Prp43 Is an Essential RNA-dependent ATPase Required for Release of Lariat-Intron from the Spliceosome*

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The essential Saccharomyces cerevisiae PRP43 gene encodes a 767-amino acid protein of the DEXH-box family. Prp43 has been implicated in spliceosome disassembly (Arenas, J. E., and Abelson, J. N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11798–11802). Here we show that purified recombinant Prp43 is an RNA-dependent ATPase. Alanine mutations at conserved residues within motifs I (118GSGKTR123), II (215DEAH218) and VI (423QRAQRGR430) that diminished ATPase activity in vitro were lethal in vivo, indicating that ATP hydrolysis is necessary for the biological function of Prp43. Overexpression of lethal, ATPase-defective mutants in a wild-type strain resulted in dominant-negative growth inhibition. The ATPase-defective mutant T123A interfered in trans with the in vitro splicing function of wild-type Prp43. T123A did not affect the chemical steps of splicing or the release of mature mRNA from the spliceosome, but it blocked the release of the excised lariat-intron from the spliceosome. We show that the lariat-intron is not accessible to debranching by purified Db1 when it is held in the T123A-arrested splicing complex. Our results define a new ATP-dependent step of splicing that is catalyzed by Prp43.

Members of the family of DEXH/D-box proteins are involved in all major RNA transactions, including transcription, translation, ribosome biogenesis, and pre-mRNA splicing (1, 2). DEXH/D-box proteins can hydrolyze NTP to NDP in a reaction that is stimulated by, or dependent on, a nucleic acid cofactor. Although several DEXH/D family members exhibit RNA helicase activity in vitro, the action of DEXH/D-box NTPases may not be limited to the unwinding of RNA duplexes. Recent studies suggest that they can act as “RNPases” to displace proteins from nucleic acids (3–6). DEXH-box proteins are defined by conserved motifs I (GXGXK), II (DEXH), III (S/TAT), and VI (QRXGXRGR), which are important for ATP hydrolysis and RNA unwinding (7, 8).

The DEXH/D-box ATPases Prp5, Brr2, Prp28, Sub2/UAP56, Prp2, Prp16, and Prp22 are involved in pre-mRNA splicing (9). Removal of introns from precursor RNAs is catalyzed by the spliceosome, which is formed by the assembly of U1, U2, and U4/U6/U5 snRNPs and non-snRNPs and is a highly conserved RNA-binding machine (10, 11). Splicing entails two successive transesterification reactions: in step 1, the 5′ splice site is cleaved and the branched lariat-intermediate is formed; in step 2, the 3′ splice site is cleaved and the exons are joined. Mature mRNA is then released, and the spliceosome components are presumed to recycle for the next round of splicing (10). Splice site recognition and positioning of the reactive nucleotides for catalysis requires dynamic remodeling of an intricate network of RNA-RNA and RNA-protein interactions (12, 13).

In vitro studies have established that ATP is required for many steps in the splicing cycle and that DEXH/D-box proteins act at those ATP-dependent steps (9, 10). For example: Prp28, Brr2, Prp5, and Sub2/UAP56 are important for spliceosome assembly; Prp2 promotes step 1 transesterification; Prp16 is required for the second transesterification step; and Prp22 triggers the release of mature mRNA from the spliceosome (9, 14–16). Prp2, Prp16, and Prp22 mutants that are defective for ATP hydrolysis are also defective in executing their ATP-dependent functions in pre-mRNA splicing in vitro (16–19). Such mutations are also invariably lethal in vivo (18, 20, 21). Moreover, overexpression of non-functional Prp2, Prp16, and Prp22 mutants impairs the growth of wild-type cells (18, 20, 21). The dominant-negative Prp16 and Prp22 phenotypes can be recapitulated in vitro with purified proteins; for example, inactive Prp16 proteins block step 2 transesterification chemistry and dominant-negative Prp22 proteins block release of mature mRNA from the spliceosome in trans (19). Thus, the steps arrested by the dominant-negative mutants illuminate the function of the wild-type proteins during pre-mRNA splicing.

*S. cerevisiae PRP43 and its mammalian homologue mDEAH9 were isolated in PCR-based screens for DEXH-box proteins (22, 23). Yeast PRP43 is an essential gene that encodes a 767-amino acid polypeptide with a predicted molecular mass of 88 kDa. Arenas and Abelson (22) isolated a temperature-sensitive mutant, prp43-1, and showed that ACT1 pre-mRNA and excised lariat-intron accumulated in prp43-1 cells that were grown at non-permissive temperature. Lariat-introns accumulated during in vitro splicing carried out in extracts prepared from the prp43-1 cells. Native gel analyses of splicing complexes revealed that the lariat-intron, but not mature mRNA, was contained within the so-called spliceosome A complex (22). These findings suggested that Prp43 plays a role in spliceosome disassembly after mRNA is released. However, it remains unclear whether Prp43 acts as a catalyst of spliceosome disassembly and whether ATP is required for Prp43 function.

