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Identification and Characterization of Mutations in the *UPF1* Gene That Affect Nonsense Suppression and the Formation of the Upf Protein Complex but Not mRNA Turnover

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To understand the relationship between translation and mRNA decay, we have been studying how premature translation termination accelerates the degradation of mRNAs. In 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. A *UPF1* gene disruption results in the stabilization of nonsense-containing mRNAs and leads to a nonsense suppression phenotype. Biochemical analysis of the wild-type Upf1p demonstrated that it has RNA-dependent ATPase, RNA helicase, and RNA binding activities. In the work described in the accompanying paper (Y. Weng, K. Czaplinski, and S. W. Peltz, Mol. Cell. Biol. 16:5477–5490, 1996) mutations in the helicase region of Upf1p that inactivated its mRNA decay function but prevented suppression of *leu2-2* and *tyr7-1* nonsense alleles are identified. On the basis of these results, we suggested that Upf1p is a multifunctional protein involved in modulating mRNA decay and translation termination at nonsense codons. If this is true, we predict that *UPF1* mutations with the converse phenotype should be identified. In this report, we describe the identification and biochemical characterization of mutations in the amino-terminal cysteine- and histidine-rich region of Upf1p that have normal nonsense-mediated mRNA decay activities but are able to suppress *leu2-2* and *tyr7-1* nonsense alleles. Biochemical characterization of these mutant proteins demonstrated that they have altered RNA binding properties. Furthermore, using the two-hybrid system, we characterized the Upf1p-Upf2p interactions and demonstrated that Upf2p interacts with Upf3p. Mutations in the cysteine- and histidine-rich region of Upf1p abolish Upf1p-Upf2p interaction. On the basis of these results, the role of the Upf complex in nonsense-mediated mRNA decay and nonsense suppression is discussed.

To understand the relationship between translation and mRNA decay, we have been studying the nonsense-mediated mRNA decay pathway, a process in which premature translation termination accelerates the degradation of mRNAs. Both *cis*-acting sequences and *trans*-acting factors involved in this decay pathway have been identified (5, 14, 16, 22, 24, 25, 27). The *UPF1*, *UPF2*, and *UPF3* genes were initially identified as allosuppressors of the *his4-38* frameshift allele (6, 25). Subsequent studies demonstrated that yeast strains harboring mutations or deletions of these genes accumulate the *his4-38* mRNA to a level greater than that observed in wild-type strains (5, 14, 16, 22, 24, 25, 27, 39). Further studies have demonstrated that, in addition to the *his4-38* frameshift allele, certain nonsense alleles such as *leu2-1*, *leu2-2*, and *tyr7-1* can also be suppressed in strains harboring mutations or deletions (*upf1*Δ) of the *UPF* genes. Furthermore, the mRNAs transcribed from these nonsense alleles are also stabilized (5, 25, 38). The *UPF1*, *UPF2*, and *UPF3* genes have been cloned and sequenced (1, 5, 16, 22, 24, 29). The *UPF1* gene and its protein product have been most extensively characterized. The predicted amino acid sequence of the Upf1 protein (Upf1p) indicates that it contains a cysteine- and histidine-rich region near its amino terminus and all of the motifs shared by the members of RNA/DNA helicase superfamily I (1, 19, 24, 25). Upf1p has been purified, and its biochemical properties have been characterized (8). Upf1p demonstrated RNA/DNA-dependent ATPase activity and helicase activity and can bind to DNA or RNA in the absence of ATP, but its RNA/DNA binding activity is reduced in the presence of ATP (8, 38). Furthermore, Upf1p has been colocalized with polyribosomes (2, 28).

