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Genetic and Biochemical Characterization of Mutations in the ATPase and Helicase Regions of the Upf1 Protein

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mRNA degradation is an important control point in the regulation of gene expression and has been linked to the process of translation. One clear example of this linkage is the nonsense-mediated mRNA decay pathway, in which nonsense mutations in a gene can reduce the abundance of the mRNA transcribed from that gene. For the yeast *Saccharomyces cerevisiae*, the Upf1 protein (Upf1p), which contains a cysteine- and histidine-rich region and nucleoside triphosphate hydrolysis and helicase motifs, was shown to be a *trans*-acting factor in this decay pathway. Biochemical analysis of the wild-type Upf1p demonstrates that it has RNA-dependent ATPase, RNA helicase, and RNA binding activities. A *UPF1* gene disruption results in stabilization of nonsense-containing mRNAs, leading to the production of enough functional product to overcome an auxotrophy resulting from a nonsense mutation. A genetic and biochemical study of the *UPF1* gene was undertaken in order to understand the mechanism of Upf1p function in the nonsense-mediated mRNA decay pathway. Our analysis suggests that Upf1p is a multifunctional protein with separable activities that can affect mRNA turnover and nonsense suppression. Mutations in the conserved helicase motifs of Upf1p that inactivate its mRNA decay function while not allowing suppression of *leu2-2* and *tyr7-1* nonsense alleles have been identified. In particular, one mutation located in the ATP binding and hydrolysis motif of Upf1p that changed the aspartic and glutamic acid residues to alanine residues (DE572AA) lacked ATPase and helicase activities, and the mutant formed a Upf1p:RNA complex in the absence of ATP; surprisingly, however, the Upf1p:RNA complex dissociated as a consequence of ATP binding. This result suggests that ATP binding, independent of its hydrolysis, can modulate Upf1p:RNA complex formation for this mutant protein. The role of the RNA binding activity of Upf1p in modulating nonsense suppression is discussed.

Many studies have indicated that the processes of mRNA turnover and translation are directly linked, and understanding this relationship is critical in elucidating the mechanism of mRNA decay (13, 38, 43, 44, 50, 53). *cis*-acting sequences that promote instability of mRNAs have been identified in 5′ untranslated regions, protein-coding regions, and 3′ untranslated regions of transcripts. Recent results demonstrate that ongoing translation is required for certain elements to be able to promote accelerated mRNA degradation (13, 38, 43, 44, 50). The role of translation in determining mRNA decay rates is direct. Certain instability elements identified in protein-coding regions and 3′ untranslated regions require translation of their respective protein-coding regions in order for the decay determinants to be active (1, 5, 7, 10, 19, 20, 32, 40, 42, 55, 64, 65).

One clear example of the relationship between translation and mRNA turnover is the observation that nonsense mutations in a gene can reduce the abundance of its mRNA in a process termed nonsense-mediated mRNA decay (reviewed in references 38, 43, and 63). Reduced mRNA levels or decreased stability of nonsense-containing transcripts has been observed in both prokaryotes and eukaryotes (38, 43). The nonsense-mediated mRNA decay pathway in the yeast *Saccharomyces cerevisiae* has been intensively investigated, and both *cis*-acting sequence elements and *trans*-acting factors involved in this decay pathway have been identified. By using the *PGK1*, *HIS4*, *CYC1*, and *GCN4* transcripts as model substrates, it has been demonstrated that specific sequences 3′ of the nonsense mutation, defined as downstream elements or sensitive sites, are required for accelerated mRNA decay (21, 42, 52, 66, 67). Analysis of downstream elements in several genes revealed a motif which was used successfully to predict downstream elements in other genes (67).

Studies on the nonsense-mediated mRNA decay pathway in *S. cerevisiae* have been particularly fruitful in the identification of genes whose products are involved in mRNA turnover. A genetic screen for translational frameshift suppressors led to the identification of a class of mutant alleles termed *upf* (for up-frameshift) (9, 36). Subsequent analysis of mutations in either *UPF1*, *UPF2*/*SUA1*, or *UPF3*/*SUA6* demonstrated that these genes are involved in controlling the abundance of nonsense-containing mRNAs (8, 9, 23, 33, 35, 36, 42, 46). Mutations in the *UPF1*, *UPF2*, and *UPF3* genes elevate the concentration of nonsense-containing mRNAs in cells by increasing their half-lives (8, 23, 33, 35, 36, 42). The *UPF1*, *UPF2*/*SUA1*, and *UPF3*/*SUA6* genes have been cloned and characterized (8, 23, 33, 36, 48). Recently, it has been demonstrated that the Upf1 and Upf2 proteins (Upf1p and Upf2p) interact (23). Similar to the case for the *upf* alleles, several mutant alleles in *Caenorhabditis elegans* affect the nonsense-mediated mRNA decay pathway. Seven *smg* alleles identified as extragenic suppressors of myosin heavy chain B mutations increase the abundance of nonsense-containing myosin transcripts while not affecting the abundance of wild-type mRNAs (25, 47).

The *UPF1* gene and its protein product have been studied most extensively (3, 11, 21, 35, 36, 42). The deduced amino acid sequence of the *UPF1* gene indicates that it encodes a 109-kDa

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protein with a cysteine- and histidine-rich region near its amino terminus and harbors the motifs characteristic of members of the RNA/DNA helicase superfamily group I (2, 29). Previous studies have demonstrated that Upf1p is polyribosome associated (3, 45). Upf1p has been purified and shown to possess nucleic acid binding as well as nucleic acid-dependent ATPase and helicase activities (11). The wild-type protein does not have any detectable ATPase activity in the absence of single-stranded RNA or DNA. Deletion of, or mutations in, the *UPF1* gene results in stabilization of nonsense-, frameshift-, and intron-containing RNAs and promotes nonsense or frameshift suppression (8, 36). For example, cells harboring nonsense mutations in the *leu2* and *tyr7* genes are unable to grow on medium lacking leucine and tyrosine, and the abundances of these mRNAs are low. Deletion of *UPF1* from this strain, however, allows growth on this medium (i.e., nonsense suppression), and the abundances of these mRNAs return to wild-type levels. Thus, nonsense and frameshift suppression was hypothesized to be a consequence of the increased concentration of these mRNAs (35, 36).

The experiments described in this paper focus on understanding the functions of Upf1p. Site-directed mutations in *UPF1* were constructed, and the effects of these mutations on Upf1p function were determined by assaying the mRNA degradation and nonsense suppression phenotypes of the mutant alleles. Certain *upf1* alleles can inactivate the nonsense-mediated mRNA decay pathway without suppressing nonsense alleles. Biochemical characterization of the wild-type and mutant Upf1 proteins indicates that the Upf1p:RNA complex is modulated by ATP binding, independent of ATP hydrolysis. Modulation of the Upf1p:RNA complex formation may be an important component in affecting nonsense suppression. These results suggest that Upf1p may influence the processes of both translation and mRNA decay.

MATERIALS AND METHODS

Strains and plasmids. *Escherichia coli* CJ236 [*dut-1 ung-1 thi-1 relA1* pCJ105 (Cm^r)] was used to prepare single-stranded uridine-containing DNA for site-directed mutagenesis. *E. coli* DH5α was used to amplify plasmid DNA. Yeast transformations were performed by the lithium acetate method (27, 56). Yeast medium was prepared as described previously (49). Minimal media lacking tryptophan or lacking tryptophan and uracil were used to select transformants and maintain plasmids containing wild-type or mutant *UPF1* alleles as well as the nonsense-containing *PGK1* alleles. The yeast 2μm plasmid and centromere plasmids pYEplac112 and pYCplac22 (16) were the vectors used to transform cells with the various *upf1* alleles. The *PGK1* alleles used in this study were inserted into low-copy-number (YCp; centromere) plasmids and were described previously (21, 42, 67).

The yeast strain PLY146 (*MAT*α *ura3-52 trp1Δ1 upf1*::*URA3 tyr7-1 leu2-2*) (36) was obtained from P. Leeds and M. Culbertson, University of Wisconsin, Madison. The yeast strain JD5ts (*MAT*α *ura3-52 trp1Δ1 his4-38 leu2-1 rpb1-1*) was obtained from A. Jacobson, University of Massachusetts Medical School. The JD5ts(−) strain (*MAT*α *ura3-52 trp1Δ1 his4-38 leu2-1 rpb1-1 upf1*::*URA3*), containing a deletion of the *UPF1* gene, was prepared as described previously (8). The *ura3*$^-$ derivative of the JD5ts(−) Ura3$^-$ strain was isolated by plating the cells on medium containing 5-fluoro-orotic acid and selecting for strains able to grow on this medium. The SWP154(−) strain was described previously (42). Cytoplasmic extracts from strain BJ5457 (*MAT*α *ura3-52 trp1-Δ1 lys2-801 leu2-1 his3Δ200 pep4*::*HIS3 prb1Δ1.6R can1 GAL*) harboring *FLAG-upf1* alleles on a high-copy-number plasmid were prepared for purification of the wild-type or mutant Upf1p (11). Yeast strain Y190 {*MAT***a** *gal4 gal80 his3 ura3-52 trp1-901 ade2-101 leu2-3,112* +*URA3*::*GAL* [*lacZ LYS2*::*GAL*(*UAS*)] *HIS3 cyh*^{r}} was obtained from S. J. Elledge, Baylor College of Medicine, and was used to detect the Upf1p-Upf2p interaction.

Materials. Restriction enzymes, ligase, T4 polynucleotide kinase, and *Taq* and T4 DNA polymerases were obtained from Boehringer Mannheim, New England Biolabs, or Bethesda Research Laboratories. Radioactive nucleotides were obtained from either NEN ([γ-^{32}P]ATP) or Amersham ([α-^{32}P]dCTP). The oligodeoxynucleotides used in these studies were prepared by the University of Medicine and Dentistry of New Jersey-Robert Wood Johnson DNA synthesis center. Helper phage R408 was purchased from Promega.