Here we have purified recombinant Prp43 and characterized its biochemical activities. We show that Prp43 hydrolyzes ATP in a divalent cation-dependent reaction that is specifically dependent on an RNA cofactor. We identify individual amino acids within conserved NTPase motifs that are crucial for Prp43 function in vivo and for ATPase activity in vitro.
expression of non-functional prp43 alleles in a PRP43 wild-type cell elicits a dominant-negative growth phenotype. In vitro, excess T123A mutant protein blocks degradation of lariat-intron, because it does not allow for release of excised intron from the spliceosome. We show that the lariat-intron is not accessible to debranching by Dbr1 and subsequent degradation when it is held in the splicing complex. We propose that T123A interferes with the ATP-dependent function of wild-type PRP43, which is to catalyze release of lariat-intron from the spliceosome.

EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Prp43 Protein—The coding region of PRP43 was amplified by PCR from genomic DNA of S. cerevisiae and inserted into the T7-based pET16b vector. Plasmid pET16-PRP43 expresses an N-terminal His-tagged version of wild-type Prp43 in bacteria under the control of a T7 promoter. The expression plasmid was transformed into Escherichia coli strain BL21-Codon Plus(DE3)RIL (Stratagene). Cultures were inoculated from single ampic colonies and maintained in logarithmic growth at 37 °C in LB medium containing 0.1 mg/ml ampicillin to a final volume of 200 ml. When the A600 reached 0.6–0.8, the culture was chilled on ice for 30 min and then adjusted to 40 ml isopropyl-1-thiogalactopyranoside (IPTG). The culture was then incubated for 5 h at 17 °C with constant shaking. Cells were harvested by centrifugation and the pellets stored at −70 °C. All subsequent operations were performed at 4 °C. The cell pellets were suspended in 20 ml of buffer A (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10% sucrose). Lysozyme was added to 0.2 mg/ml, and the suspensions were mixed gently for 40 min, then adjusted to 0.1% Triton X-100. The lysates were sonicated to reduce viscosity, and insoluble material was removed by centrifugation for 30 min at 13,000 rpm in a Sorvall SS34 rotor. The soluble lysate was mixed for 1 h with 3 ml of a 50% slurry of nickel-nitrilotriacetic acid agarose (Qiagen) that had been equilibrated in buffer A. The resin was recovered by centrifugation, resuspended in 10 ml of buffer A, and collected again by centrifugation. The washed resin was suspended in 10 ml of buffer B (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10% glycerol) containing 15 mM imidazole and poured into a column. Adsorbed proteins were eluted stepwise with 250, 500, and 300 mM imidazole in buffer B. The elution profile of recombinant Prp43 was monitored by SDS-PAGE of the column fractions. Prp43 was recovered predominantly in the 300 mM imidazole eluate.

Purification of Mutant Prp43 Proteins—pET-based plasmids were constructed for expression of His-tagged Prp43-Ala mutants. The expression and purification of the mutant proteins was carried out as described for wild-type Prp43. The yields from 200-ml cultures after nickel-agarose purification were: 1.2 mg of K122A, 3.6 mg of T123A, 1.2 mg of D215A, 0.8 mg of E216A, 2.7 mg of H218A, 3 mg of S247A, 4 mg of T249A, and 4 mg of Q423A.

ATPase Assay—Reaction mixtures (20 ml) containing 45 mM Tris-HCl (pH 8.0), 25 mM NaCl, 2.2 mM DTT, 1 mM MgCl2, 1 mM ATP (γ32P)ATP, 0.6 mM poly(A) (measured as AMP concentration), 0.01% Triton X-100, 0.1 mM EDTA, and Prp43 as specified were incubated for 15 min at 30 °C. The reactions were stopped by the addition of 200 ml of a 5% (w/v) suspension of activated charcoal in 20 ml phosphoric acid. The samples were incubated on ice for 10 min, and the charcoal was recovered by centrifugation. 32P radioactivity in the supernatant was quantified by liquid scintillation counting. All the values are averaged from at least two reaction mixtures, with a variation of less than 10% between experiments.

