MOLECULAR AND CELLULAR BIOLOGY, Aug. 1992, p. 3540–3547
0270-7306/92/083540-08$02.00/0

Vol. 12, No. 8

A Dominant Negative Mutation in a Spliceosomal ATPase Affects ATP Hydrolysis but Not Binding to the Spliceosome

BEATE SCHWER AND CHRISTINE GUTHRIE*

Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143-0448

Received 1 April 1992/Accepted 16 May 1992

PRP16 is an RNA-dependent ATPase required for the second catalytic step of splicing in vitro. A dominant suppressor of a branchpoint mutation in *Saccharomyces cerevisiae*, the *prp16-1* allele, contains a Tyr to Asp change in the nucleotide-binding site consensus sequence. We now find that cells harboring the *prp16-1* allele have a general growth defect that is exacerbated at cold temperatures. The mutant is dominant over the wild-type gene when overexpressed. Purified Prp16-1 protein binds to the spliceosome with apparently wild-type affinity; however, it only weakly complements the second-step block in a PRP16-depleted extract. Analysis of purified Prp16-1 revealed that the rate of ATP hydrolysis is greatly reduced. These results can account for the dominant negative growth phenotype and argue that the ATPase activity of PRP16 is essential for its role in splicing. Moreover, since PRP16 is a member of the DEAD/H box families, these findings have important implications for a large class of proteins.

The *PRP16* gene is essential for splicing nuclear pre-mRNAs in *Saccharomyces cerevisiae* (1). Its nucleotide sequence predicts an encoded protein of 1,071 amino acids and a relative M_r of 121,500. A central domain of about 300 amino acids contains motifs found in RNA-dependent ATPases and ATP-dependent RNA helicases. The prototype of the so-called DEAD box proteins is eIF4A (12), a protein with demonstrated RNA-dependent ATPase and RNA helicase activities (14). Several splicing proteins (designated PRP, for pre-mRNA processing; reviewed in reference 15) that belong to this class have been isolated: PRP5 (6) and PRP28 (18) are DEAD box proteins, while PRP2 (2), PRP22 (4), and PRP16 show distinct changes in conserved domains (DEAH box family) (21). We have previously described the purification of PRP16 and shown that the protein can indeed hydrolyze ATP; this activity is stimulated by single-stranded RNA (17). Our studies demonstrated that PRP16 is required specifically for the second step of the splicing reaction in vitro, since extracts that have been immunodepleted of PRP16 can perform the 5′ splice site cleavage reaction, as evidenced by accumulation of lariat intermediates. PRP16 associates with preformed spliceosomes in the absence of ATP, as was shown by immunoprecipitation of splicing intermediates. The protein together with ATP is required to promote the second catalytic step.

PRP16 was originally identified in a screen for fidelity mutants (5). The *prp16-1* allele dominantly suppresses an A to C mutation in the highly conserved intron branchpoint nucleotide. In addition, Couto et al. (5) described a recessive effect of the mutation on the splicing of wild-type pre-mRNAs: in the absence of wild-type *PRP16* gene product, lariat intermediate and precursor accumulate. Burgess et al. (1) identified the mutation which is necessary to confer the suppressor phenotype as a Tyr to Asp change in the ATP-binding domain. Here, we demonstrate that this single amino acid change is also a dominant negative mutation when *prp16-1* is overexpressed. To determine the biochemical basis for this growth defect, we purified Prp16-1 protein. Our studies show that Prp16-1 binds to the spliceosome with apparently wild-type affinity. However, the mutant protein has reduced RNA-dependent ATPase activity. The biochemical activities provide a plausible explanation for the genetic observations and support the hypothesis that PRP16 requires ATP-hydrolyzing activity in order to perform its role in splicing. The implications of these findings for the study of other DEAD and DEAH box proteins are discussed.

MATERIALS AND METHODS

Materials. Restriction enzymes and other DNA modification enzymes were purchased from Biolabs. Polynucleotides were from Sigma, and chromatography resins were from Pharmacia. [γ-^{32}P]ATP for the ATPase assay and [α-^{32}P]GTP for in vitro transcription of the actin precursor (11) were obtained from Amersham, and all reagents were used according to the supplier's protocol. The concentrations of the nucleoside triphosphates (NTPs) and polynucleotides were determined spectrophotometrically.

Yeast methods. Cells were grown in YEP medium containing glucose (YEP-glucose) or synthetic medium (e.g., SD-Trp, minimal medium containing all amino acids except tryptophan) prepared by the method of Rose et al. (13). All genetic manipulations, including yeast transformations by lithium acetate, were done as described before (13).

Strains. YTdL4 is a haploid *PRP16* deletion strain (17) (*MATa PRP16::LYS2 ura3 trp1 leu2 his3*) in which the chromosomal deletion (nucleotides +119 to +3275 of *PRP16* were replaced by *LYS2* [17]) is complemented by *PRP16* or *prp16-1* (YTdL4/1 and YTdL4/2, respectively) on a CEN plasmid. YTdL4/3 and YTdL4/4 contain *PRP16* or the mutant allele, respectively, on the 2μm plasmid. The 2μm plasmid pG1-PRP16 contains nucleotides +1 to +3275 of *PRP16* under the control of the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (16, 17). In pG1-prp16-1, the coding region is replaced by *prp16-1*; the single nucleotide change, resulting in the Tyr to Asp change of amino acid 386, was confirmed by sequencing. YWTh2 (*MATa trp1 his3 lys2 ade ura3*) was transformed with pG1-PRP16 (YWTh2/3) or pG1-prp16-1 (YWTh2/4).