Construction of *upf1* alleles harboring site-directed mutations. The *upf1* alleles harboring site-specific mutations were constructed in the *FLAG-UPF1* gene, which encodes an epitope tag at its amino terminus (DYKDDDDK) (IBI Inc.) (45). The 4.0-kb *Eco*RI-*Bam*HI fragment harboring the *FLAG-UPF1* gene was subcloned into the pBluescript KS$^-$ phagemid. pBluescript KS$^-$ phagemids harboring the *FLAG-UPF1* gene were transformed into *E. coli* CJ236. Transformants were selected on Luria-Bertani medium containing ampicillin and chloramphenicol. To obtain uridine-containing single-stranded DNA, 100 ml of Luria-Bertani liquid medium containing ampicillin and chloramphenicol was inoculated with 100 μl of overnight culture and grown to an optical density at 600 nm of 0.4 at 37°C. Helper phage R408 was added at a 10:1 ratio of helper phage to cells and grown overnight at 37°C. The single-stranded DNA was prepared by phenol extraction according to the manufacturer's protocol (Amersham). The mutant *upf1* alleles were prepared by using the oligodeoxynucleotides listed in Table 1. Oligodeoxynucleotides 1, 2, and 3 were used to prepare the DE572AA *upf1*, RR793AA *upf1*, and TR800AA *upf1* alleles, respectively (see Fig. 1A). Oligodeoxynucleotides 4 and 5 were used to prepare *upf1* alleles in the conserved lysine residue in position 436 and the RR800KK *upf1* allele, respectively (see Fig. 4A).

TABLE 1. Oligodeoxynucleotides used in this study

Oligodeoxynucleotide	Sequence[a]	Prepared mutant(s)
1	--ACTGTGTTAATTGCCGCCAGTACACAA--	DE572AA
2	--TTACGTGATCCTGCTGCTCTAAACGTG--	RR793AA
3	--AACGTGGGTCTAGCCGCCGCCAAATAT--	TR800AA
4	--CCAGGCACTGGT$^{GA}_{CC}$XACAGTTACT--	K436 series
5	--CTTACGTGATCCTAAAAAACTAAACGTGG--	RR798KK
6	--GATGGATCCCACCTATTGCGCCGCTC--	
7	--GATGGATCCATATGCCAATGGAGGGATAG--	

[a] The underlined sequences in oligodeoxynucleotides 6 and 7 are added to create *Bam*HI sites for subcloning of the *TYR7* gene.

mRNA abundance measurements and RNA preparation and analysis. To measure the RNA abundance, *upf1* alleles were transformed into yeast strain JD5ts(−) Ura3$^-$ (see Fig. 3A and B and 4B and D) or PLY146(−) (see Fig. 3C and 4C). Ten milliliters of yeast cells grown in a selective medium at 30°C to mid-log phase were harvested. Total yeast RNA was isolated as described previously (21). Equal amounts (usually 20 to 40 μg) of total RNA from each sample were analyzed by RNA blotting (59). The RNA blots were hybridized with radioactive probes prepared by random priming (see below) as described previously (21, 67). RNA blots were quantitated by using a Bio-Rad model G-250 molecular imager or model G-670 imaging densitometer. The relative mRNA abundance was obtained by assuming that the relative mRNA abundance in a *upf1*Δ strain was 100% and that that in a *UPF1*$^+$ strain was 0%. The mRNA abundances were normalized by using the wild-type *PGK1* or *CYH2* mRNA or U3 RNA.

Preparation of radioactive probes. DNA probes were labeled to high specific activity with [α-$^{32}PO_4$]dCTP (15) or by 5′ end labeling of single-stranded oligodeoxynucleotides with [γ-$^{32}PO_4$]ATP (54). Radioactive probes used to monitor the decay of mRNAs were prepared as described previously (8, 21, 42) and as follows: *CYH2* gene, 0.6-kb *Eco*RI-*Hin*dIII DNA fragment; *HIS4* gene, 4-kb *Sph*I-*Sac*I DNA fragment; *LEU2* gene, 1.5-kb *Bst*EII-*Sal*I DNA fragment; *TYR7*, 1.3-kb DNA fragment prepared by PCR corresponding to positions −342 to +1078 of the *TYR7* gene by using oligodeoxynucleotides 6 and 7; U3 gene, 0.5-kb *Bam*HI-*Hpa*I DNA fragment; *PGK1* gene, 0.5-kb *Bam*HI-*Hin*dIII fragment. Routinely, the specific activities of the radiolabeled probes were 5×10^8 cpm/μg.

Nonsense suppression assay. Plasmids containing *upf1* alleles were transformed into yeast strain PLY146, and transformants were selected in minimal medium lacking tryptophan (-Trp) to maintain the plasmid at 24°C. The cells were restreaked and replica plated twice in medium lacking leucine, tyrosine, and tryptophan (-Leu-Tyr-Trp), and growth of these cells at 24 and 30°C was monitored. To measure the growth rates, transformants as described above were grown in -Trp liquid medium to mid-log phase at 24°C and diluted to an optical density at 600 nm of 0.13 in 100 ml of -Leu-Tyr-Trp minimal medium, and the optical densities at 600 nm at various time points were determined.

SDS-PAGE and protein blotting. Standard techniques for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used as described previously (31). For immunoblotting of an SDS-PAGE gel, a polyvinylidene difluoride membrane (New England Nuclear Inc.) was used. Immunoblots were probed with the murine monoclonal M2 antibody (IBI Inc.) raised against the FLAG epitope as the primary antibody and rabbit anti-mouse immunoglobulin G antibody (whole molecule) conjugated to horseradish peroxidase (Sigma Chemical Co.) as the secondary antibody. The secondary antibody was then visualized by chemiluminescence detection (New England Nuclear Inc.).

Purification of wild-type and mutant forms of Upf1p. Purification of wild-type and mutant Upf1 proteins was done as described previously (11). All steps were performed at 0 to 4°C. In brief, crude extracts were prepared by a glass bead method with buffer X (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-eth-

anesulfonic acid]-KOH [pH 7.4], 150 mM KCl, 10% glycerol, 0.1% Triton X-100) containing 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 1 μg each of leupeptin, pepstatin A, and aprotinin per ml (buffer XA). The extracts were applied to a DEAE-Sephacel column (2.5 by 10 cm) equilibrated with buffer XA and washed with 5 column volumes of buffer XB (buffer X with the addition of 1 mM phenylmethylsulfonyl fluoride). Bound proteins were eluted with buffer XB containing 700 mM KCl in 3 column volumes. Ten microliters of each fraction was assayed by dot blot analysis (see above), and the Upf1p-containing fractions were confirmed by immunoblotting of an SDS-PAGE gel. The fractions containing Upf1p were pooled, diluted 1:2 with buffer XC (buffer X lacking KCl and containing 1 mM phenylmethylsulfonyl fluoride), and loaded onto a 1-ml anti-FLAG-M2 monoclonal antibody immunoaffinity chromatography column equilibrated with buffer XB. The flowthrough was collected and reapplied to the column. The column was washed with 50 ml of buffer XB, 50 ml of buffer XD (buffer XB containing 700 mM KCl), and 25 ml of buffer XB. Elution was performed with 10 1-ml aliquots of buffer XE (buffer XB containing 200 μg of FLAG peptide per ml). The eluted fractions were analyzed by immunoblotting and Coomassie blue staining of an SDS-PAGE gel. For long-term storage, the purified Upf1p fractions were dialyzed against storage buffer (20 mM Tris-HCl [pH 8.0], 50 mM KCl, 0.1% Triton X-100, 1 mM DTT, and 50% glycerol) for 12 h with at least two changes of buffer, and fractions were stored at −20°C. The concentration of purified Upf1p was determined from Coomassie blue-stained gels by using a Bio-Rad model GS-670 imaging densitometer with bovine serum albumin (BSA) as a protein standard.

Assay for ATPase activity. ATP hydrolysis was monitored by using a charcoal assay (6). Reactions were carried out in a total volume of 20 μl which contained 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 100 μM poly(U), 100 μM ATP, 1 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol), and various amounts of Upf1 proteins. After incubation for 20 min at room temperature, reactions were stopped and unreacted ATP was adsorbed by addition of 200 μl of 5% charcoal in 20 mM phosphoric acid. The charcoal was pelleted by centrifugation for 10 min at 13,200 × *g*, and the amount of $^{32}PO_4$ released was determined by counting the radioactivity in a 100-μl aliquot of the supernatant in a scintillation counter. For each experiment, at least five controls without Upf1p were performed in order to determine the background. Values shown are the averages from three separate experiments. The results of the experiments did not vary by more than 15%.

Nucleic acid binding assay. The ability of Upf1p to bind nucleic acid was determined by using a gel shift assay (34). A uniformly labelled 78-nucleotide RNA substrate was prepared as described previously (11). Binding reaction mixtures (20 μl) containing 20 mM HEPES-KCl (pH 7.6), 50 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 2 U of RNase inhibitor, 0.1 mg of BSA per ml, 50 fmol of RNA, and various amounts of Upf1p were incubated for 20 min at room temperature. When indicated, 2 μl of 10 mM ATP was added after 15 min of incubation. The mixtures were incubated for another 5 min. The reactions were terminated by the addition of a 5× stop buffer containing 0.1 M Tris-HCl (pH 7.4), 50% glycerol, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The RNA-protein complexes formed were separated by electrophoresis in a 4.5% PAGE gel (60:1 in 0.5× Tris-borate-EDTA containing 5% glycerol) at a 15-mA constant current for 2 to 3 h. The gels were dried, and the amount of RNA or DNA complexes was quantitated by using a Bio-Rad model GS-670 imaging densitometer.

RNA helicase assays. RNA helicase activity was examined by using a strand displacement assay (51, 60). The RNA substrate used in this assay was prepared as described previously (11). Helicase assays were performed in 20-μl reaction mixtures with 20 mM HEPES-HCl (pH 7.6), 50 mM KCl, 3 mM $MgCl_2$, 2 mM DTT, 0.1 mg of BSA per ml, 2 U of RNase inhibitor, 1 mM ATP, 50 fmol of RNA substrate, and various amounts of Upf1p. The reaction mixtures were incubated for 30 min at room temperature, and reactions were stopped by the addition of 5 μl of 5× stop buffer containing 0.1 M Tris-HCl (pH 7.4), 50% glycerol, 0.5% SDS, 0.1% Nonidet P-40, 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples from assays were electrophoresed in a 17% PAGE gel (30:1 in 1× Tris-borate-EDTA at 15-mA constant current for 2 to 3 h. The PAGE gels were dried and autoradiographed. Assay results were analyzed by using a Bio-Rad model GS-670 imaging densitometer.

