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The *Saccharomyces cerevisiae* Prp5 Protein Has RNA-dependent ATPase Activity with Specificity for U2 Small Nuclear RNA*

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The *Saccharomyces cerevisiae* protein Prp5 is a member of the "DEAD box" family of putative RNA-dependent ATPases and helicases. The protein was purified from *Escherichia coli* and determined to be an RNA-dependent ATPase. The ATPase activity is 7-fold more specific for full-length U2 than for any of the other small nuclear RNAs or nonspecific RNAs tested. An RNaseH assay in extracts was used to demonstrate that Prp5 mediates an ATP-dependent conformational change in the intact U2 small nuclear ribonucleoprotein. We propose that this conformational change makes the branch point pairing sequence of U2 RNA accessible for pairing with the intron allowing formation of the pre-spliceosome.

Splicing of introns in nuclear pre-mRNA takes place in a complex structure, the spliceosome, which is assembled *de novo* for each splicing event. Once assembled, the spliceosome catalyzes two sequential trans-esterification reactions that remove the intron from the pre-mRNA and ligates the exons. In the first reaction, the 2′-OH of a particular adenosine residue near the 3′ splice site attacks the phosphodiester bond at the 5′ splice site giving the intermediates in the reaction, exon 1, and the lariat intron, exon 2, a branched structure in which the 5′-nucleotide is connected to the branch point adenosine via a 2′-5′ phosphodiester bond. In the second reaction, the 3′-OH of exon 1 attacks the phosphodiester bond at the 3′ splice site, resulting in the formation of the spliced mRNA product and releasing the intron in lariat form (1–3).

The group II self-splicing introns are spliced by an identical pathway, leading to the principal tenets of this field that pre-mRNA splicing is an RNA-catalyzed reaction and that the two processes share a distant evolutionary ancestor (4).

The spliceosome contains five ribonucleoprotein particles, snRNPs,[1] called U1, U2, U4, U5, and U6 and is assembled stepwise in a dynamic process in which the pre-mRNA and the snRNAs undergo a series of base pairing interactions resulting finally in the catalytically competent structure, presumably a structure sharing common chemical and catalytic features with the group II self-splicing introns. The process of spliceosome assembly is quite different from the other complex ribonucleoprotein machine with which it can be compared, the ribosome (5). The ribosome can self-assemble from pure RNA and protein components *in vitro* without the requirement for ATP hydrolysis or trans-acting proteins. The unique essence of pre-mRNA splicing is to be found in the ordered and precise assembly steps all but the first of which require ATP hydrolysis.

Yeast genetic studies of proteins absolutely required for pre-mRNA splicing has thus far revealed only one source of the ATP requirement in spliceosome assembly. The proteins Prp2, Prp5, Prp16, Prp22, and Prp28 are all members of a superfamily defined by the first member of the family, the eukaryotic translation initiation factor eIF-4a (6–11). These proteins share a number of sequence motifs including a characteristic ATP binding site which includes the sequence DEAD, giving rise to one name for the family, the DEAD box proteins (12). DEAD box proteins are ubiquitous in nature. They have been found in all three major lines of descent, the bacteria, the archaebacteria, and the eukaryotes and usually in multiple copies. There are at least five in *Escherichia coli*. A recent data base search of the almost completed yeast genome found at least 66 DEAD- or DEAH-related genes, and extrapolating from the completed 2% of the *Caenorhabditis elegans* genome sequence there are probably at least 100 in higher eukaryotes. Biochemical studies of the DEAD box proteins almost invariably reveal that they are RNA-dependent ATPases, and in a few cases they have been shown to be RNA helicases, that is they can separate the strands of double-helical RNA in an ATP-dependent reaction so they have also been called RNA helicases (13–17). However, the latter reaction cannot be demonstrated for most of the proteins, so a safer term for the DEAD box proteins required for pre-mRNA splicing might be "spliceosomal ATPases."

Studies on the role of the spliceosomal ATPases in assembly suggest that each ATP-dependent step might be mediated by a single family member. The formation of the pre-spliceosome, an early intermediate in the pathway containing only the U1 and U2 snRNPs, requires Prp5 and the consecutive steps at the end of the pathway; activation of the spliceosome, activation of the second step, and release of the products require in order Prp2, Prp16, and Prp22, all members of a subclass of this family which share, instead of DEAD, a DEAH sequence and which are homologous over an additional 300 amino acids at their C termini (9–11, 18). What reaction could these proteins be catalyzing in the spliceosome assembly? The assembly pathway is punctuated by a series of RNA rearrangement that could better be understood as strand displacement reactions or structural isomerizations than helical disruptions (3). Thus a working hypothesis is that each structural isomerization is mediated by a specific spliceosomal ATPase. Clearly to understand exactly what these proteins are doing and how they do it must be a major objective for the study of pre-mRNA splicing, indeed for biochemistry since, as a class, these proteins are involved in a wide range of RNA-protein interactions.

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[1] The abbreviations used are: snRNP, small nuclear ribonucleoprotein(s); nt, nucleotide(s); PEG, polyethylene glycol; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; Mes, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; eIF-4a, eukaryotic translation initiation factor; NTA, nitrilotriacetic acid.

A possible reason why specific reactions have not been observed for any of the spliceosomal ATPases is that they work in combination with other proteins. eIF-4A has little if any helicase activity on its own, but in conjunction with eIF-4B, it has a weak but detectable helicase activity (13). However, p68 has strong RNA helicase activity on its own (15). It is possible that the spliceosomal ATPases act together with other proteins or act in the context of the snRNP or a spliceosome intermediate. In this regard, genetic studies suggest either a physical or functional interaction between Prp5 and three proteins, Prp9, Prp11, and Prp21, which have been shown by biochemical and genetic experiments to be tightly associated (19–22). The genetic studies also suggest an interaction between Prp5 and U2 RNA (21, 23, 24).

