The essential Saccharomyces cerevisiae PRP22 gene encodes a 1145-amino acid DEXH box RNA helicase. Prp22p plays two roles during pre-mRNA splicing as follows: it is required for the second transesterification step and for the release of mature mRNA from the spliceosome. Whereas the step 2 function of Prp22p does not require ATP hydrolysis, spliceosome disassembly is dependent on the ATPase and helicase activities. Here we delineate a minimal functional domain, Prp22p(262–1145), that suffices for the activity of Prp22p in vivo when expressed under the natural PRP22 promoter and for pre-mRNA splicing activity in vitro. The biologically active domain lacks an S1 motif (residues 177–256) that had been proposed to play a role in RNA binding by Prp22p. The deletion mutant Prp22p(351–1145) can function in vivo when provided at a high gene dosage. We suggest that the segment from residues 262 to 350 enhances Prp22p function in vivo, presumably by targeting Prp22p to the spliceosome. We characterize an even smaller catalytic domain, Prp22p(466–1145) that suffices for ATP hydrolysis, RNA binding, and RNA unwinding in vitro and for nuclear localization in vivo but cannot by itself support cell growth. However, the ATPase/helicase domain can function in vivo if the N-terminal region Prp22p(1–480) is co-expressed in trans.

The Saccharomyces cerevisiae PRP22 gene encodes an essential 130-kDa protein that plays two roles in pre-mRNA splicing. Prp22p promotes the second transesterification reaction, which entails attack of the 3’-OH of the exon on the 3’ splice site, and it then catalyzes the ATP-dependent release of mature mRNA from the spliceosome (1–4). Prp22p is a member of the DEXH box family of nucleic acid-dependent NTPases, which is defined by a set of six conserved motifs that are located in the central portion of Prp22p. The central NTPase region is flanked by a 505-amino acid segment N-terminal to motif I (GETGSGKT) and a 334-amino acid domain C-terminal to motif VI (QRKGRAGR) (Fig. 1). Three other yeast DEXH box splicing factors, Prp2p, Prp16p, and Prp43p, are organized likewise and share extensive sequence similarity in their central and C-terminal portions (5–7). Prp22p, Prp2p, and Prp16p are RNA-dependent ATPases. Prp22p and Prp16p, but not Prp2p, can utilize the energy of ATP hydrolysis to unwind duplex RNA (2, 3, 8–10).

ATP hydrolysis by Prp2p, Prp16p, and Prp22p drives sequential steps in the splicing pathway. Prp2p propels the first transesterification step, Prp16p the second transesterification step, and Prp22p the disassembly of the spliceosome (11). Prp43p may also participate in spliceosome disassembly (7).

The segments flanking the NTPase region of DEXH proteins are likely to contribute to their biological specificity, e.g. by facilitating protein-RNA or protein-protein interactions within the spliceosome. Deletion analysis of Prp16p showed that its unique N-terminal segment is important for Prp16p function in vivo and in vitro (12, 13). The N terminus of Prp16p appears to play a role in binding Prp16p to the spliceosome (13).

The ATPase and helicase activities are required for the function of Prp22 in pre-mRNA splicing. Alanine substitutions in motifs I–III and VI abolish ATP hydrolysis or uncouple ATP hydrolysis from duplex unwinding. The ATPase-defective Prp22 mutants are lethal in vivo and inactive in spliceosome disassembly in vitro, as are Prp22 mutants that retain ATPase activity but are helicase-defective (2–4). However, nothing is known about the role of the N- and C-terminal extensions that flank the conserved ATPase/helicase region. Of particular interest is the N-terminal segment, which contains an “S1 motif” spanning amino acids 177–256 (1). The S1 motif, which is encountered in a wide variety of RNA-associated proteins, was originally noted in the ribosomal S1 protein from Escherichia coli (14). It has been suggested that the S1 motif in Prp22p constitutes an RNA binding domain that may be important for the function of Prp22 in pre-mRNA splicing (1).

Here we examine the effects of several N-terminal deletions on Prp22p function in vivo and in vitro. We delineate a minimal functional domain, Prp22p(262–1145), that lacks the S1 motif yet suffices for the activity of Prp22p in vivo and for pre-mRNA splicing activity in vitro. We characterize an even smaller catalytic domain, Prp22p(466–1145), that suffices for ATP hydrolysis and RNA unwinding in vitro and for nuclear localization in vivo. We find that the segment 262–350, located upstream of the catalytic domain, enhances Prp22p function in vivo by targeting Prp22p to the spliceosome.