Determination of the Upf1p-Upf2p interaction. The interaction between wild-type or mutant forms of Upf1p and Upf2p was monitored by use of a two-hybrid system (17). The wild-type *UPF1* gene or its mutant alleles were subcloned into *Nco*I-*Bam*HI sites of plasmid pACT2 (pACT2-UPF1) obtained from S. J. Elledge, Baylor College of Medicine (61). The *UPF2* gene was subcloned into *Nco*I-*Sma*I sites of the pAS2 plasmid as described previously (61). Various combinations of plasmids were then transformed into yeast strain Y190. Filter and liquid assays were used to monitor the β-galactosidase activity and were carried as described before (49). In liquid assays, cells were grow to mid-log phase, extracts were prepared by glass bead lysis, and 75 μg of total protein was used to measure β-galactosidase activity.

RESULTS

Analysis of mutations in the *UPF1* gene. Previous results have demonstrated that wild-type Upf1p reduces the abundance of nonsense-containing transcripts in cells and prevents nonsense suppression (35, 36) (Table 2). On the other hand, strains in which the *UPF1* gene has been deleted (*upf1*Δ strains) increase the levels of these mRNAs and allow nonsense suppression to occur (8, 36) (Table 2). The increased levels of nonsense-containing mRNAs were thought to account for the nonsense suppression phenotype (36). For example, *UPF1*$^+$ cells harboring nonsense-containing *tyr7-1* and *leu2-2* alleles did not grow on medium lacking tyrosine and leucine (-Leu-Tyr), and the *tyr7-1* and *leu2-2* mRNA levels were low (Table 2) (see below). Conversely, a *upf1*Δ strain grew on this medium, and the abundances of these transcripts were high (Table 2) (see below). As described below, a series of mutations of the *UPF1* gene were constructed and analyzed in a *upf1*Δ strain harboring *tyr7-1* and *leu2-2* nonsense alleles. The *FLAG-UPF1* allele, which harbors an epitope tag at the amino terminus of its protein-coding region, was utilized to construct the mutant *upf1* alleles and to detect and subsequently purify Upf1p from yeast cells (11, 45). Cells harboring the *FLAG-UPF1* allele had the same mRNA turnover and nonsense suppression phenotypes as cells harboring the wild-type *UPF1* gene (11, 45). The effect of mutations in the *UPF1* gene on the Upf1p function was analyzed both by determining the abundances of nonsense-, frameshift-, and intron-containing RNAs, which are substrates for nonsense-mediated mRNA decay, and by monitoring the nonsense suppression phenotype of the *tyr7-1* and *leu2-2* alleles.

TABLE 2. Summary of genetic and biochemical properties of mutations in the helicase region of Upf1p

UPF1 allele	Nonsense suppression[a] with:		Abundance of nonsense-containing mRNAs[b]	ATPase activity[c]	Helicase activity[c]	RNA:Upf1p complex formation[d]
	YEp	YCp				
UPF1 (wild type)	−	−	Low	+	+	+
*upf1*Δ	+	+	High	−	−	−
K436Q	+	+	High	−	−	+
DE572AA	−	+	High	−	−	+
TR800AA	ts	+	High	−	−	−
RR793AA	ts	+	High	−	−	−
RR793KK	−	−	Low	+	+	+

[a] Nonsense suppression phenotypes were determined in yeast strain PLY146, which harbors *leu2-2* and *tyr7-1* nonsense alleles. −, nonsense suppression was not observed; +, nonsense suppression was observed; ts, cells were temperature sensitive in nonsense suppression (i.e., nonsense suppression was observed at 30°C but not at 24°C).

[b] the abundances of *leu2-2*, *tyr7-1*, and nonsense-containing *PGK1* (mini-*PGK*) mRNAs as well as *CYH2* precursor in cells harboring various *upf1* alleles were determined and compared with those in *UPF1*$^+$ and *upf1*Δ strains.

[c] The assay was performed as described in Materials and Methods, −, activity was not detectable by the assay used; +, activity was readily detectable.

[d] A gel shift assay was used to monitor RNA:Upf1p complex formation. −, the RNA:Upf1p complex was not observed; +, the RNA:Upfp1 complex was observed.

Characterization of the expression levels of the wild-type and mutant forms of Upf1p. A set of mutations that change amino acids located in the conserved ATPase-helicase region of Upf1p was prepared (Fig. 1). Table 2 summarizes the mutations in the *UPF1* gene and the mRNA turnover and nonsense suppression phenotypes of strains containing these mutations (see below). The aspartic acid and glutamic acid (DE) residues, which were highly conserved among both helicase superfamily groups I and II and are thought to participate in ATP hydrolysis, were changed to alanine residues (Fig. 1A; DE572AA). In addition, two arginine residues at positions 793 and 794 and the threonine and arginine residues at positions

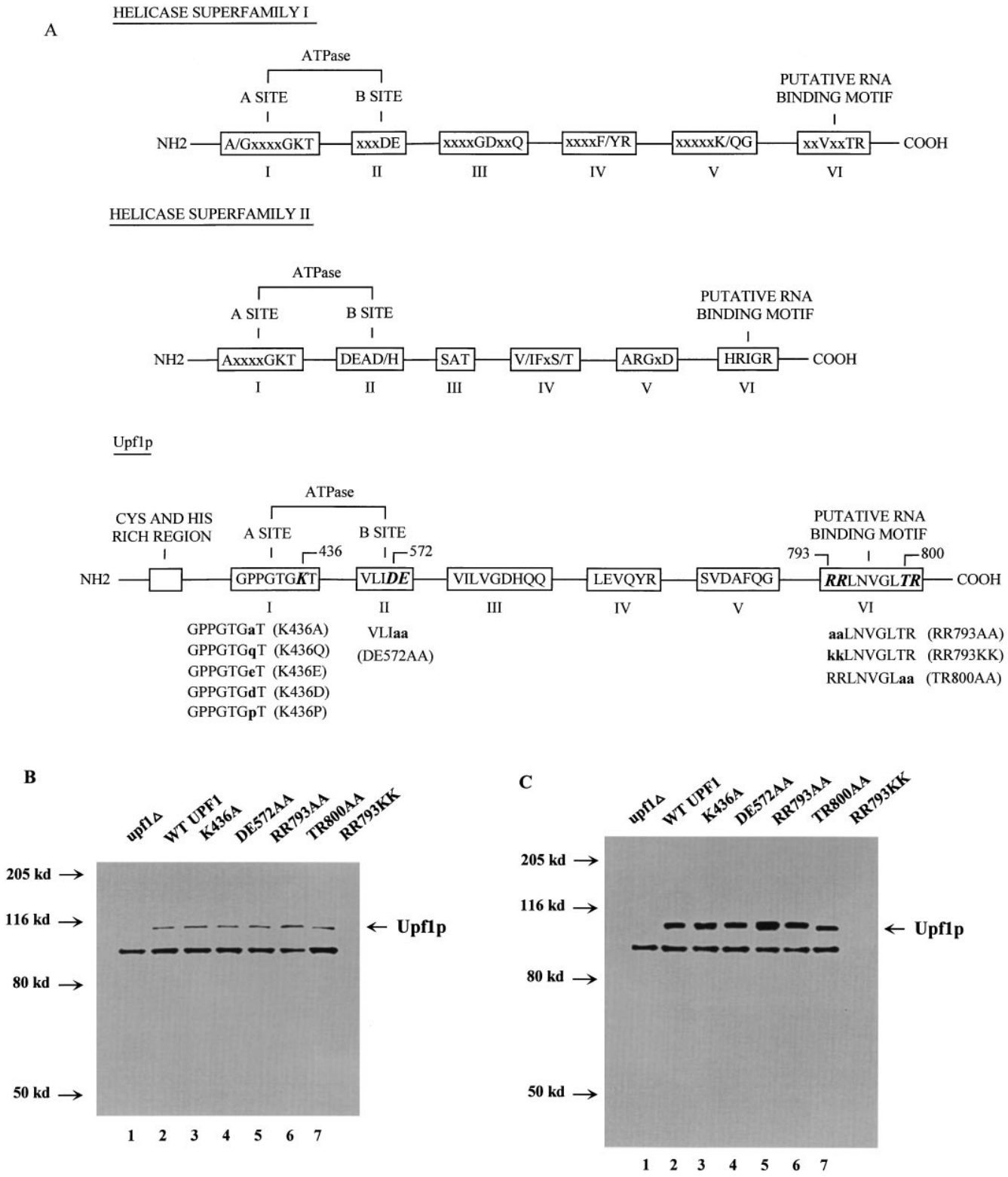

FIG. 1. Mutagenesis of the *UPF1* gene and expression of the *upf1* alleles. (A) Schematic representation of the conserved motifs in helicase superfamily groups I and II and Upf1p (29, 57). The location of the cysteine- and histidine-rich region in Upf1p is indicated by an open box. The roman numerals below the boxes indicate the nomenclature of the conserved regions as described by Hodgman (26). Mutations introduced into Upf1p are shown below the corresponding boxed motif. The numbers above the amino acids in Upf1p indicate the position of the residue. The mutated amino acids are shown as boldface italic letters, and the new amino acids in these positions are shown as boldface lowercase letters. The names of the mutant *upf1* alleles are shown in parentheses. (B and C) Western blotting analysis of yeast cell extracts harboring low-copy-number (B) and high-copy-number (C) plasmids with *upf1* alleles. Cell extracts were prepared as described in Materials and Methods. Total protein (50 μg in panel B and 20 μg in panel C) was loaded in each lane, and the expression levels of the Upf1p mutants were monitored with anti-FLAG antibody. WT, wild type.

