Characterization of Dominant-negative Mutants of the DEAH-box Splicing Factors Prp22 and Prp16*

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Saccharomyces cerevisiae Prp22 and Prp16 are RNA-dependent ATPases required for pre-mRNA splicing. Both proteins are members of the DEXH-box family of nucleic acid-dependent NTPases. Prior mutational analysis of Prp22 and Prp16 identified residues within conserved motifs I (GGKGT), II (DEAH), and VI (QRXGRXGR) that are required for their biological activity. Nonfunctional Prp22 and Prp16 mutants exerted a dominant negative effect on cell growth. Here we show that overexpression of lethal Prp22 mutants leads to accumulation of unspliced pre-mRNAs and excised introns in vivo. The biochemical basis for the lethality and inhibition of splicing in vivo was determined by purifying and characterizing recombinant mutant proteins. The lethal Prp22 mutants D603A and E604A in motif II and Q804A and R808A in motif VI were defective for ATP hydrolysis and mRNA release from the spliceosome, but were active in promoting step 2 transesterification. Lethal Prp16 mutants G378A and K379A in motif I; D473A and E474A in motif II; and Q685A, G688A, R689A, and R692A in motif VI were defective for ATP hydrolysis and step 2 transesterification chemistry. The ATPase-defective mutants of Prp16 and Prp22 bound to spliceosomes in vitro and blocked the function of the respective wild-type proteins in trans. Comparing the mutational effects in Prp16 and Prp22 highlights common as well as distinct structural requirements for the ATP-dependent steps in pre-mRNA splicing.

Nucleic acid-dependent NTPases of the DEX(H/D)-box family play important roles in many biological processes including transcription, DNA repair, and pre-mRNA splicing. They use NTP hydrolysis to remodel macromolecular interactions involving nucleic acids and proteins, and many of the DEX(H/D)-box NTPases have RNA or DNA helicase activity (1–3). DEX(H/D) proteins are defined by a set of collinear motifs (1). The importance of individual residues for ATPase and/or helicase activity has been demonstrated by mutational analysis of several prototypical DEX(H/D) proteins (4, 5). Crystal structures of the DEXH protein NS3 and other DEX(H/D)-box NTPases show that conserved residues in the motifs comprise the NTP binding site (2, 6–8).

The Saccharomyces cerevisiae genes PRP16 and PRP22 encode essential splicing factors of the DEAH-box family (9, 10). Prp16 is required for the second step of pre-mRNA splicing, whereas the 3′ splice site of the lariat-intermediate is cleaved and the exons are joined (11). This was demonstrated by in vitro reconstitution assays. In extracts depleted of Prp16, spliceosomes assemble onto precursor RNA and catalyze 5′ splice site cleavage and formation of a branched lariat-intermediate. Purified Prp16 can act on the step-arrested spliceosomes and trigger the formation of mature RNA. Complementation of splicing requires ATP hydrolysis by Prp16, which elicits a conformational change in the spliceosome that can be measured as protection of the 3′ splice site against RNase H cleavage (12). The rearrangement that leads to 3′ splice site protection and mRNA formation requires the recruitment of splicing factors Slu7, Prp18, and Prp22 (13–15). The role of Prp22 during the second step is independent of ATP. However, ATP is necessary for the function of Prp22 in catalyzing the release of mature RNA from the spliceosome (15, 16).

We have undertaken a mutational analysis to evaluate the role of the NTPase motifs of Prp16 and Prp22 in pre-mRNA splicing. Single alanine mutations at conserved residues in motifs I (GKT), II (DEAH), III (SAT), and VI (QRXGRXGR) were tested for complementation of prp16 and prp22 deletion strains. Most of the mutations were either lethal or caused conditional growth defects. Overexpression of nonfunctional proteins led to dominant-negative growth phenotypes in wild-type cells (17, 18).

Prior studies of Prp22 had focused on the analysis of cold-sensitive Prp22 mutants H606A (motif II) and S635A and T637A (motif III) (18, 19). The S635A and T637A proteins retained full ATPase activity, but were defective in catalyzing mRNA release in vitro and failed to unwind RNA duplexes. Thus, ATPase activity was insufficient for Prp22’s function in mRNA release. It was proposed that Prp22 couples the energy of ATP hydrolysis to a conformational step, either RNA unwinding or disruption of protein-RNA interactions (18). That the ATPase activity of Prp22 was necessary for mRNA release was inferred from an analysis of a mutant in which Lys-512 in motif I was replaced by alanine; the K512A mutant was lethal, defective for ATP hydrolysis and for mRNA release in vitro (15).