**EXPERIMENTAL PROCEDURES**

**Deletion Mutants of PRP22**—N-terminal deletion mutants were generated by PCR amplification, using oligonucleotide primers that either introduced NdeI restriction sites at the codons for Met-202, Met-385, Met-447, and Met-530 or introduced NdeI sites and methionine codons in lieu of the codons for Gla-50, Ser-109, Lys-261, Gln-301, Glu-350, Ile-421, Ser-465, and Asn-499. NdeI-XhoI fragments of the PCR-amplified DNA fragments were inserted into p538-PRP22 (CEN TRP1) in place of the wild-type NdeI-XhoI fragment. In this plasmid the Prp22 deletion mutants Δ50, Δ109, Δ201, Δ261, Δ301, Δ350, Δ384, Δ421, Δ446, Δ465, and Δ499 were under the control of the natural PRP22 promoter. The inserts were sequenced completely in order to exclude the acquisition of unwanted mutations during amplification and cloning. The open reading frames encoding wild-type and N-terminal deletion variants...
Δ301, Δ350, Δ384, Δ421, Δ446, and Δ465 were inserted into the multi-copy vector pYX322 (2 μm TRP1). In this plasmid, expression of PRP22 was driven by the strong constitutive TRP1 promoter.

**Test of Mutational Effects on PRP22 Function in Vivo by Plasmid Shuffling—** Viability of the prp22 strains was tested in SC-Ura medium. Colonies were streaked to agar plates containing 0.75 mg/ml 5-foorotic acid (5-FOA) to select against the URA3 plasmid. The ability of the mutant PRP22 alleles to support growth on 5-FOA was tested at 26 °C and 30 °C. After overnight incubation at 30 °C, colonies were replica-plated onto YPD medium and incubated at different temperatures from 14 to 37 °C.

**GFP Fluorescence—** The coding sequence for green fluorescent protein was amplified by PCR and inserted into pYX132 (TRP1 CEN) so that its expression is driven by the TRP1 promoter. An NdeI site was introduced at the end of the coding sequence to allow insertion of DNA fragments for in-frame fusions to GFP. The resulting plasmid is p132-GFP (TRP1 CEN). The coding sequences for PRP99 and N-terminal deletion variants were fused to the 3′ end of the GFP sequence. Wild-type PRP22 plasmid. The ability of the mutant PRP22 alleles to support growth on 5-FOA was tested at 26 °C and 30 °C. After overnight incubation at 30 °C, colonies were replica-plated onto YPD medium and incubated at different temperatures from 14 to 37 °C.

**Functional Domains of the Yeast Splicing Factor Prp22p**

Wild-type Prp22p and the Prp22 mutants were recovered predominantly in the 300 mM NaCl eluate. Aliquots (160–200 μl) of the phosphocellulose protein preparations were applied to 4.8 ml of 5-FOA to select against the URA3 plasmid. The ability of the mutant PRP22 alleles to support growth on 5-FOA was tested at 26 °C and 30 °C. After overnight incubation at 30 °C, colonies were replica-plated onto YPD medium and incubated at different temperatures from 14 to 37 °C.

**Pre-mRNA Splicing in Vivo—** Yeast whole cell extract from strain BJ2168 was prepared and incubated at 37 °C in 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM MgCl2, 1 mM γ-[32P]ATP, 0.5 μg of poly(A), and Prp22 proteins as specified were incubated for 30 min at 30 °C. The reactions were halted 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 quantitated by liquid scintillation counting. The results are average values from duplicate reaction mixes with a deviation of less than 5%.

**RNP Binding Assay—** Reaction mixtures (20 μl) contained 40 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM MgCl2, 1 mM γ-[32P]ATP, 0.5 μg of poly(A), and Prp22 proteins as specified were incubated for 30 min at 30 °C. The reactions were halted 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 quantitated by liquid scintillation counting. The results are average values from duplicate reaction mixes with a deviation of less than 5%.

