The yeast pre-mRNA splicing factor Prp22 is a member of the DEAH box family of nucleic acid-stimulated ATPases and RNA helicases. Here we report a mutational analysis of 16 conserved residues in motifs Ia (534TEDPRRVAA541), IV (695LVFLTG700), and V (757TNI-AETSIT765). Mutants T757A, I764A, and T765A were lethal, and F697A cells did not grow at ≤30 °C. The mutant proteins failed to catalyze mRNA release from the spliceosome in vitro, and they were deficient for RNA unwinding. The F697A, I764A, and T765A proteins were active for ATP hydrolysis in the presence of RNA cofactor. The T757A mutant retained basal ATPase activity but was not stimulated by RNA, whereas ATP hydrolysis by T765A was strictly dependent on the RNA cofactor. Thus Thr-757 and Thr-765 in motif V link ATP hydrolysis to the RNA cofactor. To illuminate the mechanism of Prp22-catalyzed mRNA release, we performed a genetic screen to identify extragenic suppressors of the cold-sensitive growth defect of a helicase/release-defective Prp22 mutant. We identified one of the suppressors as a missense mutation of PRP8 (R1753K), a protein component of the U5 small nuclear ribonucleoprotein. We show that PRP8-R1753K suppressed multiple helicase-deficient prp22 mutations, including the lethal I764A mutation. Replacing Arg-1753 of Prp8 by either Lys, Ala, Gln, or Glu resulted in suppression of helicase-defective Prp22 mutants. Prp8-Arg1753 mutations by themselves caused temperature-sensitive growth defects in a prp22 strain. These findings suggest a model whereby Prp22 disrupts an RNA/protein or RNA/RNA interaction in the spliceosome that is normally stabilized by Prp8.

Genetic and biochemical analyses implicate at least one member of the DEX(H/D) box protein family in each of the ATP-dependent steps of pre-mRNA splicing (1, 2). Recognition of the 5′ splice site by U1 and U6 snRNA depends on the DEX(H/D) proteins Prp28 and Br2, and the association of U2 small nuclear ribonucleoprotein with the branch site is assisted by the DEXD box proteins Sub2 and Prp5 (3–9). Once the spliceosome is assembled, ATP hydrolysis by the DEAH protein Prp2 drives the first and second transesterification steps, respectively (10, 11). The spliced mRNA and excised lariat-intron products are then released sequentially from the spliceosome by the DEAH proteins Prp22 and Prp43 (12–16). How the DEX(H/D) proteins use ATP to remodel interactions in the spliceosome is not understood, and the exact targets within the spliceosome that are subject to rearrangement by specific DEX(H/D) proteins are not known.

DEX(H/D) box proteins are defined by seven colinear peptide motifs (17–19). The conserved residues in motifs I (GTK), II (DEX(H/D)), and VI (QRXGRXRQ) are important for catalysis and likely compose the active site for NTP hydrolysis (20, 21). Motif III (S/T/AT) couples ATP hydrolysis to RNA unwinding in the exemplary DEX(H/D) helicases eIF-4A, NPHIII, and Prp22 (22–24). The roles of motifs Ia, IV, and V are less well understood.

Many of the DEX(H/D) splicing factors hydrolyze ATP in a manner that is either stimulated by or dependent on a nucleic acid cofactor (13, 14, 16, 25–28); several can unwind RNA duplexes in an NTP-dependent fashion (5, 13, 14, 26–28). ATP hydrolysis is essential for the in vivo functions of Br2, Prp2, Prp16, Prp22, and Prp43, insofar as mutations that abolish ATPase activity in vitro are invariably lethal in vivo (5, 14, 29, 30).

Prp22 plays two distinct roles during pre-mRNA splicing. It is important for the second catalytic step that results in formation of mRNA, and it is essential for releasing mature mRNA from the spliceosome (13). In yeast extract depleted of Prp22, spliceosomes assemble on actin pre-mRNA and the first step occurs, leading to accumulation of the 5′ exon and lariat intermediates. Upon addition of purified Prp22, mRNA is formed in an ATP-independent fashion. The role of Prp22 in mRNA formation is not well understood, and no mutation in Prp22 has been identified that specifically abates step 2 function without abolishing interaction with the spliceosome. Deletion analyses established that an N-terminal segment spanning residues 262–465 of the 1145-aa Prp22 protein is important for spliceosome binding (34). The interaction of Prp22 with the precursor RNA during the splicing reaction has been probed by cross-linking using chemically modified RNAs; Prp22 can be cross-linked to the last 8 nucleotides at the 3′ splice site of the intron in a sequence-independent fashion. This suggests that Prp22 might bind to the precursor RNA prior to step 2 (35, 36).

Release of mRNA from the spliceosome depends on ATP hydrolysis by Prp22 (13, 14). Although necessary, ATPase activity does not suffice for mRNA release. For example, S635A and T637A mutations in motif III (SAT) result in severe cold-sensitive growth defects that correlate with the inability of the mutant proteins to catalyze mRNA release from the spliceosome and to unwind a synthetic RNA duplex in vitro (24). The
S635A and T637A proteins are proficient in hydrolyzing ATP. These findings suggest that the chemical energy of ATP hydrolysis by Prp22 needs to be coupled to mechanical work (e.g. RNA unwinding) in disassembly of the spliceosome.