Here we address the following questions. 1) Does the correlation between biological activity, ATPase activity, and splicing activity pertain to other Prp22 mutants? 2) How do mutations in conserved residues affect the ATPase and splicing function of Prp16? 3) Are mutational effects concordant for Prp22 and Prp16? 4) Can the mutational effects explain the dominant-negative effects on cell growth?

**EXPERIMENTAL PROCEDURES**

Expression and Purification of Recombinant Prp22—pET16b-based plasmids were constructed for the expression of His-tagged Prp22 mu-

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tants D603A, E604A, Q804A, and R808A. The His_{6} tag does not interfere with the function of Prp22 in vivo, insofar as His-Prp22 complements a prp22A strain. The expression plasmids were transformed into *Escherichia coli* strain BL21-Codon Plus(DE3) RIL (Stratagene). In parallel, we transformed pET16b-PRP22 and pET16b-PRP16 into *E. coli* strain BL21(DE3) cells. The recombinant proteins were expressed and purified from soluble bacterial lysates as described (18, 20).

Expression and Purification of Recombinant Prp16—A DNA fragment carrying the coding sequence of the PRP16 gene (3278 bp) was ligated into the T7-based expression vector pET16b (Novagen). The pET16b-PRP16 plasmid encodes a protein in which 10 histidines are fused in frame to the N terminus of Prp16. This fragment does not interfere with the function of Prp16 in vivo, insofar as His-Prp16 complements a prp16A strain. Plasmids carrying mutant prp16 alleles were obtained by exchanging the corresponding mutated DNA fragments from the p358-based PRP16 plasmids (17). *E. coli* strain BL21(DE3) cells were transformed with the pET-PRP16 plasmids. Individual transformants were grown at 37 °C in LB medium containing 0.1 mg/ml ampicillin. When the *A_{600}* reached 0.6–0.7, the cultures were chilled on ice for 30 min. Sorbitol and isopropyl-1-thio-β-D-galactopyranoside were added to final concentrations of 1 M and 0.5 mM, respectively. The culture was incubated at 18 °C for 18 h. Cells were harvested by centrifugation, and the cell pellets were stored at −80 °C.

All subsequent operations were performed at 4 °C. Cell pellets from 3-liter cultures were suspended in 100 ml of lysis buffer (50 mM Tris-HCl, pH 7.6, 250 mM NaCl, 1 mM dithiothreitol, 2 mM MgCl_{2}, 0.5 mM ATP, and 3% polyethylene glycol 8000). The solution was adjusted to 200 mM NaCl and 1 mM γ-[32P]ATP, 0.5 mM dithiothreitol, 10% glycerol, and then incubated at 18 °C for 10 min. The reaction mixtures were halted by transfer to ice. Aliquots (90 μl) were layered onto 15–40% glycerol gradients containing 20 mM HEPES, pH 6.5, 100 mM KCl, 2 mM EDTA. The gradients were centrifuged at 180,000 × *g* for 15 min in a Sorvall TH641 rotor. Fractions (400 μl) were collected from the tops of the tubes. RNA was recovered from the gradient fractions by phenol extraction and ethanol precipitation. RNAs from alternate fractions were analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea. The amounts of mRNA products were quantified by scanning the gels using a STORM PhosphorImager.

**RNA Analysis**—p133-PRP22 plasmids (TRP1 CEN) carry wild-type Prp22 or the mutants K512A, D603A, and R808A under the transcriptional control of the GAL1 promoter (18). The plasmids were introduced into wild-type yeast cells. Trp⁺ transformants were selected and grown in glucose-containing synthetic medium (SD-trp) at 30 °C to mid-logarithmic phase. Cells were collected by centrifugation and suspended in SD-trp medium containing either 2% glucose or 2% galactose. The cells in glucose medium were harvested by centrifugation, washed in ice-cold water, and stored as cell pellets at −80 °C. The cells in galactose-containing medium were incubated for 12 h at 30 °C and then re-suspended in galactose buffer. A 100 μl culture was layered on to a 15%–60% glycerol gradient containing 50 mM NaCl, 1 mM β-mercaptoethanol, 10% sucrose. The gradient was centrifuged overnight in a 20% SW-41 rotor. Fractions (400 μl) were collected from the gradient fractions by phenol extraction and ethanol precipitation. RNAs from alternate fractions were analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea in 80 mM Tris borate, 2 mM EDTA. Radiolabeled RNA was visualized by autoradiography. The gel was stained with ethidium bromide, photographed, and then transferred to a Hybond mem brake (Amersham Biosciences). Radiolabeled probes were prepared with a random priming kit (Roche Molecular Biochemicals) according to the vendor’s instructions. Hybridized membranes were washed in SDS/PAGE. The gels were visualized by autoradiography. Three identical blots were generated in parallel. These were first hybridized with probes for *CYH2* and *ACT1* (exon 2 probes) and for *PGK1*. The blots were then stripped by boiling in hot water and re-probed with intron probes (*CYH2*, *ACT1*, and *SNR17A*).