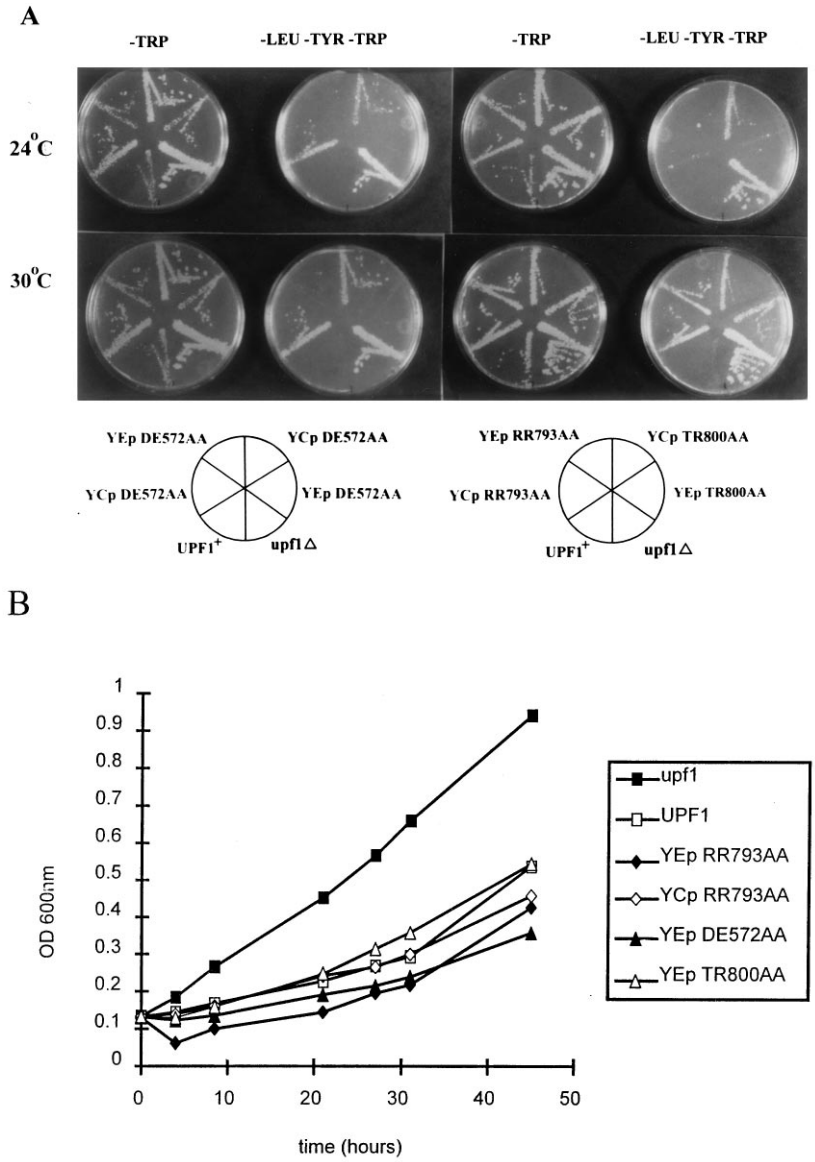

FIG. 2. Characterization of the nonsense suppression phenotype in cells harboring various *upf1* alleles. The nonsense suppression phenotypes were monitored by growing PLY146 cells harboring various *upf1* alleles on -Leu-Tyr-Trp medium at 24 or 30°C as described in Materials and Methods. (A) A photograph of the plates and a diagram indicating which of the *upf1* alleles are present in the strains are shown. The designations of the mutant *upf1* alleles are as described in the text and in Fig. 1. YEp, 2μm (high-copy-number) plasmid; YCp, centromere (low-copy-number) plasmid; *upf1*Δ, cells harboring the pYCplac22 or pYEplac112 plasmid lacking the *UPF1* gene; *UPF1*$^+$, wild-type *UPF1* gene inserted into plasmid pYCplac22 or pYEplac112. (B) Growth curves of strains harboring the indicated *upf1* alleles were determined in -Leu-Tyr-Trp minimal medium at 24°C. The designations of the *upf1* alleles are as described in Fig. 1A and in the text. OD 600nm, optical density at 600 nm.

800 and 801 were changed, in separate constructs, to alanine residues or lysine residues (Fig. 1A; RR793AA, RR793KK, and TR800AA). The amino acids at both of these positions are highly conserved among the members of helicase superfamily I, and the positive charge of this motif has been implicated in RNA binding in helicase superfamily II (37). Furthermore, the highly conserved lysine residue (K-436) in the P loop, previously demonstrated to participate in ATP binding and hydrolysis in other proteins (14, 18, 28, 30), was mutagenized to an alanine, glutamine, aspartic acid, glutamic acid, or proline (Fig. 1A).

The expression levels of various mutant *upf1* alleles inserted into centromere (YCp; low copy number) or 2μm (YEp; high copy number) plasmids were monitored by Western blotting (immunoblotting) with an antibody to the FLAG epitope. An endogenous protein of about 95 kDa cross-reacted with the FLAG antibody on a Western blot and was observed in cell extracts lacking the *FLAG-UPF1* allele (Fig. 1B and C, lanes 1) (11). Flag-Upf1p had an apparent molecular mass of approximately 110 kDa (Fig. 1B and C, lanes 2) (11) and was not present in cell extracts lacking the *FLAG-UPF1* allele (Fig. 1B and C, lanes 1). With normalization to the nonspecific band as a control, the concentrations of the wild-type and mutant forms of the Upf1p on either a high- or low-copy-number plasmid were similar (Fig. 1B and C, compare lanes 2 with lanes 3 to 7).

The DE572AA, RR793AA, and TR800AA *upf1* alleles separate the nonsense suppression and mRNA turnover functions of Upf1p. The effects of the DE572AA, RR793AA, and

TR800AA *upf1* alleles (Fig. 1A) on nonsense suppression were monitored by individually transforming these alleles on either high- or low-copy-number plasmids into a *upf1*Δ yeast strain harboring *tyr7-1* and *leu2-2* nonsense alleles and replica plating these cells to -Leu-Tyr medium. As expected, *UPF1*$^+$ strains harboring the *tyr7-1* and *leu2-2* nonsense alleles did not grow on -Leu-Tyr medium, while *upf1*Δ strains grew (Fig. 2A, compare *UPF1*$^+$ with *upf1*Δ; Table 2). The strain harboring the DE572AA *upf1* allele inserted into a low-copy-number plasmid grew on this medium, but this strain grew slowly or not at all when this *upf1* allele was present on a high-copy-number plasmid (Fig. 2A). This result indicates that overexpression of the DE572AA *upf1* allele functioned analogously to wild-type Upf1p in modulating nonsense suppression. Cells harboring the mutant RR793AA *upf1* allele did not grow at 24°C on -Leu-Tyr medium (although population was observed as a consequence of either reversion or a second-site mutation) but grew well at 30°C when present on a high- or low-copy-number plasmid (Fig. 2A), indicating that the RR793AA *upf1* allele was functional in preventing nonsense suppression only at the lower temperature. Similarly, cells harboring the TR800AA *upf1* allele on a high-copy-number plasmid were also temperature sensitive for growth on this medium, while cells with the same mutant allele on a low-copy-number plasmid grew at both 24 and 30°C (Fig. 2A). Growth curves of the strains harboring the mutant *upf1* alleles at 24°C are shown in Fig. 2B and confirm the growth phenotypes shown in Fig. 2A.

The abundances of the frameshift and intron-containing *his4-38* mRNA and *CYH2* precursor were previously demonstrated to be inversely related to the activity of the nonsense-mediated mRNA decay pathway in the cell (8, 24). To relate the nonsense suppression phenotype observed in cells harboring the DE572AA, RR793AA, and TR800AA *upf1* alleles (Fig. 2A) to their effect on the accumulation of nonsense-containing mRNAs, the levels of these RNAs were determined by Northern (RNA) blotting. As expected, the abundances of the *his4-38* mRNA and *CYH2* precursor were low in a *UPF1*$^+$ strain, while the levels of these RNAs increased in a *upf1*Δ strain [Fig. 3, compare *UPF1*(+) with *UPF1*(−)]. The abundances of the *his4-38* mRNA and *CYH2* precursor increased in cells harboring the DE572AA and TR800AA *upf1* alleles on low-copy-number plasmids, which were inactive *upf1* alleles in preventing nonsense suppression and allowed these cells to grow on -Leu-Tyr medium (Fig. 2A and 3A; Table 2). Unexpectedly, however, the *CYH2* precursor and *his4-38* mRNA accumulated in cells harboring the DE572AA and TR800AA *upf1* alleles on high-copy-number plasmids, as well as in cells harboring the RR793AA allele on high- or low-copy-number plasmids at both 24 and 30°C (Fig. 3A; Table 2). These alleles prevented cell growth on -Leu-Tyr medium, indicating that they were active in preventing nonsense suppression at the nonsense codons of the *tyr7-1* and *leu2-2* alleles. The nonsense-containing mRNAs accumulated to a greater extent in cells harboring the DE572AA and TR800AA *upf1* alleles than in those harboring the RR793AA *upf1* allele (Fig. 3A). Furthermore, the nonsense-containing *PGK1* mRNA (Fig. 3B), as well as the *tyr7-1* and *leu2-2* nonsense-containing transcripts, whose nonsense suppression phenotype and mRNA abundance are sensitive to the activity of the nonsense-mediated mRNA decay pathway in the cell, also accumulated in cells harboring these *upf1* alleles (Fig. 3C). Measurement of the half-lives of these mRNAs demonstrated that their increased accumulation was a consequence of stabilization of these RNAs in cells harboring the *upf1* alleles (data not shown). These results indicate that certain mutations in the helicase region of Upf1p result in a disparate relationship between its mRNA turnover and nonsense suppression activities. Although the mRNA levels of the *tyr7-1* and *leu2-2* transcripts are high in cells harboring the mutant *upf1* alleles and equivalent to their concentration in a *upf1*Δ strain, the synthesis of the Tyr7 and Leu2 proteins was insufficient to allow for growth on -Leu-Tyr medium. The low levels of Tyr7p and Leu2p appear to be a consequence of Upf1p functioning by modulating suppression of these nonsense alleles rather than affecting translation initiation, since previous results have demonstrated that there was no difference between the polysome profiles of *UPF1*$^+$ and *upf1*Δ strains (24, 35). Furthermore, there was no difference between the β-galactosidase activities when the ORF1 of *GCN4* was fused with the LacZ protein-coding region in *UPF1*$^+$ and *upf1*Δ strains (52). Upf1p is also not likely to be a translation elongation factor because of its low abundance (3, 36, 43, 58).