The growth and RNA unwinding defects incurred by altering motif III in Prp22 can be suppressed by single amino acid changes within the core ATPase/helicase domain (24, 37). It is thought that these mutations alter the conformation and/or flexibility of Prp22 to restore mRNA release and RNA unwinding. Four of the intragenic suppressors of cold-sensitive Prp22 mutants mapped within or close to motifs Iα (Thr757, Ile764, and Thr765) and one in motif IV (Phe697) that are important for Prp22 function in vivo, for mRNA release in vitro, and for helicase activity in vitro. The I764A and F697A proteins exhibit ATPase activity that is stimulated by RNA. ATP hydrolysis by T765A is dependent on the RNA cofactor, whereas T757A displays basal ATPase activity that is not stimulated by RNA. Thus, Thr-757 and Thr-765 are important to link ATP hydrolysis to the RNA cofactor.

Understanding the mechanism of Prp22 function in detail requires the analysis of the macromolecular interactions in the spliceosome that are remedied by Prp22. We describe the isolation of spontaneous extragenic suppressors of the cold-sensitive prp22-S635A mutant. One of these suppressors maps to the essential PRP8 gene that encodes a constituent of the U5 small nuclear ribonucleoprotein particle (38–40). The 2413-aa Prp8 protein plays important roles during spliceosome assembly and both transsterification steps (38, 41, 42). We show that a single missense mutation in Prp8, R1753K, suppresses the growth defects of multiple helicase-defective Prp22 mutants, including the lethal mutant prp22-I764A. The findings that several different substitutions for Arg-1753 of Prp8 suppress helicase-defective prp22 mutations and that these mutant PRP8 alleles are temperature-sensitive in vivo suggest that Prp8 makes a contact via Arg-1753 to either RNA or protein, which cannot be disrupted by helicase-deficient Prp22 mutants.

MATERIALS AND METHODS

Targeted Mutagenesis of Prp22—Alanine mutations were introduced into the prp22 gene by using the two-stage PCR overlap extension method. Plasmid pRS315-PRP22 (CEN TRP1 PRP22), which contains the PRP22 coding region (3435 bp) plus 232 bp upstream and 255 bp downstream of flanking yeast genomic DNA, was used as the template for the first amplification step (24). Residues targeted for Ala substitutions are as follows: Thr-534, Gln-535, Pro-536, Arg-537, and Arg-538 in motif Iα; Leu-695, Phe-697, Thr-699, and Gly-700 in motif IV; and Thr-757, Asn-758, Asp-761, Thr-762, Ser-763, Ile-764, and Thr-765 in motif V. The mutated DNA products of the second amplification were digested with restriction endonucleases and inserted into pRS315-PRP22 in lieu of the corresponding wild type restriction fragments (yielding pRS315-T534A, pRS315-Q535A, etc.). The presence of the desired mutation was confirmed by DNA sequencing, and the segments corresponding to the inserted restriction fragment were then sequenced completely in order to exclude the acquisition of unwanted mutations during amplification or cloning.

Test of Mutational Effects on Prp22 Function in Vivo by Plasmid Shuttle—Viability of the prp22 strain (MATα ura3 trp1 his3 leu2 ade2 prp22::LEU2) depends on the plasmid p360-PRP22 (PRP22 URA3 CEN), prp22Δ was transformed with TRP1 plasmids carrying the various PRP22-Ala mutants. Trp+-transformants were selected and streaked to agar medium containing 0.75 mg/ml 5-fluoroorotic acid (5-FOA) to select against the URA3 PRP22 plasmid. The ability of the mutant prp22 alleles to support growth on 5-FOA was tested at 15, 30, and 37 °C. 5-FOA survivors were streaked to YPD agar medium and incubated at different temperatures from 15 to 37 °C.

Expression and Purification of Recombinant Prp22 Protein—Plasmid pET16b-PRP22 expresses an N-terminal His-tagged version of wild type Prp22 in bacteria under the control of a T7 promoter (13). Here we constructed pET-based plasmids for expression of His-tagged Prp22 mutants F697T, T757A, I764A, and T765A. The expression plasmids were transformed into Escherichia coli strain BL21-Codon Plus (DE3) (Stratagene). Cultures were inoculated from single colonies of freshly transformed cells and maintained in logarithmic growth at 37 °C in LB medium containing 0.1 mg/ml ampicillin to a final volume of 1 liter. When the A600 reached 0.6 to 0.8, the cultures were chilled on ice for 30 min and then adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside and 2% ethanol. The cultures were incubated for 17 h at 23 °C with constant shaking. Cells were harvested by centrifugation, and the pellets were stored at -80 °C.

All subsequent operations were performed at 4 °C. The cell pellets were suspended in 100 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 and then adjusted to 0.1% Triton X-100. The lysozyme were sonicated to reduce viscosity, and insoluble material was removed by centrifugation for 30 min at 14,000 rpm in a Sorvall SS34 rotor. The solubility of the mutant proteins F697T, T757A, I764A, and T765A was similar to that of wild type Prp22.