**RESULTS**

**Effects of Prp22 Mutations on ATP Hydrolysis—Conserved residues in motifs I ([11]GKT), II ([60]DEAH), and VI ([34]QKGRAGR) are essential for the biological function of Prp22; alanine substitutions for *Lys*-512, Asp-603, Glu-604, Gln-804, and Arg-808 are lethal in vivo (18). To examine the biochemical consequences of the lethal mutations, we produced recombinant His_{6}-tagged Prp22 proteins K512A (motif I), D603A and E604A (motif II), Q804A and R808A (motif VI) and purified them from soluble bacterial lysates by nickel-agarose affinity chromatography, phosphocellulose chromatography, and glycerol gradient sedimentation. The level of purity, as gauged by SDS-PAGE analysis, was comparable for the five mutants and the wild-type Prp22 control (Fig. 1A); the Prp22 polypeptide is denoted by an asterisk.

The extent of ATP hydrolysis by wild-type Prp22 was proportional to input protein (Fig. 1B); we calculated that Prp22 hydrolyzed 420 ATP min⁻¹. The motif I K512A mutant hydrolyzed 20 ATP min⁻¹ (5% of wild type) (Fig. 1C). The motif II mutant proteins D603A and E604A exhibited no activity (<0.5% of wild type), whereas the motif VI mutants Q804A and R808A hydrolyzed 40 and 85 ATP min⁻¹ (10 and 20% of wild type), respectively (Fig. 1C). We infer that a threshold level of ATPase activity is required for the *in vivo* function of Prp22.

**Splicing Activity of ATPase-defective Prp22 Mutants**—Previous work showed that the ATPase-defective mutant K512A supported mRNA formation but not mRNA release (15). We
now tested splicing activity of the ATPase-defective mutants D603A, E604A, Q804A, and R808A (Fig. 2). Splicing intermediates were formed during a 15-min pre-incubation in extract immunodepleted of Prp22. Aliquots of the reaction mixture were then supplemented with 50 ng of wild-type or mutant Prp22. Wild-type Prp22 and each of the mutants relieved the second step block and promoted the formation of mature actin mRNA. However, excised lariat-intron accumulated in reactions supplemented with each of the ATPase-defective Prp22 mutants, but not in the wild-type Prp22 reactions, suggesting that spliceosome disassembly was affected.

To test directly whether the mutants catalyzed mRNA release from the spliceosome, we analyzed the reaction products by glycerol gradient sedimentation (Fig. 3). When the reaction mixture was supplemented with wild-type Prp22, 85% of the mature mRNA was released and sedimented near the top of the gradient (fractions 7–13). In contrast, the Q804A mutant did not catalyze mRNA release and 78% of the mRNA was found in the heavy spliceosome peak (fractions 21–27). When the reactions were supplemented with the K512A, D603A, E604A, and R808A mutants, 65, 61, 83, and 82% of the mature RNA was retained in the spliceosome fractions, respectively (data not shown). Thus, the relevant defect of the lethal Prp22 mutants with respect to splicing is their inability to promote mRNA release from the spliceosome.

**Overexpression of Prp22 Mutants Results in a Splicing Defect in Vitro**—Lethal Prp22 mutants exert dominant-negative effects when overexpressed in vivo (18). We tested whether the ATPase-defective Q804A mutant inhibits the function of wild-type Prp22 in vitro. Spliceosomes were formed in extracts immunodepleted of Prp22. The reaction mixture was then supplemented with wild-type and Q804A proteins pre-mixed at a ratio of either 1:1 or 1:10 (Fig. 4). Analysis of the splicing products by glycerol gradient sedimentation showed that, when equal amounts of wild-type and Q804A proteins were added, 68% of the mature mRNA was released and 26% was retained in the spliceosome. However, when Q804A was in 10-fold excess (500 ng) over wild-type Prp22 (50 ng), only 25% of mature mRNA was released and 71% was retained (Fig. 4A). Similar experiments performed with mutants E604A and R808A revealed that 10-fold excess of E604A and R808A led to retention of 80 and 81% of the mature RNA in the spliceosome, respectively (data not shown). Thus, excess mutant protein blocks the function of wild-type Prp22 in vitro, providing a plausible explanation for the dominant-negative phenotypes observed in vivo.