As described in the accompanying paper (38), in a *upf1*Δ strain, suppression of the *leu2-2* and *tyr7-1* nonsense alleles was observed and mRNAs transcribed from these alleles were stabilized. To understand the role of Upf1p in the nonsense-mediated mRNA decay pathway and nonsense suppression, *upf1* alleles containing mutations in the ATP binding and hydrolysis domains, as well as in the helicase motifs, were constructed. The nonsense suppression phenotype and the abundances of nonsense-containing mRNAs were monitored in cells harboring these mutant alleles (38). Mutations that abolish the ATPase and helicase activities of Upf1p result in accumulation of nonsense-containing mRNAs. Interestingly, however, certain mutations in the ATPase and helicase regions of Upf1p inactivated the nonsense-mediated mRNA decay pathway to the same degree as observed in a *upf1*Δ strain but did not result in suppression of the *leu2-2* and *tyr7-1* alleles (38). This result suggested that high levels of nonsense-containing mRNAs alone were not sufficient to allow for suppression of these alleles. We hypothesized that Upf1p is a *trans*-acting factor that can promote decay of nonsense-containing mRNAs and can be involved in preventing nonsense suppression at a premature stop codon. In a *upf1*Δ strain, the activities of Upf1p

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in both promoting mRNA decay and preventing nonsense suppression are not present. The additive effects of increased mRNA level and reduced activity in preventing nonsense suppression allowed the synthesis of sufficient levels of *tyr7-1*- and *leu2-2*-encoded readthrough protein to observe growth on medium lacking leucine and tyrosine (-Leu-Tyr medium). However, certain mutations in the ATPase and helicase motifs inactivated the mRNA decay function of Upf1p without affecting its function in preventing nonsense suppression. Thus, despite the elevated mRNA level, the amount of readthrough protein was not sufficient to allow nonsense suppression.

We predict that we should be able to identify mutations in Upf1p with the converse phenotype, i.e., that allow suppression of nonsense mutations without affecting the mRNA turnover function. In this report, we describe the identification and biochemical characterization of such mutations in Upf1p. We demonstrate that cells harboring certain mutations in the amino-terminal cysteine- and histidine-rich region have normal nonsense-mediated mRNA decay activities but are able to suppress *leu2-2* and *tyr7-1* nonsense alleles. Biochemical characterization of these proteins demonstrates that they have RNA binding, ATPase, and RNA helicase activities. The RNA:Upf1p complex formed by these mutant proteins, however, migrates more slowly on nondenaturing gels than wild-type Upf1p, suggesting that these mutant proteins either form ribonucleoproteins with altered structure or form dimers. We also show that Upf1p interacts with Upf2p and that Upf2p interacts with Upf3p but that Upf1p-Upf3p and Upf1p-Upf1p interact weakly, if at all. Furthermore, mutations in the cysteine- and histidine-rich region of Upf1p abolish Upf1p-Upf2p interaction. On the basis of these results, the role of the Upf complex in nonsense-mediated mRNA decay and nonsense suppression is discussed.

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 (18, 35). Yeast medium was prepared as described previously (30). 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 wild-type or nonsense-containing *PGK1* alleles. The yeast 2μm plasmid and centromere plasmids YEplac112 and YCplac22 (11) were the vectors used to transform cells with the various *upf1* alleles. The *PGK1* alleles used in this study were inserted into centromere plasmids and were described previously (14, 27, 32, 39).

The yeast strain PLY146 (*MAT*α *ura3-52 trp1*Δ*1 upf1*::*URA3 tyr7-1 leu2-2*) (25) was obtained from P. Leeds and M. Culbertson, University of Wisconsin, Madison. The yeast strain JD5ts (*MAT*α *ura3-52 trp1*Δ*1 his4-38 leu2-1 rpb1-1*) was obtained from A. Jacobson, University of Massachusetts Medical School. The JD5ts(−) Ura^- strain (*MAT*α *ura3-52 trp1*Δ*1 his4-38 leu2-1 rpb1-1 upf1*::*ura3*), containing a deletion of the *UPF1* gene, was prepared as described previously (38). The SWP154(−) strain was described previously (27). Cytoplasmic extracts from strain BJ5457 (*MAT*α *ura3-52 trp1*Δ*1 lys2-801 leu2-1 his3*Δ*200 pep4*::*HIS3 prb1*Δ*1.6R can1 GAL*) harboring *FLAG-upf1* alleles on a multicopy plasmid were prepared for purification of the wild-type or mutant Upf1p as described previously (8).