* Corresponding author.

Immunodepletion and complementation. Extract was immunodepleted of PRP16 as described by Schwer and Guthrie (17). The splicing reactions with Δ*PRP16* extracts were performed at 23°C for 25 to 30 min under the conditions described before (11, 17), and then 1 μl of complementing fraction (as indicated in the figure legends) was added.

Purification of Prp16-1. Prp16-1 was purified exactly as for wild-type PRP16 (17) from YTdL4/4 cells. The activity of Prp16-1 was monitored by inhibition of the wild-type protein: splicing intermediates were formed in a heat-inactivated (20 min, 37°C) extract from a strain carrying a temperature-sensitive allele of *PRP16* (*prp16-2*). We have shown previously (17; data not shown) that a splicing mutant isolated by Vijayraghavan et al., *prp23-1* (20), is allelic to *PRP16* (*prp23-1* = *prp16-2*). Intermediates formed in the heat-inactivated extract for 30 min at 23°C were incubated with column fractions for 5 min. Then, 20 ng of purified wild-type protein was added, and the incubation was continued for 5 min. The fractions containing the inhibiting activity were pooled and further fractionated (17). The inhibiting fractions from the carboxymethyl (CM)-Sepharose column were pooled (CM-Prp16-1) and used in the assays.

Sucrose gradient fractionation. Protein CM-Prp16-1 (5.2 μg) was diluted 1:4 in buffer M-glycerol (20 mM MES [morpholineethanesulfonic acid, pH 6.6], 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 10% glycerol) and separated in a 5 to 20% sucrose gradient in buffer M (20 mM MES [pH 6.6], 50 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol). The separation was done at 4°C in a Beckman TL-100 ultracentrifuge for 12 h at 55 krpm with the TL-55 rotor. Twelve fractions of 200 μl each were collected, and aliquots were analyzed.

ATPase assay. ATP hydrolysis was measured in 50 mM triethanolamine (pH 8.2)–75 mM potassium acetate–1 mM dithiothreitol–1.25 mM $MgCl_2$–1 mM ATP–1 μCi of [γ-^{32}P] ATP (10 Ci/mmol). The amounts of enzyme, the addition of polynucleotide (expressed as concentration of nucleotide), and the incubation times are indicated in the figure legends. To stop the reaction, acid-washed Norit charcoal (5% in 20 mM phosphoric acid [3, 17]) was added up to 200 μl to precipitate unreacted ATP; the assay mix was incubated on ice for 10 min and spun in a microcentrifuge for 10 min, and the supernatant was analyzed for free $^{32}P_i$ by Cerenkov counting. The background resulting from incubation in the absence of enzyme was subtracted from each value. The results are average values from duplicate reaction mixes, with a standard deviation of less than 5%.

RESULTS

The Tyr to Asp change in Prp16-1 is a dominant negative mutation. *prp16-1* was originally identified as a dominant suppressor of a branchpoint mutation (5). A single amino acid change (Tyr to Asp at amino acid position 386 [Y386D]) was shown to be necessary to confer the suppressor phenotype (1). In addition, *prp16-1* causes a growth defect. The growth of chromosomal *PRP16* deletion strains containing wild-type *PRP16* or *prp16-1* on either a CEN plasmid (YTdL4/1 and YTdL4/2, respectively) or a multicopy plasmid (TdL4/3 and TdL4/4, respectively) was tested at different temperatures on plates (Fig. 1A). Cells harboring the *prp16-1* allele grow much more slowly at 18°C and at room temperature (23°C) than the strains containing *PRP16*. The doubling time of TdL4/2 in culture is 1.4-fold longer than that of TdL4/1 at 30°C. The slow-growth phenotype is exacerbated at low temperatures; at 18°C, the doubling time of YTdL4/2 is 8.8 h (1.6-fold), whereas that of YTdL4/1 is 5.4 h. Furthermore, while overexpression of *PRP16* has no detectable effect on the doubling time, overexpression of *prp16-1* reduces the growth rate by a factor of 2.2 at 18°C.

Interestingly, Y386D is a dominant negative mutation. Figure 1B shows the effect of overexpression of the mutant allele in a haploid cell with a single (chromosomal) copy of *PRP16*. The growth rate is significantly reduced (twofold) at 18°C, suggesting that the mutant protein inhibits the activity of the wild-type gene product when overexpressed. In order to exclude the possibility that this effect is specific to the strain background, we overexpressed *prp16-1* in three unrelated wild-type strains (YWT2, BJ5457, and A364A). *prp16-1* exhibits the dominant negative effect in each of these genetic contexts (data not shown). In contrast, the growth defect was not observed in a heterozygous (*PRP16/prp16-1*) strain (not shown).

In order to determine the molecular basis for the growth defect, precursor RNAs were analyzed by primer extensions. Couto et al. (5) have previously shown that *prp16-1* causes accumulation of pre-mRNA and the lariat intermediate. This effect was enhanced when cells (YTdL4/2 and YTdL4/4) were shifted to 18°C (data not shown).