To understand how Prp5 functions, we have expressed the protein in *E. coli* and purified it to homogeneity. Like other spliceosomal ATPases Prp5 is an RNA-dependent ATPase, but unlike other members of this family it exhibits a weak but real specificity for U2 RNA, presumably its *in vivo* substrate. We also demonstrate an interesting ATP-dependent conformational change in the U2 RNA within the context of the snRNP. These studies are the basis for continuing efforts to understand just how and what Prp5 does to facilitate the formation of the pre-spliceosome.

EXPERIMENTAL PROCEDURES

Materials—Pet 19 vector was purchased from Novagen. Isopropyl-1-thio-β--galactopyranoside was purchased from Calbiochem. Ni-NTA resin was from Qiagen. Superose 12, Mono Q, and S FPLC columns were from Pharmacia Biotech Inc. Polyethyleneimine cellulose plates were from Brinkman. GeneClean was purchased from BIO101. A random priming kit was from Boehringer Mannheim. Analysis was performed on a Molecular Dynamics PhosphorImager using Imagequant software.

Oligonucleotides—4A, 5′-CTTGGTCACACACACATACGGCGCGGAAGGCGTGTTTGCTGAC-3′; 4B, 5′-GGAAACGTCAGCAAACACGCCTTCCGCGCCGTATGTGTGTGTGAC-3′; 5A, 5′-GTTTCCGAAAGGAACGGGTGGATCTTATAATTTTTGATTTATTTTAA-3′; 5B, 5′-AGCTTTAAAATAAAYCAAAAATTATAAGATCCACCCGTTCCTTTC-3′.

Expression and Purification of Prp5—An *Nde*I site was placed by site-directed mutagenesis in the 5′ end of the gene corresponding to the primary methionine. An *Nde*I-*Bam*HI fragment was then placed into a *Nde*I-*Bam*HI-cleaved pET19 vector supplied by Novagen. This plasmid contains 10 N-terminal histidines followed by the enterokinase cleavage site. The *PRP5* gene was inserted immediately following this site starting with its terminal methionine. Other constructs were made containing the N-terminal flag peptide, but the pET 19 version was used for purification. The plasmid was transformed into a BL21-pLysS strain of *E. coli*. 10 liters of the transformed cells were grown to 40 klett units and induced with 0.5 mm isopropyl-1-thio-β-galactopyranoside. The cells were grown for an additional 4.5 h, harvested, washed, and resuspended in 50 mM sodium phosphate, pH 7.0, 0.75 M NaCl, 10% glycerol, 10 mM β-mercaptoethanol, 5 mM Imidizole. The resuspended cells were lysed on ice by sonication for two 1-min bursts with a 1-min chill between bursts. The lysate was spun for 30 min to remove debris. The supernatant was cleared of nucleic acids by adding polyethyleneimine to 1% while stirring for ½ h. The lysate was cleared by ultracentrifugation for 1 h at 42,000 rpm in a Ti50 rotor.

The lysate was then loaded on a 2 ml of NTA metal chelating column. The lysate was loaded at 0.25 ml/min and washed with 10-column volumes of the loading buffer. Step elutions were carried out in 4 column volumes by increasing the imidizole to 40, 80, and then 200 mM. Prp5 mostly eluted in the 80 mM eluant as verified by immunoblot analysis.

The 80 and 200 mM eluants were pooled and dialyzed against 20 mM Mes, pH 6.5, 100 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA and loaded on a Sepharose S (Pharmacia) FPLC column equilibrated in the same buffer. The proteins were eluted in a gradient from 0.1 to 1 M NaCl. 250 1-ml fractions were collected and analyzed. Fractions containing Prp5 were pooled and dialyzed in 50 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT. The protein was then loaded on a Mono Q (Pharmacia) FPLC column equilibrated in the same buffer and eluted with a gradient of 100 to 750 mM NaCl. Fractions were analyzed for ATPase activity and the presence of Prp5 by immunoblot analysis. Peak ATPase activity correlated with the peak of Prp5 protein. Total yield of protein was about 200 μg.

Reconstitution of prp5-1 and prp5-3 Extracts—prp5-1 and *prp5-3* extracts were prepared as described (25). Extracts were inactivated for 30 min at 37 °C. Control extracts were incubated at room temperature. For RNA splicing reactions extract was diluted to 40% with buffer containing 3 mM ATP, 50 mM K_2HPO_4, pH 7.0, 2% PEG 8000, 3.5 mM $MgCl_2$, 1 nM [^{32}P]UTP-labeled actin, and Prp5 from 2 to 100 nM. Reaction mixtures were incubated at room temperature for 30 min and extracted as described (25).

Determination of Size—10 μl of 1 μM Prp5 was loaded onto a Superose 12 column equilibrated in 100 mM KCl, 50 mM Tris, pH 7.4, 5% glycerol, 1 mM DTT, 1 mM EDTA. The void volume was determined by blue dextran, and standards were run separately to determine a standard curve for size determination.

ATPase Activity—ATPase activity was determined as follows. RNAs from 50 to 500 nM were annealed in 60 mM HEPES, pH 7.4, 4% PEG 8000, 2 mM DTT, 100 mM KCl in 10 μl. This solution was heated to 70 °C for 5 min and snap-cooled on ice. 10 μl of buffer containing 7 mM $MgCl_2$, 500 μM ATP, 1 mCi of [^{32}P]ATP, 0.2 mg/ml bovine serum albumin, 20 nM Prp5 were added to start the assay. Reactions were incubated at 23 °C, and 2-μl aliquots were taken every 5 min and spotted on a polyethyleneimine TLC plate. Plates were developed in 0.75 M LiCl, 1 M formic acid for 2 h, dried, and analyzed by PhosphorImager for the fraction of ATP hydrolyzed.