Materials. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, *Taq* DNA polymerase, and T4 DNA polymerase 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.) (28). 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 at 37°C to an optical density at 600 nm of 0.4. 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 oligodeoxynucleotides 19 to 27 (listed in Table 1), as follows: oligodeoxynucleotide 19 was used to construct the C65S *upf1* allele; 20, H98R allele; 21, C122S allele; 22, C72S allele; 23, C84S allele; 24, H110R allele; 25, C148S allele; and 26 and 27, H94R and C125S *upf1* alleles, respectively. The cluster of *upf1* alleles changing charged amino acids to alanine (see Table 3) was constructed in the *FLAG-UPF1* gene as described above by using oligodeoxynucleotides 28 to 38 (Table 1), as follows: 28, *UPF1-33* allele; 29, *UPF1-184* allele; 30, *UPF1-209* allele; 31, *UPF1-257* allele; 32, *UPF1-371* allele; 33, *UPF1-608* allele; 34, *UPF1-768* allele; 35, *UPF1-829* allele; 36, *UPF1-855* allele; 37, *UPF1-933* allele; and 38, *UPF1-957* allele. Mutagenesis was carried out as described previously (20). The mutant clones were identified by DNA sequencing. After mutagenesis, the mutant *upf1* alleles were subcloned into plasmids YEplac112 and YCplac22.

PCR amplification of DNA fragments. Preparation of the *UPF1* deletion alleles shown in Fig. 1 involved amplification of the 5′ and 3′ segments of the *UPF1* gene by PCR. A typical reaction mixture contained 25 pmol of each primer, 25 ng of plasmid DNA template, and 2 U of *Pfu* DNA polymerase. Normally, 30 cycles were carried out for each reaction. The reaction products were extracted once with phenol-chloroform and once with chloroform and ethanol precipitated.