**Pre-mRNA Splicing in Vivo—** Yeast whole cell extract from strain BJ2168 was prepared and incubated at 37 °C in 50 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM MgCl2, 1 mM γ-[32P]ATP, 0.5 μg of poly(A), and Prp22 proteins as specified were incubated for 30 min at 30 °C. The reactions were halted 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 quantitated by liquid scintillation counting. The results are average values from duplicate reaction mixes with a deviation of less than 5%.
**RESULTS**

**Mutational Analysis of the S1 Motif—**Prp22p contains near its N terminus an S1 motif (aa 177-256), which is found in many RNA-associated proteins. The S1 motif is conserved in the N termini of Prp22p homologues from *S. pombe*, *Caenorhabditis elegans*, and human (19-21). The solution structure of the S1 RNA binding domain from *E. coli* polynucleotide phosphorylase (EcoS1) and ribosomal S1 protein from *E. coli* (EcoS1). The asterisks above the Prp22 sequence indicate residues that were substituted by alanines. YBST1 was transformed with the plasmid shuffle procedure. Deletion of 50, 109, 201, and 261 amino acid residues from the N terminus did not affect the biological activity of Prp22p as the deletion mutants all formed colonies on 5-FOA medium (Fig. 1), and they grew as well as wild-type cells on YPD medium at 14, 25, 30, 34, and 37 °C (not shown). Thus, elimination of the S1 motif had no effect on Prp22p function in vivo.

**Deletion alleles prp22Δ301 and prp22Δ350, which were lethal when expressed from the natural PRP22 promoter on a CEN plasmid, could complement the prp22Δ strain when provided on a 2-μm plasmid under the transcriptional control of the strong TPI1 promoter. The more extensively truncated mutants prp22Δ384, prp22Δ421, prp22Δ446, and prp22Δ465 failed to sustain growth of prp22Δ on 5-FOA even when the mutants were expressed at high gene dosage (not shown).**

**Determinants of Nuclear Localization of Prp22p—**The green fluorescent protein (GFP) was fused to the N terminus of wild-type Prp22p and truncated Prp22 variants. The GFP-PRP22 alleles were placed on CEN TRP1 plasmids under the control of the TPI1 promoter and then tested for complementation of the prp22Δ strain. The fusion of GFP to full-length Prp22p was functional in vivo as was GFP-Prp22Δ350. In contrast, GFP-Prp22Δ384, GFP-Prp22Δ421, GFP-Prp22Δ446, and GFP-Prp22Δ465 were unable to sustain growth of prp22Δ cells (data not shown).

Fluorescence microscopy showed that GFP itself was distributed throughout the cell (Fig. 2). However, when GFP was fused to wild-type Prp22p, the fluorescence was concentrated in the nucleus. Truncated variants GFP-Prp22Δ350 and GFP-Prp22Δ446 were also localized to the nucleus (Fig. 2) as was GFP-Prp22Δ465 (not shown). The GFP fluorescence coincided with 4,6-diamidino-2-phenylindole staining of DNA (not shown). These findings show that (i) a nuclear localization (and/or retention) signal resides in Prp22(466–1145) and (ii) the lethality of the truncated alleles Δ384, Δ421, Δ446, and Δ465 is dependent on a (and/or retention) signal residing in Prp22(466–1145) and (ii) the lethality of the truncated alleles Δ384, Δ421, Δ446, and Δ465 is dependent on a nuclear targeting signal residing in Prp22(466–1145) and (ii) the lethality of the truncated alleles Δ384, Δ421, Δ446, and Δ465 is dependent on a nuclear targeting signal residing in Prp22(466–1145).

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**Fig. 1. The minimum functional domain of Prp22p.** The 1145-amino acid Prp22p is depicted as a horizontal bar. The ATPase/helicase motifs I–VI, which are conserved among members of the DEXH box family, are indicated as blocks. The S1 motif spans residues 177–256, and a segment of this region in Prp22p is aligned with S1 motifs from polynucleotide phosphorylase (EcoPNP) and ribosomal S1 protein from *E. coli* (EcoS1). The asterisks above the Prp22 sequence indicate residues that were substituted by alanines. YBST1 was transformed with TPI1 plasmids containing the indicated alleles encoding full-length (WT) and N-terminal truncation mutants (the designations refer to the number of residues that are deleted from the N terminus). The PRP22 alleles were either expressed from the natural PRP22 promoter on CEN plasmids or from the TPI1 promoter on 2-μm plasmids. Trp⁺ transformants were selected and then streaked to medium containing 5-FOA. Growth was scored as + and failure to form colonies after 10 days is indicated by −.