Transcription and Purification of RNAs—T7 transcripts of U1, U2, U2Δ107, U4, U5, and U6 were made. U1ΔNC is an internal deletion of U1 created by a cleavage of the DNA template at the *Sty*I and *Hin*dIII sites of the yeast U1 gene and religation with the four oligonucleotides, 4a 4b, 5a, and 5b. Oligonucleotides 4a and 5a constitute the coding strand. The sequence of U1ΔNC is similar to the biologically active U1 constructs prepared by Siliciano *et al.* (26). Truncations of U2 were made by cutting the T7U2 plasmid at the *Ban*I and *Eco*N1 sites. The 3′ end of U2, U2 3′ end, was created by PCR from nucleotide 126 of U2Δ107 (27) to its 3′ end. This PCR product, also containing the T7 promoter, was then cloned and transcribed (see Fig. 5). Transcription reactions typically contained 50 μg of plasmid, 80 mM HEPES, pH 7.4, 12 mM $MgCl_2$, 3 mM NTPs in each, 40 nM DTT, 0.1 units of pyrophosphatase, and 1200 units of T7 RNA polymerase/ml. Reactions were carried out at 37 °C for 4 h. Transcripts were loaded on a 4-mm 6–8% denaturing polyacrylamide gel (20:1, acrylamide:bisacrylamide). The transcripts were visualized by UV shadowing, excised, eluted in 500 mM sodium acetate, pH 5.3, 10 mM EDTA. Eluants were filtered, extracted with 1:1 phenol/chloroform equilibrated in 0.2 M NaOAc, pH 5.3, extracted with chloroform, and precipitated twice. Concentrations were determined by optical density.

Isolation of Polyuridine—Polyuridine obtained from Sigma was dissolved to 10 mg/ml in water. Samples were prepared at 0.1 mg/ml in 50 mM Na_2CO_3:$NaHCO_3$, pH 9.2. The samples were incubated at 90 °C for 10 min. The reaction was stopped by addition of sodium acetate. A small sample was radiolabeled with [γ-^{32}P]ATP and polynucleotide kinase and then added to the reaction to visualize bands. Samples were loaded onto a 20% denaturing sequencing gel with several small labeled RNAs to calibrate the size. Bands were excised as 5–8-, 9–11-, 12–15-, 16–18-, 19–21-, 22–25-, 26–30-, 60–80-, 80–100-, and 100–120-mers. Longer pieces were similarly isolated by running the samples on an 8% gel. ATPase activity was assayed as described above except for the following alterations. 10 nM RNA was assayed in 20 mM sodium phosphate, pH 7.0, 4% PEG 8000, 2.5 mM $MgCl_2$, and 1 mM DTT. ATP concentrations remained the same.

RNaseH Probing—Extracts were inactivated at 37 °C for 30 min as described previously. Extracts were diluted in splicing buffer with or without the presence of 10 nM Prp5. The Sru2 oligonucleotide complementary to nucleotides 29–43 of U2 RNA was added to 220 nM to start the reaction. The RnaseH is endogenous to the extract (28). Assays were incubated at 30 °C for the indicated amount of time. In the same manner oligonucleotide d1 was added for RNaseH probing of U6 RNA as described by Fabrizio *et al.* (29). RNAs were extracted as described previously, ethanol-precipitated, and electrophoresed on a 4% 20:1 gel for 3 h at 400 V. The gel was blotted onto a nylon membrane (GeneScreen) in 25 mM K_2HPO_4, pH 6.5, overnight at 25 V. The blot was cross-linked, washed in water, and pre-hybridized in 1 × HYB (100 mM PO_4 buffer, pH 7.0, 1 M NaCl), 1% SDS, 100 (μg/ml sonicated salmon sperm DNA) at 65 °C for 2 h. Random primed probes (Boehringer Mannheim) were prepared using as template excised fragments of U1, U2, U4, and U6 DNA purified on glassmilk or GeneClean (BIO-101). A random primed probe was added to the blot and incubated overnight at 65 °C. The blot was washed once in ½ × HYB 1% SDS for 30 min at

