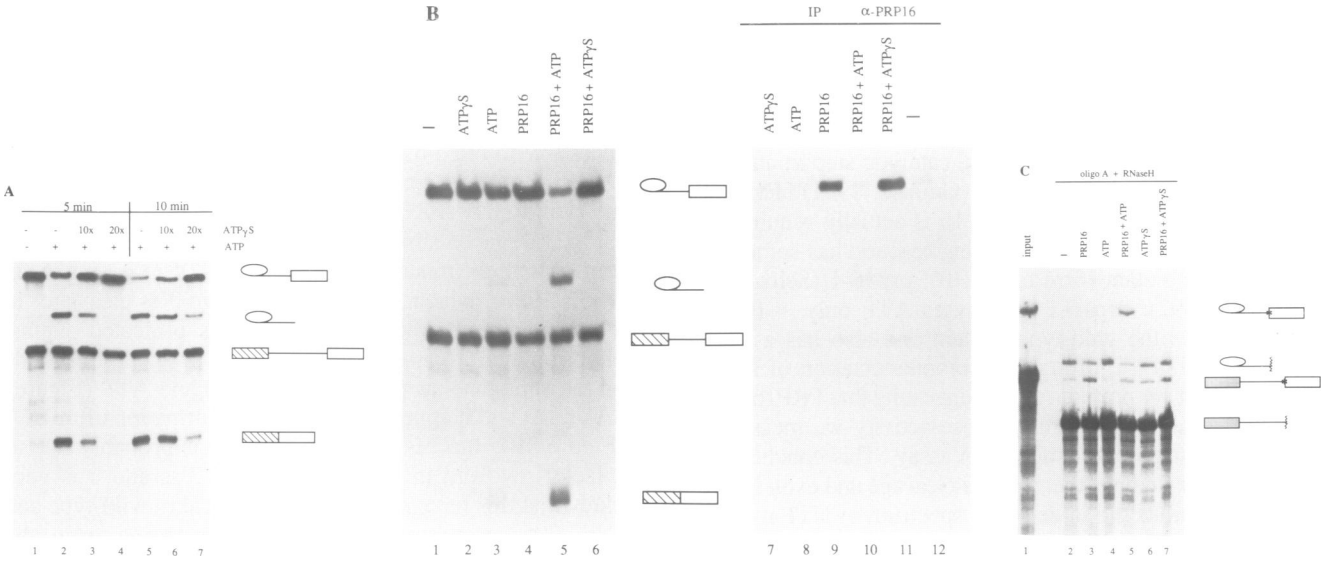

Fig. 5. ATPγS does not support mRNA formation and 3′ss protection. (**A**) Splicing intermediates were formed in ΔPRP16 extract for 25 min at 24°C using wild-type precursor. ATP was then removed and PRP16 protein was added. Aliquots were further incubated in the absence (lane 1) or presence of 250 μM ATP (lanes 2–7), mixed with a 10- (lanes 3 and 6) or 20- (lanes 4 and 7) fold molar excess of ATPγS. After 5 or 10 min at 24°C, the reactions were stopped and the products analysed by denaturing PAGE. (**B**) Using wild-type precursor, intermediates were formed in ΔPRP16 extract, then ATP was removed. Aliquots of the reaction were incubated with buffer (−) or the components indicated on top of each lane for 10 min at 24°C. Aliquots of the reactions shown in the left panel (lanes 1–6) were subjected to immunoprecipitation (in IPP_{150}) with anti-PRP16 antibodies (lanes 7–12). (**C**) Intermediates were formed in ΔPRP16 extract using the mutant C303/305 precursor, then ATP was removed (input, lane 1). Aliquots were supplemented with the components indicated above each lane for 10 min. Then 25 ng of oligo A and 1 U of RNase H were added. After an incubation of 10 min, the reactions were stopped and the RNA analysed by denaturing PAGE.

protection of the 3′ss only in the presence of ATP. Aliquots of each reaction were subjected to immunoprecipitation [in IPP_{150} (see Materials and methods) without NP40] by α-PRP16 antibodies (Figure 4B, lanes 9–12). PRP16 binds stably to the spliceosome in the absence of ATP (lane 11), as evidenced by the specific precipitation of lariat intermediates, as shown previously for wild-type pre-mRNA (Schwer and Guthrie, 1991). However, using wild-type pre-mRNA, we had shown that in the presence of PRP16 and ATP, mRNA is formed readily and that the protein does not remain associated with the products of the reaction (Schwer and Guthrie, 1991). Interestingly, when a similar experiment was performed with the mutant C303/305 precursor RNA, the protein remained bound to spliceosomes after the addition of ATP (Figure 4, lane 12). However, the binding of PRP16 to the spliceosome appears to be less stable in the presence than in the absence of ATP: for example, when immunoprecipitation is assayed in the presence of 0.1% NP40 (IPP_{150}), the binding appears to become destabilized although not completely abolished in the presence of ATP (data not shown). Since the bound protein alone does not promote the protection of the 3′ss, these experiments demonstrate that PRP16 does not bind directly to the 3′ss in the absence of ATP. In conclusion, the change in the 3′ss protection pattern requires both PRP16 and ATP.