Pre-mRNA Splicing in Vitro— Yeast whole cell extracts from strain B2168 (Mata leu2 trpl-52 prb1-1122 pep4–3 prc1–407 gal2) or an isogenic dbr1Δ mutant (YH3) were prepared using the liquid nitrogen method (24). Splicing reaction mixtures (100 ml) contained 50% whole cell extract, −400 fmol of [32P]GMP-labeled actin precursor RNA, 2.5 mM magnesium acetate, 2.5 mM MgCl2, 0.5% polyethylene glycol 8000, and 2 mM ATP. The reaction mixtures were incubated for 25 min at 23 °C, then halted by transfer to ice. An aliquot (5 ml) was removed from each mixture and added directly to 200 ml of STOP solution (50 mM sodium acetate (pH 5.2), 1 mM EDTA, 0.1% SDS, 30 μg/ml RNA). The remaining aliquot (95 ml) was layered onto 15–40% glycerol gradients containing 20 mM HEPES (pH 6.5), 100 mM KCl, 2 mM EDTA, and then centrifuged at 4 °C for 14 h at 35,000 rpm in a Sorvall TH641 rotor. Fractions (400 μl) were collected from the tops of the gradients. RNA was recovered from the gradient fractions by precipitation. RNA from alternate gradient fractions was analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 mM urea in TBE. Radiolabeled RNA was visualized by autoradiographic exposure of the dried gel.

Chromosomal Deletion of DBR1— DNA segments from the regions flanking the DBR1 ORF were PCR-amplified from genomic DNA and inserted upstream and downstream of the LEU2 gene on a pUC8 plasmid. The plasmid containing the dbr1::LEU2 disruption cassette was transformed into B2168 (Mata leu2 trpl-52 prb1-1122 pep4–3 prc1–407 gal2) and an isogenic dbr1Δ mutant (YH3) were prepared using the liquid nitrogen method (24). Splicing reaction mixtures (100 ml) contained 50% whole cell extract, −400 fmol of [32P]GMP-labeled actin precursor RNA, 2.5 mM magnesium acetate, and 0.5% polyethylene glycol 8000, and 2 mM ATP. The reaction mixtures were incubated for 25 min at 23 °C, then halted by transfer to ice. An aliquot (5 ml) was removed from each mixture and added directly to 200 ml of STOP solution (50 mM sodium acetate (pH 5.2), 1 mM EDTA, 0.1% SDS, 30 μg/ml RNA). The remaining aliquot (95 ml) was layered onto 15–40% glycerol gradients containing 20 mM HEPES (pH 6.5), 100 mM KCl, 2 mM EDTA, and then centrifuged at 4 °C for 14 h at 35,000 rpm in a Sorvall TH641 rotor. Fractions (400 μl) were collected from the tops of the gradients. RNA was recovered from the gradient fractions by precipitation. RNA from alternate gradient fractions was analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 mM urea in TBE. Radiolabeled RNA was visualized by autoradiographic exposure of the dried gel.
Recombinant Prp43 Has ATPase Activity—The DEXH-box protein Prp43 is most closely related in primary structure to the splicing factors Prp2, Prp16, and Prp22, all of which exhibit RNA-stimulated NTPase activity. To investigate the enzymatic activities of Prp43, we expressed His<sub>10</sub>-tagged Prp43 in bacteria under the transcriptional control of T7 RNA polymerase and purified the protein from soluble bacterial lysates by nickel-agarose chromatography and glycerol gradient sedimentation (Fig. 1). IPTG induction of T7 polymerase resulted in the accumulation of a polypeptide of ~90 kDa (consistent with the calculated molecular mass of His-Prp43) that was recovered predominantly in the soluble fraction of a crude cell lysate (Fig. 1A). The polypeptide bound to nickel-agarose and was eluted with 300 mM imidazole. Prp43 sedimented as a single discrete peak in a glycerol gradient (Fig. 1B). Aliquots of the gradient fractions were tested for the ability to catalyze the hydrolysis of ATP in the presence of an RNA cofactor, poly(A). A single peak of ATPase activity was detected that coincided with the abundance of the Prp43 protein (Fig. 1C).

Characterization of the ATPase Activity—The rate of ATP hydrolysis in the presence of poly(A) was measured as a function of protein concentration (Fig. 2A). Reaction velocity was proportional to enzyme concentration, and the reactions proceeded to an end point at which 85% of the input [γ<sup>32</sup>P]ATP was converted to <sup>32</sup>P<sub>i</sub>. From the initial rates, and assuming that 100% of the Prp43 protein in the preparation was active, we calculated that Prp43 hydrolyzed 700–720 ATP per min.

We next tested whether the ATPase activity was dependent on a nucleic acid cofactor. Increasing amounts of Prp43 were incubated with 1 mM ATP in the absence of nucleic acid or with 0.6 mM poly(A), poly(C), or poly(dA) for 15 min at 30 °C (Fig. 2B). In the presence of saturating concentrations of either poly(A) or poly(C), the extent of ATP hydrolysis varied linearly with the amount of input Prp43. The specific activity with poly(C) was 38% of the activity with poly(A). In contrast, no activity was detected when nucleic acid was omitted. The DNA homopolymer poly(dA) failed to stimulate ATP hydrolysis. Thus, the ATPase activity of Prp43 is specifically dependent on an RNA cofactor.

