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Mutational Analysis of the Prt1 Protein Subunit of Yeast Translation Initiation Factor 3

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The *Saccharomyces cerevisiae PRT1* gene product Prt1p is a component of translation initiation factor eIF-3, and mutations in *PRT1* inhibit translation initiation. We have investigated structural and functional aspects of Prt1p and its gene. Transcript analysis and deletion of the *PRT1* 5 end revealed that translation of *PRT1* mRNA is probably initiated at the second in-frame ATG in the open reading frame. The amino acid changes encoded by six independent temperature-sensitive *prt1* mutant alleles were found to be distributed throughout the central and C-terminal regions of Prt1p. The temperature sensitivity of each mutant allele was due to a single missense mutation, except for the *prt1-2* allele, in which two missense mutations were required. In-frame deletion of an N-terminal region of Prt1p generated a novel, dominant-negative form of Prt1p that inhibits translation initiation even in the presence of wild-type Prt1p. Subcellular fractionation suggested that the dominant-negative Prt1p competes with wild-type Prt1p for association with a component of large Prt1p complexes and as a result inhibits the binding of wild-type Prt1p to the 40S ribosome.

Mutations in the *PRT1* gene of the yeast *Saccharomyces cerevisiae*, as exemplified by the temperature-sensitive *prt1-1* mutant allele, cause a conditional impairment of translation initiation in vivo (26, 27; see also reference 18); in vitro, *prt1-1* mutant cell extracts are defective for the interaction of the eIF-2 GTP Met-tRNA$_i$ ternary complex with the 40S ribosomal subunit (16), suggesting that the *PRT1* polypeptide (Prt1p) may be a component of the yeast translation initiation factor 3 (eIF-3) complex (37, 38). Recently, this suggestion has been validated by the identification of Prt1p in purified yeast eIF-3 (40).

Mutant forms of the *PRT1* gene have been found several times by selection for temperature-sensitive growth mutants (26, 27, 58) and also through searches for more-specific effects. The *prt1-63* allele (formerly *cdc63* [22]) was obtained by selection for mutants conditionally unable to perform the G_1 cell cycle regulatory step (2), whereas the *prt1-26* allele (formerly *dna26* [15]) was identified in a mutant defective for DNA synthesis (13). The identification of *prt1* mutant alleles by the latter criteria can be understood from the effects of *prt1* mutations on the cell cycle. At appropriate restrictive temperatures, all temperature-sensitive *prt1* mutations can cause regulated cell cycle blockage (23), and the DNA synthesis impairment caused by the *prt1-26* mutation was shown to be an indirect consequence of a concerted arrest of mutant cells in the G_1 phase of the cell cycle (15). Thus, *prt1* mutant alleles can allow sufficient protein synthesis for completion of an ongoing cell cycle (7) while blocking initiation of the next cell cycle and DNA replication.

Prt1p has been characterized by analysis of the cloned *PRT1* gene (24). In this report, we extend the characterization of Prt1p by showing that the open reading frame (ORF) of *PRT1* does not accurately predict Prt1p and by identifying the mutational change(s) in Prt1p caused by each of six temperature-sensitive *prt1* mutant alleles. We also characterize a novel dominant-negative mutant form of Prt1p and show that this mutant Prt1p inhibits translation initiation by competing with wild-type Prt1p for a component that mediates interaction of eIF-3 with the 40S ribosome.

MATERIALS AND METHODS

Strains, culture conditions, and determination of cellular parameters. The yeast strains used in this study are listed in Table 1. Yeast cells were grown and monitored as described elsewhere (24). Standard procedures were employed for DNA manipulations (53) and genetic analysis (55). Yeast transformations followed the method of Schiestl and Gietz (54).

Transcript mapping. Primer extension reactions (19) were carried out with total yeast RNA and reverse transcriptase. Primers were 20-mers (purchased from C. Hew, University of Toronto) complementary to nucleotides 630 to 649 and 599 to 618 of the *PRT1* sequence (24) and were end labeled with [γ-^{32}P]ATP (Amersham) by using T4 polynucleotide kinase (New England Biolabs). A sequencing ladder generated by using end-labeled primers identified the 3′ termini of primer extension products. Densitometry of autoradiograms was done with a Bio-Rad model 620 video densitometer.

Marker rescue and gap repair. Marker rescue was carried out as described previously (48) by transforming *prt1* mutant cells with mixtures of 1 μg of the *URA3* plasmid YEp352 (28) and 30 μg of a fragment of the wild-type *PRT1* gene and then selecting for growth at 22°C on medium lacking uracil. At least 500 of these transformant colonies were then collected and spread onto selective medium, and papillations of growth at 36°C were scored after 4 days of incubation.

For gap repair, plasmid pDE-PGR was constructed (Fig. 1B) to contain a *PRT1* gene deleted for the 1.7-kbp region to which temperature-sensitive mutations had mapped by marker rescue. For this construction, two fragments, the 1.0-kbp *Pst*I-*Cla*I fragment from the *PRT1* plasmid pJ53 (24) and a 1.1-kbp *Sst*I-*Spe*I fragment consisting of the *Sst*I-*Acc*I fragment of the pUC19 multiple cloning site (MCS) joined to nucleotides 1 to 1002 of a wild-type *PRT1* gene (24), were sequentially inserted into the MCS of pRS304 (56). The 2.1-kbp *Sal*I-*Sst*I fragment from this plasmid was then transferred to the MCS of pRS315 (56), creating pDE-PGR. Before transformation, pDE-PGR was linearized with *Pst*I and *Spe*I to generate a 1.7-kbp gap in the *PRT1* gene amenable to repair by gene conversion (41). Repaired plasmids from Leu$^+$ transformants were identified by

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype or description	Source or reference
GR2	*MAT***a** *his6 ura1*	30
W303-1A	*MAT***a** *ade2-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 can1-100*	J. Friesen
W303-1B	*MAT*α *ade2-1 leu2-3,112 ura3-1 his3-11,15 trp1-1 can1-100*	J. Friesen
A364A	*MAT***a** *ade1 ade2 ura1 lys2 tyr1 his7 gal1*	25
TP11-4-1	*MAT***a** *prt1-1 ade1 ade2 leu2-3,112 ura3-52*	24
TP12-7-0[a]	*MAT*α *prt1-2 ade1 leu2-3,112 ura1 ura3-52 his6*	
TP13-1-2[b]	*MAT***a** *prt1-3 ade1 leu2-3,112 ura1 ura3-52 his6*	
TC3-212-3[c]	*MAT*α *prt1-63 leu2-3,112 ura3-52*	
TR1-1-1[d]	*MAT***a** *prt1-1564 leu2-3,112*	
TDE/16A	*MAT***a** *prt1-26 ade2 leu2-3,112 ura3-11,15 trp1-1*	15
GDU4	*MAT***a** */MAT*α *PRT1/prt1*::*URA3 ade1/ade1 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	24
GDE4/1C[e]	*MAT*α *prt1*::*URA3 ura3-52 ade1 leu2-3,112* YEpDE-303 (*LEU2 PRT1*)	
GDE4-303	*MAT*α *prt1*::*URA3 ade leu2-3,112 ura3 his3-11,15 trp1-1* YEpDE-303 (*LEU2 PRT1*)	GDE4/1C × W303-1A
GDE303-88[f]	GDE4-303 with replacement plasmid pDE-31 (*HIS3 PRT1*)	
GDE1-1[e]	*MAT*α *prt1*::*URA3 ade1 ade2 ura3 leu2-3,112* YEpDE-TP11 (*LEU2 prt1-1*)	
GDE1-3[e]	*MAT***a** *prt1*::*URA3 ade1 ura3 leu2-3,112* YEpDE-TP13 (*LEU2 prt1-3*)	
GDE1-63[e]	*MAT***a** *prt1*::*URA3 ade1 ura3 leu2-3,112* YEpDE-TC3 (*LEU2 prt1-63*)	
GDE1-26[e]	*MAT***a** *prt1*::*URA3 ade1 ura3 leu2-3,112* YEpDE-16A (*LEU2 prt1-26*)	
GDE-PRT1[e]	*MAT*α *prt1*::*URA3 ade1 ade2 ura3 leu2-3,112* YEpDE-303 (*LEU2 PRT1*)	
GDE88-1-2[g]	GDE303-88 with replacement plasmid pDE-TP12 (*LEU2 prt1-2*)	
GDE-G1154[g]	GDE303-88 with replacement plasmid pDE-A1154 (*LEU2 prt1-21*)	
GDE-A2114[g]	GDE303-88 with replacement plasmid pDE-G2114 (*LEU2 prt1-22*)	
GDE-A2228[g]	GDE303-88 with replacement plasmid pDE-A2228 (*LEU2 prt1-1564*)	
GDE-303[g]	GDE303-88 with replacement plasmid pDE-303 (*LEU2 PRT1*)	