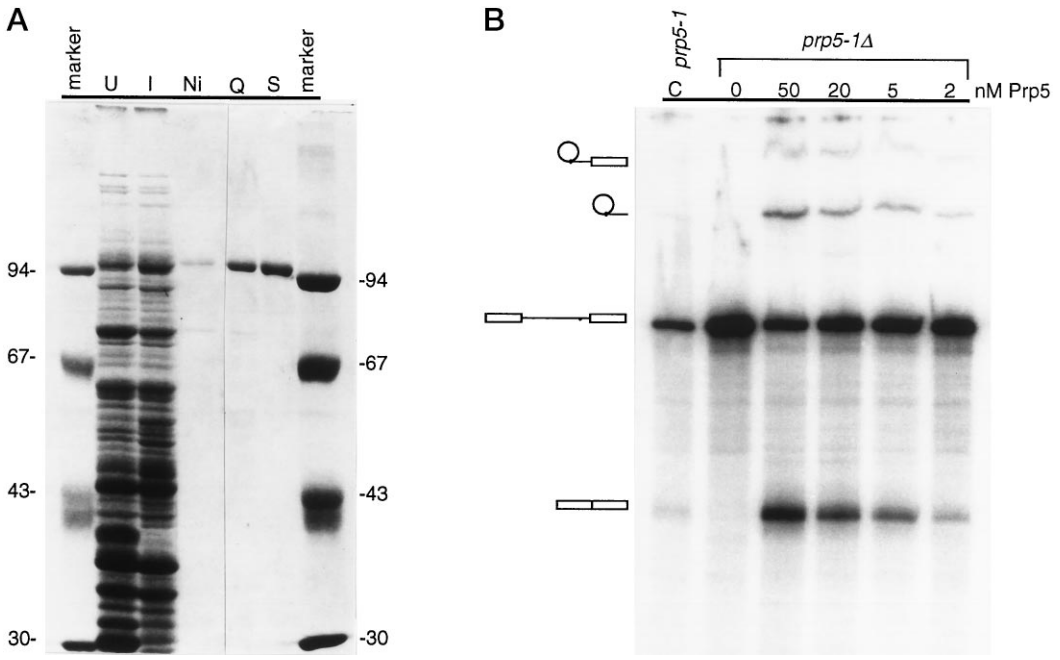


FIG. 1. ***A,* SDS-PAGE of protein fractions from Prp5 purification.** From *left* to *right*: molecular weight markers, *U*, 10 μg of uninduced cell extract; *I*, 10 μg of induced cell extract; *Ni*, 5 μg of Ni-NTA elution; *Q*, 2.5 μg of Mono Q elution; *S*, 2.5 μg of Mono S elution. Protein was visualized by silver staining. *B,* reconstitution of pre-mRNA splicing activity with recombinant Prp5. Reconstitution was performed with the indicated concentration of Prp5 in *prp5-1* extract preheated to 37 °C for 30 min. *C* denotes *prp5-1* extract without heat or added protein. Splicing activity was assayed using the actin pre-mRNA substrate.

65 °C. The blot was washed two more times in ½ × HYB at 65 °C and autoradiographed.

RESULTS

Purified Prp5 Protein Is a Monomer That Reconstitutes Splicing Activity in Vitro—Previously Dalbadie-McFarland and Abelson (8) reported the sequence of *PRP5* gene clones from *Saccharomyces cerevisiae*. Utilizing the cloned gene, we constructed vectors for expression of Prp5 in *E. coli* and yeast. Fig. 1*A* shows an SDS-PAGE of histidine-tagged Prp5 expressed in *E. coli* and purified by chromatography on Ni-NTA, Sepharose S, and Mono Q as described under "Materials and Methods." Expression levels for Prp5 protein were consistently low under a variety of growth conditions. However, we were able to purify significant quantities of the protein using affinity tagged constructs (Fig. 1). Immunoblot analysis of Prp5 produced in *E. coli* using anti-Prp5 sera revealed multiple sized polypeptides and very little full-length protein (data not shown). Analysis of the nucleotide sequence of *PRP5* indicated that several sites within the gene could be prokaryotic translation start sites. Probably the Prp5 fragments represent initiation at internal sites because when Prp5, N-terminally tagged with the flag peptide, was expressed only full-length protein was detected by the anti-flag antibody.

Prp5 protein was overexpressed in 10 liters of LB broth. Cells were lysed by sonication; the nucleic acids precipitated by polyethyleneimine, and the debris removed with a high speed spin. The extract was passed over a Ni-NTA column in pH 7.0 phosphate buffer. Attempts to purify protein at a higher pH or with Tris buffer resulted in very low recovery. The histidine tag may have been only partially accessible to the affinity ligand. The pI of Prp5 is approximately neutral, and this was exploited by successive chromatographic separations on Sepharose S at pH 6.5 and Mono Q at 8.0. Analyses of the pooled fractions from the different column separations by SDS-PAGE are shown in Fig. 1*A*. The purified protein was stored at −70 °C. Storage at −20% in 50% glycerol as well as freezing and thawing of the protein resulted in partial loss of activity.

Purified Prp5 was assayed for the ability to reconstitute the splicing activity of heat-inactivated yeast extracts from the *prp5-1* mutant strain. As seen in Fig. 1*B*, the splicing activity on the *prp5-1* extract was evident but poor before heat treatment. Incubation at 37 °C abolished all activity. Addition of increasing concentrations of purified Prp5 to the heat-treated extract resulted in increasing levels of splicing activity. Recombinant Prp5 can reconstitute splicing activity at concentrations as low as 2 nM, but splicing activity is enhanced at higher concentrations (Fig. 1*B*). Purified Prp5 also reconstituted splicing activity of heat-treated extracts from the *prp5-3* mutant strain (data not shown). Purified His-tagged Prp5 expressed in yeast was equally as active as the *E. coli* expressed protein (data not shown). Taken together, these results indicate that purified Prp5 contains all of the activities required to complement extracts in which the protein has been inactivated.

The native molecular weight of Prp5 was determined by size exclusion chromatography. A Superose 4B column was calibrated, and Prp5 was loaded in 100 mM NaCl to determine its multimeric state. Although Prp5 had some tendencies to aggregate during the purification procedure, size exclusion chromatography detected a protein of about 100 kDa, the approximate size of Prp5. Although more experiments would have to be done to determine whether or not Prp5 functions as a dimer as do many DNA helicases (30, 31), in the absence of substrate it is primarily a monomer.

Prp5 Is an RNA-dependent ATPase—Most members of the DEAD family of proteins are RNA-dependent ATPases. On its own, purified Prp5 showed virtually no ATPase activity. However, in the presence of RNA homopolymers, significant levels of ATPase activity were seen with the purified protein. Analysis of several different nucleic acid homopolymers indicated that poly(rU) and poly(rI) were best able to stimulate activity. In general the activity of poly(rI) is greater than or equal to poly(rU) > poly(rA) > poly(rC) (Fig. 2). This activity is relative since the average size of the homopolymers was not controlled. Deoxyribonucleic acids were not active (data not shown). Ac-

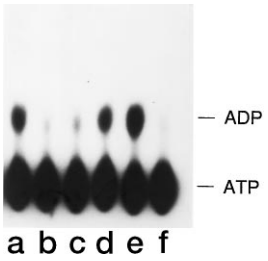


FIG. 2. **ATPase activity of recombinant Prp5 using various homopolymers.** 0.2 μg of various homopolymers was added to a 20-μl reaction with 100 nM Prp5 and [γ-^{32}P]ATP. TLC was performed as stated under "Materials and Methods." Substrates used from *left* to *right*: *a*, poly(rU); *b*, poly(rC); *c*, poly(rA); *d*, poly(rUG); *e*, poly(rI); *f*, no RNA.