The requirement for divalent cations was assessed by measuring ATP hydrolysis in the presence of increasing concentrations of MgCl<sub>2</sub> (Fig. 3A). Activity was optimal at 0.75–1.5 mM MgCl<sub>2</sub> and declined progressively at 2–5 mM ATP hydrolysis without added MgCl<sub>2</sub> was 4% of the optimal value. Addition of 1 mM EDTA to the reaction mixture reduced the level of ATP hydrolysis under the transcriptional control of T7 RNA polymerase, poly(A). A single peak of ATPase activity was detected that coincided with the abundance of the Prp43 protein (Fig. 1C).

PRP43 promoter, and their in vivo function was tested in a prp43Δ strain using the plasmid shuffle technique. Growth of prp43Δ is dependent on a CEN URA3 PRP43 plasmid. Trp<sup>+</sup> transformants were selected and then tested for growth on medium containing 5-FOA, a drug that selects against the URA3 PRP43 plasmid. The results are summarized in Fig. 4A. Deletion of up to 90 amino acids from the N terminus did not affect the function of Prp43 insofar as the deletion mutant

![Fig. 1](http://www.jbc.org/)

**Fig. 1.** Expression and purification of Prp43. **A**, induced expression of His-Prp43 in bacteria. Aliquots (the equivalent of 0.4 A<sub>260</sub> units) of the bacterial culture before induction (bl) and after induction with 0.4 mM IPTG for 5 h at 17 °C (al) were analyzed by SDS-PAGE and Coomassie Blue staining. Aliquots of the cell lysate (T), the soluble supernatant of a high-speed spin (S), and of the insoluble material (P) were also analyzed by SDS-PAGE. The soluble lysate was mixed with nickel-nitritriacetic acid-agarose and the polypeptide composition of the flowthrough fraction (FT), and the fractions upon stepwise elution with 25, 300, and 500 mM imidazole were analyzed (5 μl of each fraction was loaded). The His-Prp43 protein is abundant in the first 300 mM imidazole fraction (2.2 μg/μl). The positions and the sizes (in kDa) of marker proteins are indicated on the left. B, glycerol gradient sedimentation of His-Prp43. Aliquots (5 μl) of the indicated glycerol gradient fractions were analyzed by SDS-PAGE. Fractions 9, 11, and 13 contain 1.3, 0.2, and 0.6 μg/μl of protein, respectively. C, ATPase activity. Aliquots (0.2 μl) of the indicated glycerol gradient fraction were assayed for ATPase activity in the presence of poly(A). ATPase activity is expressed as nanomoles of P<sub>i</sub> released from ATP during a 15-min incubation at 30 °C.
PRP43(91–767) formed colonies on 5-FOA medium. The PRP43(91–767) cells grew as well as wild-type cells on rich medium (YPD, 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) at 15, 25, 30, and 37 °C. However, further deletion of the segment from residues 91–104 abrogated function at all temperatures. Removal of 35 residues from the C terminus did not affect cell growth, but deletion of 45 residues resulted in temperature-sensitive growth. Truncation of 55 amino acids from the C terminus was lethal at all temperatures. We conclude that the N-terminal 90 and the C-terminal 45 residues in the Prp43 protein are not essential for in vivo function. A strain carrying the PRP43(91–732) allele that was truncated from both the N and C termini was viable at all temperatures.

Conserved Residues in Motifs I, II, III, and VI Are Important for Prp43 Function in Vivo—DEHX-box proteins contain six conserved collinear motifs. Mutational studies of several DEHX-box proteins, including the splicing factors Prp2, Prp16, and Prp22, and the viral helicases NS3 and NPH-II, have established that conserved amino acids in motifs I (GXXGKT), II (DEXXH), III (S/TAT), and VI (QRXXGRXXGR) are important for biological activity and for ATP hydrolysis or RNA unwinding (7, 16–21, 30, 31). Here, we performed an Ala scan of 13 positions in Prp43 (Fig. 4B). The function of the PRP43-Ala alleles was tested by plasmid shuffle. Cells containing wild-type PRP43 on a TRP1 CEN plasmid grew on 5-FOA medium, whereas the K122A, T123A, D215A, E216A, Q423A, G426A, R427A, and R430A alleles were lethal, i.e. the Trp⁺ cells did not form colonies on 5-FOA medium at 15, 30, or 37 °C. In contrast, H218A in motif II, S247A and T249A in motif III, and R424A and G429A in motif VI, were viable. These mutants were streaked to rich medium (YPD) and incubated at 15, 30, and 37 °C. H218A, S247A, T249A, R424A, and G429A dis-
Prp43: An ATPase Required for Lariat-Intron Release