[a] Derived from *prt1-2* mutant strain ts 171 (26).
[b] Derived from *prt1-3* mutant strain 19-29.
[c] Derived from *prt1-63* mutant strain S44 (2).
[d] Derived from *prt1-1564* strain ts 1564 (58).
[e] Segregant of a transformant of GDU4.
[f] From plasmid shuffling in strain GDE4-303.
[g] From plasmid shuffling in strain GDE303-88.

the *Eco*RI restriction pattern of the intact *PRT1* gene. These plasmids include pDE-TP11 (*prt1-1*), pDE-TP13 (*prt1-3*), pDE-16A (*prt1-26*), and pDE-TC3 (*prt1-63*). To confirm the recovery of sequences conferring temperature sensitivity, each gap-repaired *prt1* allele was transferred as a *Sal*I-*Sst*I fragment to YEp351 (28) (generating YEpDE-TP11, YEpDE-TP13, YEpDE-16A, and YEpDE-TC3, respectively) and separately introduced into diploid strain GDU4, and resultant transformants were sporulated and subjected to random spore analysis. Spores were germinated at 22°C and then replica plated onto yeast extract-peptone-dextrose medium at 22 and 36°C to test for temperature sensitivity and onto medium lacking either uracil or leucine to test for the presence of the chromosomal *prt1*::*URA3* disruption and the plasmid-borne *prt1* allele, respectively. For mutant alleles containing more than one change from the published *PRT1* sequence, a restriction fragment containing a base change of interest was used to replace the homologous fragment of the wild-type *PRT1* gene (Fig. 1).

Construction of mutations in vitro. A 0.6-kbp *Ssp*I-*Xba*I fragment containing *PRT1* 5′ ORF sequences (nucleotides 406 to 1053 [24]) was used to replace the 1.1-kbp *Sma*I-*Xba*I fragment from pDE-303 (Fig. 1A), generating pDE-440, harboring a modified *PRT1* allele (*PRT1-440*) lacking the first 33 nucleotides of the *PRT1* ORF and upstream genomic sequences (see Fig. 2B). The *PRT1-440* ORF is 5′ extended by 177 nucleotides of vector DNA, but this 5′ extension of the ORF contains no ATG and is preceded by two closely spaced TAA stop codons, making the second ATG of the wild-type *PRT1* ORF the first ATG in the *PRT1-440* ORF.

In-frame deletions were made by removal of *Eco*RI fragments, religation, and verification by DNA sequencing across the junctions. Plasmids pDE-1E, pDE-12E, and pDE-123E, containing the *PRT1-Δ100*, *prt1-Δ101*, and *prt1-Δ102* alleles, respectively, were constructed by *Eco*RI partial digestion and religation of pDE-303 (see Fig. 4A). To construct pDE-3E containing the *prt1-Δ103* allele, the 1.7-kbp *Spe*I-*Pst*I fragment from pDE-303, containing part of the *PRT1* ORF, was inserted into the *Spe*I-*Pst*I site of pBSII KS$^+$, which had been modified to abolish the *Eco*RI site. The resulting plasmid, pDE-63S, was cleaved with *Eco*RI and religated to delete the 0.58-kbp *PRT1 Eco*RI fragment. The resulting 1.1-kbp *Spe*I-*Pst*I fragment was then used to replace the 1.7-kbp *Spe*I-*Pst*I fragment of pDE-303, generating *prt1-Δ103*. The *PRT1* ORF was truncated by filling and religating the *Bam*HI site in pDE-63S, thus creating a frameshifted ORF encoding Arg-Ser-Ile-Trp beyond the *Bam*HI site. The resulting 1.8-kbp *Spe*I-*Pst*I fragment was then used to replace the homologous fragment from YEpDE-TC3, generating the *prt1-581* plasmid YEpDE-2220.

Functional tests of *prt1* mutant alleles by plasmid shuffling. The activity of plasmid-borne alleles of the *PRT1* gene was tested by plasmid shuffling (4). Cloned alleles in *LEU2* vectors were introduced into the *prt1*::*URA3* disruption strain GDE303-88, which is kept alive by the *PRT1 HIS3* plasmid pDE-31. These transformants were grown in nonselective medium (to allow plasmid loss) at 22 or 37°C and then replica plated onto media lacking uracil, histidine, or leucine and incubated at 22°C to test for loss of the *HIS3* plasmid or onto yeast extract-peptone-dextrose medium to test for temperature sensitivity.

DNA sequencing. Sequencing was performed with Sequenase version 2.0 (United States Biochemicals) and ^{35}S-dATP. Single-stranded DNA was prepared directly from gap-repaired pRS315-based phagemids (50). In-frame deletions of the *PRT1* gene were verified by direct sequencing of plasmid DNA (35).

Immunoblotting. Cells were grown in yeast nitrogen base medium at 30°C to 5×10^6 to 8×10^6 cells per ml, washed twice with ice-cold H_2O, resuspended in ice-cold lysis buffer consisting of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.0)–100 mM NaCl–1 mM dithiothreitol containing protease inhibitors (11), and lysed by agitation with glass beads. The cell extract was resolved and immunoblotted as described previously (11) with anti-Prt1p primary antibody against Prt1p (11) or eIF-2α (a gift from Alan Hinnebusch).

Subcellular localization of Prt1p. Sucrose gradient centrifugation was carried out as described previously (1, 11, 45), using buffers containing 3 mM Mg^{2+} for immunoblot experiments. Differential centrifugation, with an added 800 mM KCl wash, was done as described elsewhere (11).