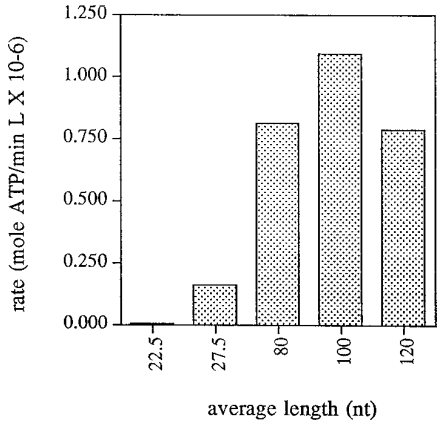
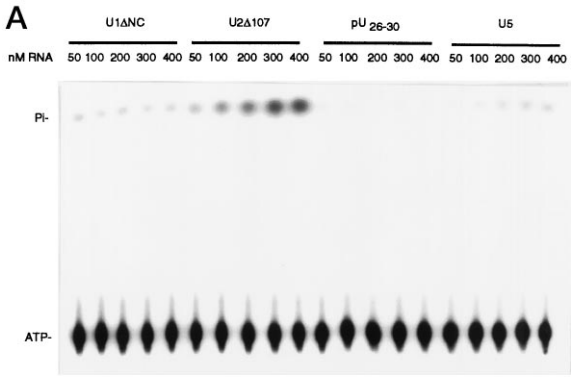


FIG. 3. **ATPase activity of recombinant Prp5 using various length uridine polymers.** ATPase was assayed as described under "Materials and Methods" using 20 nM Prp5 and 10 μM RNA polymers. ▨, rate (mol ATP/minliter $\times 10^{-6}$.



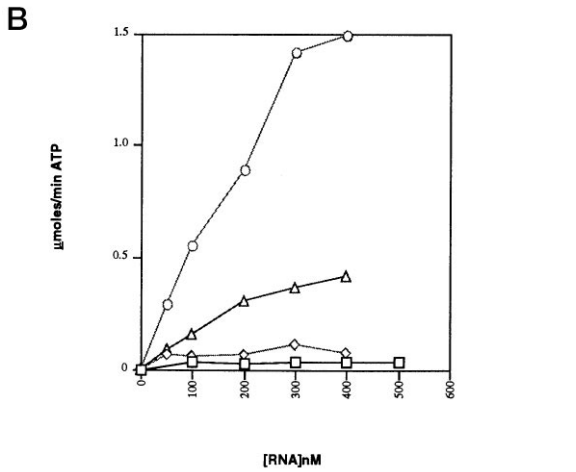


FIG. 4. **ATPase activity of recombinant Prp5 using various snRNAs and a homopolymer.** *A*, TLC of reaction mixtures containing various concentrations of RNA after 30 min incubation. *B*, rate of hydrolysis *versus* [RNA]. Note that hydrolysis of U1ΔNC is *above background* but does not increase with increasing concentrations of RNA. □, mol/min pU (26–30); ◇, mol/min U1ΔNC; ○, mol/min U2Δ107; △, mol/min U5.

tivity stimulated by poly(U,G) was found to be similar to poly(U). Using size-fractionated rU homopolymers, we investigated the size dependence of the RNA stimulatory activity. Polyuridine was partially base-hydrolyzed, and polymers of defined lengths were isolated. At saturating levels of RNA, Prp5 had virtually no ATPase activity until the polymer length was approximately 25–30 nt, and maximum activity was observed at about 100 nt (see Fig. 3). Longer polymer mixtures (~800 nt average length) did not stimulate the ATPase activity to a greater extent than the 100-nt polymer (data not shown). Apparently the optimum binding site is about 100 nt in length.

The Prp5 ATPase Activity Is Stimulated Specifically by U2 RNA—We tested the ability of the five spliceosomal snRNAs to stimulate ATPase activity. The sizes of these snRNAs are extremely variable; therefore, to facilitate our analysis we utilized truncated versions of the U1 and U2 snRNAs, U1ΔNC and U2Δ107, respectively, to make RNAs of similar lengths. The U1ΔNC clone is similar in size to mammalian U1 and to a similar deletion studied by Siliciano *et al*. (26) and found to be active *in vivo*. U2Δ107 is a truncation of the yeast U2 RNA which is also active *in vivo* (27). Of the five snRNAs tested, U2Δ107 exhibited the highest ATPase stimulatory activity (Fig. 4*A*). The U5 snRNA showed a level of stimulation similar to the 100-nt poly(rU) fragment. A comparison of the RNAs tested and their apparent K_m and k_{cat} values are tabulated in Table I. As seen in Fig. 4 and in Table I, U2 is about 2.5 times better in overall specificity as compared with the next best substrate, U5. When K_m and k_{cat} values were calculated, it was found that U5 bound slightly tighter, but had a 4-fold lower k_{cat}. Similarly poly(U100) has a similar K_m but has a 4-fold lower V_{max}. The activities of the other snRNAs were too low to determine with any reliability.