Prp16-1 protein complements the second-step block with reduced activity. We sought to compare the phenotype of the mutant Prp16-1 protein with that of the wild type in a cell-free splicing system. We have shown previously that in extracts immunodepleted of PRP16 (ΔPRP16), 5′ splice site cleavage and lariat formation occur efficiently, but the reaction is blocked after the formation of the lariat intermediate (17). Immunoprecipitation experiments showed that PRP16 binds to the spliceosome in the absence of ATP. We further demonstrated that splicing intermediates are "chased" into mRNA by PRP16 in an ATP-dependent fashion. In order to assess the mutant protein for these functions, we prepared fxtI/Prp16-1, a 40% ammonium sulfate fraction from splicing extract (17) which is enriched for the protein from YTdL4/4, a strain overexpressing *prp16-1* on a 2μm plasmid under the control of the strong constitutive glyceraldehyde-3-phosphate dehydrogenase promoter. First, we tested whether Prp16-1 could complement the second-step block (Fig. 2). Splicing was performed for 30 min at room temperature with ΔPRP16 cell extracts. Then, fxtI/PRP16 or fxtI/Prp16-1 was added, and the reaction was allowed to proceed for 10 min at the temperatures indicated in the figure. The amounts of PRP16 and Prp16-1 in these fractions are comparable, as determined by Western immunoblot analysis (data not shown). When fxtI/Prp16-1 is the source of the complementing activity, only low levels of mRNA are formed. Moreover, this experiment showed that complementation by PRP16 and Prp16-1 is less efficient at 15 and 23°C; the optimal temperature is 30°C (data not shown).

Prp16-1 inhibits complementation of the second-step block. In order to determine the basis for the complementation deficiency, and given that Y386D is a dominant negative mutation in vivo, we sought to determine whether the mutant protein retained partial functional activity. Spliceosomes formed in ΔPRP16 extracts were incubated with fxt I/Prp16-1 for 5 min. Subsequent addition of wild-type protein did not promote the formation of mRNA (Fig. 3 and 4A with purified Prp16-1 protein and data not shown). This result could be explained if Prp16-1 can bind stably to the spliceosomes but is defective in a subsequent activity. Based on this inhibition of wild-type activity in preincubation experiments, Prp16-1 was purified to apparent homogeneity by the protocol used for the purification of PRP16 (17) (data not shown).