RESULTS

Transcription initiation sites for the *PRT1* gene. As a first step of a structure-function analysis of Prt1p, we determined sites of transcription initiation. By primer extension, multiple transcript 5′ ends were mapped to the region spanning nucleotides 436 to 480 of the *PRT1* sequence (24), with major transcripts initiating around nucleotides 461 and 466 (Fig. 2A). This multiplicity of transcription start sites is typical in *S. cerevisiae* (10), but the positions of these transcription start sites were surprising: all of the transcripts initiate downstream of the ATG at position 373, which begins the *PRT1* ORF. A similar result was obtained by S1 nuclease mapping (data not shown). RNAs from cells grown on glucose or the nonferment-

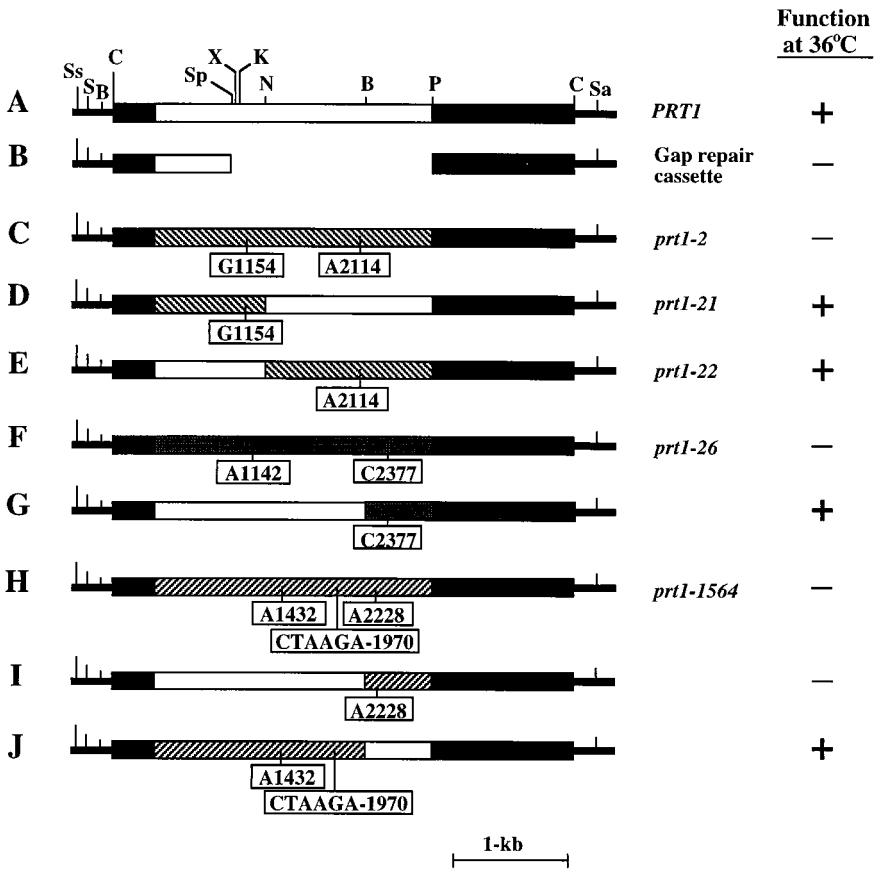

FIG. 1. Plasmid-borne alleles of *PRT1*. (A) Plasmid pDE-303 containing wild-type *PRT1* genomic sequences recovered by gap repair in strain W303-1A. The *Sst*I-*Sal*I fragment was transferred to YEp351, generating YEpDE-303 for high-copy-number expression of *PRT1*, and to pRS313, generating pDE-31 for plasmid shuffling. (B) Gap repair plasmid pDE-PGR, used to clone mutant sequences. (C through E) Plasmids used to identify the temperature-sensitive mutations in *prt1-2*: pDE-TP12, containing *prt1-2* sequences (C); pDE-G1154, constructed by replacing the 2.6-kbp *Nsi*I-*Sal*I fragment of pDE-TP12 with the homologous fragment from pDE-303 (D); and pDE-A2114, constructed by replacing the 2.6-kbp *Nsi*I-*Sal*I fragment of pDE-303 with the homologous fragment from pDE-TP12 (E). (F and G) Plasmids used to characterize the T2377→C base substitution present in several alleles: YEpDE-16A, containing *prt1-26* sequences (F), and YEpDE-C2377, constructed by replacing the 2.2-kbp *Bam*HI fragment of YEpDE-16A (containing the *prt1-26* mutation) with the homologous wild-type fragment (G). (H through J) Plasmids used to identify the *prt1-1564* mutation: pDE-TR1, containing *prt1-1564* sequences (H); pDE-A2228, constructed by replacing the 2.2-kbp *Bam*HI fragment of pDE-TR1 with the homologous wild-type fragment (I); and pDE-UPS, generated by replacing the 2.2-kbp *Bam*HI fragment of pDE16A with the homologous fragment from pDE-TR1 (J). Open boxes, wild-type *PRT1* ORF; black boxes, flanking genomic sequences; left-handed diagonals, *prt1-2* sequences; gray boxes *prt1-26* sequences; right-handed diagonals, *prt1-1564* sequences; Black bars, vector sequences from the MCS of pRS315 (A through E and H through J) or YEp351 (F and G). Changes from the *PRT1* sequence (24) are identified in boxes by the nucleotide present in the mutant allele followed by the nucleotide position number; CTAAGA denotes the insertion at 1970. Restriction sites: Ss, *Sst*I; S, *Sma*I; B, *Bam*HI; C, *Cla*I; Sp, *Spe*I; X, *Xba*I; K, *Kpn*I; N, *Nsi*I; P, *Pst*I; and Sa, *Sal*I.

able carbon source ethanol showed identical primer extension patterns (data not shown). Densitometry of autoradiograms indicated that detection was possible for a band 1% as intense as the total signal generated by all bands. Therefore, any transcription initiating upstream of the first ATG in the *PRT1* ORF constitutes <1% of the total.

To determine if the first ATG in the *PRT1* ORF is required for cell growth, we constructed the *PRT1-440* allele, which is missing the first 33 nucleotides of the ORF, including the first ATG (Fig. 2B). In cells lacking any other functional *PRT1* allele, the plasmid-borne *PRT1-440* allele allowed wild-type growth on medium containing either glucose or glycerol as a carbon source (data not shown). (*PRT1-440*, which lacks most of the *PRT1* promoter, is most likely expressed from adjacent vector sequences.) The protein product of *PRT1-440* comigrated with wild-type Prt1p during denaturing gel electrophoresis (Fig. 2C). These results suggest that coding sequences upstream of the second ATG in the *PRT1* ORF are dispensable for wild-type growth and that the second ATG in the ORF encodes the translation initiator for the *PRT1* gene. Accordingly, we have numbered the 724 (or 726; see below) residues of the predicted Prt1p to reflect this finding.

Cloning and sequence analysis of *prt1* mutant alleles. We took advantage of six temperature-sensitive *prt1* mutant alleles (see the introduction) to identify structural alterations in Prt1p that affect function. Each temperature-sensitive mutation was localized by transforming *prt1* mutant cells with fragments of a wild-type *PRT1* gene and scoring for temperature-resistant growth (Fig. 3). This assay determines whether wild-type function can be restored by recombination of a transforming DNA fragment with the chromosomal locus to replace mutant sequences with wild-type DNA (48). As shown in Fig. 3A, the *prt1-26* temperature-sensitive mutation was mapped in this way to a central 0.17-kbp *Kpn*I-*Eco*RI fragment within the ORF, while mutations in the *prt1-1*, *prt1-2*, *prt1-3*, *prt1-63*, and *prt1-1564* alleles all mapped to a 0.92-kbp *Eco*RI-*Pst*I fragment at the 3′ end of the gene. The temperature-sensitive mutations in the six different *prt1* mutant alleles all fall within a 1.7-kbp *Kpn*I-*Pst*I fragment, which was therefore cloned from each mutant allele by the technique of gap repair (41).