Although U1ΔNC-stimulated ATPase activity above background, ATPase activity did not increase linearly with substrate (Fig. 4, *A* and *B*). It is possible that this RNA is saturating at low concentration but has very little activity. However, in competition experiments (not shown) U1ΔNC RNA was not an effective competitor of U2 RNA in the RNA-dependent ATPase reaction. The experiments shown in Fig. 4 and Table I were repeated a number of times, and although the absolute value for K_m/k_{cat} varied as much as 2-fold from experiment to experiment the ranking of RNAs in activity remained the same. U2 RNA always was most effective in stimulating the ATPase activity.

To identify specific regions of the U2 snRNA responsible for stimulation of Prp5 ATPase activity, we assayed several U2 deletions for activity. Two 3′ deletions and one 5′ deletion were prepared as described under "Materials and Methods." A diagram of these RNAs as well as the full-length U2 snRNA (U2FL) are shown in Fig. 5. ATPase assays with Prp5 and the various U2 RNAs were performed, and the kinetic parameters K_m and k_{cat} were determined if possible for each RNA (Table II). Both U2Δ107 and U2FL had similar K_m values; the full-length having a 2–3-fold larger k_{cat} than U2Δ107 and almost

TABLE I
Prp5 substrates

RNA	Length	K_m	V_{max}	k_{cat}/K_m
	nt	*nM*	*μmol ATP/min liter*	
U2Δ107	283	438	2.56	5.8
U1ΔNC	223	NA[a]	NA	<0.43
U5	267	267	0.60	2.25
U4	161	NA	NA	<0.43
U6	113	NA	NA	<0.43
pU (25–30)	25–30	NA	NA	<0.43
pU (80–100)	80–100	644	0.73	1.14

[a] NA, activity too low to be determined.

TABLE II
U2 RNA variants

RNA	Length	K_m	V_{max}	k_{cat}/K_m
	nt	*nM*	*μmol ATP/min liter*	
U2Δ107	283	438	2.56	5.8
U2FL	1177	574	8.69	15
U2Ban	125	184	0.08	0.43
U2EcoN1	78	NA[a]	NA	<0.43
U23end	159	NA	NA	<0.43

[a] NA, activity too low to be determined.

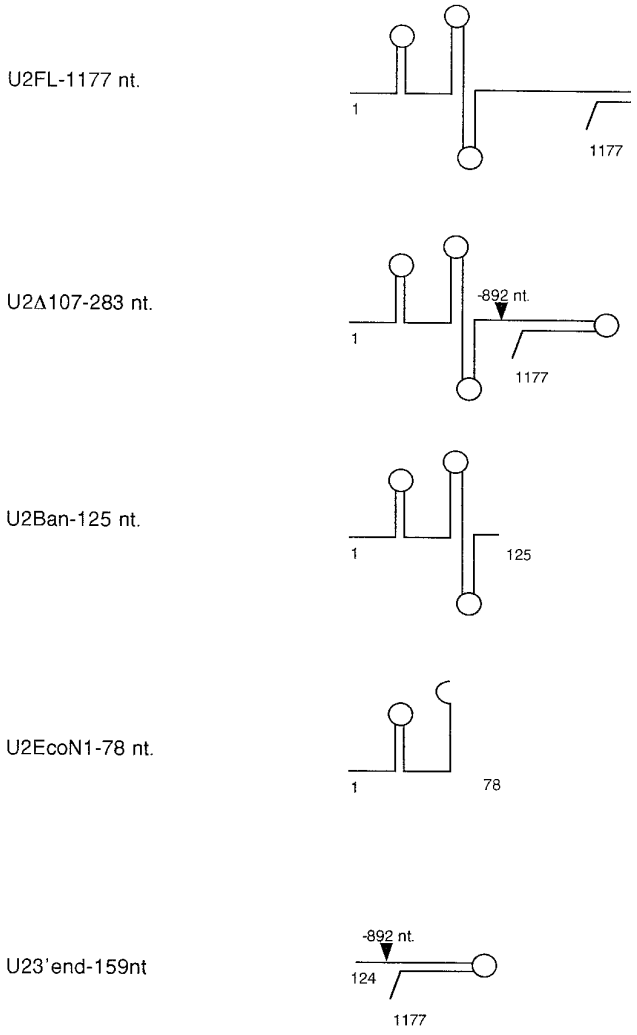


FIG. 5. **List of the various U2 variants used for the ATPase assay.** The length and the proposed secondary structures are listed. The numbering given after the schematic of the RNA is the numbering system for the full-length U2. In U2Δ107 and U2 3′ end, the 3′ end contains an 892-base pair deletion which is religated with a 10-base pair linker as described (27).

7-fold greater specificity than the next best substrate, U5. With the U2Ban deletion, the k_{cat} lowered at least 10-fold, and no activity was seen with U2EcoN1. These values agree with the activity of these U2 variants in the reconstitution assay (28). Full-length U2 is best able to reconstitute activity but U2Δ107 can also reconstitute activity to at least 50% of wild type. U2Ban has both a low reconstitution activity and a low ATPase stimulatory activity. *Eco*N1 and the 3′ end fragment were inactive in both assays. Thus a good correlation can be made between splicing activity and ATPase activity, but we were unable to delineate a specific region of U2 that interacts with Prp5 to activate the ATPase.

A number of different substrates were constructed to test for helicase activity including the pGEM/pSP runoff transcript and the U4/U6 RNA used by Claude *et al.* (16), the rGC transcript used by Rozen *et al.* (14) on eIF-4A, and substrates representing the branch point and hairpin/pseudoknot regions (A A′, B′ B, and C C′ of U2) proposed by Ares and Igel (35). No helicase activity was seen in any of these assays under a variety of conditions (data not shown).