A conservative arginine-to-lysine change in the putative RNA binding motif did not alter the activity of Upf1p in promoting nonsense-mediated mRNA decay and preventing nonsense suppression. The positively charged areas of region VI of the putative helicase motifs of superfamily groups I and II are putative RNA/DNA binding domains for RNA/DNA helicases (37) (Fig. 1A). The results shown in Fig. 2 and 3 indicated that changing the arginine residues to alanine residues at position 793 and 794 in this motif affected both the mRNA decay and nonsense suppression phenotypes in these cells. We asked whether a conservative change of the arginine residues to lysine residues would affect the activity of Upf1p. To test this possibility, an RR793KK *UPF1* allele was prepared by site-directed mutagenesis, and the effect of these changes on nonsense suppression and mRNA abundance of nonsense-containing alleles was monitored. The abundances of the *CYH2* precursor and *his4-38* mRNA were low in cells harboring either the wild-type *UPF1* gene or the RR793KK *UPF1* allele (Fig. 4D; Table 2), indicating that the RR793KK lesion did not inactivate the decay activity of Upf1p. Furthermore, cells harboring the RR793KK *UPF1* allele did not grow on -Leu-Tyr medium (Fig. 4D). Collectively, these results demonstrate that the RR793KK mutation in the *UPF1* gene did not affect either the nonsense suppression or mRNA decay activity of Upf1p.

Mutations in the conserved lysine residue in the putative ATP binding domain stabilize nonsense-containing mRNAs and cause translational suppression of nonsense codons. The highly conserved lysine residue (K-436) in the P loop, previously demonstrated to participate in ATP binding and hydrolysis in other proteins (14, 18, 28, 30) was mutagenized to five other amino acids (alanine, aspartic acid, glutamic acid, glutamine, and proline) (Fig. 4A). The effects of these *upf1* alleles on the nonsense suppression phenotype and mRNA abundance were determined as described above. The *CYH2* precursor, *his4-38* frameshift mRNA, and *tyr7-1* and *leu2-2* transcripts accumulated in strains harboring the mutant *upf1* alleles containing an altered lysine codon, indicating that these mutations inactivated the nonsense-mediated mRNA decay pathway (Fig. 4B and C). Furthermore, cells containing the various K-436 mutant *upf1* alleles grew on -Leu-Tyr medium, demonstrating suppression of the *leu2-2* and *tyr7-1* nonsense alleles (Fig. 4B). Thus, mutating the lysine residue in position 436 to any of the five amino acids inactivated the functions of Upf1p in promoting mRNA decay and preventing nonsense suppression. These phenotypes were not the consequence of the concentration of Upf1p within the cell, since the amount of the K-436 mutant Upf1p in cells was similar to the quantity of the other mutant Upf1 proteins in the cell (Fig. 1B and C and data not shown).

Purification of the variant forms of Upf1p. The biochemical properties of the mutant forms of Upf1p were analyzed and

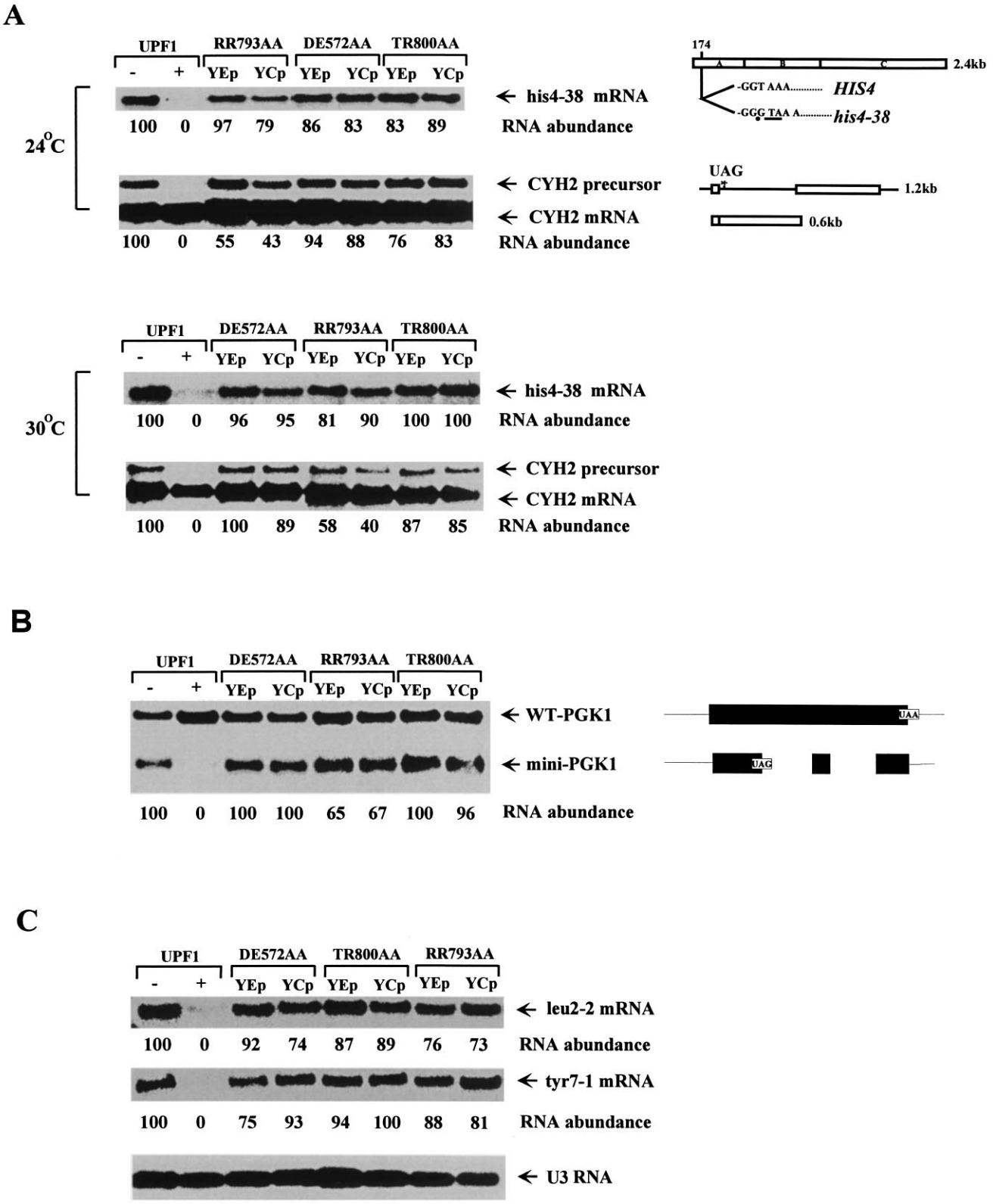

FIG. 3. Effect of the mutant *upf1* alleles on the abundance of nonsense-containing RNAs. (A) The abundances of wild-type and nonsense-containing mRNAs were determined by Northern blotting analysis of RNAs isolated from cells [JD5ts(−) Ura3$^-$ strain] harboring the varoius *upf1* alleles grown at 24 or 30°C. The membrane was hybridized with radiolabeled DNA fragments from either the *CYH2* or *HIS4* gene and quantitated as described in Materials and Methods. The relative RNA abundances in cells harboring *upf1* alleles are indicated below the autoradiograph. To the right of the autoradiograph is a schematic representation of the *CYH2* precursor and its spliced product, as well as the *HIS4* gene and the location of the frameshift insertion. (B) The abundances of the wild-type (WT) *PGK1* and nonsense-containing *PGK1* (mini-*PGK1* [21, 67]) transcripts were determined at 24°C in the yeast strain JD5ts(−) Ura3$^-$ harboring plasmids containing the *upf1* alleles. To the right of the autoradiograph is a schematic representation of the wild-type and nonsense-containing mini-*PGK1* alleles. The transcripts were quantitated and normalized to the abundance of the endogenous *PGK1* transcript. The relative RNA abundances in cells harboring *upf1* alleles were determined as described in Materials and Methods and are indicated below the blot. (C) the abundances of the nonsense-containing *leu2-2* and *tyr7-1* mRNAs were determined at 24°C in PLY146 as described above by using a radiolabeled DNA fragment harboring the *LEU2* or *TYR7* gene and normalized to U3 RNA. The relative RNA abundances in cells harboring *upf1* alleles are indicated below the blot.