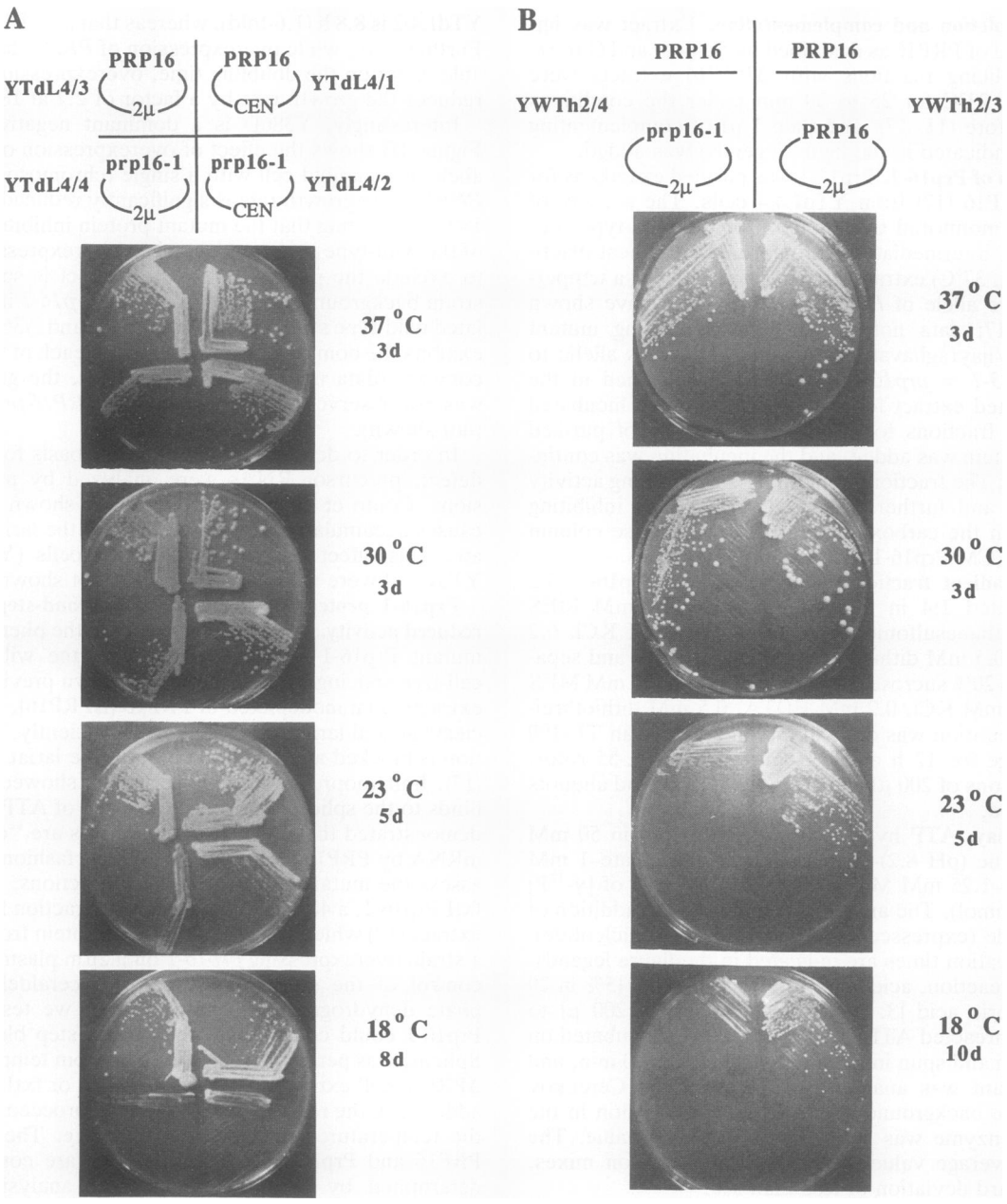

FIG. 1. Growth phenotype at different temperatures. (A) Growth of YTdL4 (*PRP16* deletion strain, *PRP16*::*LYS2*) containing wild-type *PRP16* on a CEN (YTdL4/1) or 2μm (YTdL4/3) plasmid or *prp16-1* on a CEN or 2μm plasmid (YTdL4/2 and YTdL4/4, respectively). Cells were streaked on YEP-glucose plates and incubated for the times (in days) indicated at different temperatures. (B) A haploid wild-type strain, YWTh2, was transformed with the 2μm plasmid pG1-PRP16 (YWTh2/3) or pG1-prp16-1 (YWTh2/4) (see Materials and Methods). Cells were grown on SD-Trp to select for the plasmids.

The Prp16-1 protein, detected by Western blotting, cofractionates with the inhibiting activity and an RNA-dependent ATPase activity in a 5 to 20% sucrose gradient (Fig. 3).

Figure 4A shows the results of the preincubation experiment with equimolar amounts of purified proteins. Preincubation with Prp16-1 inhibits complementation by subsequently added wild-type PRP16. Furthermore, mixing of Prp16-1 and PRP16 reduced the amount of mRNA formed in the complementation assay proportional to the amount of Prp16-1 protein present (Fig. 4B). Finally, the most direct evidence that Prp16-1 binds to preformed spliceosomes came from immunoprecipitation experiments (Fig. 4C). We incubated spliceosomes formed in ΔPRP16 extracts with either Prp16-1 or PRP16 in the absence of ATP (lanes 1 to 4) or with ATP (lanes 5 and 6) and subjected them to immunoprecipitation with anti-PRP16 antibodies (lanes 7 to 12). When spliceosomes are incubated with either protein in the absence of ATP, lariat intermediates and exon I are selectively precipitated. Prp16-1 but not PRP16 remains bound upon the addition of ATP (lanes 12 and 11, respectively). These experiments indicate that Prp16-1 binds to spliceosomes with apparently wild-type efficiency. Exact quantifi-

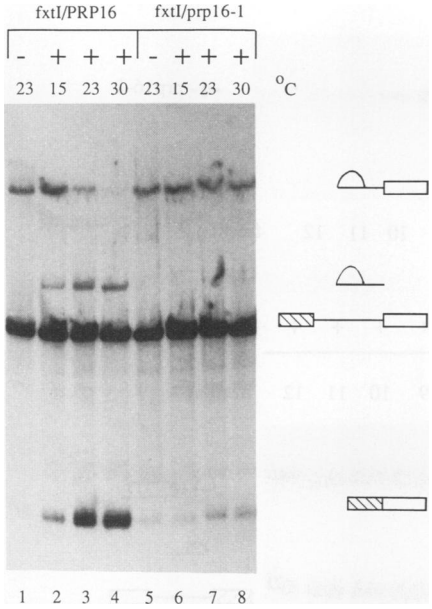

FIG. 2. Complementation of the second-step block at different temperatures. Intermediates were formed in ΔPRP16 extracts at 23°C (Materials and Methods) and then buffer (lanes 1 and 5), fxtI/PRP16 (lanes 2 to 4), or fxtI/Prp16-1 (lanes 6 to 8) was added, and the incubation was continued at the temperatures indicated. After 10 min, the reaction was stopped, and RNA was extracted, analyzed by denaturing gel electrophoresis, and autoradiographed. The structures of the various RNA species are indicated on the right. Exon 1 is represented by a hatched box, exon 2 is represented by an open box, and the intron is represented by a line.

cation of the affinities was difficult, however, since Prp16-1 does complement to some extent and the immunoprecipitation experiments are not quantitative. Our results show unequivocally that Prp16-1 binds to the spliceosome but is impaired in its ability to promote the 3′ splice site cleavage and exon ligation. The finding that Prp16-1 retains its binding activity argues against the uninteresting possibility that the complementation deficiency is due to a gross structural disruption in the protein.

Prp16-1 has reduced ATPase activity. Wild-type PRP16 protein has RNA-dependent ATPase activity (17). The mutation in *prp16-1* conferring the suppressor and slow-growth phenotype resides within the ATP-binding domain, and this change was proposed to alter the rate of ATP binding and/or hydrolysis (1). As shown in Fig. 3, Prp16-1 cofractionates with an ATPase activity; however, the specific activity appears to be lower than that of wild-type protein. We set out to compare the ATPase activities of the two enzymes. The activity, measured at a saturating poly(U) RNA concentration for 20 min, increased with the amount of protein added (Fig. 5A). However, Prp16-1 exhibited only 7 to 12% of wild-type activity. Assaying the ATPase activities in the presence and absence of RNA over a period of 90 min (Fig. 5B) showed the same result; in the presence of poly(U) RNA, Prp16-1 hydrolyzes ATP only about 10% as efficiently as wild-type enzyme (e.g., at 60 min). While the ATPase activity of PRP16 was stimulated about 10- to 12-fold upon the addition of poly(U) RNA, the ATPase activity of Prp16-1 was stimulated only 2- to 3-fold at 60 to 90 min.