3′ss protection requires ATP hydrolysis

It was then of interest to ask whether ATP hydrolysis was necessary to promote protection of the 3′ss or whether binding of ATP was sufficient. To address this question we made use of the ATP analogue adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS). ATPγS is not a substrate for the PRP16 RNA-dependent ATPase activity, since [^{35}S]ATPγS is hydrolysed to ADP less than 0.05% as efficiently as ATP (data not shown). However, ATPγS is a competitive inhibitor of the RNA-dependent ATPase activity with a K_i of 460 μM; the K_m for ATP is ~200 μM. In order to test whether ATPγS also competes with ATP for binding when PRP16 is bound to the spliceosome, we mixed ATP (final concentration of 250 μM) with a 10- or 20-fold molar excess of ATPγS and assayed the formation of mRNA in 5 and 10 min (Figure 5A). The addition of ATPγS clearly reduced the formation of mRNA (Figure 5A, lanes 3, 4, 6 and 7). Probably due to nucleoside diphosphate kinase activity and various ATPase activities (that can hydrolyse ATPγS) in the crude extract, and thereby regenerating ATP, mRNA is formed over a longer period of time of incubation even in the presence of excess ATPγS (lanes 6 and 7). Nonetheless, it is clear that ATPγS strongly inhibits the formation of mRNA. If binding of ATP was sufficient to cause the conformational change assayed by targeted RNase H cleavage, the addition of ATPγS and PRP16 should promote 3′ss protection and formation of mRNA. Figure 5B shows that ATPγS, in the presence of PRP16, does not allow the formation of mRNA (Figure 5B, lane 6) and PRP16 remains stably bound (immunoprecipitations performed in the presence of 0.1% NP40) to the spliceosome when ATP is replaced by ATPγS (lane 11). Aliquots of each reaction (as shown in lanes 1–6) were incubated with 25 ng of oligo A and RNase H for 10 min: in all cases, the intermediates were specifically cleaved and the products migrated at the size of lariat (data not shown). In order to include a positive control for the assay (oligo A and RNase H), we performed an identical experiment using C303/305 precursor RNA and the result of the 3′ss protection assay is shown in Figure 5C: when ATP plus PRP16 was added, the 3′ss was protected against RNase H cleavage (Figure 5C, lane 5); however, ATPγS does not cause this reaction (lane 7). These results argue that hydrolysis of ATP is required to promote the conformational rearrangement resulting in the protection of 3′ss.

Discussion

PRP16 has broad nucleotide specificity

We have previously demonstrated that PRP16 is an RNA-dependent ATPase that associates with the spliceosome after the first cleavage–ligation reaction is completed. PRP16 then promotes the second catalytic step in an ATP-dependent fashion (Schwer and Guthrie, 1991). Evidence that the ATPase activity of PRP16 is actually required for its function in step 2 of the splicing reaction has come from studies of a mutant form of PRP16, prp16-1 (Schwer and Guthrie, 1992): prp16-1 hydrolyses ATP only ~10% as efficiently as the wild-type protein and also has a highly reduced activity in promoting complementation of step 2. In this report we present results suggesting that PRP16 might in fact be the only NTPase whose activity we measure in the second step complementation assay. This conclusion is based on our findings that the 3′ss cleavage and exon ligation reaction exhibits the same broad spectrum of NTP usage as does the purified PRP16 protein in the RNA-dependent ATPase assay (Figure 1A and B). However, we cannot rule out the possibility that another, as yet unidentified ATPase acts at the same step as PRP16 and exhibits the same NTP specificity. Splicing has been shown to be very inefficient if ATP is replaced by any of the four NTPs in the *in vitro* reaction (Lin *et al.*, 1985). In contrast, as shown here for the PRP16-dependent step, UTP, CTP or GTP can also efficiently substitute for ATP in the PRP2-dependent reaction, i.e. 5′ss cleavage and lariat formation (Kim *et al.*, 1992). It is curious that two other enzymes that contain amino acid motifs indicative of DEAH-box proteins (Company *et al.*, 1991) are also known to exhibit a broad spectrum of nucleotide usage: the potyvirus PPV-CI protein (Lain *et al.*, 1990) and vaccinia NPH-II (Shuman, 1992); these proteins are RNA-dependent NTPases, and in addition have been shown to be NTP-dependent RNA helicases. NTP-dependent RNA unwinding activity has not been demonstrated for the purified splicing factors PRP2 (Kim *et al.*, 1992) and PRP16 (our unpublished results). Whether the broad nucleotide specificity for hydrolysis is characteristic of all DEAH-box proteins remains to be examined. In particular, we would expect that PRP22, which is required for the release of mRNA from the spliceosome after the second catalytic reaction (Company *et al.*, 1991), shows similar biochemical properties to PRP2 and PRP16.