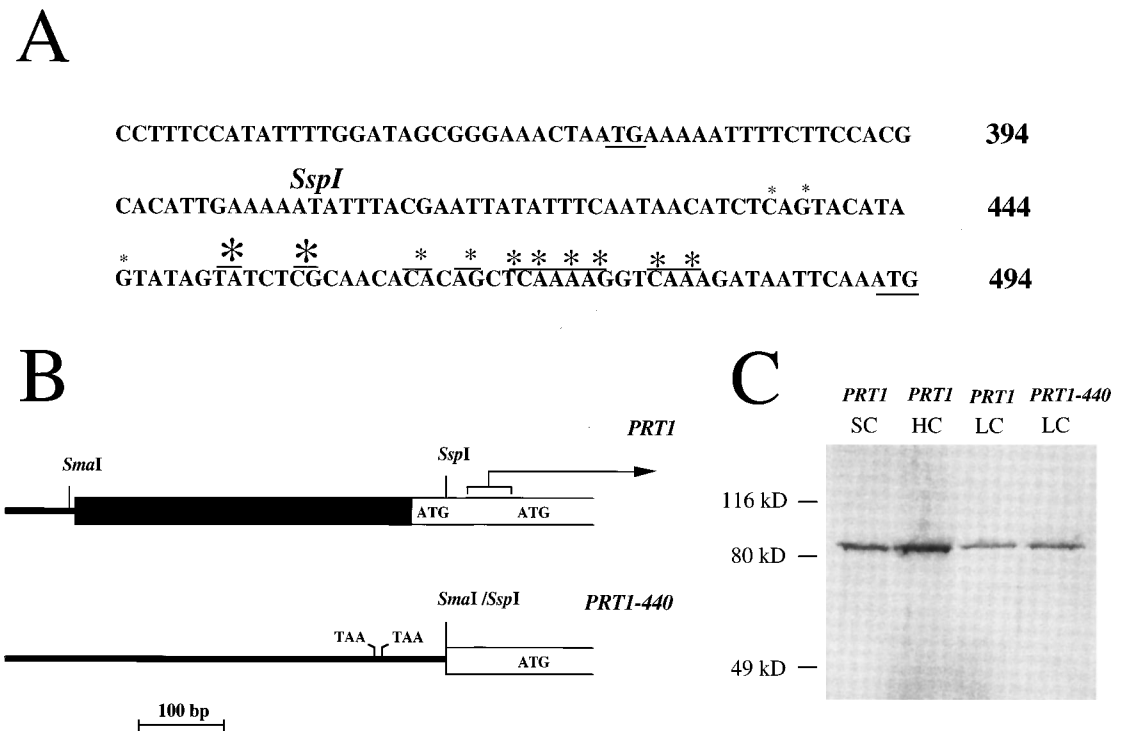

FIG. 2. Transcript 5′ ends map within the *PRT1* ORF. (A) Sites of transcription initiation indicated by primer extension and confirmed by S1 nuclease mapping are marked by asterisks, with asterisk size proportional to transcript abundance. The overlines define the resolutions of 5′ end determinations. The first and second ATG codons in the *PRT1* ORF are underlined. Nucleotides are numbered on the right. (B) The *PRT1-440* allele, lacking the first 33 nucleotides of the *PRT1* ORF (including the first ATG codon) and upstream genomic sequences. Open boxes, *PRT1* ORF sequences; filled box, flanking genomic sequences; filled bars, pRS315 vector sequences; TAA, stop codon; arrow, sites of transcription initiation. (C) The *PRT1-440* gene product. Extracts were prepared from cells (strain W303-1A) containing a single chromosomal *PRT1* gene (lane *PRT1* SC) or a high-copy-number *PRT1* plasmid (lane *PRT1* HC) as a control for antiserum specificity and from *prt1*::*URA3* cells (strain GDE303-88) containing pDE-303 (lane *PRT1* LC) or pDE440 (lane *PRT1-440* LC) and immunoblotted. Positions of molecular mass markers (in kilodaltons) are shown on the left.

Each plasmid-borne allele recovered by gap repair was shown to support the growth of cells disrupted for the chromosomal copy of *PRT1*, demonstrating that intact versions of each gene had been recreated. Furthermore, the growth allowed by each plasmid-borne allele was temperature sensitive, and at a restrictive temperature each gap-repaired *prt1* mutant allele caused the accumulation of cells in the G_1 interval of the cell cycle (data not shown), a phenotype conferred by the corresponding chromosomal *prt1* mutant alleles (15, 24). These results indicated that mutant sequences causing the mutant phenotype had been recovered from each of the chromosomal *prt1* mutant alleles.

Mutant sequences recovered by gap repair (and wild-type *PRT1* DNA from strain W303-1A recovered in the same way) were determined. The recovered region of the wild-type *PRT1* allele from strain W303-1A was identical in sequence to the original *PRT1* clone (24) derived from strain S288C (8). In contrast, each of the cloned mutant alleles contained a mutational change from the wild-type sequence (Table 2), as well as a number of strain-specific polymorphisms (see below). With the exception of *prt1-2* (see below), each cloned *prt1* mutant allele contained a single missense mutation within the region of marker rescue (Table 2). Despite their independent origins, the cloned *prt1-3* and *prt1-1564* alleles contained the same temperature-sensitive mutation, while the independent derivations of these two alleles were confirmed by the polymorphisms surrounding the common mutation (Table 2).

The *prt1-2* allele contained two mutations within the region of gap repair (Table 2). To identify the sequence change(s) responsible for temperature sensitivity, fragments of a plasmid-borne wild-type *PRT1* gene were replaced with *prt1-2* fragments encoding each mutation individually, generating alleles *prt1-21* and *prt1-22* (Fig. 1D and E). Both of these alleles supplied apparently wild-type *PRT1* gene function at the restrictive temperature of 36°C (data not shown). Therefore, neither amino acid substitution encoded by *prt1-2* is sufficient individually to cause growth inhibition at the restrictive temperature; both are required for the temperature sensitivity of *prt1-2* mutant cells.

This conclusion is supported by the marker rescue experiments described above. One of the wild-type gene fragments that was used overlapped the *prt1-2* mutation at nucleotide 2114 but not the second nucleotide change upstream (Fig. 3A). Recombination with this wild-type gene fragment restored temperature resistance, because both *prt1-2* mutations must be present for temperature sensitivity. (None of the wild-type gene fragments used for marker rescue included only the mutation at position 1154, in the *Kpn*I-*Eco*RI section.)

***PRT1* polymorphisms.** Each of the *prt1* mutant alleles recovered by gap repair contained a number of strain-specific polymorphisms (Table 2). Most were recognized because they were present in all mutant alleles derived from the same parental strain. DNA sequence polymorphisms that alter the encoded amino acid sequence were confirmed as polymorphisms (and not temperature-sensitive mutations) by replacement of a fragment of the wild-type *PRT1* gene with a fragment containing the sequence polymorphism followed by tests for temperature sensitivity (Fig. 1F to J).