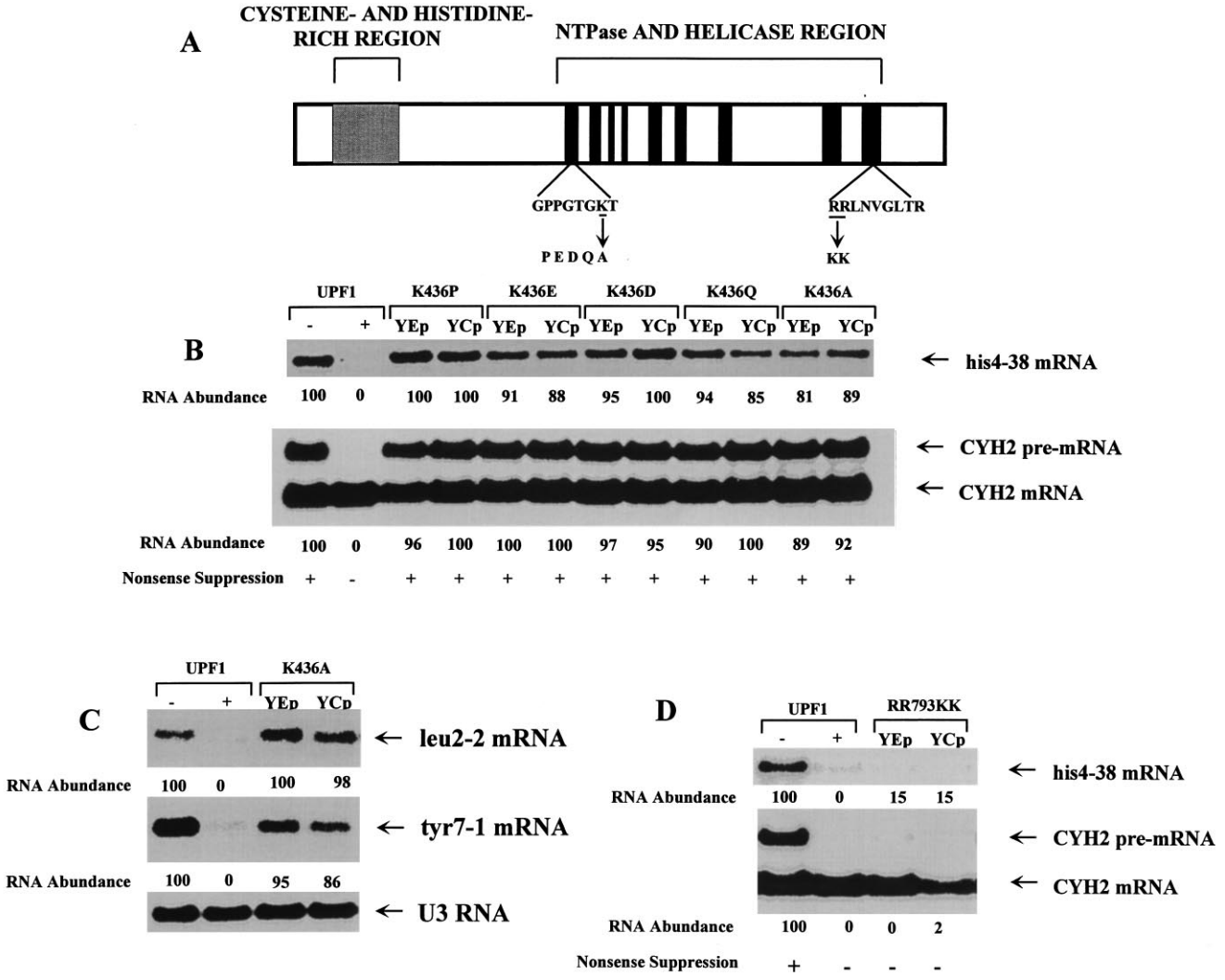

FIG. 4. RNA abundance and nonsense suppression in cells containing mutations in the ATPase and helicase motifs of the *UPF1* gene. (A) Schematic representation of the *UPF1* gene depicting the locations of the cysteine- and histidine-rich region (shaded region) and the conserved regions of the nucleoside triphosphatase (NTPase) and helicase motifs (solid vertical bars). The mutagenized amino acids in the putative ATP binding/hydrolysis and RNA/DNA binding domains are underlined, and the new amino acids in these positions are indicated. (B) The abundances of *CYH2* precursor and *his4-38* mRNAs were measured in strain JD5ts(−) Ura3$^-$ harboring various *upf1* alleles at 24°C and normalized to *CYH2* mRNA. The same results were obtained if the cells were grown at 30°C (data not shown). The relative RNA abundances in cells harboring these mutant alleles were obtained as described for Fig. 3. The nonsense suppression phenotypes for the *leu2-2* and *tyr7-1* alleles were determined as described for Fig. 2. The same growth characteristics were observed at both 24 and 30°C. +, nonsense suppression was observed (the cells grew on -Leu-Tyr-Trp medium); (−), nonsense suppression was not observed (the cells did not grow on -Leu-Tyr-Trp medium). (C) The abundances of the *leu2-2* and *tyr7-1* transcripts in the yeast strain PLY146 harboring the K436A *upf1* allele were determined as described above. (D) RNA abundances and nonsense suppression phenotypes in cells harboring the RR793KK *upf1* allele, the wild-type *UPF1* gene (*UPF1*$^+$), or the vector (*upf1*$^-$). The *CYH2* precursor and *his4-38* mRNA abundances and nonsense suppression phenotypes were determined in yeast cells harboring mutant RR793KK as described for panel B.

compared with the activities observed for wild-type Upf1p. The wild-type, K436Q, RR793AA, RR793KK, TR800AA, and DE572AA forms of Upf1p were purified by immunoaffinity chromatography directed against the FLAG epitope as described previously (11). SDS-PAGE of the various Upf1p mutants eluted from the antibody column are shown in Fig. 5. Samples contained only one band, with an apparent molecular mass of approximately 110 kDa (Fig. 5A), which reacted with the anti-FLAG antibody as detected by immunoblotting (Fig. 5B).

Characterization of the ATPase and helicase activities of Upf1p mutants. The ATPase activity of the purified K436Q, RR793AA, RR793KK, TR800AA, and DE572AA Upf1p proteins was monitored as described previously (11) and compared with the ATPase activity of the wild-type Upf1p. The RR793KK Upf1p was an active ATPase, albeit with reduced activity compared with that of the wild-type Upf1p (Fig. 6). No ATP hydrolysis was observed, however, for the DE572AA, K436Q, RR793AA, and TR800AA mutant proteins (Fig. 6; Table 2).

The RNA helicase activities of the wild-type and mutant forms of Upf1p were examined. The RNA substrate used for monitoring helicase activity is illustrated in Fig. 7A. Previous results indicated that Upf1p is a 5′→3′ RNA helicase (11). The present results demonstrate that although the wild-type Upf1p promoted displacement of the radiolabeled double-stranded RNA substrate (Fig. 7A and B), the K436Q, DE572AA, RR793AA, and TR800AA forms of Upf1p had no helicase

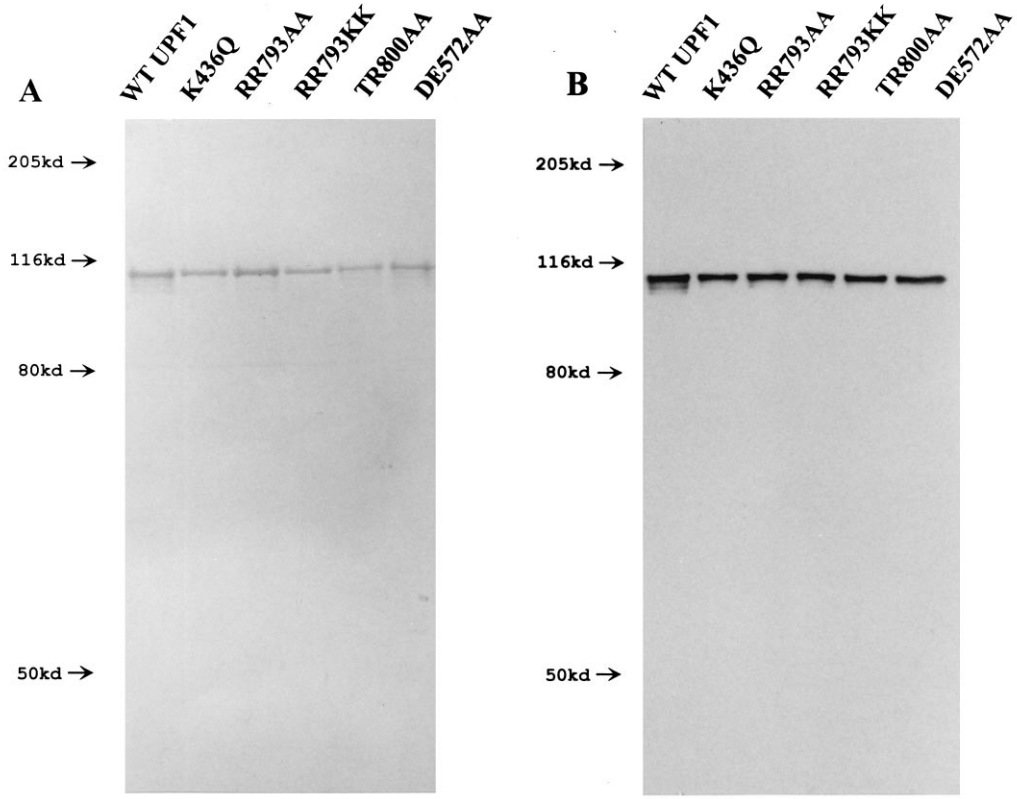

FIG. 5. Purification of wild-type and mutant Upf1 proteins. The wild-type (WT) and mutant forms of Upf1p were purified as described in Materials and Methods. The purified proteins were analyzed by SDS-PAGE and either stained with Coomassie blue (A) or immunoblotted with a monoclonal antibody against the FLAG epitope (B).

activity (Fig. 7A; Table 2). The RR793KK Upf1p resulted in the displacement of the double-stranded RNA substrate, which increased with greater protein concentrations (Fig. 7B and C). The helicase activity of the RR793KK mutant Upf1p, however, was significantly reduced compared with that of the wild-type Upf1p (Fig. 7B and C). Interestingly, yeast cells containing this allele had wild-type mRNA turnover and nonsense suppression phenotypes. The RNA helicase activity was dependent on ATP: reaction mixtures containing a high concentration of Upf1p but no ATP lacked RNA helicase activity (Fig. 7B, lanes 5 and 10).

RNA binding activities of the wild-type and mutant forms of Upf1p. The RNA binding characteristics of the wild-type and mutant Upf1 proteins were investigated by using a gel shift assay as described previously (11). The substrate for the RNA binding assay was a uniformly radiolabeled, in vitro-prepared 78-nucleotide transcript (see Materials and Methods). In the absence of ATP, the wild-type, K436Q, RR793KK, and DE572AA Upf1 proteins form a more slowly migrating band, indicating formation of a Upf1p:RNA complex (Fig. 8, lanes 2 to 6, 10 to 15, 17 to 21, and 23 to 27; Table 3). The RR793KK Upf1p, which demonstrated wild-type nonsense suppression and mRNA turnover phenotypes in vivo, had a reduced RNA binding activity compared with the wild-type Upf1p (Fig. 8, compare lanes 23 to 27 with lanes 2 to 6, 10 to 15, and 17 to 21). The RR793AA and TR800AA mutant forms of Upf1p did not bind to RNA (Fig. 8, lanes 8 and 9).