What is the function of NTP hydrolysis by PRP16?

It has been proposed that one role of NTP hydrolysis in splicing is to induce a series of conformational changes that drive the reaction forward and thus promote accuracy (Guthrie, 1991). We sought to detect conformational changes in the spliceosome in response to PRP16 by probing the accessibility of precursor RNA to targeted RNase H cleavage. A similar approach has been used successfully by Ray and coworkers, who showed that eIF-4F, and to a lesser extent eIF-4A, elicit conformational changes in the mRNA structure as monitored by a nuclease sensitivity assay (Ray *et al.*, 1985). In the case of splicing, it has been demonstrated previously that (i) the 3′ss is dispensible for 5′ss cleavage and lariat formation in the yeast cell-free splicing system (Rymond and Rosbash, 1985), and (ii) there is a change in the accessibility of the 3′ss to targeted RNase H cleavage after the first and before the second catalytic reaction of mammalian splicing (Sawa and Shimura, 1991). These reports prompted us to look at the protection pattern of the 3′ss as a means to detect conformational rearrangements in the spliceosome in response to PRP16.

We demonstrated that in lariat intermediates which were formed in extracts immunodepleted of PRP16, the 3′ss is accessible to oligo-directed RNase H cleavage (Figure 2A). Due to a rapid conversion of intermediates into mRNA upon the addition of PRP16 using a wild-type substrate, we assayed the accessibility of the 3′ss in lariat intermediates formed with the mutant precursor C303/305 (Vijayraghavan *et al.*, 1986), which is unable to undergo 3′ss cleavage; in these mutant intermediates, the 3′ss is protected against oligo-directed RNase H cleavage. This protection depends on the simultaneous presence of PRP16 and ATP (Figures 2A and 4) and it appears to parallel the formation of mRNA (Figure 2B). Inaccessibility of the 3′ss is probably due to a factor involved in recognition of the 3′ss and it is very likely that this recognition step is identical in wild-type and C303/305 precursor. This assumption comes from the observation that a very small amount of mRNA can be formed using the mutant precursor and in this case splicing occurs at the correct position; that is, the site is chosen correctly, but the reaction is very inefficient (Vijayraghavan *et al.*, 1986). Taken together, our data are consistent with the hypothesis that the change in the 3′ss protection pattern is a functional step preceding the second cleavage ligation reaction.

In the mammalian system, the pattern of 3′ss protection is more complicated: (i) the 3′ss is inaccessible before step 1; (ii) after the first cleavage ligation reaction it becomes unprotected; and (iii) the addition of protein factor(s) is required to render it inaccessible again. Then, upon addition of ATP and no additional factors, mRNA is formed (Sawa and Shimura, 1991). Several mammalian proteins that meet the criteria of binding to the polypyrimidine tract and 3′ss have been described (Gerke and Steitz, 1986; Tazi *et al.*, 1986; Ruskin *et al.*, 1988; Swanson and Dreyfuss, 1988; Garcia-Blanco *et al.*, 1989, 1990), but given the complexity of the 3′ss recognition in mammals it remains to be determined which factor(s) bind(s) at which stage of the splicing reaction. In mammalian splicing, the protection of the 3′ss requires the presence of certain protein factors but no ATP (Sawa and Shimura, 1991). In contrast, we show in this report that in the yeast splicing system, the 3′ss protection requires the presence of both ATP and the protein PRP16. In fact, we have demonstrated that ATP hydrolysis is required (Figure 5), by using the ATP analogue ATPγS, which cannot be hydrolysed by PRP16 and which is a competitive inhibitor of the RNA-dependent ATPase activity.