Fig. 4. Mutational analysis of Prp43. A, truncation mutants. The truncated Prp43 proteins are depicted as horizontal bars with the numbers indicating the first and last amino acid residues. ATPase motifs I–VI are indicated by boxes. The function of the truncated proteins in complementation of prp43Δ was tested by plasmid shuffle: (−) indicates that the mutants did not form colonies on 5-FOA at 15, 30, or 37 °C. The 5-FOA survivors were streaked to rich YPD medium and growth of the mutants was compared with wild-type Prp43: (+) indicates growth comparable to wild-type at all temperatures; Prp43(1–722) grew well at 15 and 30 °C but did not form colonies at 37 °C (ts). B, alanine substitution mutants. Motifs I, II, III, and VI are shown at the top, the numbers indicate the positions of the amino acids in Prp43. The ability of the mutated Prp43 alleles to complement a prp43Δ strain was tested at 15, 30, and 37 °C. “Lethal” indicates that the cells did not form colonies on 5-FOA medium at any temperature. Growth of viable mutants was assessed on YPD medium and scored after 2 days at 30 and 37 °C and after 8 days at 15 °C; (+ + +) indicates wild-type growth; (+) indicates slow growth; (−) indicates no growth. C, dominant-negative growth phenotypes caused by overexpression of lethal prp43 alleles. Wild-type Prp43 and the mutant alleles T123A, D215A, Q423A, and R430A under the control of a GAL1 promoter on CEN TRP1 plasmids were transformed into wild-type PRP43 cells. Transformants were grown in liquid cultures (SD-Trp, 2% raffinose). The cultures were diluted to an A600 of 10−1, 10−2, 10−3, and 10−4, and 3 μl was spotted to plates containing glucose or galactose (2%) in SD-Trp medium. The plates were photographed after 5 days of incubation at 19 °C.

played conditional growth defects, however, the severity of the defects varied (Fig. 4B). For example, the S247A mutant, which grew slowly at 30 °C, did not grow at 15 °C. H218A, T249A, and Q429A formed pinpoint colonies after 9 days at 15 °C. The R424A mutant grew slowly at 30 °C, failed to grow at 37 °C, and formed pinpoint colonies at 15 °C. We conclude that individual residues in the conserved motifs are important for the function of Prp43.

Non-functional Prp43 Mutants Elicit Dominant-negative Phenotypes in Vivo—Four of the lethal prp43 alleles (T123A, D215A, Q423A, and R430A) were placed under the transcriptional control of the GAL1 promoter on TRP1 CEN plasmids and transformed into wild-type PRP43 cells (Fig. 4C). All four GAL-prp43-Ala strains grew on glucose-containing medium. However, galactose-induced expression of the T123A, D215A, Q423A, and R430A proteins resulted in dominant-negative inhibition of growth (Fig. 4C). We have analyzed RNAs from cells in which the non-functional Prp43 mutants were induced in galactose-containing medium; Northern blot analysis showed that excised intron and precursor RNA of ACT1 and CYH2 accumulated (data not shown). Thus, overexpression of non-functional Prp43 mutants leads to dominant-negative growth inhibition and to a splicing defect in vivo.

APase Activities of the Prp43 Mutant Proteins—Conserved residues in motifs I (121GKT123), II (215DEAH218), III (247SAT249), and VI (423QRAGAGR430) are important for in vivo function of Prp43 (Fig. 4). To examine the biochemical consequences of the mutations, we produced recombinant His6-tagged Prp43 proteins K122A and T123A (motif I); D215A, E216A, and H218A (motif II); S247A and T249A (motif III); and Q423A (motif VI) and purified them from soluble bacterial lysates by nickel-agarose affinity chromatography and glycerol gradient sedimentation. The level of purity, as gauged by SDS-PAGE analysis, was comparable for the eight mutants and the wild-type Prp43 control (Fig. 5A). We surmise that the minor polypeptide of ~84 kDa is a proteolytic fragment of Prp43, insofar as it cross-reacts with anti-Prp43 serum on a Western blot (data not shown).

The extent of ATP hydrolysis by wild-type and mutant Prp43 was proportional to input protein (Fig. 5B); we calculated that wild-type Prp43 hydrolyzed 740 ATP min⁻¹ in the presence of poly(A) (Fig. 5C). The lethal motif I mutants K122A and T123A hydrolyzed 31 and 47 ATP min⁻¹, respectively (4 and 6% of wild-type). The motif II mutant proteins D215A and E216A exhibited less than 1% of wild-type activity, and the motif VI mutant Q423A hydrolyzed 93 ATP min⁻¹ (13% of wild-type). The viable, albeit cold-sensitive mutants exhibited ATPase activity; H218A (motif II) hydrolyzed 200 ATP min⁻¹ (27% of wild-type) and S247A and T249A (motif III) hydrolyzed 480 and 650 ATP min⁻¹ (65 and 87% of wild-type, respectively) (Fig. 5C). We infer that ATPase activity is necessary for the in vivo function of Prp43 and that there may be a threshold level of ATPase activity required for viability.