In order to assess whether Prp16-1 has an altered RNA specificity, we tested a variety of RNAs and DNAs at 250 μM for their ability to stimulate the ATPase activity (Fig. 6). At the concentrations tested, poly(U) RNA stimulated the activity of wild-type protein about 10-fold (high concentrations were inhibitory; data not shown). In contrast, single- or double-stranded DNA did not stimulate it at all, and double-stranded RNA caused only a slight increase in the ATPase activity. The mutant Prp16-1 protein appears to be less responsive not only to RNA stimulation (two- to threefold), but also to the kind of RNA, since the levels of activity were low in all cases (<0.21 nmol of P_i). We attempted to determine whether the defect in Prp16-1 was caused by reduced ATP and/or RNA binding (data not shown). ATP binding was assayed with [α-^{32}P]ATP or [^{35}S]ATPγS by gel filtration or filter binding, but the binding appears to be unstable in both assays, as we were not able to detect bound ATP. Because of high variability between duplicate reaction mixes in the filter-binding assay, it was not possible to reliably measure and compare the rates of RNA binding of PRP16 and Prp16-1. Thus, we cannot yet distinguish whether the reduction in the rate of ATP hydrolysis of Prp16-1 is also due to a decrease in its ability to bind ATP and/or RNA.

DISCUSSION

A suppressor of an intron branchpoint mutation, Prp16-1, contains a single amino acid change, Y386D, which was shown to be necessary to confer the suppressor phenotype (1). In addition to its dominant suppression phenotype, *prp16-1* has been shown to affect the splicing of wild-type precursor RNAs; precursor and intermediates accumulate (5). We have now shown that this single amino acid change in the nucleotide-binding domain of PRP16 also affects cell growth (Fig. 1A). Cells containing the mutant allele *prp16-1* in single or multiple copies grow at a reduced rate. The slow-growth phenotype is strongly exacerbated at 18°C. Primer extension analysis of pre-mRNAs extracted from cells shifted to 18°C revealed that this phenotype is manifested in accumulation of precursor RNA and lariat intermediates (data not shown). Interestingly, Y386D is a dominant negative mutation, since overexpression of *prp16-1* in wild-type cells causes the same slow-growth phenotype as when *prp16-1* is the only copy in the cell (Fig. 1B). This means that the overexpressed mutant protein interferes with the function of the wild-type protein. Many examples of such dominant negative phenotypes are known, including several involving mutations in NTP-binding domains (7, 8). These phenotypes are likely to be particularly informative when they arise by mutation in subunits of multiprotein machines (9).

In order to determine the basis of the dominant negative effect on splicing, we analyzed the biochemical defect of Prp16-1. We took advantage of several assays which we had developed previously to determine the function of wild-type protein (17): (i) complementation of the second-step block in PRP16-immunodepleted extracts; (ii) spliceosome binding; and (iii) RNA-dependent ATPase activity. We were thus able to demonstrate that preincubation of spliceosomes with Prp16-1 abolishes complementation by subsequently added wild-type protein. This finding implies that Prp16-1 binds to the spliceosome but cannot efficiently carry out its requisite function to promote the second catalytic step. Binding of Prp16-1 to the spliceosome was then tested directly (Fig. 4C). When spliceosomes formed in ΔPRP16 extracts are incubated with PRP16 or Prp16-1 and then subjected to immunoprecipitation with anti-PRP16 antiserum, lariat intermediates and the 5′ exon are specifically precipitated. Inter-