Previous studies showed that the K512A mutant remained associated with the spliceosome after completion of step 2, i.e. immunoprecipitation of the reaction products under native conditions with Prp22-specific antibodies resulted in the recovery of mature mRNA and lariat-intron, but not pre-mRNA (15). Here, using the same immunoprecipitation assay, we found that the ATPase-defective Prp22 mutants D603A, E604A, Q804A, and R808A also remained bound to the spliceosomes after step 2 transesterification (data not shown). We surmise that the ATPase-defective mutants inhibit wild-type Prp22 because they occupy a Prp22 binding site on the spliceosome, but do not function in spliceosome disassembly subsequent to their action in forming mature mRNA.

**Overexpression of Prp22 Mutants Results in a Splicing Defect in Vivo**—The in vitro analysis (Fig. 4) would suggest that overexpression of the nonfunctional Prp22 mutants in vivo...
**Fig. 3. Release of mRNA from the spliceosome.** Spliceosomes that were formed in Prp22-depleted extract were chased by the addition of recombinant WT or mutant (Q804A) Prp22 proteins. The reaction mixtures were analyzed by zonal velocity sedimentation. RNA was isolated from aliquots of the odd-numbered fractions (fractions 3–29) of each glycerol gradient, analyzed by denaturing PAGE and visualized by autoradiography. The arrow indicates the position of mature mRNA. The amount of mRNA was quantified using a PhosphorImager, and the sum of mRNA in all lanes was set to 100%. Fractions 7–13 contained released mRNA, and fractions 21–27 contained mRNA that was retained in the spliceosome.

should elicit a splicing defect. To test this directly, we analyzed RNAs from wild-type cells in which PRP22 or the prp22 alleles K512A, D603A, or R808A were placed under the transcriptional control of the GAL1 promoter. RNA was isolated from the strains grown either in glucose-containing medium (expression repressed) or in galactose-containing medium (expression induced). Northern blots were probed for RNAs derived from the ACT1, CYH2, and SNR17A (U3 small nuclear RNA) genes (Fig. 5). PGK1 RNA is not spliced and served as a loading control. Hybridization to probes that specifically recognized intron sequences showed the accumulation of CYH2 and of ACT1 pre-mRNA upon galactose-induced overexpression of K512A, D603A, and R808A. Note that galactose-induced overexpression of wild-type PRP22 did not lead to increased levels of pre-mRNA (Fig. 5, lane 5). The intron probe also detected the excised intron RNA product of splicing of ACT1, CYH2, and U3 (indicated by arrows in Fig. 5). The excised intron is undetectable under normal circumstances (Fig. 5, lanes 1–5). Exon 2 probes were used to detect mRNA and pre-mRNA of ACT1 and CYH2. Although low levels of unspliced pre-CYH2 RNA were detectable in uninduced cells, the galactose-induced overexpression of K512A, D603A, and R808A resulted in the accumulation of unspliced pre-CYH2 RNA (Fig. 5, lanes 6–8). We conclude that overexpression of nonfunctional Prp22 mutants leads to a pre-mRNA splicing defect in vivo. This defect, which is manifest in increased steady-state levels of pre-mRNA and of excised intron, is probably caused by the inhibition of spliceosome disassembly and subsequent recycling of splicing factors.

**Effects of Prp16 Mutations on Pre-mRNA Splicing in Vitro—** The DEAH-box ATPase Prp16 is required for the second step of splicing. Prior mutational analysis identified 10 positions in the NTPase motifs at which alanine substitutions abrogated Prp16 function in vivo (17). These residues were Gly-378, Lys-379, and Thr-380 in motif I (378GKT); Asp-473 and Glu-474 in motif II (473DEAH); and Gln-685, Arg-686, Gly-688, Arg-689, and Arg-692 in motif VI (685QRSGRA). We next assessed the splicing activity of purified recombinant mutant Prp16 proteins in vitro (Fig. 6). Yeast extracts immunodepleted of Prp16 catalyzed the first step of splicing of pre-mRNA leading to accumulation of exon 1 and lariat-exon 2 intermediate (11). The pre-formed splicing intermediates could be readily chased into mature RNA upon addition of 50 ng of wild-type Prp16 protein, but mutants G378A, K379A, D473A, E474A, Q804A, G688A, and R692A were unable to complement the step 2 defect (Fig. 6A).