Prp43 Functions at a Late Step in Pre-mRNA Splicing in Vivo—Having determined that Prp43 is an RNA-dependent ATPase, we wished to explore the role of Prp43 in pre-mRNA splicing. Specifically, we sought to identify a distinct step within the splicing cycle that was dependent on ATP hydrolysis by Prp43. Our preliminary efforts to immunodeplete yeast whole cell extract of the Prp43 protein were unsuccessful, thereby precluding simple depletion/reconstitution studies, as had been done for Prp16 and Prp22 (15, 16).

Prior in vitro studies of Prp16 and Prp22 showed that the ATP-dependent functions of these splicing factors could be specifically blocked in trans by adding purified ATPase-defective mutant proteins (19). Here we showed that lethal Prp43 mutants exert dominant-negative effects when overexpressed, leading to a splicing defect in vivo. We therefore tested the effect of the purified T123A mutant in splicing of actin pre-mRNA in vitro. Yeast whole cell extract containing endogenous wild-type Prp43 (~10–15 ng/μl as estimated from Western blots) was supplemented with recombinant wild-type Prp43 or the T123A mutant protein (0.6 μg of each) and then reacted with 32P-labeled actin precursor RNA for 25 min at 37 °C. The splicing products were extracted from aliquots of the reaction mixtures and analyzed by denaturing PAGE and au-
Prp43: An ATPase Required for Lariat-Intron Release

Fig. 5. Mutational effects on Prp43 ATPase activity. A, aliquots (1 μg) of the indicated glycerol gradient preparations of Prp43 were analyzed by electrophoresis in an 8% polyacrylamide gel containing 0.1% SDS. Polypeptides were visualized by staining with Coomassie Blue dye. The positions and sizes (in kDa) of marker proteins are indicated at the left. B, the extents of ATP hydrolysis by the indicated Prp22 preparations in the presence of poly(A) are plotted as a function of input protein. C, ATPase activity is expressed as turnover number (min⁻¹) and as a percentage of wild-type activity, which was set to 100%.

Fig. 6. Effects of exogenous Prp43 and T123A proteins on splicing. Yeast whole cell extract was supplemented with 0.6 μg of either wild-type Prp43 or the T123A mutant and reacted with actin pre-mRNA for 25 min at 23°C. A, aliquots (5 μl of 100 μl) were extracted and the reaction products were resolved by denaturing PAGE and visualized by autoradiography (Input). The symbols on the left indicate the positions of the following labeled RNA species, proceeding from the top to the bottom of the gel: lariat-exon 2 intermediate; lariat-intron; pre-mRNA substrate; mature spliced mRNA; 5' exon. B, the remainder of the reaction mixtures (95 μl) were analyzed by zonal velocity sedimentation through a 15–40% glycerol gradient (fractions 23–27). The lariat-intron product cosedimented with the residual lariat-exon 2 splicing intermediate, implying that it remained associated with the spliceosomes. In contrast, when wild-type Prp43 was added, the amount of lariat-intron cosedimenting with the spliceosomes was less than with T123A. There is no corresponding increase in “free” lariat-intron, because, as shown below, as the released lariat-intron is quickly debranched and degraded in the extract.

We hypothesize that the ATPase-defective Prp43 mutant T123A blocks the function of wild-type Prp43 in trans and infer that it reveals a step in spliceosome disassembly that is normally triggered by Prp43’s ATPase activity. This provides a plausible explanation for the dominant-negative phenotypes observed in vivo. Inactivation of the Debranching Enzyme Shows That the T123A Mutant Blocks Release of Lariat-Intron from the Splicing Complex—To prevent rapid degradation of lariat-intron in wild-type extract, we performed splicing in extracts lacking the debranching enzyme Dbr1. Dbr1 specifically cleaves the branched 2'-5' phosphodiester bond (26–28). A chromosomal deletion of DBR1 is viable, and excised lariat-introns are present at high levels in the dbr1Δ cells (26). We prepared whole cell extract from the dbr1Δ strain and reacted it with 32P-labeled actin pre-mRNA (Fig. 7). The splicing reaction mixtures were supplemented with wild-type Prp43 or T123A protein and incubated for 25 min at 23°C. Fig. 7A shows that mature mRNA was formed in every case and that the lariat-intron was stable. When the splicing reaction products generated in Δdbr1 extract were analyzed by sedimentation in a glycerol gradient (Fig. 7B), the majority of the lariat-intron (70%) sedimentsed near the top of the gradient (fractions 7–13). The addition of exogenous wild-type Prp43 did not alter the sedimentation profile. In contrast, excess T123A resulted in the retention of 80% (with 0.6 μg of T123A) and 90% (with 1.8 μg of T123A) of the lariat-intron in the heavier spliceosome fractions (fractions 23–27). Mature mRNA was released in every case, showing that mRNA release precedes intron release. These findings
Prp43: An ATPase Required for Lariat-Intron Release