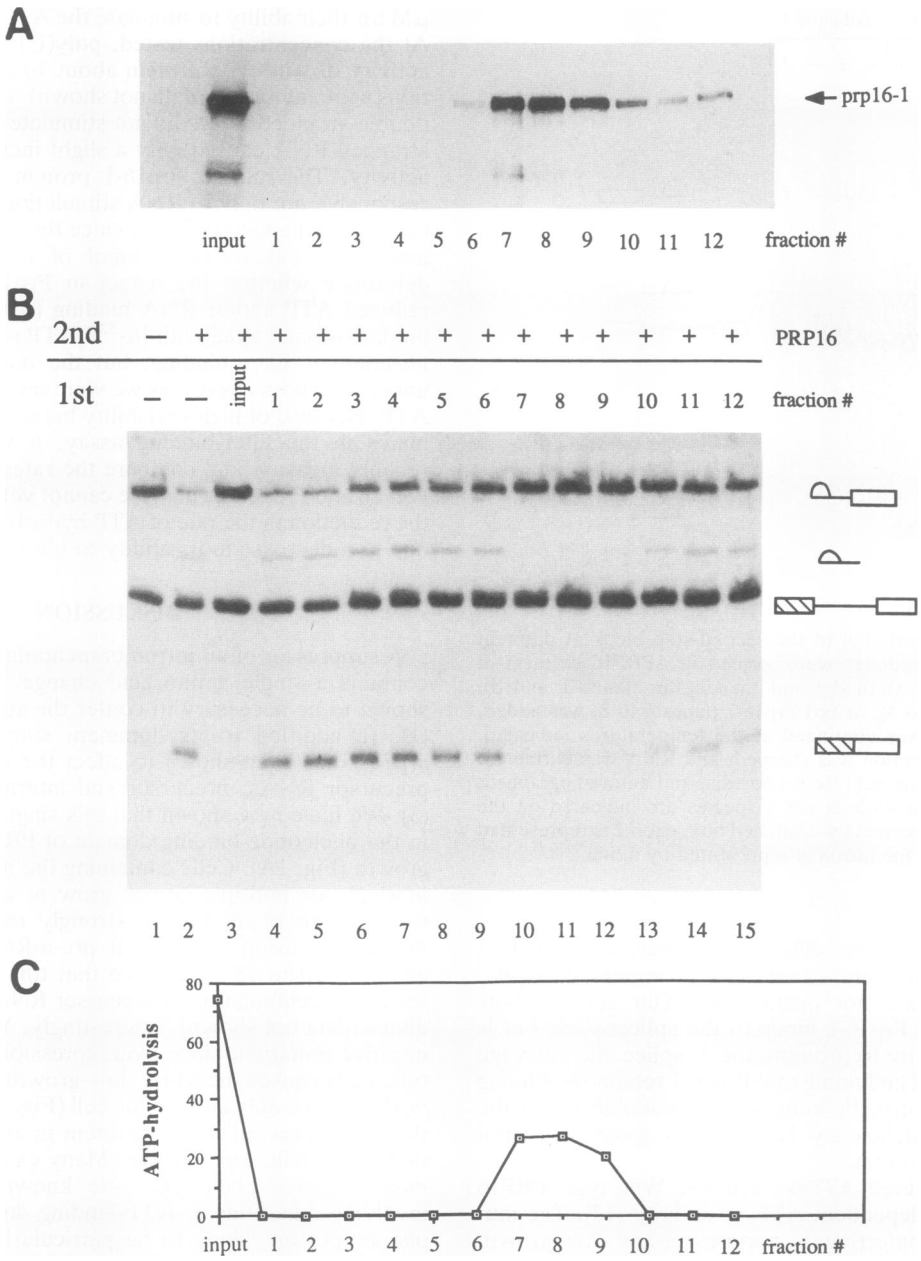

FIG. 3. Cofractionation of Prp16-1 protein (A), complementation-inhibiting activity (B), and RNA-dependent ATPase activity (C) in a 5 to 20% sucrose gradient. (A) CM-Prp16-1 (Materials and Methods) (17) were separated on a 5 to 20% sucrose gradient. Then, 10-μl aliquots of each gradient fraction and 2.5 μl of CM-Prp16-1 (input) were separated by 7.5% polyacrylamide–sodium dodecyl sulfate electrophoresis and transferred to nitrocellulose. The filter was incubated with anti-PRP16 antiserum (1:1,500 dilution in Tris-buffered saline) and then with goat anti-rabbit immunoglobulin secondary antibodies coupled to horseradish peroxidase (Bio-Rad). The enhanced chemiluminescence system (Amersham) was used to detect Prp16-1. (B) Inhibition in the splicing assay. Intermediates were formed during a 30-min incubation of heat-inactivated Prp16-2 extracts (Materials and Methods) (17) under splicing conditions. Aliquots (10 μl) were then incubated with 1 μl of buffer (lanes 1 and 2), CM-Prp16-1 (input; diluted 1:4; lane 3), or gradient fractions (lanes 4 to 15) for 5 min, followed by the addition of 20 ng of purified PRP16 protein (lanes 2 to 15). After 5 min, the reactions were stopped, and RNA was extracted and analyzed by denaturing gel electrophoresis and autoradiography. The structures of the various RNA species are indicated on the right. Exon 1 is represented by a hatched box, exon 2 is represented by an open box, and the intron is represented by a line.(C) RNA-dependent ATPase activity was measured in 20-μl assay mixes (Materials and Methods), with 5 μl of each fraction (fractions 1 to 12) and 1.2 μl of CM-Prp16-1 (input). The activity (ATP hydrolysis) is given as picomoles of P_i released after 20 min of incubation at 23°C. The background obtained during incubation without enzyme was subtracted.

estingly, Prp16-1, unlike wild-type PRP16, remains bound to the intermediates even after the addition of ATP, suggesting that the ATP requirement is linked to the function and subsequent release of PRP16. Although the mutant Prp16-1 protein binds to the spliceosome with apparently wild-type affinity, it is severely defective in its ATPase activity; the rate of hydrolysis is only about 10% of that of the wild-type protein (Fig. 5). While PRP16 is stimulated about 10-fold by