Step 2 complementation correlated with ATPase activity. In the linear range of enzyme dependence, wild-type Prp16 hydrolyzed 270 ATP min⁻¹ in the presence of poly(A) (data not shown). The Prp16 mutants that were nonfunctional in splicing had reduced ATPase activity as follows: G378A (2% of wild type), K379A (9% of wild type), D473A (1% of wild type), E474A (2% of wild type), Q804A (12% of wild type), R689A (4% of wild type), and R692A (24% of wild type) (data not shown). These findings suggest that a threshold level of ATPase activity
Motif III Mutants of Prp16 Are Active in Pre-mRNA Splicing—Prp16 mutants S505A and T507A in motif III and H476A in the DEAH-box (motif II) are functional in vitro (17). These mutants are of particular interest in light of studies of NPH-II, NS3, and eIF-4A, which suggested that motif III and the DEAH-box histidine/aspartate are responsible for coupling NTP hydrolysis to RNA unwinding (23–25). Thus, we purified the recombinant H476A, S505A, and T507A proteins and tested their activity in vitro. H476A, S505A, and T507A supported step 2 trans-esterification in extracts immunodepleted of Prp16 (Fig. 6A). H476A, S505A, and T507A displayed 65, 71, and 34% of wild-type ATPase activity, respectively. We conclude that the Ser and Thr hydroxyls of motif III and the His in motif II are not essential for Prp16 function in splicing in vitro, for ATP hydrolysis, or for cell growth.

Prp16 Mutants Inhibit Splicing in Trans—We noted previously that overexpression of lethal Prp16 mutants blocked the growth of wild-type yeast cells (17). Exogenous K379A mutant protein was shown to inhibit splicing of actin pre-mRNA in whole cell lysates in vitro (26). Here we tested whether other Prp16 mutants that were inactive for splicing would affect splicing step 2 in the presence of wild-type Prp16. Whole cell extract containing endogenous wild-type Prp16 was supplemented with 50 ng of recombinant Prp16. Mature actin mRNA was efficiently generated in un-supplemented mixtures, and additional wild-type Prp16 had no impact on the reaction (Fig. 6B). The H476A, S505A, and T507A mutants also had no effect on splicing. However, each of the eight mutant proteins that were nonfunctional in complementing splicing in immunodepleted extracts were inhibitory to splicing in extract containing Prp16. We surmise that the G378A, K379A, D473A, E474A, Q685A, G688A, R689A, and R692A proteins can bind to spliceosomes and thus block the function of wild-type Prp16 in trans.

Mutant Prp16 Proteins Bind to Spliceosomes—To determine directly whether the Prp16 mutants bind to spliceosomes, we performed immunoprecipitation experiments (Fig. 7). Because wild-type Prp16 had been shown to stably associate with spliceosomes only in the absence of ATP (11), we used an actin pre-mRNA (C303/305) in which the 3′ splice site is mutated. The 3′ splice site mutation in C303/305 precursor RNA does not interfere with the first step of splicing, however, it effectively blocks step 2 chemistry (12, 27). C303/305 RNA was reacted with extract immunodepleted of Prp16 to allow for spliceosome assembly and for the first catalytic step to occur. Glucose was added to deplete ATP (by the action of endogenous hexokinase), and then aliquots of the reaction mixture were supplemented with Prp16 proteins in the absence or presence of ATP. An aliquot (25%) of each reaction mixture served as the
FIG. 7. Binding of Prp16 to the spliceosome. Mutated actin precursor RNA (C303/305) was incubated for 20 min in extract depleted of Prp16. Glucose was then added to a final concentration of 2.5 mM to allow for depletion of ATP by endogenous hexokinase for 7 min. Aliquots were supplemented with wild-type, K379A, or R692A proteins. As a control, buffer was used instead of the protein fractions (−). The reaction mixtures were incubated for another 10 min either with ATP (5 mM final concentration) or without addition of ATP. One quarter of each reaction was analyzed by denaturing PAGE (INPUT), and three quarters were used for immunoprecipitation with anti-Prp16 antibodies bound to Protein A-Sepharose (IP v-Prp16). The RNA products were analyzed by denaturing PAGE and autoradiography. The symbols at the left indicate the positions of the labeled RNA species. These are (from top to bottom) lariat-exon 2, precursor RNA, and exon 1. The asterisk indicates the 3′ splice site mutation in C303/305 actin precursor RNA.