We next determined whether addition of ATP to the reaction mixture altered complex formation of Upf1p with RNA. Binding mixtures were prepared as described above, and ATP was subsequently added to the mixture. As observed previously (11), addition of ATP to the mixtures markedly reduced complex formation for the wild-type and RR793KK Upf1 proteins (Fig. 8, lanes 7 and 28). As expected, addition of ATP had no affect on RNA:K436Q Upf1p complex formation (Fig. 8, lane

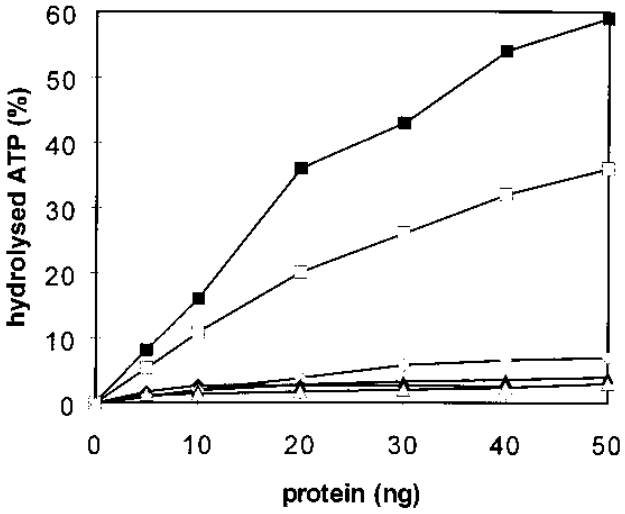

FIG. 6. ATPase activities of mutant forms of Upf1p. The ATPase activities of the wild-type and mutant forms of Upf1p were determined by using a charcoal assay (see Materials and Methods). ■, wild-type *UPF1*; □, RR793KK; ◆, DE572AA; ◇, K436Q; ▲, RR793AA; △, TR800AA.

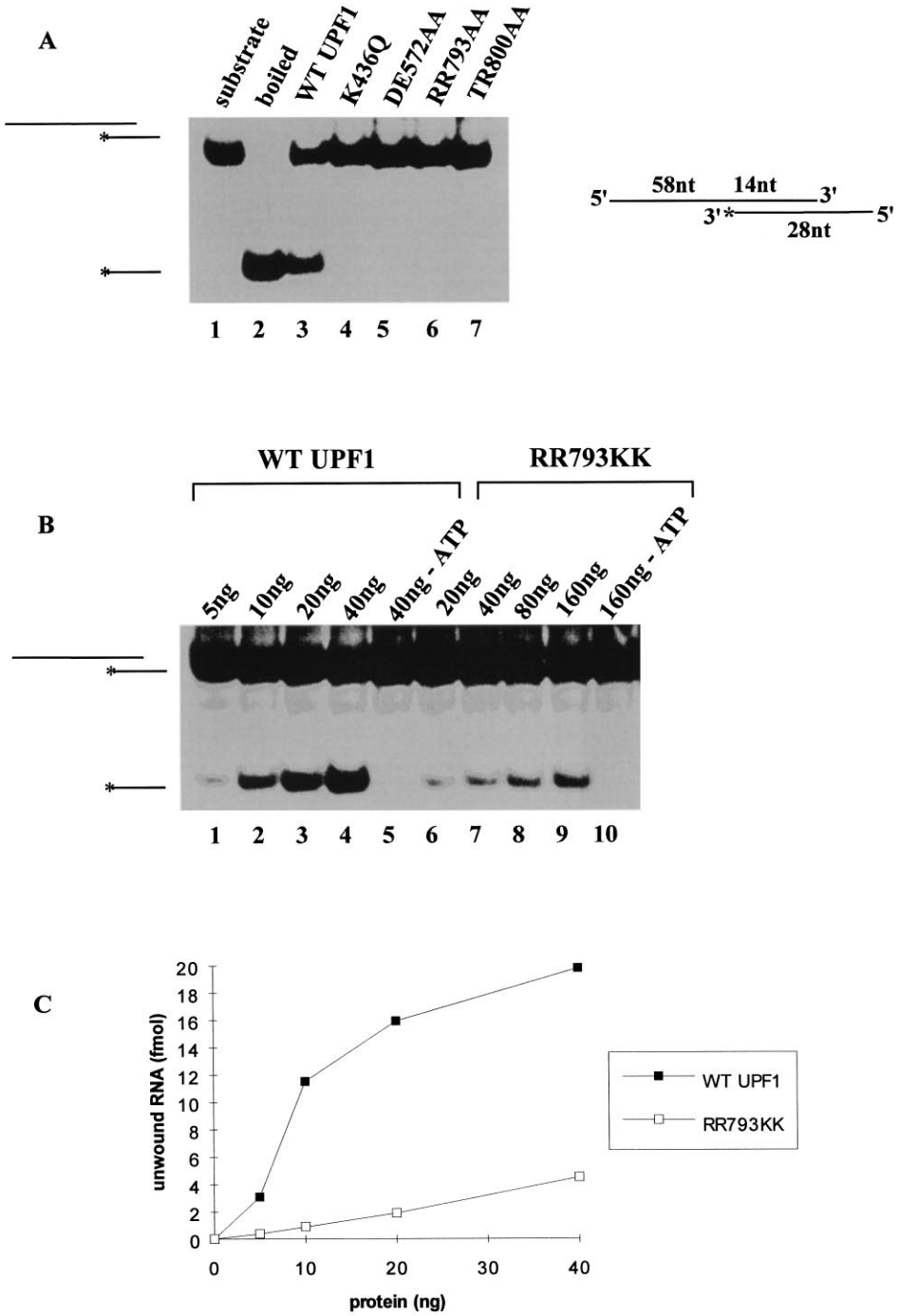

FIG. 7. RNA helicase activities of mutant Upf1 proteins. (A) A strand displacement assay was used to determine the relative helicase activities of the mutant forms of Upf1p. The substrate used was prepared as described in Materials and Methods, and a schematic representation is illustrated to the right. The asterisk depicts the radiolabeled transcript. The helicase reactions were carried out with 50 fmol of the RNA substrate and 20 ng of wild-type (WT) or 200 ng of mutant Upf1p under the standard assay conditions as described in Materials and Methods. The product of the helicase reaction runs as a faster-migrating band. nt, nucleotide. (B) Conservative changes in the putative RNA binding motif (RR798KK) reduced the helicase activity of the Upf1p. The helicase reactions were performed under standard conditions with various amounts of wild-type or RR793KK Upf1p. (C) The results shown in panel B were quantitated as described in Materials and Methods.

22). This mutation would be expected to reduce or abolish the ATP binding of Upf1p. Surprisingly, ATP addition also reduced complex formation with DE572AA Upf1p (Fig. 8, lane 16). This result suggested that although the DE572AA Upf1p does not have ATPase or helicase activity, it is still capable of binding ATP and that ATP binding, independent of its hydrolysis, is sufficient to cause dissociation of the RNA:DE572AA Upf1p complex.

Mutations in the ATPase or helicase region of Upf1p do not affect Upf1p-Upf2p interaction. By using the two-hybrid system to genetically monitor protein-protein interactions, it has been shown that Upf1p and Upf2p interact (22, 23). To determine

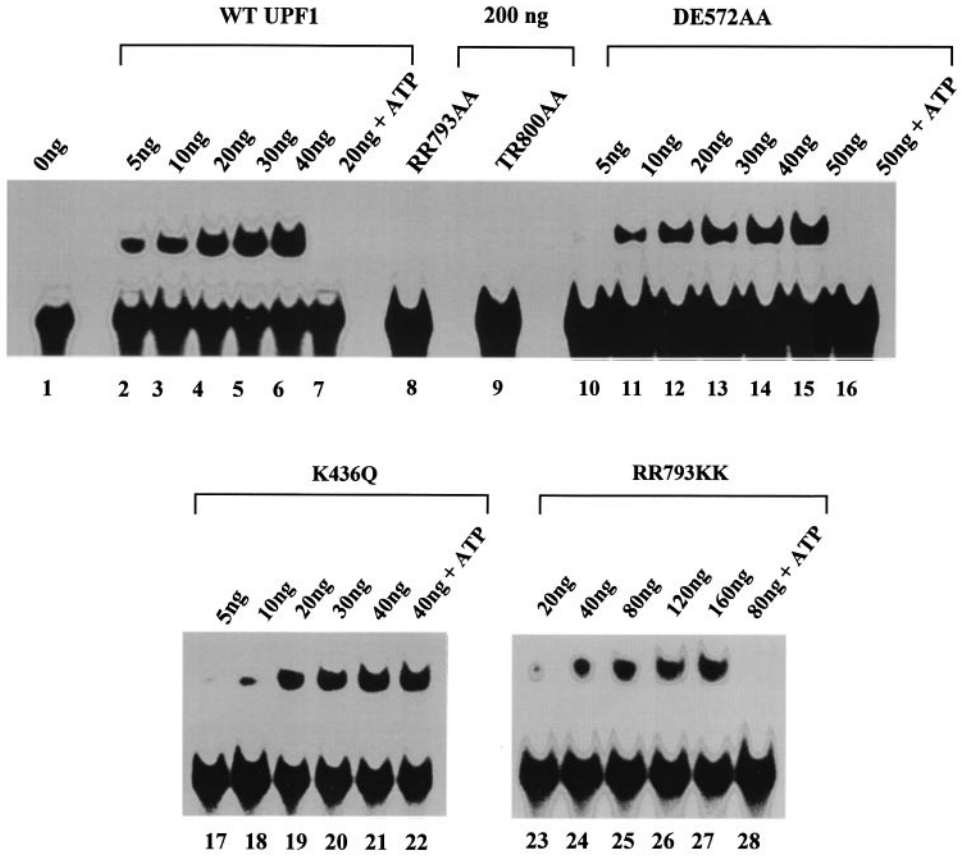

FIG. 8. Comparison of the RNA binding activities of the wild-type (WT) and mutant forms of Upf1p. RNA binding assays were performed under standard conditions with various amounts of protein as described in Materials and Methods. When indicated, 2 μl of 10 mM ATP was added after 15 min of incubation. An autoradiograph of a gel shift assay is shown. The mutant forms of Upf1p are shown above the autoradiograph and are designated as in the text.

whether the mutations in the ATPase and helicase regions of Upf1p altered its interaction with Upf2p, the mutant *upf1* alleles were fused in frame to the *GAL4* DNA binding domain or activation domain. Plasmids harboring *GAL4-UPF1* and *GAL4-UPF2* fusions were transformed into yeast cells harboring a UAS-LacZ fusion. Cell extracts were prepared, and the β-galactosidase activities were measured as a monitor of protein-protein interactions. Consistent with previous findings, Upf1p interacts with Upf2p (Table 3) (22, 23). The DE572AA, RR793AA, and TR800AA forms of Upf1p interact with Upf2p as monitored by this assay, with activity similar to that observed for the wild-type Upf1p. These results suggest that the mutations in the *UPF1* gene that inactivated the nonsense-mediated mRNA decay pathway did not cause disruption of the Upf1p-Upf2p interaction.