| PRP22 allele | Growth | ATPase/helicase |
|--------------|--------|----------------|
| WT           | +      | I, II, III, IV, V, VI |
| Δ50          | +      | II, III, IV, V, VI |
| Δ109         | +      | I, II, III, IV, V, VI |
| Δ201         | +      | I, II, III, IV, V, VI |
| Δ261         | +      | I, II, III, IV, V, VI |
| Δ301         | −      | I, II, III, IV, V, VI |
| Δ350         | −      | I, II, III, IV, V, VI |
| Δ384         | −      | I, II, III, IV, V, VI |

GFP-Prp22(1–480) was excised and inserted into an ADE2 CEN plasmid (pSA360) to yield pSA/GPD-prp22(1–480). The DNA fragment encoding Prp22(466–1145) was inserted into pYN132 (TRP1 CEN). In the plasmid p132-Δ465, expression of prp22(466–1145) was driven by the TPI1 promoter. The prp22Δ strain was transformed with combinations of TRP1 and ADE2 plasmids as follows: PRP22, pSA360 and p358-PRP22 (TRP1 CEN); prp22(466–1145), pSA360 and p132-Δ465; prp22(1–480), pSA/GPD-prp22(1–480) and pYN132; prp22(466–1145) + prp22(1–480), p132-Δ465 and pSA/GPD-prp22(1–480). Trp⁺ Ade⁺ transformants were selected and analyzed by plasmid shuffle.

In order to assess the importance of these conserved residues for the biological function of Prp22p, we substituted Gly-183, Arg-186, Phe-191, Phe-194, His-210, Gly-229, Gln-240, and Lys-244 (Fig. 1) by alanine. The mutant PRP22—Ala alleles were cloned into CEN TRP1 plasmids, and their function was tested in vivo in a prp22Δ strain using the plasmid shuffle procedure. Growth of prp22Δ is dependent on a CEN URA3 PRP22 plasmid (4). Trp⁺ transformants were selected and then tested for growth on medium containing 5-FOA, a drug that selects against URA3. Wild-type PRP22 cells and the mutant strains G183A, R186A, F191A, F194A, H210A, H210A/G229A, and K244A grew under counterselection conditions. The 5-FOA survivors all formed colonies on YPD medium at 15, 25, 30, and 37 °C (data not shown), indicating that individual residues within the S1 motif of Prp22p are not important for growth. This finding prompted us to determine the minimal domain that was essential for Prp22p function.

**Prp22 Deletion Mutants Define a Minimum Essential Domain—**A series of N-terminal deletion mutants was designed to progressively truncate the 1145 amino acid Prp22 protein. The in vivo function of the truncated alleles, under the transcriptional control of the natural PRP22 promoter, was tested using the plasmid shuffle procedure. Deletion of 50, 109, 201, and 261 amino acid residues from the N terminus did not affect the biological activity of Prp22p as the deletion mutants all formed colonies on 5-FOA medium (Fig. 1), and they grew as well as wild-type cells on YPD medium at 14, 25, 30, 34, and 37 °C (not shown). Thus, elimination of the S1 motif had no effect on Prp22p function in vivo.

Deletion alleles prp22Δ301 and prp22Δ350, which were lethal when expressed from the natural PRP22 promoter on a CEN plasmid, could complement the prp22Δ strain when provided on a 2-μm plasmid under the transcriptional control of the strong TPI1 promoter. The more extensively truncated mutants prp22Δ384, prp22Δ421, prp22Δ446, and prp22Δ465 failed to sustain growth of prp22Δ on 5-FOA even when the mutants were expressed at high gene dosage (not shown).

**Determinants of Nuclear Localization of Prp22p—**The green fluorescent protein (GFP) was fused to the N terminus of wild-type Prp22p and truncated Prp22 variants. The GFP-PRP22 alleles were placed on CEN TRP1 plasmids under the control of the TPI1 promoter and then tested for complementation of the prp22Δ strain. The fusion of GFP to full-length Prp22p was functional in vivo as was GFP-Prp22Δ350. In contrast, GFP-Prp22Δ384, GFP-Prp22Δ421, GFP-Prp22Δ446, and GFP-Prp22Δ465 were unable to sustain growth of prp22Δ cells (data not shown).