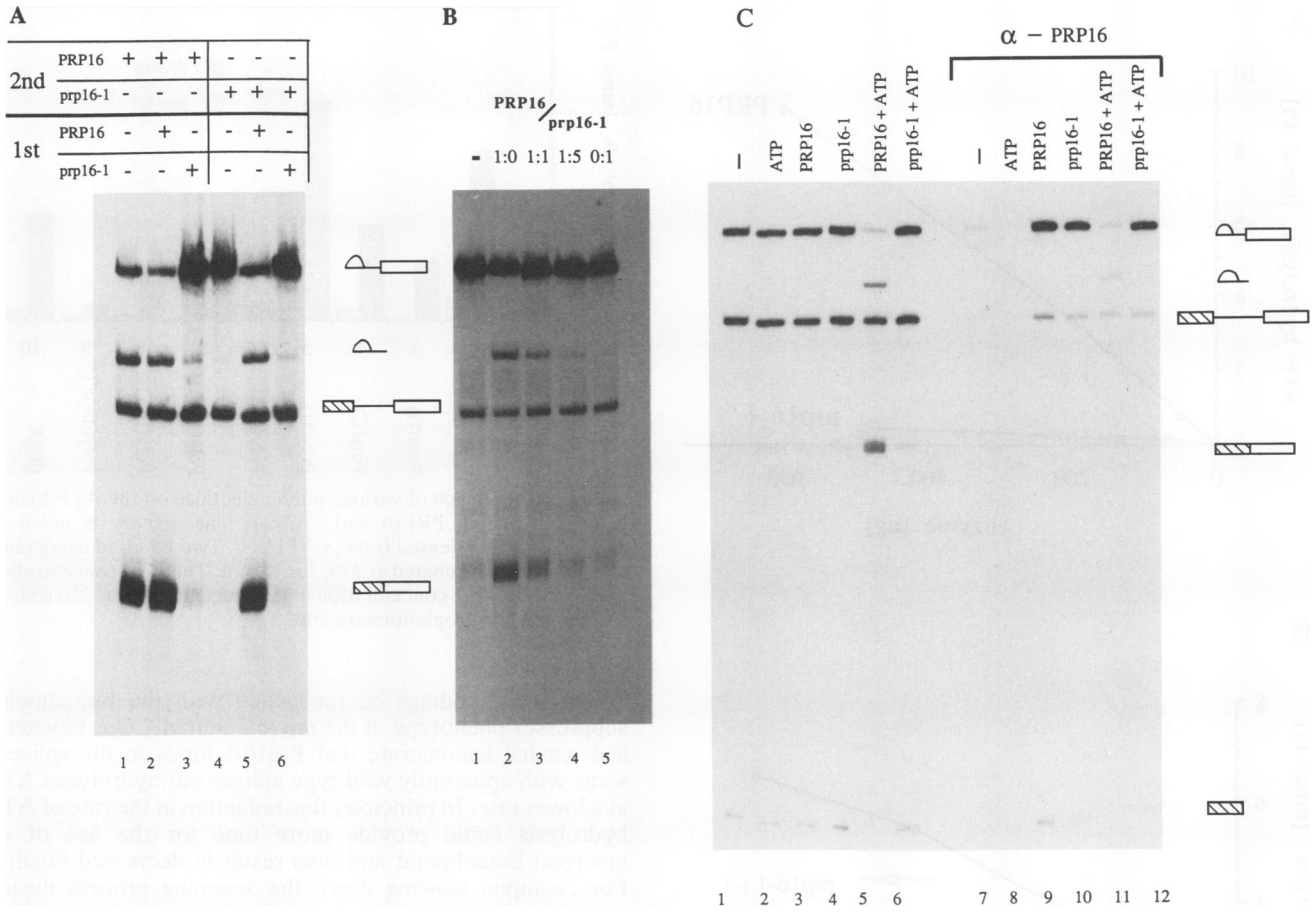

FIG. 4. Prp16-1 inhibits complementation by wild-type protein. (A) Preincubation. Spliceosomes formed in ΔPRP16 extracts were incubated for 5 min (1st) with buffer (lanes 1 and 4), purified PRP16 (lanes 2 and 5), or purified Prp16-1 (lanes 3 and 6). Then (2nd), wild-type PRP16 protein (lanes 1 to 3) or purified Prp16-1 (lanes 4 to 6) was added, and the incubation was continued for another 10 min, the reactions were stopped, and the RNA was analyzed. (B) Mixing. Preformed spliceosomes were incubated for 5 min in the presence of buffer (lane 1), PRP16 (lane 2), Prp16-1 (lane 5), or a mixture of both proteins in the ratios indicated (lanes 3 and 4). The total amount of protein added was 50 ng. (C) Immunoprecipitation. Splicing reactions were performed in ΔPRP16 extracts for 30 min at 23°C, ATP was removed by gel filtration, and then buffer, ATP, purified protein, or ATP plus purified protein was added as indicated and incubated for 7 min. One-third of each reaction mix was extracted and analyzed (lanes 1 to 6); two-thirds were subjected to immunoprecipitation with anti-PRP16 antiserum (lanes 7 to 12) as described before (17). Diagrams to the right of panels A and C are as described in the legend to Fig. 2.

poly(U) RNA, addition of RNA stimulates the ATPase activity of Prp16-1 only 2- to 3-fold. Since the rate of hydrolysis is lower even in the absence of RNA, however, a change in the affinity for RNA does not seem to be solely responsible for the reduction in ATPase activity. In order to test whether Prp16-1 has an altered RNA specificity, we assayed various RNAs for stimulation (Fig. 6). While PRP16 reacts differently to these RNAs, Prp16-1 is stimulated to a small extent by all RNAs tested.

Taken together, these results are consistent with a scenario in which Prp16-1 becomes incorporated into the spliceosome but can then carry out its ATPase-dependent function only at a greatly reduced level. Although the loss of Prp16-1 activity in the second-step complementation assay is almost certainly caused by the inability to efficiently hydrolyze ATP, we cannot distinguish at this time whether Prp16-1 also has altered ATP- and/or RNA-binding affinities. In any case, Prp16-1 can efficiently occupy the binding site in the spliceosome and thereby prevent wild-type protein from functioning; this can explain the dominant negative effect observed in vivo. We believe that these findings can be extrapolated to predict the behavior of comparable mutants in other members of the DEAD and DEAH box families. For example, mutations which specifically compromise the ATPase function in PRP2 and PRP22 (DEAH) or PRP5 and PRP28 (DEAD) (15, 21) are also likely to result in the accumulation of "trapped" spliceosomal intermediates, which can then be probed biochemically. Moreover, the generation of dominant negative mutations in the putative NTP-binding domains of DEAD and DEAH box proteins of unknown biochemical function (21) should be a generally powerful strategy for elucidating the diverse roles of this family.