The soluble lysate was mixed for 1 h with 10 ml of a 50% slurry of Ni2+-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A. The resin was washed and eluted by centrifugation in 40 ml of buffer A, and collected again by centrifugation. The washed resin was suspended in 40 ml of buffer A and poured into a column. Adsorbed proteins were eluted stepwise with 25, 100, and 500 mM imidazole in buffer B (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 10% glycerol). The elution profiles of recombinant Prp22 were monitored by SDS-PAGE of the column fractions. Wild type Prp22 and the mutated polypeptides were recovered predominantly in the 100 mM imidazole eluate (containing 5–8 mg of protein). Aliquots (160–200 μg) of the nickel-agarose preparations were applied to 4.8 ml of 15–30% glycerol gradients containing 250 mM NaCl, 50 mM Tris-HCl (pH 8.0), 2 mM DTT, 1 mM EDTA, 0.1% Triton X-100. The gradients were centrifuged for 47 hours rpm in a SW50 rotor. Fractions were collected from the tops of the tubes. The elution profiles of the Prp22 proteins were gauged by SDS-PAGE. Protein concentrations were determined by using the Bio-Rad dye-binding reagent with bovine serum albumin as the standard.

ATPase Assay—Reaction mixtures (20 μl) containing 40 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM MgCl2, 1 mM γ32P-ATP (0.5 μg of poly(A)), and Prp22 proteins as specified were incubated for 30 min at 30 °C. The reactions were stopped by the addition of 280 μl of a 5% (w/v) suspension of activated charcoal (Sigma) in 20 mM phosphoric acid. The samples were incubated on ice for 10 min, and the charcoal was recovered by centrifugation. 32P radioactivity in the supernatant was determined by using the Bio-Rad assay (13). The results are representative of at least 5–10 experiments from duplicate reaction mixtures with a deviation of less than 10%.

Helicase Assay—To prepare the RNA/DNA hybrid substrate, an in vitro synthesized 79-mer RNA molecule (5'-CGCAAUUGGGCCCCUCUCUGAGUCAUCCUGACCGCCACGUGAAGGUAUGCAGC-3') was annealed to a 32P-labeled DNA oligonucleotide (40-mer) complementary to the RNA (underlined) using a 3:1 molar ratio of the DNA to RNA. The RNA/DNA hybrid substrate was then purified by native gel electrophoresis. Radiolabeled double-stranded RNA substrate was prepared as described previously (13). The helicase assays were performed as described previously (13).

Pre-mRNA Splicing in Vivo—Yeast whole cell extract from strain BY2168 was prepared using the liquid nitrogen method (43). The extract was immunodepleted using polyclonal, affinity-purified anti-Prp22 antibodies. Splicing reaction mixtures (30 μl) contained 50% (v/v) of the depleted extract, 10 μl of the reaction buffer (25 mM Tris-HCl, (pH 8.0), 10 mM MgCl2, 5 mM KCl), 0.05 mM [γ-32P]-labeled actin precursor RNA, 60 mM potassium phosphate, 3% (w/v) PEG8000, 2.5 mM MgCl2, and 2 mM ATP. The reaction mixtures were incubated for 15 min at 23 °C, and then 4–5 μg of native or mutant Prp22 protein was added and incubation continued for 15 min at 30 °C.
halted by transfer to ice. For analyses of spliceosome disassembly, the reaction volumes were increased to 100 μl, and the amount of Prp22 protein added was ~200 ng. Reaction mixtures were layered onto 15–40% glycerol gradients containing 20 mM HEPES, pH 6.5, 100 mM KCl, 2 mM MgCl₂. The gradients were 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 tubes. RNA was recovered by phenol extraction and ethanol precipitation. RNA from alternate fractions was analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea in 1× TBE. Radiolabeled RNA was visualized by autoradiographic exposure of the dried gel.

**Extragenic Suppressors of the prp22-S635A cs Phenotype—Spontaneous suppressors were selected as described (24).** In brief, 80 independent transformants were selected at 37 °C and incubated at temperatures from 15 to 37 °C. Eight strains that grew at 30 °C were transformed with a wild type genomic library on 2-μm YEp24 plasmids. 5-FOA survivors were patched to YPD agar and incubated at 30 °C. Selection of suppressing mutations was linked to the original prp22 allele. Each of the eight strains, that grew at 30 °C, the suppressing mutation was linked to the original prp22 allele. The genotype of the strain used for testing prp8 mutant alleles by the plasmid shuffle procedure was MATa ura3 ade2 trp1 his3 leu2 can1 prp22 (prp8::ura3/S635A prp8::H606A/shm1-1 prp8:: R538A). The same plasmids were transformed into strain YBST3 (prp8::URA3 CEN URA3), generating 2-μm yeast strain W303 by insertion of the wild type PRP22 (24) to yield p360-PRP22/PRP8. In this plasmid, both wild type genes are expressed from their natural promoters in a tail-to-tail configuration.