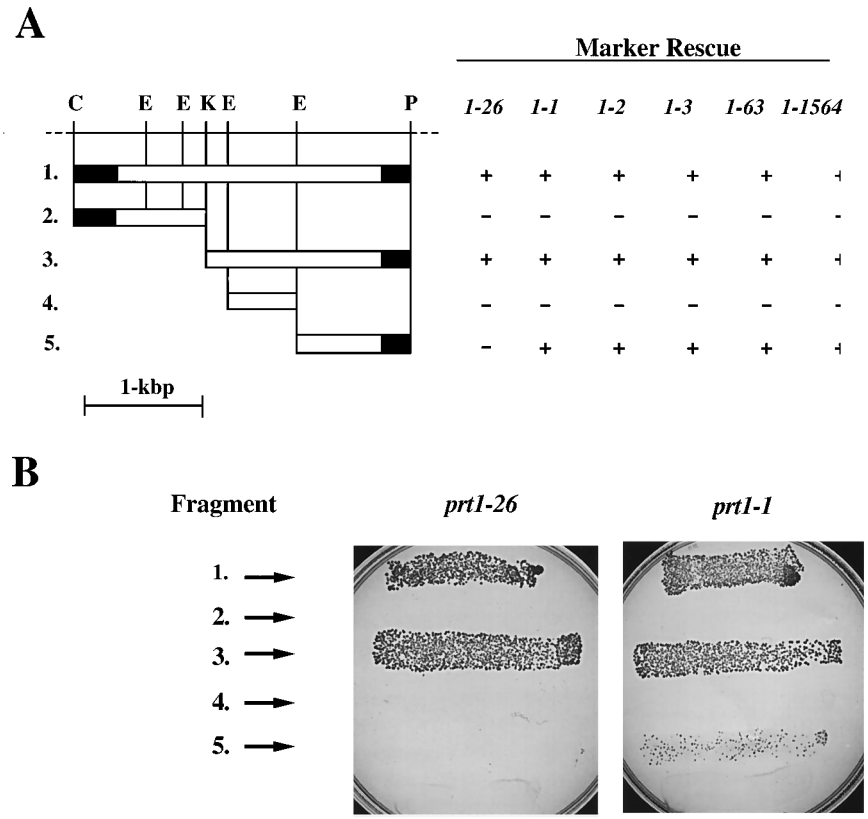

FIG. 3. Marker rescue of temperature-sensitive *prt1* alleles. (A) Fragments of the wild-type *PRT1* gene that are shown below the restriction map were introduced into *prt1* mutant cells (allele numbers indicated) and scored for the ability (+) to produce temperature-resistant recombinants. Open bars, *PRT1* ORF; closed bars, flanking genomic sequences. Restriction sites: C, *Cla*I; E, *Eco*RI; K, *Kpn*I; and P, *Pst*I. (B) Growth of *prt1-26* and *prt1-1* transformants at 37°C. The fragment numbers correspond to the numbers in panel A. Temperature-resistant recombinants produce papillations of growth (fragments 1 and 3 for *prt1-26* [strain TDE/16A] and fragments 1, 3, and 5 for *prt1-1* [strain TP11-4-1]).

A *PRT1* sequence polymorphism found in all mutant alleles derived from strain A364A was an insertion of 6 bp (5′-CCAAGG) at nucleotide 1970 (Table 2). This insertion encodes an additional alanine and lysine residue within Prt1p and also produces a *Sty*I restriction site. Southern analysis verified the presence of this *Sty*I site in the wild-type *PRT1* locus in strain A364A (data not shown) and also in strain SKQ-2n (reference 15 and data not shown).

The *prt1-1564* allele came from strain Σ1278b (58), which has a genealogy (39) different from that of strains S288C (the source of the original *PRT1* clone) and A364A (the source of the other *prt1* mutant alleles). Accordingly, the cloned *prt1-1564* allele contained more sequence polymorphisms (Table 2). Like the other *prt1* alleles, the cloned *prt1-1564* sequences also contained an insertion of 6 bp at position 1970, although in this case the sequence (5′-CTAAGA) differed from that found in alleles derived from strain A364A. This insertion also encodes an additional alanine and lysine residue and changes an adjacent aspartate codon to one encoding asparagine. However, experiments with chimeric genes (Fig. 1H to J) revealed that the *prt1-1564* allele contains only one temperature-sensitive mutation, the same as that in *prt1-3* (Table 2).

Deletions within the *PRT1* gene. The temperature-sensitive *prt1* mutations described above were found only in central and 3′ portions of the *PRT1* gene. To investigate whether changes at the 5′ end would affect *PRT1* gene function, in-frame deletions that removed 5′ and central fragments from the ORF were constructed (Fig. 4A). For comparison, the 147 codons encoded by the 3′ end of the *PRT1* ORF were in effect deleted by creation of a frameshift mutation (Fig. 4A). Each of these *prt1* mutant alleles failed to complement the *prt1-1* mutation at 36°C or support growth at any temperature as the only *prt1* allele (data not shown). Thus, sequences essential for *PRT1* gene function had been removed from each allele.

Immunoblotting (Fig. 4B) failed to detect the predicted product of *prt1-581*, the C-terminally frameshifted *prt1* allele. The absence of C-terminal residues may therefore destabilize the mutant protein, although we cannot rule out the possibility that the polyclonal antiserum used in this study is directed mainly against the missing C terminus. In contrast, lysates from strains containing the internally deleted *PRT1-Δ100*, *prt1-Δ101*, *prt1-Δ102*, or *prt1-Δ103* allele all contained mutant Prt1p of the expected abundance and molecular weight (Fig. 4B).

***PRT1-Δ100*, a dominant-negative allele, interferes with translation initiation.** Unlike other *prt1* mutant alleles, the plasmid-borne *PRT1-Δ100* allele (on a low-copy-number vector) inhibited cell growth even in the presence of the wild-type *PRT1* gene: the generation time of *PRT1-Δ100* transformants was threefold longer than that of cells with the wild-type *PRT1* gene on the same vector. This slow-growth phenotype was exacerbated with *PRT1-Δ100* on a high-copy-number vector (data not shown). *PRT1-Δ100* is therefore a dominant-negative allele.

The dominant-negative *PRT1-Δ100* allele encodes an altered form of the eIF-3 component Prt1p. To determine if *PRT1-Δ100* affects translation, the polyribosome profiles for

TABLE 2. Sequence changes in *prt1* mutant alleles[a]

Allele	Strain[b]	Origin[c]	Polymorphisms		Ts$^-$ mutations	
			Base change	Amino acid change	Base change	Amino acid change
prt1-26	TDE/16A	A364A	T1500→C		G1142→A	G218D
			1970 CCAAGG[d]	+ Ala-Lys[e]		
			T2377→C	S630P		
prt1-2	TP12-7-0	A364A	T1500→C		T1154→G	F222C
			1970 CCAAGG[d]	+ Ala-Lys	T2114→A	M544K
			T2377→C	S630P		
prt1-63	TC3-212-3	A364A progeny	T1500→C		G1855→A	E456K
			1970 CCAAGG[d]	+ Ala-Lys		
			T2377→C	S630P		
prt1-1	TP11-4-1	A364A	T1500→C		C2036→T	S518F
			1970 CCAAGG[d]	+ Ala-Lys		
			T2377→C	S630P		
prt1-3	TP13-1-2	A364A	T1500→C		G2228→A	G582D
			1970 CCAAGA[d]	+Ala-Lys		
			T2377→C	S630P		
prt1-1564	TR1-1-1	Σ1278b	T1299→C		G2228→A	G582D
			T1419→C			
			G1432→A	D315N		
			T1596→C			
			T1869→C			
			1970 CTAAGA[d]	+ Ala-Lys		
				D494N		
			T2037→C			
			G2043→A			
			T2377→C	S630P		
			C2574→T			

[a] Amino acids (represented by single letters) are counted from the second ATG in the *PRT1* ORF. Nucleotide numbers are from reference 24.
[b] Transformation host for gap repair.
[c] Strain in which the *prt1* mutant allele was generated.
[d] Six-base insertion at position 1970.
[e] + Ala-Lys, addition of an Ala and a Lys.

wild-type cells harboring either *PRT1-Δ100* or a wild-type *PRT1* gene (also on a low-copy-number vector) were determined by sucrose density gradient centrifugation (Fig. 5A). This analysis showed that cells containing the plasmid-borne *PRT1-Δ100* allele were depleted of large polyribosomes and enriched in 80S monoribosomes. This profile is similar to that observed for temperature-sensitive *prt1-1* mutant cells at a restrictive temperature (1, 18, 27), suggesting that the mutant Prt1p encoded by *PRT1-Δ100* interferes with Prt1p function in translation initiation.