Fluorescence microscopy showed that GFP itself was distributed throughout the cell (Fig. 2). However, when GFP was fused to wild-type Prp22p, the fluorescence was concentrated in the nucleus. Truncated variants GFP-Prp22Δ350 and GFP-Prp22Δ446 were also localized to the nucleus (Fig. 2) as was GFP-Prp22Δ465 (not shown). The GFP fluorescence coincided with 4,6-diamidino-2-phenylindole staining of DNA (not shown). These findings show that (i) a nuclear localization (and/or retention) signal resides in Prp22(466–1145) and (ii) the lethality of the truncated alleles Δ384, Δ421, Δ446, and Δ465 is dependent on a nuclear targeting signal residing in Prp22(466–1145).
Functional Domains of the Yeast Splicing Factor Prp22p

**Fig. 2. Localization of GFP-Prp22p.** Green fluorescent protein (GFP) was fused to the N terminus of wild-type Prp22p (GFP-Prp22) and the truncated variants Δ350 (GFP-Δ350) and Δ446 (GFP-Δ446). The right panel shows GFP fluorescence, and the left panel shows the outline of the same cells with Nomarski optics. The photographs were taken with a Nikon SPOT digital camera at ×1,000 magnification.

Δ465 is not attributable to the failure of nuclear localization of the truncated polypeptides but most likely to a functional defect of these proteins.

**Dominant-negative Growth Inhibition by ATPase-defective Mutants**—Previous work showed that ATPase-defective Prp22 proteins inhibit growth of wild-type cells when they are overexpressed (4). The dominant-negative effects arise because ATPase-defective mutant proteins bind to spliceosomes and compete with wild-type Prp22p but then fail to release mature RNA. We reasoned that introduction of the ATPase-inactivating D603A mutation (in motif II, the DEAH box) into Δ261 and Δ350 alleles might uncover effects of the N-terminal region on spliceosome binding. The Prp22 genes were cloned into CEN TRP1 plasmids under the transcriptional control of a GAL1 promoter and then introduced into wild-type PRP22 cells. Trp+ transformants were selected and streaked on agar medium containing either glucose or galactose (Fig. 3). All of the strains grew on glucose-containing medium as determined by Western blotting using polyclonal Prp22-specific antibodies (data not shown). We infer from these findings that Δ261-D603A was capable of competing with wild-type Prp22p. However, Δ350-D603A was less effective, suggesting a role for residues 262–350 in the interaction of Prp22p with the spliceosome. This finding agrees with the observed requirement for increased expression of Δ350 for cell growth (Fig. 1).

**Effect of N-terminal Deletions on Prp22 Function in Pre-mRNA Splicing**—We assayed the deleted Prp22 proteins for their ability to splice actin pre-mRNA in vitro. Prp22p and the truncated variants were expressed in bacteria as His-tagged fusions (2, 4). The proteins were purified from soluble lysates by Ni²⁺-nitrilotriacetic acid-agarose and phosphocellulose chromatography and glycerol gradient sedimentation.

SDS-PAGE gel analysis of the peak glycerol gradient fractions showed that the protein preparations were substantially pure (Fig. 4A).

We tested whether the truncated Prp22 proteins were capable of complementing the step 2 defect in extracts depleted of Prp22p (Fig. 4B). 32P-Labeled actin pre-mRNA was incubated in depleted extract to allow for spliceosome assembly and step 1 transesterification. Aliquots of the reaction mixture were then supplemented with buffer, wild-type Prp22p, or the mutant proteins Δ261, Δ301, Δ350, Δ384, Δ421, or Δ446. After 15 min at 30 °C, the RNAs were extracted, and the products analyzed by denaturing PAGE. In the absence of added protein, very little mature RNA was formed and lariat-exon 2 persisted. Wild-type Prp22p and the Δ261 protein both promoted step 2 efficiently. The more extensively truncated Prp22 mutants Δ301, Δ350, Δ384, Δ421, and Δ446 were inactive for step 2 complementation in vitro, i.e., lariat-exon 2 persisted and little mRNA was produced (Fig. 4B).