The remainder (75%) was subjected to immunoprecipitation with Prp16-specific antibodies (Fig. 7). As expected, wild-type Prp16 bound specifically to spliceosomes containing the RNA products of step 1 (but not precursor RNA) in the absence of ATP. However, the association was destabilized upon ATP hydrolysis and only background levels of RNAs were co-precipitated. In contrast, the K379A and R692A mutants, which bound to spliceosomes in the absence of ATP, remained stably associated in the presence of ATP. When similar experiments were carried out with actin pre-mRNA, wild-type Prp16 promoted mRNA formation and was released from the spliceosome. However, the Prp16 mutants K379A and R692A, and also G378A, D473A, E474A, Q655A, G688A, and R689A, co-precipitated lariat-intermediates in the presence of ATP (data not shown). These findings are consistent with the hypothesis that ATPase-defective mutants block the function of wild-type Prp16 protein by occupying the Prp16 binding site on the spliceosome.

DISCUSSION

We have conducted an inquiry into the basis for the lethality and the dominant-negative phenotypes elicited by alanine substitutions in motifs I, II, and VI of Prp16 and Prp22. The principle conclusion from the in vitro analysis is that lethality arises from a defect in the execution of ATP-dependent steps in pre-mRNA splicing, these being step 2 transesterification chemistry in the case of Prp16 and the release of mature mRNA from the spliceosome in the case of Prp22. The dominant-negative inhibition of splicing can be recapitulated in vitro for those Prp16 and Prp22 Ala mutants that inhibit cell growth when overexpressed in vivo. This inhibition, together with direct studies of spliceosome binding by immunoprecipitation, indicates that the splicing-defective Ala mutants occupy specific sites on the spliceosome and thereby block the action of the wild-type Prp16 and Prp22 proteins.

For mutations of the motif I lysine (GKT) and the Asp and Glu in motif II (DEAH/D), there is a clear correlation between lethality and diminished ATPase activity. Substitution of Asp and Glu by Ala abrogate ATP hydrolysis by both Prp16 and Prp22. This is consistent with studies of other NTPases, including vaccinia virus NPH-I and NPH-II, HCV NS3, and eIF-4A, which show that the Asp and Glu residues are essential for ATP hydrolysis (28–30). The motif II aspartic acid has also been replaced in yeast Brr2 and Sub2, both of which play a role in spliceosome assembly (31, 32). In Brr2, replacing the aspartic acid in the DEIH-box by glycine resulted in a protein that was nonfunctional in vivo and in vitro (31). A Sub2 mutant, in which motif II (DECD) was changed to EECD, was lethal in vivo (32).

Mutation of the invariant lysine in motif I to alanine caused 10- and 20-fold decrements in the ATPase activities of Prp16 and Prp22. This is again in agreement with other studies, although the magnitude of the decrease in ATPase activity can vary between different NTPases. For example, ATP hydrolysis by a motif I mutants of eIF-4A (Lys → Asn mutant) and of the Drosophila MLE (Lys → Glu mutant) were undetectable (23, 33). Alanine substitutions of the lysine in NPH-I and NPH-II reduced ATPase activity by more than 10,000-fold in the case of NPH-I and over 500-fold for NPH-II (24, 28). Replacing the motif I Lys in HCV-NS3 by Ala caused a more modest reduction in the protein’s ATPase activity (4–5-fold) (30). Crystal structures of NTPases show that the lysine of motif I contacts the β- and γ-phosphates of the bound nucleotide (2, 4).

Individual residues within motif VI are important for ATP hydrolysis by eIF-4A, NS3, NPH-I, and NPH-II (28, 30, 34, 35). Structural studies have suggested that motif VI is involved in nucleotide binding (2, 7). Motif VI mutants of Prp16 and Prp22 are inactive in splicing; however, they suffer a more modest decrement in ATPase activity than mutants in motifs I and II. Does this indicate that splicing/viability requires a threshold level of ATP hydrolysis that is above 20–24% of wild-type activity for Prp22 and Prp16? Prior studies of several Prp16 mutants, isolated in a genetic screen, suggest that low levels of ATPase activity can suffice for function. Despite a 3–20-fold decrement in ATP hydrolysis by those Prp16 mutants, which were purified from yeast, the Prp16 mutants did not exhibit any splicing or growth defect in vivo (36). It is possible that the lack of a tight correlation between lethality and ATPase activity hints at a more complex role for motif VI in splicing that is not limited to ATP hydrolysis. Alternatively, the requirements for ATP hydrolysis by Prp16 and Prp22 in the context of the spliceosome may differ from those of the isolated enzymes.