Prt1p associates with large complexes. The inhibition caused by *PRT1-Δ100* was investigated by immunoblotting of sucrose gradient fractions and by fractionation of extracts by differential centrifugation. Although Prt1p is a component of eIF-3 that associates with the 40S ribosomal subunit, wild-type cells contained significant amounts of Prt1p in large complexes that migrated more slowly than 40S subunits (Fig. 5B), with only a minority of Prt1p in the 40S region of the gradient. An internal marker for a protein associated with the 40S ribosome was the translation initiation factor subunit eIF-2α (11, 45). As expected, much of the eIF-2α was found in the 40S region of the gradient, but eIF-2α was also found in complexes smaller than 40S, and the distributions of eIF-2α and Prt1p overlapped significantly. Both Prt1p and eIF-2α were markedly diminished in the 40S region if cell extracts were made with a buffer of greater ionic strength (30 mM Mg^{2+}–100 mM NaCl; data not shown), a property of ribosome-associated initiation factors. Like eIF-2α, Prt1p was virtually undetectable lower in the gradients (data not shown), suggesting that the ribosome association of Prt1p is limited to the 40S subunit.

In cells containing a *PRT1* low-copy-number plasmid, and therefore containing increased amounts of Prt1p, Prt1p was also detected at the top of the gradient and thus was not in large complexes (Fig. 5B). Similarly, a smaller proportion of Prt1p in these extracts pelleted with ribosomes (data not shown). The exclusion of excess Prt1p from large complexes suggests that there is a limited amount of complex that can be assembled and that Prt1p is not a limiting component.

Competition of the dominant-negative Prt1p. Gradient analysis showed that the dominant-negative mutant form of Prt1p could also associate with large nonribosomal complexes (Fig. 5B). However, the size distribution of mutant Prt1p was skewed towards smaller complexes, and some mutant Prt1p remained at the top of the gradient, observations suggesting impaired assembly or stability of complexes containing the dominant-negative Prt1p. The absence of a low-molecular-weight form of wild-type Prt1p in these cells suggests that incorporation of wild-type Prt1p into complexes is not impaired by competition from the mutant Prt1p. Complexes containing wild-type Prt1p were distributed as in wild-type cells, except that even less Prt1p could be found in the 40S region (Fig. 5B). This finding suggests that the dominant-negative Prt1p competes for an important component that allows the association of wild-type complexes with the ribosome.

Differential centrifugation of extracts gave similar findings. Under these experimental conditions, Prt1p in wild-type extracts was pelleted along with ribosomes and was released from the pellet by 0.5 M KCl (data not shown). In extracts of *PRT1-Δ100* transformants, the dominant-negative Prt1p significantly decreased the amount of wild-type Prt1p that pelleted with ribosomes and was itself found predominantly in the ribosome supernatant (data not shown). These results support the sug-

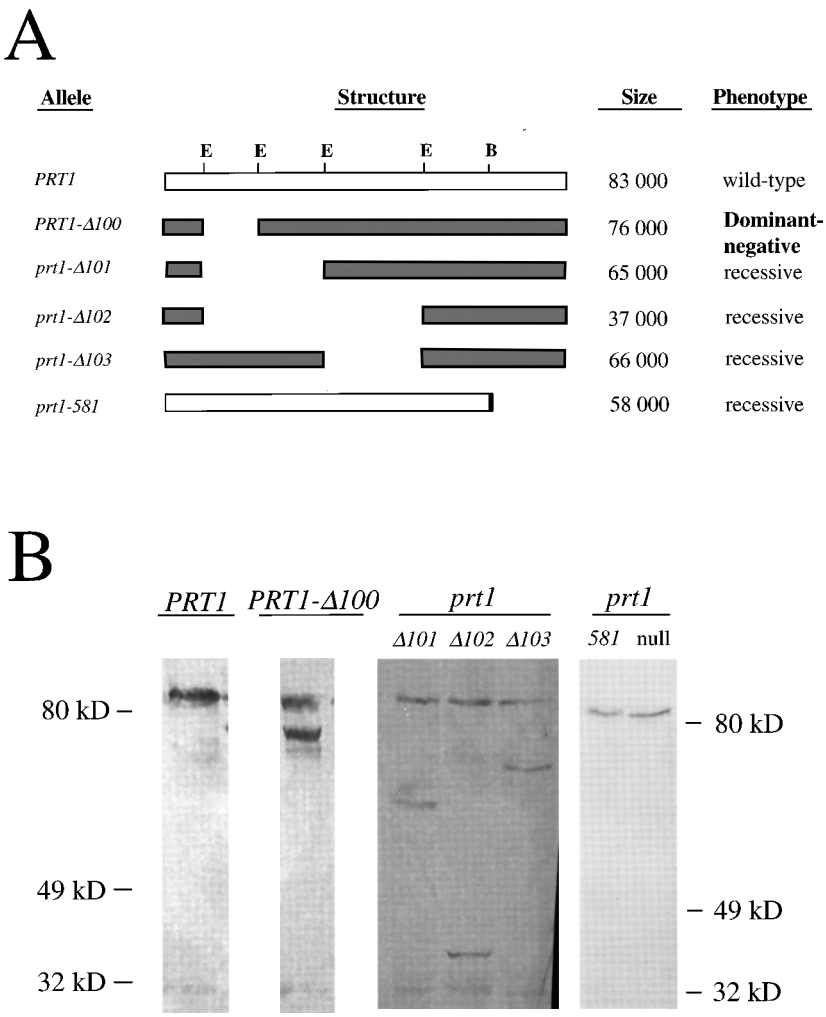

FIG. 4. *prt1* deletion alleles. (A) The wild-type *PRT1* ORF (open box) is depicted above in-frame deletions (shaded boxes) and a frameshift (open box joined to a black box). (B) Immunoblotting of *prt1* mutant proteins. Extracts from untransformed cells containing a single chromosomal *PRT1* gene or from cells harboring mutant or wild-type *PRT1* alleles were analyzed. Lane null, strain GDE303-88 containing the *prt1*::*URA3* disruption and the *PRT1* plasmid pDE31. Molecular mass markers are shown on the left.

gestion that the dominant-negative mutant form of Prt1p competes for a component that allows ribosome association.

A mutant form of Prt1p that does not interfere with the function of wild-type Prt1p fractionated aberrantly during differential centrifugation. This mutant Prt1p from the recessive deletion allele *prt1-Δ101*, lacking the same amino acids missing from the dominant-negative Prt1p plus the adjacent C-terminal 125 residues (Fig. 4A), pelleted along with ribosomes but was not released by a salt wash, showing that it does not associate like the wild-type Prt1p (data not shown). Sequences immediately C terminal to those deleted from the *PRT1-Δ100* polypeptide therefore facilitate the interactions responsible for the dominant-negative phenotype.

DISCUSSION

S. cerevisiae Prt1p has been consistently implicated in the process of translation initiation (see the introduction) and recently has been shown to be a component of eIF-3 (40). In this study, we have confirmed that Prt1p is found in large complexes (11) and have shown that a portion of Prt1p is associated with the 40S ribosomal subunit. We also characterized the temperature-sensitive alteration in each of six *prt1* mutant alleles and identified an essential N-terminal region of Prt1p that is important for ribosome association.