Several lines of evidence indicate that PRP16 does not bind directly to the 3′ss. First, the addition of PRP16 (even in 1000-fold excess over precursor) does not result in 3′ss protection in the absence of ATP. Second, PRP16 binds to spliceosomes that lack a 3′ss, as evidenced by immunoprecipitation of intermediates containing truncated pre-mRNA (data not shown). We propose that by hydrolysing ATP, PRP16 induces a conformational change which allows another factor to bind to the precursor. It is tempting to speculate that this change leads to the recognition of the 3′ss and is necessary to bring exon 1 in close proximity to the 3′ss. Experiments to test this hypothesis are under way.

To our knowledge, this is the first report showing that ATP hydrolysis by a spliceosomal factor leads to a struc-

tural rearrangement in the spliceosome. The challenge is now to determine the exact mechanism by which PRP16 promotes the protection of the 3'ss.

Materials and methods

RNase H, T7 polymerase and ATPγS were purchased from Boehringer Mannheim, organomercurial agarose for purification of ATPγS from Bio-Rad and protein A–Sepharose from Pharmacia. All α-^{32}P-labelled NTPs and dNTPs were obtained from Amersham, other NTPs/dNTPs, AMP-PNP and AMP-PCP from SIGMA.

The purification of PRP16 protein used in all assays as well as the immunodepletion of PRP16 was described in Schwer and Guthrie (1991). The extracts used in these studies were prepared according to Lin *et al.* (1985) from the wild-type strain BJ 2168 (Jones, 1991). Templates for both wild-type and C303/305 (Vijayraghavan *et al.*, 1986) precursor were kindly provided by Art Zaug; they contain the actin sequences (Gallwitz and Sures, 1980; Ng and Abelson, 1980; Lin *et al.*, 1985) under the control of a T7 promoter. Transcription was done using T7 polymerase (Boehringer) according to the supplier's protocol. The amount of transcript used per splicing assay was ~2 fmol.

Immunoprecipitations were performed by pre-incubating 5 μl of anti-PRP16 antiserum with protein A–Sepharose beads (40 μl of 1 g protein A–Sepharose per 10 ml) in 400 μl IPP_{500} (10 mM Tris, pH 8, 500 mM NaCl, 0.1% NP40) for 1 h at 24°C. The beads were washed extensively (three times with 1 ml each) in IPP_{150} (10 mM Tris, pH 8, 150 mM NaCl, 0.1% NP40). Then 30 μl of the splicing reactions [performed as described by Lin *et al.* (1985) and Schwer and Guthrie (1991)] were incubated for 1 h at 4°C on a nutator. The beads were then washed three times with 400 μl of IPP_{150} and phenol-extracted, and the RNA was analysed by denaturing PAGE. When IPP_{150} without NP40 was used, this is mentioned in the text and the figure legends.

Prior to use, the ATPγS was purified by affinity chromatography on organomercurial agarose (Smith *et al.*, 1978) according to the protocol supplied by Bio-Rad for use of Affigel 501.

NTPase activity was assayed in the presence or absence of the nucleic acid cofactor poly(U) RNA (250 μM, measured spectrophotometrically as the nucleotide concentration) as the formation of [α-^{32}P]NDP from [α-^{32}P]NTPs. The reactions were carried out at 24°C in a buffer containing 50 mM triethanolamine (pH 8.3), 75 mM potassium acetate, 1 mM dithiothreitol, 1.25 mM $MgCl_2$, 1 mM of the corresponding NTP/dNTP and 1 μCi of α-^{32}P-labelled NTP/dNTP (10 Ci/mmol). An aliquot was analysed by polyethyleneimine cellulose chromatography, developed with 1 M $LiCl_2$ (for CTP, UTP, dCTP and dTTP) or 750 mM potassium phosphate buffer (pH 3.5) (for ATP, GTP, dATP and dGTP). The dried plates were then scanned and the radioactivity in the resolved NDP/dNDP spots was quantified using a Phosphor-imager (Molecular Dynamics). The values in Figure 1 are expressed as pmol NDP/dNDP formed after 15 min incubation using ~1.5 pmol of PRP16 protein per assay.

The sequences of the oligos used in the assays are: oligo A, 5'-TAAACATATATATATAG-3' (upstream of 3' AG); oligo B, 5'-ACATACCAGAA-3' (starts 29 nucleotides downstream of 3'ss AG) and oligo C, -GGAGGTTATGGGAGAGTG-3' (starts 99 nucleotides downstream of 5'ss).

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