Preparation of *upf1* alleles harboring deletions within their protein-coding regions. The *upf1* deletion alleles were constructed as follows. The 4.0-kb *Eco*RI-*Bam*HI fragment from YEp-UPF1-FLAG was subcloned into pUC19, yielding the plasmid pUC19-UPF1-FLAG. The 5′ and 3′ DNA fragments that border the desired deletions of the *UPF1* protein-coding region (Fig. 1A) were amplified by PCR with oligodeoxynucleotides listed in Table 1. The DNA fragments used to construct the *upf1*Δ*1* allele were prepared by PCR with oligodeoxynucleotides 1 and 2 to amplify the 5′ DNA fragment and oligodeoxynucleotides 3 and 18 to amplify the 3′ DNA fragment. The 5′ DNA fragment was digested with *Eco*RI and *Xho*I, and the 3′ segment was digested with *Xho*I and *Bam*HI. The two DNA fragments were ligated together with plasmid pUC19 cleaved with *Eco*RI and *Bam*HI, and the plasmid pUPF1Δ1-pUC19 was isolated. A *Bst*XI-*Bsa*BI fragment was then cleaved from pUPF1Δ1-pUC19 and ligated together with a 6.6-kb *Bst*XI-*Bsa*BI fragment of pUC19-UPF1-FLAG, yielding pUC19-FLAG-UPF1Δ1. The DNA fragments used to construct the *upf1*Δ*2* allele were prepared by PCR with oligodeoxynucleotides 1 and 4 to amplify the 5′ DNA fragment and oligodeoxynucleotides 5 and 18 to amplify the 3′ DNA fragment. The 5′ DNA fragment was digested with *Eco*RI and *Sac*II, and the 3′ segment was digested with *Sac*II and *Bam*HI. The two DNA fragments were ligated with plasmid pUC19 cleaved with *Eco*RI and *Bam*HI, and the plasmid pUPF1Δ2-pUC19 was isolated. A *Bst*XI-*Bsa*BI fragment was then cleaved from pUPF1Δ2-pUC19 and ligated together with a 6.6-kb *Bst*XI-*Bsa*BI fragment of pUC19-UPF1-FLAG, resulting in pUC19-FLAG-UPF1Δ2. The DNA fragments used to construct the *upf1*Δ*3* allele were prepared by PCR with oligodeoxynucleotides 1 and 6 to amplify the 5′ DNA fragment and oligodeoxynucleotides 7 and 18 to amplify the 3′ DNA fragment. The 5′ DNA fragment was digested with *Eco*RI and *Mlu*I, and the 3′ segment was digested with *Mlu*I and *Bam*HI. The two DNA fragments were ligated with plasmid pUC19 cleaved with *Eco*RI and *Bam*HI, and the plasmid pUPF1Δ3-pUC19 was isolated. A 23-bp *Bst*BI-*Bgl*I DNA fragment was ligated with the *Bst*BI-*Bgl*I fragment of pUC19-UPF1-FLAG, yielding pUC19-FLAG-UPF1Δ3. The DNA fragments used to construct the *upf1*Δ*4* allele were prepared by PCR with oligodeoxynucleotides 17 and 8 to amplify the 5′ fragment and oligodeoxynucleotides 9 and 18 to amplify the 3′ fragment. The 5′ fragment was digested with *Eco*RI and *Ecl*XI, and the 3′ fragment was digested with *Ecl*XI and *Bam*HI. The two fragments were ligated with plasmid pUC19 cleaved with *Eco*RI and *Bam*HI, and plasmid pUPF1Δ4-pUC19 was isolated. A 0.3-kb *Asp*718-*Apa*I fragment from pUPF1Δ4-pUC19 was used to replace the 0.7-kb *Asp*718-*Apa*I fragment of pUC19-UPF1-FLAG, resulting in pUC19-FLAG-UPF1Δ4. The DNA fragments used to construct the *upf1*Δ*5* allele were prepared by PCR with oligodeoxynucleotides 17 and 10 to amplify the 5′ fragment and oligodeoxynucleotides 11 and 18 to amplify the 3′ fragment. The 5′ fragment was digested with *Eco*RI and *Bss*HII, and the 3′ fragment was digested with *Bss*HII and *Bam*HI. The two DNA fragments were ligated with plasmid pUC19 cleaved with *Eco*RI and *Bam*HI, and plasmid pUPF1Δ5-pUC19 was isolated. A *Sau*I-*Msc*I fragment was cleaved from pUPF1Δ5-pUC19 and ligated together with *Sau*I- and *Msc*I-digested pUC19-UPF1-FLAG, yielding pUC19-FLAG-UPF1Δ5. The DNA fragments used to construct the *upf1*Δ*6* allele were prepared by PCR with oligodeoxynucleotides 17 and 12 to amplify the 5′ fragment and oligodeoxynucleotides 13 and 18 to amplify the 3′ fragment. The 5′ fragment was digested with *Eco*RI and *Avr*II, and the 3′ fragment was digested with *Avr*II and *Bam*HI. The two DNA fragments were ligated with plasmid pUC19 cleaved with *Eco*RI and *Bam*HI, and plasmid pUPF1Δ6-pUC19 was isolated. An *Apa*I-*Bgl*II fragment was cleaved from pUPF1Δ6-pUC19 and used to replace the *Apa*I-*Bgl*II fragment of pUC19-UPF1-FLAG, resulting in pUC19-FLAG-UPF1Δ6. The DNA fragments used to construct the *upf1*Δ*7* allele were prepared by PCR with oligodeoxynucleotides 17 and 14 to amplify the 5′ fragment and oligodeoxynucleotides 15 and 18 to amplify the 3′ fragment. The 5′ fragment was digested with