### RESULTS

**Conserved Residues in Motifs Ia, IV, and V Are Important for Prp22 Function in Vivo—**Prp22 (466–1145) encompasses an autonomous catalytic ATPase/helicase domain that includes the seven defining motifs of the DEAH box protein family (34). The importance of motifs I, II, III, and VI for NTP hydrolysis and RNA unwinding has been demonstrated for the spliceosomal DEAH proteins and several viral DEXH box RNA helicases (5, 22, 23, 33, 45, 46). However, the roles of motifs Ia, IV, and V have not been analyzed in depth. In order to assess the significance of these motifs for Prp22 function, we substituted 16 individual amino acids by alanines (Fig. 1) and tested the function of the mutants in vivo using the plasmid shuffle procedure. We found that the prp22 alleles T757A, I764A, and T765A (motif V) failed to complement the prp22 mutation at all temperatures tested between 15 and 37 °C (Fig. 2). Mutants R538A (motif Ia), F697A (motif IV), and N758A, E761A, and S763A (motif V) exhibited cold-sensitive growth defects of varying magnitude. A strain with the prp8 and prp22 genes was both disrupted was generated by mating, sporulation, and dissection. The prp22Δ prp8Δ strain YBS229 (MATa ura3 ade2 trp1 his3 leu2 can1 prp22::LEU2 prp8::kanMX) depends on the plasmid p360-PRP22/PRP8 (URA3 CEN) for viability. Transformation of YBS228 with either p358-PRP22/PRP8 (TRP1 CEN) or prp8::PRP8 (His3 CEN) alone did not yield viable cells on 5-FOA medium; cotransformation with both genes was necessary for growth of the prp22Δ prp8Δ cells on 5-FOA.

**Targeted Mutagenesis of Prp8—**Mutations were introduced into the prp8 gene using the two-step PCR overlap extension method. Residues targeted for Ala substitution were Tyr-1751, Arg-1753, Lys-1755, and Lys-1756. We also replaced Arg-1753 by Lys, Gln, and Glu and Lys-1756 by Glu. A BsEI fragment (2.1 kb) containing the mutation was exchanged for the wild type fragment in pRS-PRP8. The plasmids were sequenced to confirm the desired mutation and exclude the introduction of unwanted mutations during amplification and cloning.

**Suppression of Helicase-defective Prp22 Mutants**

| Prp22 | prp8 | prp22 | prp22 |
|-------|------|-------|-------|
|       |       |       |       |
|       |       |       |       |

**Suppression of Cold-sensitive and Lethal prp22 Mutant Alleles by shm1-1 CEN TRP1 carrying either PRP22 or the cold-sensitive prp22-Ala alleles under control of the natural PRP22-promoter were introduced into YBDST4 and YBDST5. Trp⁺ transformants were selected and streaked to 5-FOA medium at 30 and 37 °C to eliminate the URA3 prp22-S635A plasmid. 5-FOA survivors were patched to YPD agar plates and incubated at temperatures from 15 to 37 °C.**

**Cloning of SHM1 by Complementation—**prp22-H606A/shm1-1 cells were transformed with a wild type genomic library on 2-μm URA3 (in YEP24). Ura⁺ transformants were selected at 37 °C. The plasmids were isolated from the individual strains, amplified in E. coli, and re-tested to confirm their ability to complement the ts phenotype of prp22-H606A/shm1-1 cells. A total of 7 clones that re-tested were analyzed by restriction analysis and sequencing. The 2-μm plasmids contained either PRP22 or PRP8.

A DNA fragment (~8.3 kb) encompassing the PRP8 coding region plus 718 bp of upstream and ~400 bp of downstream sequence was inserted into the yeast plasmids pSE360 (CEN URA3), pSE358 (CEN TRP1), and pRS413 (CEN HIS3) to yield p360-PRP8, p358-PRP8, and pRS-PRP8. The same PRP8 fragment was also inserted into p360-PRP22 (24) to yield p380-PRP22/PRP8. In this plasmid, both wild type genes are expressed from their natural promoters in a tail-to-tail configuration.

**Prp22 Function in Vivo**

**Fig. 1.** A schematic drawing of Prp22 indicating conserved motifs I–VI as boxes. Sequences of N-terminal segments of Prp22 proteins from S. cerevisiae (Sc), S. pombe (Sp), human (Hu), and D. melanogaster (Dm) are aligned. Residues that were targeted by alanine replacement in pairs of two are indicated by dots. Below the schematic drawing of Prp22, the sequences of motifs Ia and II–V of Prp22 are aligned with those of Prp16, Prp2, and Prp43. Amino acid residues that were individually replaced by alanine in Prp22 are indicated by asterisks and carets indicate previously identified lethal or cold-sensitive prp22-Ala mutants, respectively.

| Sp | 335–TSPEIWELQLAASGAIAATDIPLEN | 374–EDEDVEIEILR–EEBEPFL | 494–SYGKRRTLSMKRQREGL |
| Hu | 395–SDPEKELWIKMAANVLSKEEPFDE | 432–EBEDDELEIEVL–EEBEPFL | 549–SYGKTQGSMFILQREGL |
| Dm | 417–SPERWEIRKMQSISGVRDENSEMPFDE | 453–DDEADIRIEIV–EEBEPFL | 570–SFPGKTDLTLVQERQGL |
| Sc | 292–TPERWEIRQLAASGAIDQYDELPKLD | 354–DTEIDEDVNTTDGGPKFL | 467–SYGKRFRPLTSAQROTLP |
Suppression of Helicase-defective Prp22 Mutants

| Prp22 Mutant | Growth |
|--------------|--------|
|              | 15°    | 19°    | 30°    | 37°    |
| T534A        | +++    | +++    | +++    | +++    |
| Q535A        | +++    | +++    | +++    | +++    |
| P536A        | +++    | +++    | +++    | +++    |
| R537A        | +++    | +++    | +++    | +++    |
| R538A        | +      | +      | +++    | +++    |
| L695A        | +++    | +++    | +++    | +++    |
| F697A        | -      | -      | -      | +++    |
| T699A        | +++    | +++    | +++    | +++    |
| G700A        | +++    | +++    | +++    | +++    |
| T757A        | -      | -      | -      | -      |
| N758A        | -      | +      | +++    | +++    |
| E761A        | -      | -      | +      | +++    |
| S763A        | +      | +++    | +++    | +++    |
| I764A        | -      | -      | -      | -      |
| T765A        | -      | -      | -      | -      |