A mutant Prt1p missing N-terminal sequences inhibits translation initiation. Removal of a 0.32-kbp fragment from the 5′ end of the *PRT1* ORF produced a mutant allele, *PRT1-Δ100*, with a dominant-negative effect. Polysome analysis revealed that the *PRT1-Δ100* polypeptide, missing 102 residues from its N-terminal region, prevents the proper function of intact Prt1p and inhibits translation initiation. We showed that this dominant-negative mutant Prt1p does not seem to affect the association of the 40S ribosome with the ternary complex, as indicated by eIF-2α distribution, and does not prevent the incorporation of intact Prt1p into large nonribosomal complexes. However, the translation inhibition in this situation suggests that these Prt1p complexes do not function effectively. The mutant Prt1p is also found in complexes, indicating that Prt1p lacking the N-terminal domain is competent to undergo many normal interactions but cannot supply full Prt1p function to these complexes. We presume that the mutant complexes, which are smaller than the wild-type Prt1p complexes, are inactive. These complexes containing the mutant Prt1p may

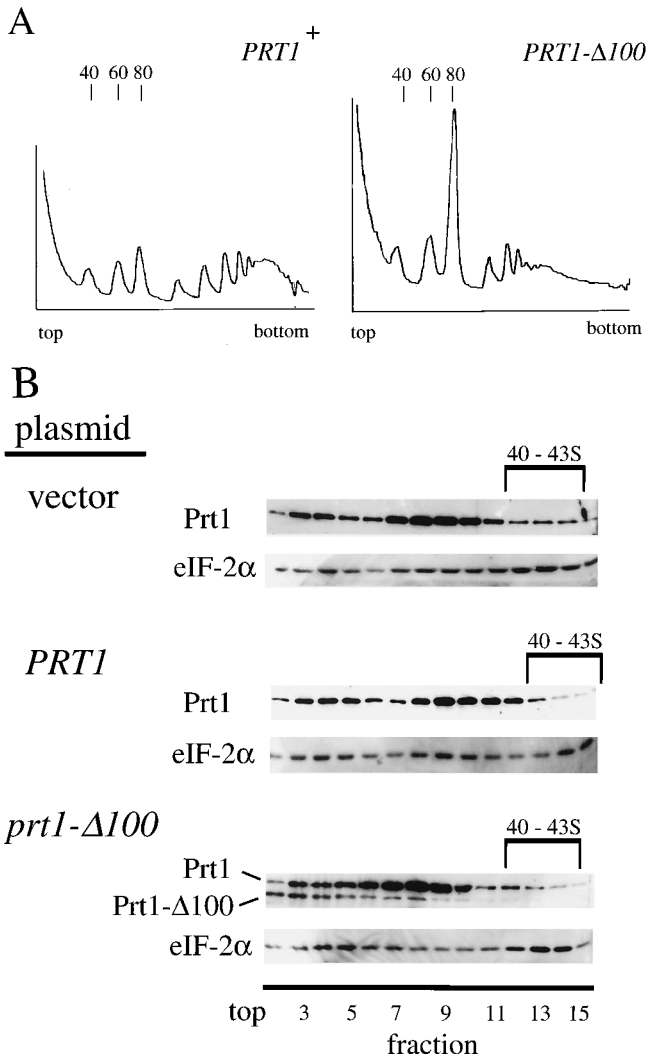

FIG. 5. Distributions of wild-type and dominant-negative Prt1p complexes and association with the 40S ribosomal subunit. (A) Transformants (strain W303-1A) containing plasmid pDE-1E (*PRT1-Δ100*) or pDE-303 (*PRT1*) were grown at 30°C to 8×10^6 cells per ml and harvested for polysome analysis. The positions of the 40 and 60S ribosomal subunits and 80S monoribosomes (40, 60, and 80, respectively) are indicated. (B) Immunoblots of lysates from cells harboring the indicated plasmids, resolved by sucrose gradients and fractionated. Little Prt1p and eIF-2α was in fractions 16 to 24 from lower in the gradient (data not shown). Positions of 40 and 60S ribosome subunits and 80S monoribosomes were verified by RNA analysis (data not shown).

inhibit the function of complexes containing intact Prt1p by competing for an important component, perhaps a component of eIF-3, thereby limiting the number of functional complexes available for translation initiation.

The N-terminal region of Prt1p that we show is crucial for function encompasses a eukaryotic RNA recognition motif (12) (Fig. 6A). This motif is a region of 80 to 90 amino acids with a highly conserved βαββαβ secondary structure, many conserved amino acid positions, and two conserved motifs: a highly conserved octamer designated RNP1 and the less conserved hexamer RNP2 (44; reviewed in references 6 and 32). *S. cerevisiae* Prt1p shows a good match to the RNP1 motif and many of the conserved amino acids of the overall motif consensus, while an RNP2 motif is less evident (Fig. 6A). The predicted secondary structure of this region of Prt1p matches the βαββαβ structure of the RNA recognition motif (14, 49). The deletion in *PRT1-Δ100* removes this motif (plus 4 residues upstream and 10 residues downstream). The motif is therefore essential for Prt1p function.

A polypeptide similar to *S. cerevisiae* Prt1p, including the RNA recognition motif, is encoded by a gene from another yeast. A gene fragment from the methylotrophic yeast *Hansenula polymorpha* (5) encodes only the N terminus of a polypeptide, but this N terminus is homologous to the N terminus of Prt1p, including the RNP1 motif and conserved amino acids of the RNA recognition motif (Fig. 6A). The RNP1 and presumptive RNP2 motifs are spaced farther apart in Prt1p of both yeasts than in most proteins containing the RNA recognition motif because of a longer loop 1, which has been implicated in RNA-binding specificity (32). This essential motif may confer to Prt1p an RNA-binding ability.

eIF-3 may be involved in the binding of mRNA to the 40S ribosomal subunit (3, 59). Indeed, another component of yeast eIF-3 can bind an uncapped 5′ fragment of mRNA, but Prt1p had no such activity (40). Nonetheless, Prt1p may be involved in other eIF-3 interactions in translation initiation. eIF-3 binds 40S ribosomal subunits, thereby preventing their association with 60S subunits, and is also thought to promote the binding of the eIF-2 GTP Met-tRNA$_i$ ternary complex to the 40S subunit (for a review, see reference 16). Prt1p, through its RNA recognition motif, may supply one of these functions.

Other proteins involved in translation initiation contain RNA recognition motifs. eIF-4B contains a single domain closely matching the consensus (36), while Tif4631p and Tif4632p, the yeast homologs of the mammalian p220 subunit of eIF-4F, each contain a domain related to an RNA recognition motif (20). Poly(A)-binding protein, which plays a role in translation initiation (52), contains tandem repeats of the motif (51). Both p220 and eIF-4B are thought to be required for mRNA binding to ribosomes, while poly(A)-binding protein may mediate the joining of 60 and 40S ribosomal subunits (34).

***prt1* temperature-sensitive mutations.** We dissected the *prt1* mutant alleles to identify the temperature-sensitive mutation(s) in each. With the exception of the mutations present in the *prt1-2* allele (discussed below), each of the temperature-sensitive *prt1* mutations is a G→A transition, which is typical for mutations caused by ethyl methanesulfonate or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (21, 43), the mutagens used to generate these alleles (2, 13, 25, 58). In contrast, the temperature-sensitive mutations in the *prt1-2* allele are unusual for nitrosoguanidine. Each *prt1-2* mutation is a transversion: a T→G transversion at nucleotide 1154 and a T→A transversion at 2114 (Table 2). Although nitrosoguanidine can induce transversions at low frequency (21, 47), the origin of the two transversions in *prt1-2* is unclear.

Marker rescue and nucleotide sequence analysis pinpointed the *prt1-1* temperature-sensitive mutation as a C→T change at nucleotide 2036. This location is consistent with the results of Keierleber et al. (31), who mapped the *prt1-1* mutation upstream of position 2220 by integrative transformation. The same study also suggested that, in the absence of gene conversion, the *prt1-1* mutation lies upstream of position 1052; our findings indicate that gene conversion may indeed have occurred during that work.