The mutant allele *prp16-1* can support growth at 30°C, but the growth of cells harboring *prp16-1* is strongly impaired at 18°C. The analysis of precursor RNAs showed that *prp16-1* causes the accumulation of pre-mRNA and lariat intermediates at all temperatures in vivo (5; unpublished results). This indicates that the *prp16-1* gene product has a constitutive defect which becomes rate-limiting for growth only at low temperatures. Our results, obtained with the cell-free splicing system and purified protein, support this hypothesis: (i)

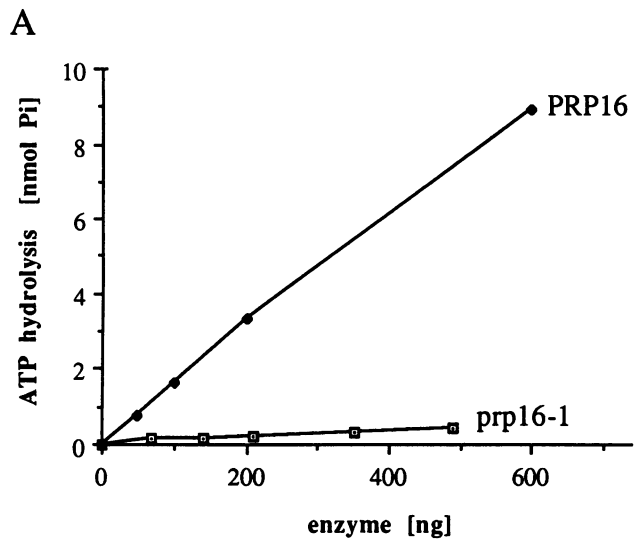

FIG. 5. ATPase activity of PRP16 and Prp16-1. (A) Increasing amounts of enzyme were incubated in 20-μl assay mixes as described in Materials and Methods [250 μM poly(U), 1 mM ATP] for 20 min at 23°C. The activity is given as nanomoles of P_i released from [γ-^{32}P]ATP. (B) Time course of ATP hydrolysis. Two hundred nanograms of enzyme was incubated in the presence (+) or absence (−) of poly(U) RNA in 10-μl assay mixes [250 μM poly(U), 1 mM ATP] at 23°C for increasing times. The release of P_i from [γ-^{32}P]ATP was measured.

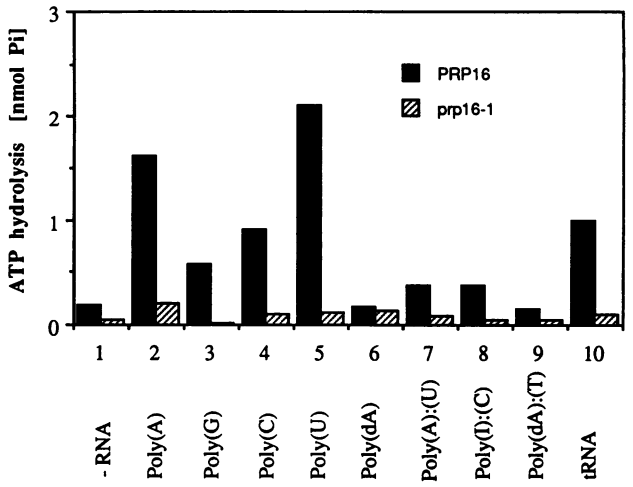

FIG. 6. Influence of various polynucleotides on the ATP-hydrolyzing activity of PRP16 and Prp16-1. The activity is given as nanomoles of P_i released from [γ-^{32}P]ATP. Two hundred nanograms of enzyme was incubated at 23°C for 15 min. The ATP concentration was 1 mM, and the concentration of polynucleotides was 250 μM, as determined spectrophotometrically.

the ATPase and the complementation activity of Prp16-1 are low at all temperatures tested, and (ii) complementation by wild-type protein shows temperature dependence, with an optimum at 30°C. These findings provide evidence that the step at which PRP16 functions, not the protein itself, is especially sensitive to cold temperatures. The rate-limiting steps in other macromolecular assembly processes have been shown to be temperature dependent (19), and it is likely that such temperature-dependent conformational rearrangements occur during spliceosome assembly and during the splicing reaction itself (18).

Can these findings be reconciled with the branchpoint suppressor phenotype of the *prp16-1* mutant? Our biochemical results demonstrate that Prp16-1 binds to the spliceosome with apparently wild-type affinity but hydrolyzes ATP at a lower rate. In principle, this reduction in the rate of ATP hydrolysis could provide more time for the use of an incorrect branchpoint and thus result in decreased fidelity. For example, slowing down the scanning process during translation initiation was shown to allow use of nonoptimal and even non-AUG start codons (10). At present, however, we cannot rule out more complicated models in which the loss-of-function and suppressor phenotypes reflect two different consequences of a single amino acid change. The isolation of novel suppressor alleles should allow these hypotheses to be distinguished. Ultimately, determination of the molecular basis for suppression will require the development of an in vitro system.

ACKNOWLEDGMENTS

We thank Dan Frank and Sean Burgess for their comments on the manuscript. We are grateful for the excellent technical assistance of Lucita Esperas.

This work was supported by National Institutes of Health grant GM21119. B.S. was supported by a NATO fellowship and a grant from the Cancer Research Coordinating Committee. C.G. is an American Cancer Society Research Professor in Molecular Genetics.

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