TABLE 3. Interactions of the wild-type or mutant forms of Upf1p and Upf2p[a]

Plasmids	β-Galactosidase activity
ACT2-UPF1/AS2-UPF2	130
ACT2-DE572AA/AS2-UPF2	139
ACT2-TR800AA/AS2-UPF2	181
ACT2-RR793AA/AS2-UPF2	157
ACT2-UPF1/AS2	0.25
AS2-UPF2/ACT2	0.25

[a] The interactions of wild-type and mutant forms of Upf1p with Upf2p were determined by the two-hybrid system. The wild-type and mutant *UPF1* alleles were fused to plasmid ACT2 (which contains the *GAL4* activation domain), and the *UPF2* gene was fused in frame to plasmid AS2 (which contains the *GAL4* DNA binding domain). Plasmids with various combinations were transformed into yeast strain Y190, and β-galactosidase activities in these cells were determined as described in Materials and Methods.

DISCUSSION

Utilizing the nonsense-mediated mRNA decay pathway to understand how mRNA decay and translation are linked. We have been investigating the relationship between translation and mRNA turnover by utilizing the observation that premature translation termination accelerates the decay of nonsense-containing mRNAs. Both *cis*-acting sequences and *trans*-acting factors involved in the nonsense-mediated mRNA decay pathway are currently being studied (8, 21, 23, 35, 36, 67). Recent results have suggested that a subset of the steps in the decay of both nonsense-containing and wild-type transcripts are similar (12, 13, 21, 39). For example, an important cleavage event both in the nonsense-mediated mRNA decay pathway and for a subset of wild-type transcripts is the removal of the 5′ cap structure followed by degradation by a 5′→3′ exoribonuclease (12, 21, 39). Thus, we anticipate that much of what we learn concerning the nonsense-mediated mRNA decay pathway will be relevant to other turnover pathways.

The studies presented here have taken both genetic and biochemical approaches to understand how Upf1p functions in the nonsense-mediated mRNA decay pathway. Upf1p is a

member of the superfamily group I helicases (2, 11, 29). Although a systematic mutagenesis analysis for superfamily group II helicases has been performed, an equivalent analysis for superfamily group I helicases has not been previously undertaken. The results from our experiments are summarized in Table 2. Our results have identified important regions in Upf1p that are required for ATP binding and hydrolysis, helicase activity, and RNA binding activity. Interestingly, certain mutations in these regions inactivate the nonsense-mediated mRNA decay pathway and allow nonsense suppression, while other mutations inactivate nonsense-mediated mRNA decay but do not allow nonsense suppression to occur. The mutations described above separate the translation termination and mRNA decay activities of Upf1p. These results indicate that Upf1p is involved in modulating both translation termination and mRNA turnover. The biochemical activities of the mutant forms of Upf1p were investigated, and the mutants in which the mRNA turnover phenotype was separated from the nonsense suppression phenotype demonstrated an unusual RNA binding activity. This result indicates that ATP binding, independent of ATP hydrolysis, may be a cofactor that regulates the RNA binding activity of Upf1p.

Mutations in the ATPase and helicase regions of the *UPF1* gene can affect both mRNA turnover and nonsense suppression activities of Upf1p. Site-directed mutations of the *UPF1* gene that converted the highly conserved lysine residue in the ATP binding and hydrolysis P-loop site to alanine, aspartic acid, glutamic acid, glutamine, or proline increased the abundance of the *his4-38*, *leu2-2*, and *tyr7-1* transcripts and *CYH2* intron-containing RNA and promoted suppression of the *tyr7-1* and *leu2-2* nonsense alleles (Fig. 4). Thus, both the nonsense-mediated mRNA decay pathway was inactivated and nonsense suppression was observed in cells harboring these alleles. Furthermore, purification and characterization of the K436Q form of Upf1p demonstrated that it no longer had ATPase or helicase activity, and it bound RNA in the presence or absence of ATP (Fig. 6 to 8). Consistent with these results, we have demonstrated that wild-type Upf1p binds to ATP efficiently but that the K436Q mutant form of Upf1p does not (62).

The DE572AA, RR793AA, and TR800AA *upf1* alleles were interesting because they separated the mRNA decay and nonsense suppression phenotypes of Upf1p. Strains containing these alleles were inactive in nonsense-mediated mRNA decay and led to increased cellular concentrations of nonsense-containing mRNAs without suppressing the *leu2-2* and *tyr7-1* nonsense alleles. Cells harboring the DE572AA *upf1* allele on a high-copy-number plasmid did not allow suppression of the *leu2-2* and *tyr7-1* nonsense alleles (Fig. 2). Similarly, the RR793AA *upf1* allele inserted into either a high- or low-copy-number plasmid and the TR800AA *upf1* allele on a high-copy-number plasmid was temperature sensitive for nonsense suppression. Using the two-hybrid system to monitor protein-protein interaction, we demonstrated that the mutations in the ATPase and helicase regions of Upf1p did not affect the Upf1p-Upf2p interaction.

ATP binding, independent of its hydrolysis, caused the DE572AA Upf1p:RNA complex to disassociate. The DE572AA, RR793AA, and TR800AA mutant proteins did not demonstrate any ATPase or helicase activities (Fig. 6 and 7; Table 2). In addition, the RR793AA and TR800AA proteins did not bind RNA (Fig. 8; Table 2). The DE572AA Upf1p bound RNA in the absence of ATP, but, surprisingly, in the presence of ATP the Upf1p:RNA complex disassociated (Fig. 8). This result indicated that the binding of ATP, rather than its hydrolysis, modulated the RNA binding activity of Upf1p. Consistent with this view, the nonhydrolyzable analog of ATP, ATPγS, was also able to destabilize both the mutant and wild-type Upf1p:RNA complexes (62). Furthermore, a nitrocellulose binding assay has demonstrated that the DE572AA Upf1p binds to ATP (62).

It is possible that the RNA binding activity of Upf1p is important in regulating its nonsense suppression. The DE572AA, RR793AA, and TR800AA *upf1* alleles, whose protein products did not bind RNA (RR793AA and TR800AA) or whose binding activity can be modulated by interacting with ATP, were inactive in nonsense-mediated mRNA decay activity but were still capable of preventing the suppression of the *tyr7-1* and *leu2-2* nonsense alleles (Fig. 2; Table 2). Conversely, the protein product of the K436Q *upf1* allele bound RNA in the presence or absence of ATP, and strains harboring this allele inactivated the nonsense-mediated mRNA decay pathway and allowed nonsense suppression. Recent results suggest that the K436Q form of Upf1p did not bind ATP (62). Taken together, these results demonstrate that the lysine residue is absolutely necessary for ATP binding. The DE residues, however, may be involved in modulating ATP binding and are required for ATP hydrolysis. The DE572AA mutant had no detectable ATPase activity but still was able to bind ATP. This causes a change in the DE572AA protein, resulting in dissociation of the mutant DE572AA Upf1p:RNA complex. Similarly, mutating the DE residues in the translation initiation factor eIF-4A inactivates its ATPase activity, but the mutant protein can still bind ATP (41). Similar results were also observed for *E. coli* DNA helicase II (4). DNA helicase II is also a member of the helicase superfamily I and is involved in DNA repair. When the DE residues were mutated to glutamine and asparagine, the mutant protein demonstrated less than 0.5% of the ATPase activity of the wild-type protein. The affinity of binding of the mutant protein to ATP, however, was not affected (4).

Mutations in the *UPF1* gene affect nonsense suppression. Previous results demonstrated that deletion of the *UPF1* gene from the yeast chromosome resulted in stabilization of nonsense-containing mRNA and nonsense suppression (36). The increased concentration of nonsense-containing mRNAs in a *upf1*Δ strain was initially believed to account for the nonsense suppression phenotype (36). The disparate phenotypes caused by a subset of mutations in the helicase region of the *UPF1* gene described in this study, however, suggest that Upf1p may also function in modulating nonsense suppression. The phenotypes of the DE572AA, RR793AA, and TR800AA *upf1* alleles were not simply gain-of-function mutations, since other mutations in the ATP binding motif (i.e., K-436 *upf1* alleles) cause accumulation of nonsense-containing mRNAs to the same level as these mutations and lead to nonsense suppression (Figs. 4A and B). Furthermore, biochemical analysis of Upf1p does not support the view that these mutations are gain-of-function mutations (Fig. 6 to 8; Table 2). Rather, these results indicate that altered modulation of RNA binding by ATP may change the nonsense suppression activity of Upf1p. Although not proven, it is possible that the status of the RNA binding activity of Upf1p determines whether the protein functions in modulating nonsense suppression.

The genetic studies presented here demonstrated a strong link between translation and mRNA decay and that factors identified in one pathway can affect the other. One interesting idea is that there is cross talk between the *trans*-acting factors involved in these processes. The hypothesis that Upf1p is a *trans*-acting factor that can modulate nonsense-mediated mRNA decay and nonsense suppression suggests that mutations with converse phenotypes should be identified. As described in the accompanying paper (61), such mutations have been identified.

Future experiments to identify and investigate other factors that interact with Upf1p, as well as to understand how the biochemical properties of Upf1p function in mRNA turnover and translation, will address the relationship between these two processes.

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