**Effect of N-terminal Deletion Mutants on Spliceosome Disassembly**—Prp22p is also important for the release of mature mRNA from the spliceosome (2). In order to test whether the truncated Prp22 proteins were active for mRNA release, we used a customized precursor RNA (Act7) that does not require the spliceosome still does require Prp22p (2). We incubated Act7 pre-mRNA substrate in Prp22-depleted extract and supplemented the reactions either with wild-type Prp22p, buffer, or the truncated polypeptides Δ261, Δ350, and Δ465. Mature mRNA was formed in every case (Fig. 5A). Aliquots of the reaction mixtures were sedimented in 15–40% glycerol gradients to assess whether mRNA was released from the spliceosome complex. Fractions were collected, and the RNA species in every odd fraction were analyzed by denaturing PAGE. The distribution of spliced RNA across each gradient from top to bottom is shown in Fig. 5B. When the reactions were carried out in the presence of wild-type Prp22p or the Δ261 mutant, released mature mRNA sedimented near the top of the gradient in fractions 7–11. In contrast, when Prp22p was omitted, mRNA sedimented in two peaks, one corresponding to the released mRNA (fractions 7–11) and a second heavier peak (fractions 19–23) corresponding to the spliceosomes. The same two-peak profile was observed when extracts were supple-

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*2. S. Schneider and B. Schwer, unpublished observations.*
Functional Domains of the Yeast Splicing Factor Prp22p

Fig. 4. N-terminal Prp22p deletion mutants. A, protein gel. Wild-type (WT) and the indicated mutant variants of Prp22p were expressed in bacteria and purified by Ni²⁺-nitrilotriacetic acid-agarose and phosphocellulose chromatography, followed by glycerol gradient sedimentation. One µg of the glycerol gradient preparations was separated 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, effects of N-terminal deletion mutants on actin pre-mRNA splicing in vitro. Prp22-depleted extract catalyzed the formation of lariat-exon 2 and exon 1 during 10 min of incubation at 23 °C. Aliquots of the reaction mixtures were supplemented with either buffer (−) or 5 ng of wild-type (WT) and the indicated truncated Prp22 polypeptides for 15 min at 30 °C. The reaction products were resolved by denaturing PAGE and visualized by autoradiography. The symbols at the left indicate the positions of the labeled RNA species (from top to bottom) as follows: lariat-exon 2; intron lariat, actin precursor RNA; mature spliced mRNA.

Fig. 5. Effects of Prp22p deletion mutants on the release of mature RNA from the spliceosome. Prp22-depleted extract was reacted with ACT7 pre-mRNA in the presence of wild-type Prp22p (WT), or the mutants Δ261, Δ350, and Δ465. A, aliquots of the reaction mixtures were analyzed by denaturing PAGE. B, the remainders were sedimented in 15–40% glycerol gradients. Fractions were collected from the tops of the gradients, and the RNA species from odd-numbered fractions were analyzed by denaturing PAGE. The amount of mature RNA in each fraction was quantitated using a PhosphorImager and is expressed as percent of total mRNA (sum of mRNA in all fractions). The % of mRNA in each fraction is plotted as a function of the fraction number (top to bottom).

ATP Hydrolysis by Truncated Prp22 Polypeptides—Spliceosome disassembly is dependent on ATP hydrolysis by Prp22p. To determine if loss of disassembly function was attributable to the effects of deletions on ATP hydrolysis, we measured the ATPase activities of the Prp22p deletion variants (Fig. 4). The proteins were incubated with 1 mM ATP in the presence of poly(A) homopolymer as the RNA cofactor for 30 min at 30 °C. The limits of ATP hydrolysis increased linearly with the amounts of Prp22 proteins added (Fig. 6A). Titrations of the Δ261, Δ350, Δ44, and Δ465 polypeptides indicated that the mutants were more active than the wild-type Prp22p (Fig. 6A). The turnover numbers are shown in Fig. 6B. Wild-type Prp22p hydrolyzed 43 and 400 ATP per min in the absence and presence of an RNA cofactor, respectively. The Δ446 mutant protein hydrolyzed 870 ATP per min in the presence of poly(A) and 128 without RNA cofactor. Further N-terminal deletions of Prp22p to positions 500 or 530 were insoluble when expressed in bacteria. These mutants were thus refractory to purification and biochemical analysis. We conclude that the N-terminal 465 amino acids of Prp22p are not part of the ATPase domain. Indeed, deletion of the N-terminal 465 aa results in increased ATPase activity with and without the poly(A) cofactor.