Fig. 2. In vivo function of the various Prp22 mutants was tested using the plasmid shuffle procedure. Mutants that did not form colonies on 5-FOA medium at temperatures ranging from 15 to 37 °C are indicated by −. The 5-FOA-resistant strains were streaked on YPD agar, and growth was scored after 4 (30 and 37 °C), 6 (19 °C), and 8 days (15 °C). + + + indicates growth comparable with wild type Prp22 cells; + + indicates reduced growth; + signifies that only pinpoint colonies formed and mutant cells that did not form colonies are scored as −.

ATP Hydrolysis by Mutant Prp22 Proteins—To examine the biochemical basis for the growth defects of the lethal and most severe cs mutants, we produced recombinant His10-tagged Prp22 mutant proteins F697A, T757A, T764A, and T765A in bacteria and purified them from soluble bacterial lysates by nickel-agarose chromatography and glycerol gradient sedimentation, in parallel with wild type Prp22. The peak glycerol gradient fractions were nearly homogeneous with respect to the 130-kDa Prp22 protein (Fig. 3A). The proteins were assayed for ATP hydrolysis in the presence and absence of poly(A). The extent of ATP hydrolysis during a 30-min reaction at 30 °C was proportional to the amount of input Prp22 (Fig. 3B). The turn-over numbers for ATP hydrolysis with and without poly(A), calculated from the slopes of protein titration curves, are listed in Fig. 3C. The extent of ATP hydrolysis was near 10-fold at 30 °C.

The ATPase activity of wild type Prp22 was stimulated 12-fold by poly(A) (Fig. 3C). I764A and F697A were stimulated by RNA to similar extents (9–10-fold). However, ATP hydrolysis by T765A was undetectable in the absence of poly(A), i.e. the T765A protein was strictly dependent on an RNA cofactor. In contrast, the Thr-757 to Ala change had no impact on basal RNA-independent ATP hydrolysis but abated the stimulation by RNA. These findings suggest that residues Thr-757 and Thr-765 in motif V play a role in linking ATP hydrolysis to the nucleic acid cofactor.

RNA Binding and Unwinding of Duplex RNA by Prp22 Mutants—To test mutational effects on Prp22 helicase activity, an RNA/DNA hybrid substrate containing single-stranded RNA overhangs and a 40-bp duplex region was incubated with wild type Prp22 and the F697A, T757A, T764A and T765A mutants in the presence of Mg2+ (data not shown). The products were analyzed by native PAGE after disruption of RNA-protein interactions by addition of SDS. The free [32P]-labeled RNA strand migrated faster than the RNA/DNA hybrid substrate (Fig. 4A, lane ΔT). Wild type Prp22 was active in displacing the RNA strand; 50% of the substrate was unwound upon incubation with 40 ng of Prp22 at 37 °C for 15 min. Unwinding was dependent on ATP and Mg2+ (data not shown). F697A, T757A, and T765A were inactive in disrupting the RNA/DNA duplex, and T764A showed weak helicase activity (~5% of wild type) (Fig. 4A). We also tested mutational effects on an RNA/RNA substrate with 3′-single-stranded tails (illustrated in Fig. 4B). This RNA/RNA substrate was unwound by wild type Prp22 but not by F697A, T757A, T764A, and T765A (data not shown).

In order to gauge mutational effects on RNA binding, a native gel mobility shift experiment was performed using the 3′-tailed RNA/RNA substrate as the ligand (Fig. 4B). Prp22 proteins were incubated with labeled RNA and ATP in the absence of Mg2+ (to prevent RNA unwinding), and the mixtures were analyzed by native PAGE. In the absence of protein, the [32P]-labeled RNA molecule migrated as a single species in the native gel (Fig. 4B, free RNA). Addition of increasing amounts of wild type or mutant Prp22 proteins resulted in the formation of RNA-protein complexes that migrated slower than the free RNA substrate. The appearance of two shifted bands may reflect the binding of one and two molecules of Prp22 to a single RNA molecule. The T757A-RNA and F697A-RNA complexes migrated slightly slower than the complexes formed by wild type, I764A, and T765A proteins. The reason for the difference in mobility is unclear. We conclude that the observed lack of helicase activity is not attributable to an inability of the Prp22 mutant proteins to bind to the RNA substrate.

The finding that T757A formed a stable RNA-protein complex was of particular interest in light of the fact that the ATPase activity of T757A was insensitive to stimulation by RNA (Fig. 3C). This may indicate that (i) the binding detected in the RNA mobility shift assay does not reflect the RNA/protein interaction that enhances ATP hydrolysis, or (ii) Thr-757 plays a role in coupling RNA binding to ATP hydrolysis.