**DISCUSSION**

We have presented an analysis of the DEXH-box protein Prp43. Our results demonstrate that: (i) Prp43 is an RNA-dependent ATPase; (ii) ATPase activity is necessary for Prp43 function in vivo; (iii) the N-terminal 90 amino acids and the C-terminal 45 amino acids are not essential in vivo; (iv) the dominant-negative ATPase-defective mutant T123A blocks release of excised lariat-intron from the spliceosome in vitro; and (v) debranching enzyme can access the branched RNA only after Prp43 acts.

Prp43 is closely related in primary structure to the DEAH-box splicing factors Prp2, Prp16, and Prp22 (22). The mutational analysis of Prp43 highlights the common structural requirements for ATP hydrolysis, insofar as conserved residues in motifs I, II, and VI that are important for NTP hydrolysis by Prp16 and Prp22, are also important for ATP hydrolysis by Prp43. Structural studies of several DEXH/D-box proteins, including HCV NS3, eIF-4A, and MjDEAD corroborate biochemical data showing that motifs I, II, and VI constitute the active site for NTP hydrolysis (2, 7, 8).

The side-chain hydroxyls of motif III (S/TAT) are not important for NTP hydrolysis, however, they play a crucial role for RNA unwinding by the eIF-4A, NPH-II, NS3, and Prp22 proteins (21, 29–31). Alanine replacements in motif III of these DEXH/D-box helicases result in proteins that retain ATPase activity but do not unwind duplex RNA in vitro, indicating that motif III is responsible for coupling the energy from ATP hydrolysis to a conformational step required for RNA unwinding. Prp22 motif III mutants exhibit severe growth defects, and they fail to release mRNA from the spliceosome in vitro (21, 25). In contrast, alanine substitutions in motif III of Prp16 result in proteins that are functional in vivo and that promote the second chemical step of splicing in vitro (19, 20).

The cold-sensitive growth phenotypes of Prp43 mutants S247A and T249A suggest that the side-chain hydroxyls in motif III are important for Prp43 function in vivo, however, the molecular basis for the growth defect remains to be determined. We found show that release of lariat-intron is a **bona fide** step in the yeast spliceosome disassembly pathway.

Dbr1 Gains Access to the Branched 2'-5' Phosphodiester Bond after Prp43 Acts—Lariat-intron RNA is stable when spliceosome disassembly is blocked by a non-functional Prp43 mutant, suggesting that Dbr1 cannot function when the RNA products are held in the spliceosome. To test this directly, we performed splicing in Δdbr1 extract in the absence and presence of T123A protein (Fig. 8). Aliquots of the reaction mixtures were then supplemented with recombinant purified Dbr1 enzyme and incubated at 30 °C for 25 min (Fig. 8, lanes 2 and 6). Excised actin lariat-intron, which accumulated to high levels in Δdbr1 extract (lane 1), was a substrate for Dbr1 activity (lane 2). In contrast, the lariat-intron that was generated in Δdbr1 extract in the presence of exogenous T123A was not debranched by Dbr1 (lane 6). To show that this was not due to a difference in the branched RNA itself, we phenol-extracted the reaction products prior to treatment with Dbr1 (lanes 4 and 8). Deproteinized lariat-introns were debranched (lanes 4 and 8). Note that, after phenol extraction, the lariat-exon 2 RNA is also debranched (lanes 4 and 8, indicated by the asterisk). Lariat-exon 2 intermediates and lariat-introns that accumulate when Prp43 fails to release mRNA from the spliceosome

**FIG. 7.** T123A blocks the release of lariat-intron from the spliceosome. Δdbr1 extract was supplemented either with buffer (−), wild-type Prp43 (WT), or the T123A mutant protein and then reacted with 32P-labeled actin pre-mRNA. A. input. Aliquots (5 μl) of the reaction mixtures were analyzed by denaturing PAGE and autoradiography. B, the remainder (95 μl) of the reaction mixtures were sedimented in 15–40% glycerol gradients. RNA from odd-numbered fractions was analyzed by denaturing PAGE and autoradiography. The arrow at the right highlights the position of lariat-intron.