RNA Binding and Unwinding of Duplex RNA by Prp22-deletion Mutants—The ATPase activity of full-length and truncated Prp22p variants was stimulated by an RNA cofactor. We surmised that the proteins retained their capability to bind to RNA and to unwind duplex RNAs. In order to test this directly, a 3′-tailed RNA substrate containing a 25-bp duplex (Fig. 7A) was incubated with the Δ261 and Δ446 proteins in the absence of ATP. The mixtures were analyzed by native PAGE (Fig. 7B). In the absence of protein, the labeled RNA molecule migrated as a single species in the native gel. Addition of increasing amounts of Δ261 and Δ446 proteins resulted in the appearance of protein-RNA complexes of reduced mobility. We presume that the appearance of two shifted bands at higher protein concentrations reflects the sequential binding of one and two molecules of protein to a single RNA molecule. Note that the Δ261-RNA complex migrates more slowly than the complexes formed with Δ446 polypeptide, which we presume reflects the difference in size of the RNA-bound Prp22 proteins. Other truncated polypeptides of intermediate size formed protein-RNA complexes that migrated between the Δ261-RNA and Δ446-RNA complexes (data not shown).

The same duplex RNA substrate (Fig. 7A) was used to assess the helicase activity of the truncated Prp22 proteins. Substrate RNA was incubated with the Prp22p variants in the presence of ATP, and the products were analyzed by PAGE after disruption.
Functional Domains of the Yeast Splicing Factor Prp22

Prp22p variants.

The ATPase activity of wild-type (WT) and the truncated mutant versions (as indicated) of Prp22p was measured in the presence of poly(A) cofactor and is plotted as a function of input Prp22p (in ng). A, the ATPase activity of wild-type Prp22p, measured in the absence of the RNA cofactor, is also shown (WT(-RNA)). B, ATPase activities are expressed as turnover numbers (per min) for the various Prp22p variants.

A

B

C

FIG. 6. ATP hydrolysis by N-terminal deletion mutants of Prp22p. The ATPase activity of wild-type (WT) and the truncated mutant versions (as indicated) of Prp22p was measured in the presence of poly(A) cofactor and is plotted as a function of input Prp22p (in ng). A, the ATPase activity of wild-type Prp22p, measured in the absence of the RNA cofactor, is also shown (WT(-RNA)). B, ATPase activities are expressed as turnover numbers (per min) for the various Prp22p variants.

Prp22p

\[
\text{ATPase (min}^{-1})
\]

| Prp22p | -RNA | +RNA |
|--------|------|------|
| WT     | 43   | 400  |
| \(\Delta261\) | 43   | 460  |
| \(\Delta350\) | 41   | 500  |
| \(\Delta350\) | 51   | 500  |
| \(\Delta384\) | 79   | 580  |
| \(\Delta421\) | 142  | 700  |
| \(\Delta446\) | 128  | 870  |
| \(\Delta465\) | 101  | 630  |

all were active in RNA unwinding. Their helicase activities were dependent on ATP (data not shown). The helicase activities of truncated proteins (adjusted to measure equal molar amounts) were increased compared with wild-type Prp22p. This increase may be due, at least in part, to the higher ATPase activities of the Prp22 deletion mutants. We conclude that the N-terminal 465 amino acids of the Prp22 protein are not involved in ATP hydrolysis, RNA binding, and helicase activities.

Prp22(1–480) and Prp22(466–1145) Can Function in Trans to Support Cell Viability—Neither the ATPase/helicase domain Prp22(466–1145) nor the N-terminal polypeptide Prp22(1–480) were active in pre-mRNA splicing (Fig. 4B and not shown). As expected, Prp22(1–480) did not hydrolyze ATP (not shown). Neither Prp22(1–480) nor Prp22(466–1145) alone could support growth of prp22Δ cells even when the N- and C-terminal domains were overexpressed at high gene dosage (not shown and Fig. 8). However, the two segments Prp22(1–480) and Prp22(466–1145) did support growth of the prp22Δ strain when they were expressed in trans on different plasmid vectors (Fig. 8). The 5-FOA survivors were streaked to YPD medium at temperatures from 14 to 37 °C. The strain carrying the prp22(1–480) and the prp22(466–1145) alleles grew at 25, 30, and 34 °C, but the cells exhibited cold-sensitive and heat-sensitive growth phenotypes; they failed to grow at 14 and 19 °C and formed only pinpoint colonies at 37 °C. Wild-type PRP22 cells grew well at all temperatures. We conclude that the N- and C-terminal domains can function in trans to sustain cell growth at temperatures from 25 to 34 °C.