Pre-mRNA Splicing Activity of the Prp22 Mutants—Prp22 is important for the second transesterification step and is essential for release of mRNA from the spliceosome (13). In order to determine the splicing activities of the mutant proteins, we depleted yeast whole cell extract of Prp22 and incubated the Δ22 extract with labeled actin precursor RNA for 15 min at room temperature. To aliquots of the reaction mixture, wild type or mutant Prp22 proteins were added, and the incubation was continued. The reaction products were analyzed directly by denaturing PAGE (Fig. 5A) and by sedimentation in a 15–40% glycerol gradient (Fig. 5B). In the absence of Prp22 protein, the lariat-intermediate and exon 1 products of the first transesterification reaction accumulated, and little mRNA was formed. Wild type Prp22 and each of the mutant proteins (F697A, T757A, I764A, and T765A) promoted the second transesterification step, as evinced by the production of mature RNA (Fig. 5A). To assess whether mRNA was released from the splicing
complex, we separated the products by glycerol gradient sedimentation, followed by denaturing PAGE analysis of the gradient fractions (Fig. 5B). Mature RNA that was formed in the presence of wild type Prp22 sedimented near the top of the gradient. In contrast, mRNA formed in the presence of F697A or T757A sedimented near the bottom of the gradient, indicating that the spliced mRNA was retained within the spliceosome (Fig. 5B). Similar results were obtained for I764A and T765A (data not shown). Thus, although each of the mutant proteins sufficed for step 2 complementation, they did not catalyze mRNA release. In the case of T757A, the splicing defect was most likely attributable to a lack of ATPase activity, whereas the ATPase-proficient F697A, I764A, and T765A mutants were unable to couple ATP hydrolysis to a conformational rearrangement that leads to mRNA release.

Suppression of Helicase-defective Prp22 Mutants—To better understand how Prp22 elicits mRNA release, we sought to identify extragenic suppressors of helicase-defective Prp22 mutants. The rationale was that if Prp22 disrupts an RNA/RNA, RNA/protein, or protein/protein interaction, then a mutation in a spliceosomal component that weakens this interaction might rescue a helicase-defective Prp22 mutant. A screen for spontaneous suppressors of the cold-sensitive phenotype caused by the S635A mutation in motif III had yielded 18 intragenic suppressors and 8 extragenic suppressors (24). We provisionally named the extragenic suppressor genes **SHM1-8** (suppressor of helicase mutant).

We focused on **shm1-1**, a semi-dominant mutation that segregated 2:2 in genetic analyses (data not shown) and suppressed the growth defect of **prp22-S635A** cells at 30 °C (Fig. 6A). When the **prp22-S635A** allele was replaced by wild type **PRP22** (**CEN TRP1**), the **shm1-1** mutation conferred a slight temperature-sensitive phenotype, i.e. **PRP22/shm1-1** cells formed smaller colonies than **PRP22/SHM1** cells at 37 °C (Fig. 6B).

To test allele specificity of suppression, we replaced **prp22-S635A** in **prp22-S635A/shm1-1** cells by various other **prp22-Ala** mutant alleles. The **shm1-1** mutation suppressed the **cs** phenotypes of **prp22-N758A** and **prp22-E761A** at 19 °C (Fig. 6B). The permissive temperature for growth of the **prp22-F697A/shm1** strain was 37 °C, but **prp22-F697A/shm1-1** cells grew at 30 and at 25 °C (Fig. 6C). Remarkably, **prp22-I764A/shm1-1** cells formed colonies at 37 °C (Fig. 6C), showing that the **shm1-1** mutation suppressed the lethality of **prp22-I764A** (Fig. 2). In contrast, the lethal phenotype of **prp22-T765A** was not suppressed by the **shm1-1** mutation (data not shown).
shown). Note that the T765A protein, like I764A, was ATPase-proficient; however, its ATPase activity was strictly dependent on an RNA cofactor (Fig. 3).

We also tested whether shm1-1 could rescue the lethality of Prp22 mutants that were defective for ATP hydrolysis, including T757A (motif V), K512A (motif I), D603A (motif II), and Q804A (motif VI) (34). We found that shm1-1 did not suppress the lethality of ATPase-deficient prp22-Ala mutants, nor did it bypass the prp22 deletion (data not shown). These findings indicate that suppression occurs at the step during Prp22 function at which ATP hydrolysis is coupled to mechanical work.

The shm1-1 mutation suppressed the cs growth phenotype of prp22-H606A at 14 °C (Fig. 6D). However, the prp22-H606A/shm1-1 strain was unable to grow at 37 °C. Genetic analyses established that the ts phenotype of prp22-H606A/shm1-1 was recessive and cosegregated with the shm1-1 suppression phenotype (data not shown).

Cloning of SHM1—We exploited the recessive ts phenotype of prp22-H606A/shm1-1 to isolate SHM1 from a 2-μm plasmid library of wild type yeast genomic DNA. prp22-H606A/shm1-1 cells were transformed, and Ura+ transformants were selected at 37 °C. As expected, PRP22 was recovered multiple times. We also isolated clones containing the PRP8 gene, suggesting either that PRP8 is SHM1 or PRP8 is a dosage suppressor of the ts phenotype. In order to distinguish between these possibilities, we compared growth of prp22-H606A/shm1-1 cells that were transformed with PRP8 on CEN and 2-μm plasmids (Fig. 7A). PRP22 (URA3 CEN) and the URA3 CEN vector served as controls. PRP8 suppressed the growth defect of prp22-H606A/shm1-1 cells at 37 °C at low and high gene dosage. These findings suggested that PRP8 was SHM1.