**FIG. 8.** Dbr1 acts on released lariat-introns. Splicing products were formed in Δdbr1 extract without and with exogenous T123A protein. The reaction mixtures were then treated as indicated, and Dbr1 enzyme was either added directly to the splicing reaction mixtures or added after phenol-extraction (α extr.) and ethanol precipitation of the RNA. The symbols on the left indicate the positions of the following labeled RNA species, proceeding from the top to the bottom of the gel: lariat-exon 2 intermediate; lariat-intron; pre-mRNA substrate; mature spliced mRNA. The arrow on the right highlights the lariat-intron, and the asterisk indicates the position of the debranched lariat-exon 2. Debranched lariat-intron (indicated by an arrowhead) migrates close to mature mRNA and is visible in shorter exposures above mRNA (inset at the bottom) in lanes 4 and 8.
that recombinant Prp43 is unable to unwind the 3′-tailed 25-bp duplex RNA used to demonstrate helicase activity of Prp22 (21). This may indicate that Prp43 requires a different RNA structure to reveal a putative helicase function, that it may require another protein cofactor for unwinding, or that Prp43 couples ATP hydrolysis to an event other than RNA unwinding.

The DEAH-box splicing factors Prp2, Prp16, and Prp22 act sequentially in the splicing pathway (9). Prp2, Prp16, and Prp22 associate with the spliceosome at distinct stages and dissociate after they hydrolyze ATP (15, 16, 32). Their functional specificity is determined at least in part by unique interactions with the spliceosome. In the case of Prp16 and Prp22, it was shown that their unique N-terminal segments are involved in spliceosome binding and that the C-terminal region enhanced or stabilized spliceosome association (20, 33, 34). Deletion analyses established that more than 145 amino acids upstream of motif I was essential for the in vivo functions of Prp16 and Prp22 (20, 33, 34). The segment N-terminal to motif I is smaller in Prp43, comprising 118 amino acids, of which 90 can be deleted without deleterious effect on cell growth. Thus, Prp43 is fully active in vivo with an N-terminal extension of only 28 residues. Whether the 28-amino acid segment plays a role in Prp43’s association with the spliceosome or whether it is simply an integral part of the ATPase domain is not clear. The latter suggestion arises from the finding that DEAH-box ATPases contain several conserved residues, including an Leu-Pro dipeptide 26 residues upstream of motif I (35).

ATPase activity is crucial for the biological functions of Prp2, Prp16, Prp22, and Prp43. Non-functional mutants of the DEAH-box splicing factors exert dominant-negative phenotypes in vivo and in vitro (18, 19–21). Such Prp2, Prp16, and Prp22 mutants specifically block pre-mRNA splicing at the ATP-dependent steps that are catalyzed by the respective wild-type proteins (18, 19). An ATPase-defective Prp43 mutant blocks the release of excised lariat-intron from the spliceosome, revealing that intron-release is an ATP-dependent step catalyzed by Prp43. However, these experiments do not address whether Prp43 carries out an additional, possibly ATP-independent, function during splicing. For example, Prp22 catalyzes mRNA release from the spliceosome, but it also plays an ATP-independent role during the second step of splicing. This latter function was revealed in depletion/reconstitution studies and was unaffected by dominant-negative mutants (16).

In wild-type cells, the lariat-intron products of pre-mRNA splicing are not detectable, but they accumulate in prp22, dbr1, prp27, and prp43 mutant cells (22, 33, 34). In vitro studies showed that, when the splicing reaction is blocked at the Prp22 or the Prp43-dependent steps, lariat-intron remains associated with the spliceosome and is therefore protected from debranching and degradation (19, 21, 22) (Fig. 8). In contrast, stable lariat-intron is released from the splicing complex in extracts lacking debranching enzyme Db1 (Fig. 7). There was evidence of two forms of intron-containing complexes from the mammalian splicing system. Sucrose gradient fractionation of splicing complexes generated in a HeLa cell nuclear extract revealed small (<30 S) and large (50–60 S) lariat-intron-containing RNP complexes (37). The latter complex appeared to contain snRNPs, insofar as the lariat-intron could be immunoprecipitated with anti-Sm antibodies. Electrophoretic separation of splicing complexes in native gels showed that excised lariat-intron remained associated with U2, U5, and U6 snRNPs at the end of the splicing reactions in HeLa extracts (38).

The initial steps in spliceosome disassembly, which entail release of the mature mRNA and lariat-intron splicing products, are catalyzed by the DEAH-box ATPases Prp22 and Prp43. The fate of the snRNP complex after release of lariat-intron by Prp43 is not known. It has been suggested that the complex disassembles and that the snRNPs are recycled for the next round of splicing. This view is based on a model for spliceosome assembly in which the U1, U2, and the U4/U6-U5 snRNPs assemble onto the pre-mRNA in a stepwise fashion (10). However, the recent description of the penta-snRNP suggests that a large preassembled complex may associate with the pre-mRNA to form the spliceosome (11). In either case, the snRNP complex at the end of splicing reaction likely requires extensive remodeling, such as annealing of U4/U6 by Prp24 (39), before it can engage in a new round of splicing. Whether some of these remodeling steps are driven by ATPases remains to be determined.

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