DISCUSSION

Prp22(466–1145) Constitutes an ATPase/Helicase Domain—Prior studies have shown that conserved residues in motifs I–III and VI are essential for the function of Prp22p in pre-mRNA splicing. Mutational analyses of DEX(H/D) RNA helicases, including Prp22p, vaccinia virus NPH-II, eIF-4A, and hepatitis C virus NS3 highlighted the importance of conserved amino acids in these motifs for NTP hydrolysis and duplex unwinding (2–4, 26–32). Structural studies of the NS3 helicase show that the catalytic core consists of three globular domains. Motifs I and II are located in domain 1 and motif VI in domain 2. Domains 1 and 2 are connected via a flexible linker segment that includes motif III (33, 34). Motif III (T/Y SAT) couples ATP hydrolysis to duplex unwinding (4, 27, 28, 30). Kim et al. (34) surmised from their crystal structure of NS3 that (i) ATP bridges domains 1 and 2 and (ii) a conformational change upon ATP hydrolysis leads to opening of the interdomain cleft and translocation of the protein along the polynucleotide.

Whereas the importance of the conserved motifs for ATPase/
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The Biologically Active Domains of Prp22p—The in vivo and in vitro activities of Prp22(261–1145) are indistinguishable from those of wild-type Prp22, demonstrating that the N-terminal 260 residues, which include an S1 motif (aa 177–256), are not essential for Prp22p function. However, the minimal ATPase/helicase domain Prp22(2466–1145) does not suffice for the role of Prp22p in pre-mRNA splicing, thereby indicating the importance of residues N-terminal to position 465 in Prp22p. However, Prp22p(466–1145) can function in vivo if Prp22(1–480) is co-expressed, demonstrating that the protein domains can act in trans. We suggest that both domains are required for the productive interaction of Prp22p with the splicing apparatus.

The inference that the N-terminal region in Prp22p from aa 262–465 is involved in spliceosome binding is based on the findings that deletion of 350 and 465 amino acids in the ATPase-deficient Prp22-D603A reduced and abolished, respectively, its effectiveness as a dominant-negative growth inhibitor, whereas Δ261-D603A was a potent inhibitor. Although the segment from aa 262 to 465 in Prp22p appears to be necessary, it is not sufficient to afford spliceosome binding because Prp22p(1–480) did not compete effectively with wild-type Prp22p. Overexpression of Prp22p(1–480) in wild-type Prp22 cells did not lead to growth inhibition, and preincubation of spliceosomes with Prp22p(1–480) protein did not prevent splicing by subsequently added Prp22p in vitro (not shown). Thus, Prp22p requires both the ATPase/helicase and a region within the N-terminal domain for spliceosome association. How the two domains cooperate to provide biological activity in trans remains to be investigated. It is possible that they interact directly, or indirectly via another splicing component, to provide a functional Prp22p protein. Alternatively, the N-terminal domain and the ATPase/helicase domain may bind independently to the splicing apparatus and effect splicing.

The DEAH box splicing factor Prp16p also requires its N terminus for spliceosome binding (13). Prp16p(1–300) alone is capable of binding to the spliceosome; however, the binding is stabilized by the C-terminal region (13). As is the case for Prp22p, the two domains of Prp16p do not need to be physically linked, and the N-terminal 300-aa segment functions in trans with Prp16p(301–1071) to sustain growth of a prp16Δ strain (13).

The DEAH box ATPases, Prp2p, Prp16p, and Prp22p act sequentially; each of the proteins associates with the spliceosome at a distinct stage during the splicing pathway and dissociates upon ATP hydrolysis (2, 9, 36). It will be interesting to determine whether each of these proteins makes similar contacts within the splicing apparatus and to analyze the nature of these contacts at the molecular level.

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