TABLE 1. Oligodeoxynucleotides used in this study

Oligodeoxynucleotide	Sequence[a]	Prepared mutant	Introduced restriction site
1	--CCGGAATTCATGAACGGGAAA--	Deletions	
2	--ACCGCTCGAGTGAATTGTCTGAAG--	*upf1Δ1*	*Xho*I
3	--ACCGCTCGAGGCCCAGACGAAAAATGC--	*upf1Δ1*	*Xho*I
4	--ATCCCCGCGGACAAGGTATTCTACAAAG--	*upf1Δ2*	*Sac*II
5	--ATCCCCGCGGGAGTTGAAAGTTGCCATC--	*upf1Δ2*	*Sac*II
6	--ACGTACGCGTGTTAGATTCGAAAGTAGA--	*upf1Δ3*	*Mlu*I
7	--ACGTACGCGTTCTTTAATTCAAGGCCCA--	*upf1Δ3*	*Mlu*I
8	--GACCGGCCGTAACGGACGTTGTAATACAT--	*upf1Δ4*	*Ecl*XI
9	--GACCGGCCGGTTGGTGCTGGTGATAAG--	*upf1Δ4*	*Ecl*XI
10	--GACGCGCGCACATGTGCAACATACGAC--	*upf1Δ5*	*Bss*HII
11	--GACGCGCGCCGAAAGGCGGCAGACGCT--	*upf1Δ5*	*Bss*HII
12	--GACCCTAGGTTCCAATATGACTGGGCC--	*upf1Δ6*	*Avr*II
13	--GACCCTAGGCGTGATCCTCGTCGTCTA--	*upf1Δ6*	*Avr*II
14	--GACACTAGTTAAGAAACCAATGGCCTGTTG--	*upf1Δ7*	*Spe*I
15	--GACACTAGTGTTATTCTTGGTAATCCTAG--	*upf1Δ7*	*Spe*I
16	--GACGTCGACCTAACCATATTTGGCACGGGT--	*upf1Δ8*	*Sal*I
17	--GACGAATTCATCTGGAAAGGTACCTCT--	Deletions	*Eco*RI
18	--GACGGATCCAAAGTATATTGGAC--	Deletions	
19	--TCATGTGCGTATTCTGGTATAGAT--	C65S	
20	--CACATTGTTAATAGATTAGTTTTA--	H98R	
21	--ACCGTTTTGGAAAGTTATAACTGT--	C122S	
22	--TAGATTCTGCAAAGTCTGTCATCAAA--	C72S	
23	--ATCAAATGTAATTCATCTAAGAAATGG--	C84S	
24	--CGTAGTTTCTTTACGTCCAGATTCT--	H110R	
25	--GGTTGTTTTACTTTCTAGAATACCT-	C148S	
26	--ACAAGCAGCTCCCGCATTGTTAAT--	H94R	
27	--GGAATGTTATAACTCTGGACGTAAG--	C125S	
28	--TCCACCTTGGTGGCGGCTGCCGATGTAGAT--	*UPF1-33*	
29	--GAGCAACCAACTGCAGCAGCAAAATTGAAA-	*UPF1-184*	
30	--TGGAGATCCAATGCAGCCGCTACAATT--	*UPF1-209*	
31	--GATAAACAACTCGCGGCATCTCAAGCT--	*UPF1-257*	
32	--AAATTTGCCATTGCTGCAGCATCTATTTCA--	*UPF1-371*	
33	--CAAGTCATATTGGCAGCAGCGGCGGCAGAC--	*UPF1-608*	
34	--GCATTCCAAGGTGCTGCAAAGGATTAC--	*UPF1-768*	
35	--GTTAATCCACTTCGCAGCGGCGGGTTGTTTAG--	*UPF1-829*	
36	--CGTCCTCAGCCAGCAGCGACTGAACGG--	*UPF1-855*	
37	--CGCAATGAAATTGCCGCTGCAAATTTGTAC--	*UPF1-933*	
38	--GAGAGAAGAACAAGCGGCTGCATTGTCAAAAG--	*UPF1-959*	
39	--AAAAAACCATGGAGCAGAAGCTGATTAGCGAGGAAGATCTGAATATG GACGATGGACGGAAAAAAGAATTGGATG--		*Nco*I
40	--ATGTCAACAGAGGGGTTC--		
41	--CATTCCATGGAGAGCAATGTGGCTGGGGAATT--		*Nco*I
42	--CTAAGGATCCGAAGCCATGAGCTTTTACTAC--		*Bam*HI

[a] The introduced restriction sites in some oligodeoxynucleotides are underlined.