A difference between Prp16 and Prp22 that is reinforced in the current study concerns the function of the hydroxyls of the serine/threonine residues in motif III (SAT) and the histidine in the DEXH-box. Alanine substitutions at these positions in Prp16 resulted in proteins that were functional in splicing in vitro and in vivo. This is in contrast to the findings for Prp22 and for vaccinia virus NPH-II. Alanine substitutions at the His residue in the DEXH-box or either of the threonines in motif III (TAT) of NPH-II were lethal in vivo (24). The NPH-II mutants retained ATPase activity, but were unable to unwind an RNA duplex in vitro, suggesting uncoupling of ATPase and helicase activity (24, 29). The Prp22 mutants H606A (motif II) and S635A and T637A (motif III) retained ATPase activity, but showed severe growth phenotypes, e.g. S635A failed to grow at temperatures <34 °C (18). The growth phenotype reflects the deficiency of the Prp22 mutants in releasing mature mRNA from the spliceosome (18, 19). Notably, both Prp22 motif III mutants also uncoupled ATP hydrolysis from RNA unwinding (18).

It is thought that Prp16 and Prp22 disrupt or remodel macromolecular contacts within the spliceosome, which may involve RNA/RNA, protein/RNA, or protein/protein interactions. The distinct molecular phenotypes exhibited by motif III Ala mutants may indicate a difference in the nature of the rearrangements triggered by Prp22 and Prp16.

Conserved ATPase motifs are important for Prp16 and Prp22’s function in splicing; however, they are not involved in
spliceosome binding. Nonfunctional Prp16 and Prp22 mutants elicit dominant-negative effects, presumably by occupying binding sites on the spliceosome and thus blocking access by the respective wild-type protein.

Dominant-negative phenotypes have been described for numerous mutants in DEX(H/D)-box splicing factors, including Prp2, Brr2, and Sub2 (32, 31, 37, 38). The biochemical analysis of dominant-negative Prp2 mutants showed that, although many of the mutant proteins bound to the spliceosome and blocked the function of wild-type Prp2 \textit{in vitro}, the nonfunctional Prp2 mutant H349K (in motif II) was dominant-negative \textit{in vivo}, but failed to bind to spliceosomes or to inhibit splicing by wild-type Prp2 \textit{in vitro} (37). Thus, dominant-negative effects do not inevitably arise from binding of a nonfunctional DEX(H/D)-box protein to the spliceosome.

It is also not axiomatic that all inactivating mutations in the ATPase domain of DEX(H/D)-box splicing factors elicit dominant-negative phenotypes, as might be suggested by studies of mutants of Prp16, Prp22, Prp2, and Brr2 (21, 31, 37, 38). For example, lethal mutants in motifs I (GKT \rightarrow GNT) and II (DECD \rightarrow EECD) of Sub2 did not inhibit growth of wild-type prp16 \textit{in vivo} (32). However, the Sub2-LAT mutant (in motif III) exhibits dominant-negative effects upon overexpression. Because motifs I and II are implicated in nucleotide binding, whereas motif III is not, it is possible that only Sub2 mutants that can bind ATP elicit dominant-negative phenotypes.

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Characterization of Dominant-negative Mutants of the DEAH-box Splicing Factors Prp22 and Prp16
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Figs. 1–7 are reprinted here.

Fig. 1. Mutational effects on Prp22 ATPase activity. A, aliquots (0.5 μg) of the indicated glycerol gradient preparations of Prp22 were analyzed by electrophoresis in an 8% polyacrylamide gel containing 0.1% SDS. Polypeptides were visualized by staining with Coomassie Blue dye. Prp22 is indicated by an asterisk. The positions and sizes (in kDa) of marker proteins are indicated at the left. B, the extents of ATP hydrolysis by wild-type Prp22 (○), R808A (●), Q804A (●), K512A (■), D603A (▲), and E604A (×) in the presence of poly(A) are plotted as a function of input protein. C, ATPase specific activities in the presence and absence of poly(A) are expressed as turnover numbers (min⁻¹).

| Prp22        | ATPase (min⁻¹) |
|--------------|---------------|
|              | + RNA | -RNA |
| WT           | 420    | 36   |
| K512A        | 20     | 7    |
| D603A        | <1     | <1   |
| E604A        | 2      | <1   |
| Q804A        | 40     | 5    |
| R808A        | 85     | 1    |

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FIG. 2. Effects of Prp22 mutations on actin pre-mRNA splicing in vitro. Spliceosomes were formed on 32P-labeled actin pre-mRNA in extracts depleted of Prp22 during a 15-min pre-incubation at 23 °C. Aliquots of the reaction mixture were supplemented with buffer (−) or 50 ng of wild-type Prp22, D603A, E604A, K512A, Q804A, or R808A and then incubated for 10 min at 30 °C. The reaction products were resolved by denaturing PAGE followed by autoradiography. The symbols at the left indicate the positions of the labeled RNA species, which are (from top to bottom) lariat-exon 2, intron-lariat, pre-mRNA, and spliced mRNA (exon 1 is not shown). Intron-lariat is indicated by an arrow at the right.