The shm1-1/prp8) mutation suppressed the cs defect of prp22-H606A at 14 (Fig. 6D) and 19 °C (Fig. 7A). Suppression was semi-dominant insofar as provision of a PRP8 (CEN) plasmid only partially reversed growth of prp22-H606A/shm1-1 at 19 °C, whereas a PRP8 (2 μm) plasmid negated suppression by the shm1-1 allele (Fig. 7A). We sequenced the prp8 gene from the shm1-1 strain and found a single mutation resulting in an Arg-1753 to Lys coding change of the 2413-amino-acid Prp8 protein. Prp8 is highly conserved throughout its length from primitive eukaryotic organisms such as Giardia lamblia, to yeast, and human (47). A short segment of Prp8 encompassing Arg-1753 is aligned with the corresponding Prp8 sequences from diverse organisms (Fig. 7B).

The Single Amino Acid Change in Prp8-R1753K Is Necessary and Sufficient to Suppress Prp22-S635A—In order to test whether the change from Arg to Lys at position 1753 was sufficient for suppression, we generated a prp8Δ prp22Δ strain in which the chromosomal PRP8 and PRP22 genes were disrupted. Growth of prp8Δ prp22Δ was contingent on the presence of wild type PRP8 and PRP22 genes on URA3 CEN plasmids. prp8Δ prp22Δ cells were transformed simultaneously with a prp22-S635A (TRP1 CEN) and a prp8-R1753K (HIS3 CEN) plasmid. After 5-FOA selection against the PRP8 prp22 URA3 CEN plasmid, the cells were tested for growth at 37, 34, and 30 °C (Fig. 8A). The prp22-S635A prp8-R1753K cells grew at 30 °C, demonstrating that the prp8-R1753K mutation sufficed to suppress the cs phenotype of prp22-S635A. PRP8-R1753K also suppressed the growth defects of other helicase-defective Prp22 mutants (T637A, F697A, and I764A) and it rendered prp22-H606A unable to grow at 37 °C in the prp8Δ prp22Δ strain background (data not shown).

Arg-1753 is conserved in Prp8 from yeast to human with two reported exceptions; in Arabidopsis thaliana and in G. lamblia, a lysine occupies the equivalent position (Fig. 7B). In order to test whether suppression was specific to R1753K, we replaced Arg-1753 by Ala and Gin, and we also substituted conserved vicinal residues Tyr-1751, Lys-1755, and Phe-1756 by alanines. The PRP8 mutant alleles were introduced into prp8Δ prp22Δ together with prp22-S635A or with wild type PRP22. 5-FOA-resistant derivatives of these transformants were tested for growth at 30, 34, and 37 °C (Fig. 8). The cs phenotype of prp22-S635A was suppressed not only by prp8-R1753K but also by the R1753A and R1753Q mutations in Prp8. In contrast, prp8 alleles Y1751A and F1756A did not suppress prp22-S635A (Fig. 8A). K1755A exacerbated the phenotype of prp22-S635A, and cells carrying the prp8-K1755A and prp22-S635A alleles were inviable at temperatures ranging from 15 to 37 °C (data not shown). The prp8-K1755A allele was viable in combination with other prp22-Ala mutants, but it did not suppress their growth defects (data not shown). In combination with PRP22, the PRP8 mutant alleles Y1751A, K1755A, and F1756A grew

![Image](8622_Suppression_of_Helicase-defective_Prp22_Mutants.png)
as well as wild-type PRP8, whereas R1753A, R1753K, and R1753Q cells were ts at 37 °C (Fig. 8B). Reversing the charge of the Arg-1753 side chain by replacement with Glu resulted in a mutant strain that was inviable at 37 °C, and like the other mutations at position 1753, R1753E suppressed the cs phenotype of prp22-S635A cells (data not shown).
cells (data not shown). We conclude that alanine replacements of the targeted residues in the N-terminal segment of Prp22 are not deleterious to the function of the protein in vivo, although deletion of this segment causes lethality.

**DISCUSSION**

**Motifs IV and V of Prp22 Link ATP Hydrolysis to the RNA Cofactor**—Mutational and structural analyses of exemplary RNA helicases eIF4A, NS3, NPHII, and Prp22 have consistently shown that amino acids in motifs I, II, and VI are essential for NTP binding and hydrolysis (22, 23, 33, 45, 48, 49). Such studies have also shown that the hydroxyamino acids in motif III are involved in coupling ATP hydrolysis to RNA unwinding. Furthermore, mutations of His/Asp in the DEX(H/D) motif result in increased ATPase activity in the absence of a nucleic acid cofactor (22, 37, 45, 50). The roles of motifs Ia, IV, and V have received relatively little attention.

Here we report that alanine substitutions at three positions in motif V of Prp22 (757PNIAETS765) cause lethality that correlates with a failure of the mutant proteins to catalyze mRNA release and RNA unwinding. The T757A mutant retained basal ATPase activity but was not stimulated by RNA, whereas ATP hydrolysis by T765A became strictly dependent on the RNA cofactor. Thus, mutations in motif V do not impair the ability to hydrolyze ATP but rather affect the responsiveness to an RNA cofactor. Similar effects were observed for equivalent motif V mutations in the HCV NS3. In the crystal structure of NS3 bound to (dU)₈ oligonucleotide, the motif V Thr-411 that corresponds to Thr-757 of Prp22 donates a hydrogen bond to a phosphate oxygen of the nucleic acid (48). Replacing NS3 Thr-411 by Ala abolished helicase activity (51). Like the Prp22-T757A mutant described here, the NS3 T411A mutant retained basal ATPase activity but was not stimulated by nucleic acid (51).