ATP-dependent Changes in the U2 snRNP Structure Are Mediated by Prp5—Further evidence that Prp5 interacts with U2 snRNA was obtained from an RNaseH structure probing experiment. Previously McPheeters and Abelson (28, 29) used an oligonucleotide complementary to the branch point interaction site in U2 to target the RNaseH-dependent destruction of U2 RNA for complementation experiments. This reaction was discovered to be partially ATP-dependent (28, 29). To address the mechanism of ATP-stimulated cleavage, we tested whether degradation of the U2 RNA mediated by the branch point oligo was also Prp5-dependent. We performed RNaseH cleavage reactions in the *prp5-1* mutant extract and monitored the cleavage of the U2 snRNA. Fig. 6 shows a time course of degradation in the heat-treated *prp5-1* extract. RNaseH activity in the absence of Prp5 protein is slow, with significant amounts of full-length U2 remaining after 20 min. When Prp5 protein was added, cleavage of U2 was significantly faster, with little or no full-length U2 remaining after 10 min of degradation.

There is always some background or ATP-independent degradation of U2 RNA. We believe that this is due to alternate and perhaps less active or incomplete forms of the U2 snRNP in extracts. Extracts that splice poorly contain more of this form of the snRNP than do more active extracts.

To verify that the Prp5 effect was specific to U2, we assayed the ATP-dependent oligonucleotide-directed degradation of U6 RNA in the presence and absence of Prp5. (Fig. 7). In *prp5* extracts d1-directed degradation is ATP-dependent, but the addition of Prp5 had no effect (29).

DISCUSSION

In this paper, we describe the initial experiments in a program to explore the role of the spliceosomal ATPase, Prp5 in the process of spliceosome assembly. We have adapted the enzyme for purification by adding 10 histidine residues to the N terminus allowing for a powerful metallo-affinity step in the purification. Overall the purification is simple, rapid, and reproducible, and the protein retains activity during storage at −20 °C. The N-terminal histidines did not affect the activity of the protein in an *in vitro* complementation assay. The histidine tagged Prp5, whether purified from yeast or *E. coli*, is fully able to restore activity to a *prp5-1* heat-inactivated extract.

Purified Prp5 behaves as a monomer as judged by its mobility in size exclusion chromatography. Many DNA helicases act as dimers although they too can be monomers in the absence of substrate (30, 31). More experiments are necessary to deter-

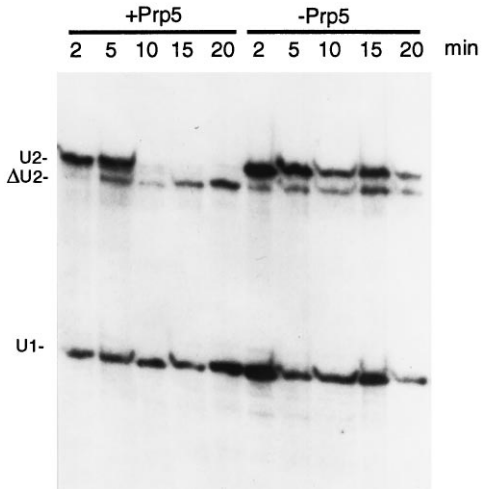


FIG. 6. **Time course of RNaseH digestion of the endogenous U2 snRNA using the oligonucleotide Sru2 (28), in the presence and absence of recombinant Prp5 in a heat-inactivated 5-1 extract.** 4% PAGE gels were blotted and probed for U2 and U1 snRNAs. ΔU2 denotes the degraded form of U2 after RNase digestion which is known to be the 3′ end of U2 up to the Sm binding site. U1 is probed as a control.

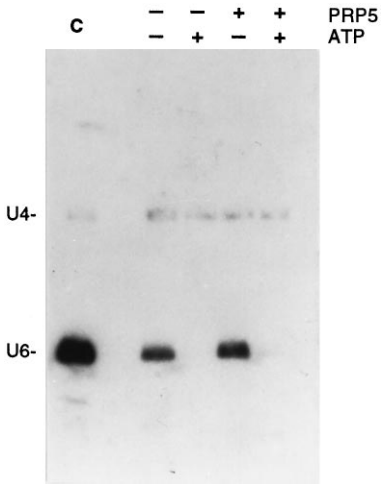


FIG. 7. **RNaseH digestion of the endogenous U6 snRNA using the oligonucleotide d1, in the presence and absence of recombinant Prp5 and ATP (29).** 7% PAGE gels were blotted and probed for U4 and U6 snRNAs. The degraded U6 cannot be visualized, but U4 was probed as a control.

mine whether Prp5 acts as a multimer.

Some of the DEAD box family members can act as RNA helicases, that is they possess the ability to separate the strands of double strand RNA (13–17). It is this activity that naturally attracts attention to this group of proteins in the context of RNA splicing. During spliceosome assembly, a number of changes take place in the interactions between snRNAs and the pre-mRNA substrate and between snRNAs. For example, U4 and U6 RNA enter the spliceosome as a complex joined together by 21 base pairs, but at a later step in the assembly process, this helical interaction is displaced, U4 leaves the spliceosome and U6 enters into a new set of interactions with the substrate, with U2 RNA, and with itself (3). This is a reaction which is probably catalyzed, and since ATP hydrolysis is required for this step, one imagines that it could be catalyzed by a member of this family, although as yet none has unambiguously been assigned. Consequently we have tested the ability of Prp5 to act as a helicase using a variety of substrates that have been previously described. No helicase activity was seen. As outlined in the introduction, there is strong evidence that implicates Prp5 in the formation of the pre-spliceosome. There are as yet no known RNA transformations at this stage of spliceosome assembly. Possible rearrangements include a destabilization in the base pairing interaction of U1 RNA with the 5′ splice site or some change in the conformation of U2 RNA which allows it to form a base pairing interaction with the branch point sequence near the 3′ splice site of the intron. More information will be necessary to allow us to design an assay for the relevant RNA transformation catalyzed by Prp5.