*Eco*RI and *Spe*I, and the 3′ fragment was digested with *Spe*I and *Bam*HI. The DNA fragments were ligated together with plasmid pUC19 cleaved with *Eco*RI and *Bam*HI, and plasmid pUPF1Δ7-pUC19 was isolated. An *Msc*I-*Bgl*II fragment was cleaved from pUPF1Δ7-pUC19 and ligated with *Msc*I- and *Bgl*II-digested pUC19-UPF1-FLAG, yielding pUC19-FLAG-UPF1Δ7. The DNA fragment used to construct *upf1Δ8* was prepared by PCR with oligodeoxynucleotides 16 and 17. In oligodeoxynucleotide 16, a stop codon was introduced in frame into the coding region of the *UPF1* gene. The DNA fragment amplified by PCR was digested with *Eco*RI and *Sal*I and ligated with plasmid pUC19 DNA cleaved with *Eco*RI and *Sal*I, resulting in pUPF1Δ8-pUC19. An *Msc*I-*Sal*I fragment was cleaved from pUPF1Δ8-pUC19 and ligated with *Msc*I- and *Sal*I-digested pUC19-UPF1-FLAG, resulting in pUC19-FLAG-UPF1Δ8. The *upf1* alleles constructed as described above were cloned into YEplac22 and YCplac22 shuttle vectors (11), and their effects on decay of nonsense-containing mRNAs and nonsense suppression were determined.

RNA preparation and analysis. To measure the RNA abundance, *upf1* alleles were transformed into yeast strain SWP154(−) (Fig. 1B and C), JD5ts(−) Ura3$^-$ (see Fig. 5B and C), or PLY146(−) (see Fig. 1D and 5C). mRNA abundances were determined as described previously (14, 36, 39). The RNA blots were prepared and hybridized with radioactive probes prepared as described previously (10, 14, 33, 39). 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 in a *UPF1*$^+$ strain was 0%. The mRNA abundances were normalized by using the *PGK1* or *CYH2* mRNA or U3 RNA.

Nonsense suppression assay. Plasmids containing *upf1* alleles were transformed into yeast strain PLY146, and transformants were selected at 30°C as described previously (38).

SDS-PAGE, protein blotting, and ATPase, RNA binding, and helicase activities. Standard techniques for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used as described previously (21). 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.) as described previously (38). ATPase, helicase, and RNA binding activities of the wild-type and mutant forms of Upf1p were determined as described previously (3, 38).

Purification of wild-type and mutant forms of Upf1p. Purification of wild-type and mutant Upf1 proteins was done as described previously with slight modifications (8, 38). All steps were performed at 0 to 4°C. In brief, crude extracts were prepared by glass bead lysis with buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid]-KOH [pH 7.4], 150 mM KCl, 10% glycerol, 0.1% Triton X-100) containing 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1 μg each of leupeptin, pepstatin A, and aprotinin per ml (buffer XA). Cell extracts were diluted with 2 volumes of buffer XC (buffer X lacking KCl and containing 1 mM phenylmethylsulfonyl fluoride, 1 μg of proteinase inhibitor per ml, and 50% glycerol) and loaded onto a 1-ml anti-