Prt1p structure-function relationships are shown schematically in Fig. 6B. The segments, labeled by amino acid numbers above the schematic polypeptide, are defined by the restriction sites used for deletion analysis (Fig. 4A); the sites of the two-residue insertions are also indicated. Immunoblotting suggests that the C-terminal, charged region of Prt1p may be necessary for polypeptide stability. In contrast, the dominant-negative

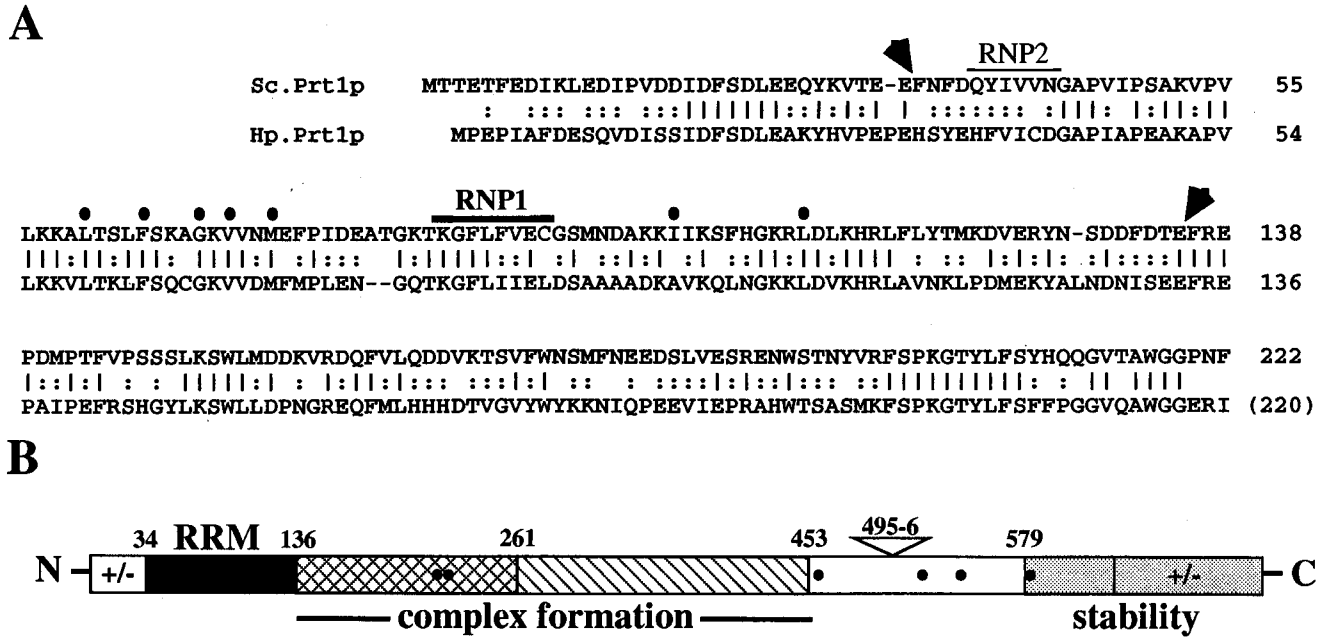

FIG. 6. Prt1p sequences and the RNA recognition motif. (A) Comparison of Prt1p from *S. cerevisiae* (Sc.Prt1p) with a putative Prt1p from *H. polymorpha* (Hp.Prt1p) predicted by the sequence of a 3′-truncated (at codon 220) clone (5). Identities (vertical lines), conservative changes (paired dots), and gaps inserted by FASTA (42) (dashes) are indicated. This alignment gives 45% identity over 209 amino acids. Residues deleted from the *PRT1-Δ100* polypeptide are bounded by arrowheads. The RNP1 and presumed RNP2 motifs (overlines) and conserved residues in the overall consensus that are found in Prt1p (large dots) are shown. The presence of these motifs is considered significant, for RNP1 motifs were not found in 10 random sequences generated from the marked region of Prt1p. (B) Prt1p structural features. Black box, RNA recognition motif (RRM); cross-hatched and singly hatched boxes, regions shown or inferred from phenotype, respectively, to facilitate complex formation; shaded box, region which may be necessary for polypeptide stability; +/−, charged region, numbers, amino acid residues; inverted triangle, the two-residue insertion; dots, temperature-sensitive amino acid substitutions.

phenotype caused by deleting the domain encoding the RNA recognition motif shows that this domain is necessary for some but not all Prt1p interactions. The adjacent region mediates other interactions involved in complex formation, as evidenced by mutant Prt1p distribution and the recessive nature of the *prt1-Δ101* phenotype (Fig. 6B). Deletion of the central region also results in a detectable polypeptide and a similar recessive phenotype, suggesting that this region also mediates Prt1p interactions. The amino acid substitutions encoded by the six temperature-sensitive mutant alleles all occur near predicted secondary structure discontinuities in Prt1p (14, 49), which is a general characteristic of temperature-sensitive mutations (9, 29, 33). Each of these substitutions causes a recessive phenotype; therefore, these alterations may also prevent the incorporation of mutant Prt1p into complexes under restrictive conditions.

The *PRT1* gene 5′ end. Remarkably, the *PRT1* ORF specified by the nucleotide sequence does not accurately predict Prt1p, as shown by transcript analysis and deletion of *PRT1* 5′ sequences. Transcription of the *PRT1* gene was found to initiate downstream of the first in-frame ATG of the ORF. This result is consistent with earlier work that localized *PRT1* transcription initiation to a region 0.57 kbp upstream of the internal *Xba*I site (31). The first 39 codons of the *PRT1* ORF are therefore not translated; the translation start site is most likely encoded by the second in-frame ATG.

In keeping with these findings, the similarity between *S. cerevisiae* Prt1p and the putative Prt1p from *H. polymorpha* (5) begins close to the methionine encoded by the first ATG in the *H. polymorpha* gene but downstream of the second ATG in the *S. cerevisiae* gene (Fig. 6A). The 5′-extended ORF in the *S. cerevisiae PRT1* gene is not conserved in any reading frame in the putative homolog from *H. polymorpha*, despite being found in two independently isolated, polymorphic genomic clones from different *S. cerevisiae* strains, S288C and SKQ-2n (14, 15, 24).

An unused ORF at the 5′ end of a gene is not without precedent in *S. cerevisiae*. For example, the *STE11* gene, which encodes a protein kinase necessary for mating, contains two in-frame but nonessential ATGs at its ORF 5′ end: a frameshift mutation downstream of the first and second in-frame ATGs in the *STE11* ORF (but upstream of the third) does not affect *STE11*-dependent mating efficiency (46). A frameshift mutation downstream of the third ATG generates a nonfunctional allele, indicating that the third in-frame ATG in the *STE11* ORF encodes the translation initiator.

Perhaps under growth conditions not tested here some transcription may initiate upstream of the first ATG in the *PRT1* ORF, resulting in the expression of an alternative, N-terminally extended form of Prt1p. Alternative translation initiation codons are used for genes that encode both a cytoplasmic and a mitochondrial form of a protein (57). Such genes initiate transcription both upstream and downstream of the most 5′ ATG of the ORF, with the longer transcript encoding a presequence required for mitochondrial targeting of the longer form of the protein. However, it is unlikely that a mitochondrial form of Prt1p exists: the 5′-truncated version of the *PRT1* gene supported apparently wild-type growth under conditions requiring mitochondrial function, and for the wild-type gene there was no indication that any transcription initiated upstream of the first ATG in the ORF. These findings support the observation that the *prt1-1* mutation affects cytoplasmic but not mitochondrial translation initiation (17).

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