Whereas the T411A mutation of NS3 motif V reduced RNA binding about 5-fold as measured by filter binding assays (51), Prp22 does not rely on residues in motif V for stable RNA binding; as measured by electrophoretic mobility shift experiments. This suggests that either a single mutation does not disrupt RNA binding or that Prp22 contains additional RNA-binding sites. Prp22 contains in its N terminus a so-called S1 motif that is dispensable for the functions of the protein in vivo and in vitro (34). We sought to test whether the S1 domain and motif V had redundant function with respect to RNA binding, by introducing motif V mutations into Prp22(262–1145). Deletion of the S1 domain did not alter the biochemical activities of motif V mutants (not shown), arguing against functional redundancy between motif V and the N-terminal portion of Prp22.

Motif IV (S467LVFLTG700 in Prp22) is only loosely conserved, but a phenylalanine is present in motif IV of most DE(X/H/D) proteins (17, 19). Substitution of this Phe in the yeast DEAD box splicing factor Prp28 by glycine or serine elicited ts and cs growth phenotypes (52). We show here that the Prp22 mutant F697A grows at 37 °C but is inviable at 30 °C. This cs phenotype and the biochemical properties of the F697A mutant (ATPase-proficient, helicase-deficient) are concordant with prior findings that Prp22-catalyzed mRNA release is a temperature-dependent process and that thermal energy may compensate for a deficiency in coupling ATP hydrolysis to a mechanical step that leads to mRNA release (37).

The role of motif Ia (S447QPRHRVA460) for Prp22 function is less clear. A V539I mutation had been shown to suppress the cold-sensitive phenotype of prp22-S635A; the secondary mutation revived the helicase activity of the S635A protein and concomitantly restored mRNA release activity (24). Although these results suggested a role for motif Ia in helicase function,
we find that alanine substitutions of individual amino acids did not impair the biological activity of Prp22, except for R538A, which caused a growth defect at 15 °C. This is in contrast to severe mutational effects in motif Ia of the DEH helicase NPHP III and the DEAD box ATPases eIF4A and Prp28 (23, 52, 53). Mutations in motif Ia (228PRI230) of vaccinia NPHP III have been analyzed in vitro and in vivo. These studies showed that R229A mutation is deleterious to ATPase and helicase activities, whereas substitution of Pro-228 elicits milder effects. Marker rescue experiments suggested that Arg-229 in motif Ia of NPHP III is essential for virus replication, whereas Pro-228 is not (23). In the DEAD box subfamily, the conserved peptide motif Ia is PTRELA (17, 19). Replacement of PTRELA by Prp8 by PRVRA abolishes ATP hydrolysis and RNA unwinding activity of the mouse eIF4A protein (55). In the yeast DEAD box splicing factor Prp28, mutation of Arg-264 in motif Ia (262PTRELA267) to Glu and Asp caused lethality (52). Prp22 is apparently less reliant on motif Ia than are other DEH(D) ATPases.

**Suppression of Helicase-deficient Prp22 Mutants by Prp8**—We report that a mutation in Prp8 (R1753K) suppresses the growth phenotypes of several Prp22 mutants. Prp8-R1753K does not bypass the requirement for the Prp22 gene product, which is sensible, given that Prp22 not only affects mRNA release, but has an additional ATP-independent role during the second transesterification step (13). In contrast, the essential functions of the DEAD proteins Prp28 and Sub2 can be bypassed in vitro (54, 55). Prp28 becomes dispensable when U1-C, a protein that binds to the 5′ splice site, is mutated or when the U1–5′ splice site pairing is weakened (54). Deletion of the non-essential Mud2 renders Sub2 dispensable, and sub2Δ mud2Δ cells are viable (55). These studies suggest that the essential functions of Prp28 and Sub2 are to counteract RNA-binding proteins and thus enable conformational switches at the 5′ splice site and branch point, respectively (3, 56).

Prp8-R1753K specifically suppressed Prp22 mutants that are ATPase-proficient but impaired in their ability to unwind synthetic duplex RNA substrates in vitro. Neither ATPase-deficient Prp22 mutants nor the ATPase-proficient T765A mutant that is strictly dependent on an RNA cofactor for ATP hydrolysis are revived by Prp8-R1753K. These findings are consistent with the idea that Prp22 normally uses the energy of ATP hydrolysis to effect a conformational change and thereby mRNA release, and that this conformational step is eased by the mutation in Prp8.

Changing Arg-1753 in Prp8 to either Lys, Ala, Glu, or Gln allows suppression of helicase-defective Prp22 mutations. Every Prp8-R1753K mutant tested exhibits a temperature-sensitive growth phenotype in a prp22 strain, suggesting that Arg-1753 of Prp8 makes a stronger contact to RNA or protein than does Ala, Glu, or Gln. We hypothesize that Prp22 normally uses the energy of ATP hydrolysis to effect a conformational change and thereby mRNA release, and that this conformational step is eased by the mutation in Prp8.

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