FIG. 3. Release of mRNA from the spliceosomes. Spliceosomes that were formed in Prp22-depleted extract were chased by the addition of recombinant WT or mutant (Q804A) Prp22 proteins. The reaction mixtures were analyzed by zonal velocity sedimentation. RNA was isolated from aliquots of the odd-numbered fractions (fractions 3–29) of each glycerol gradient, analyzed by denaturing PAGE, and visualized by autoradiography. The amount of mRNA (indicated by arrow) in each lane was quantified using a PhosphorImager. The sum of mRNA in all lanes was set to 100%.

FIG. 4. Q804A inhibits release of mRNA by wild-type Prp22. Spliceosomes were formed in extracts depleted of Prp22. Aliquots of the reaction mixture were chased with 50 ng each of WT and mutant Prp22 (Q804) (1:1) or with WT and Q804A proteins pre-mixed at a ratio of 1:10 (50 ng of WT and 500 ng of Q804A). The reaction mixtures were analyzed by zonal velocity sedimentation. RNA was isolated from aliquots of the odd-numbered fractions of each glycerol gradient, analyzed by denaturing PAGE, and visualized by autoradiography. The amount of mRNA (indicated by arrow) in each lane was quantified using a PhosphorImager. The sum of mRNA in all lanes was set to 100%.
Fig. 5. **Northern blot analysis.** RNA was isolated from cells grown in either glucose (lanes 1–4) or galactose (lanes 5–8) medium. Aliquots (30 μg) of total RNA were separated on a 1% agarose/formaldehyde gel and then transferred to a membrane, which was hybridized with denatured 32P-labeled DNA fragments of the indicated genes. The blots were analyzed by autoradiography. The left panel shows the membranes after hybridization with probes that detect exon 2 plus intron sequences. The relative levels of ACT1 pre-mRNA are very low compared with ACT1 mRNA, so that precursor RNA is not detectable on this exposure using the exon 2 probe. The hybridization results using intron probes are shown in the right panel. Asterisks (*) indicate the position of precursor RNA, and arrows mark the position of excised introns, deduced from co-electrophoresed RNA size markers and the positions of ribosomal RNAs visualized with ethidium bromide.

Fig. 6. **Splicing activity of Prp16 proteins.** A, complementation of Prp16-depleted splicing extract with wild-type and mutant proteins. Yeast whole cell extract was immunodepleted of Prp16 and incubated with actin pre-mRNA for 20 min at 23 °C. Aliquots were supplemented with buffer (−) and 50 ng of each wild-type or mutant Prp16 proteins and incubated for another 10 min. B, inhibition of splicing by mutant Prp16 proteins. Yeast whole cell extract was incubated with labeled actin pre-mRNA and 50 ng of wild-type or mutant Prp16 polypeptides for 20 min at 23 °C. As a control, buffer (−) was used instead of the protein fraction. The RNA products were analyzed by denaturing PAGE and autoradiography. The symbols at the left indicate the positions of the labeled RNA species, which are (from top to bottom) lariat-exon 2, intron-lariat, pre-mRNA, spliced mRNA (note that exon 1 is not shown).
FIG. 7. Binding of Prp16 to the spliceosome. Mutated actin precursor RNA (C303/305) was incubated for 20 min in extract depleted of Prp16. Glucose was then added to a final concentration of 2.5 mM to allow for depletion of ATP by endogenous hexokinase for 7 min. Aliquots were supplemented with wild-type, K379A, or R692A proteins. As a control, buffer was used instead of the protein fractions (→). The reaction mixtures were incubated for another 10 min either with ATP (5 mM final concentration) or without addition of ATP. One quarter of each reaction was analyzed by denaturing PAGE (INPUT), and three quarters were used for immunoprecipitation with anti-Prp16 antibodies bound to Protein A-Sepharose (IP α-prp16). The RNA products were analyzed by denaturing PAGE and autoradiography. The symbols at the left indicate the positions of the labeled RNA species. These are (from top to bottom) lariat-exon 2, precursor RNA, and exon 1. The asterisk indicates the 3' splice site mutation in C303/305 actin precursor RNA.