Like Prp5, most of the proteins in this class that have been tested do not exhibit RNA helicase activity. Possibly they too have specific substrates that have not yet been identified. However, most members of the family are RNA-dependent ATPases, and this is true of Prp5 as well. In the absence of RNA, Prp5 exhibits no ATPase activity over background. However in the presence of a number of different RNAs there is a low but clearly detectable level of ATPase. Among the homopolymeric substrates we tested, poly(U) and poly(I) are the most effective stimulant of the ATPase activity. We tested a size fractionated set of poly(U) oligomers, and the optimum size was about 100 nucleotides. This might indicate a rather large binding site in the enzyme, or it could be that for optimum ATPase activity more than one enzyme molecule must interact with the substrate.

We tested all of the yeast spliceosomal snRNAs for activity in stimulating the Prp5 ATPase activity. Of those tested only U5 RNA and U2 RNA showed activity, with U2 RNA being most effective. Specificity is 7-fold greater for the full-length U2 than U5 RNA but only 2.5-fold greater for U2Δ107. Although the degree of specificity that is seen here is modest, this was an encouraging result since much previous work had indicated an interaction between Prp5 and U2 RNA. We constructed a number of deletions of U2 RNA and tested them for activity in this assay. This approach could have identified a sub-domain of U2 RNA responsible for the stimulatory activity. No such domain was identified. The most active RNA is the full-length 1175-nt U2 RNA. We observed a correlation between the activity of variants of U2 RNA in a reconstitution assay and the ability of that species to stimulate Prp5 ATPase activity (28). This might be expected if only a particular conformation of U2 is active in both assays, and this conformation cannot be achieved in single domains.

The Prp5 specificity for U2 RNA does not appear to be due to RNA length. Referring to Table I, U2Δ107 has more than a 10-fold higher k_{cat}/K_m than U1ΔNC which is about the same size (283 *versus* 223 nts) and U4 RNA which is more than half the size (283 *versus* 161 nts). For U5 RNA (283 *versus* 267 nts) the difference is over 2-fold. Furthermore, the ATPase activity stimulated by poly(U) RNA is not increased by increasing the average size of the RNA from 100 to ~800 nts (data not shown). Although full-length U2 RNA is about 3-fold more active than Δ107, deletion variants about half the length of U2Δ107 (*cf.* U2Ban and U2EcoN1, Table II) show no detectable activity despite the fact that they are larger than the optimum poly(U) substrate. These results taken together with the observation that the activity of U2 RNA in stimulating Prp5 ATPase activity is highly dependent on the salt and buffer conditions in the reaction suggest that the specificity, although modest, is due to Prp5 recognition of some feature of U2 secondary or tertiary structure, perhaps a state that is not fully occupied under the conditions of the *in vitro* assay but is more represented in the population of full-length U2 RNA molecules.

It is encouraging that some RNA specificity can be seen in the Prp5 RNA-dependent assay since this had not yet been observed for any spliceosomal ATPase. However, the degree of

specificity, at best 10-fold over poly(U), is very modest when compared with the specificity of the *E. coli* DEAD box protein DbpA (32). DbpA is an RNA-dependent ATPase that is absolutely dependent on 23 S rRNA. Furthermore, all of the activity can be traced to a 93-nucleotide segment of the RNA in domain V near the 530 loop. Clearly this subdomain can fold into an optimum substrate for DbpA in the absence of the complete RNA or any accessory protein. It is ironic, however, that the biological function of DbpA is unknown and its substrate beautifully accessible, while the biological function of Prp5 is well understood and its substrate comparatively obscure.

Earlier, we had developed a U2 RNA complementation system in which the endogenous U2 RNA in the extract is destroyed by incubation of the extract with a deoxyoligonucleotide complementary to the branch point interaction region in U2 RNA (28). In this assay, U2 RNA is cleaved by endogenous RNaseH, and the deoxyoligonucleotide is destroyed in time by endogenous DNases. Activity is restored by addition of synthetic U2 RNA. In developing this assay David McPheeters noted that the RNaseH destruction of U2 is partially dependent on added ATP. In this paper we demonstrate that this effect is mediated by Prp5. No ATP effect on RNaseH cleavage of U2 RNA is seen in a heat-inactivated *prp5-1* extract, but the effect can be restored by addition of purified Prp5 and ATP. In the accompanying paper (33) we demonstrate that the same effect extends to the U2cs mutants, G53A and C62U (34), and also to *prp9, prp11,* and *prp21* extracts. Although this is not a quantitative assay, we believe that it is a biologically relevant one because all of the factors seem to interact with *prp5-1* genetically (21, 23, 24); the U2cs mutants in stem II, *prp9, prp11,* and *prp21* also affect this assay. Addition of purified Prp9·Prp11·Prp21 to the Prp5 U2 RNA-dependent ATPase assay had very little effect on ATP hydrolysis levels (data not shown). Contaminating ATPase activity of the complex, however, made interpretation difficult. In light of the results in the accompanying paper (33), this is a promising avenue to explore under more defined reaction conditions. The RNaseH potentiation effect suggests that Prp5, in conjunction with Prp9, Prp11, and Prp21, acts on the U2 snRNP, most likely through the stem II structure (35), and induces a conformational change that exposes the branch point pairing sequence. This is indeed a reaction that would be relevant to the formation of the prespliceosome. What remains to be discovered is precisely what Prp5 does to U2 RNA in that reaction. It is possible that the transformation in U2 RNA structure only takes place in the context of the U2 snRNP.

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