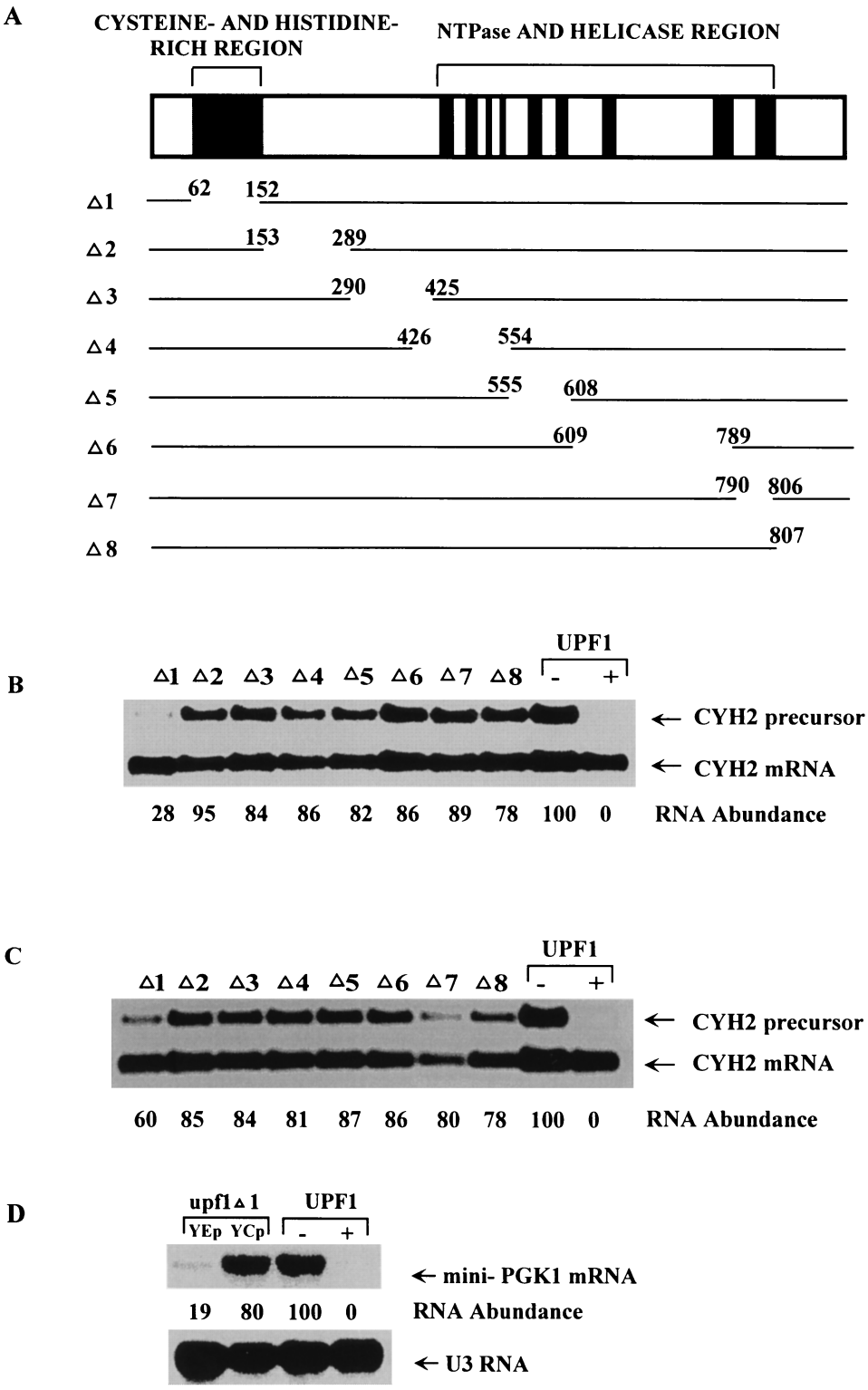

FIG. 1. Deletion of the cysteine- and histidine-rich region of the *UPF1* gene does not affect the decay function of Upf1p. (A) Schematic representation of the *UPF1* gene and eight *upf1* alleles that harbor in-frame deletions of segments of the Upf1p-coding region. NTPase, nucleoside triphosphatase. The numbers indicate the 5′ and 3′ amino acids that demarcate the deleted region, and the absence of a line represents the region deleted. (B and C) *CYH2* precursor abundance in cells harboring *upf1* deletion alleles on high-copy-number (2μm, YEp) (B) and low-copy-number (centromere, YCp) (C) plasmids. The abundances of these RNAs were measured and quantitated as described in Materials and Methods and are indicated below the autoradiographs. (D) Abundance of the nonsense-containing *PGK1* (mini-*PGK1* [14]) mRNA in cells harboring the *upf1Δ1* allele. The *upf1Δ1* allele (panel A) was inserted into either a low- or high-copy-number plasmid. The abundance of the *PGK1* mRNA was determined as described in Materials and Methods and normalized to the concentration of the U3 RNA. The relative abundances in cells harboring *upf1Δ1* were quantitated as described in Materials and Methods and are indicated below the autoradiograph. *upf1*$^{-}$, cells harboring the YEplac112 or YCplac22 plasmid lacking the *UPF1* gene; *UPF1*$^{+}$, wild-type *UPF1* gene inserted into plasmid YCplac22 or YEplac112.

FLAG-M2 monoclonal antibody immunoaffinity chromatography column equilibrated with buffer XB (buffer X with the addition of 1 mM phenylmethylsulfonyl fluoride and 1 μg of proteinase inhibitors per ml). The flowthrough was collected and reapplied to the column. The column was washed with 50 ml of buffer XD (buffer XB containing 50 mM KCl and 40% glycerol), 50 ml of buffer XE (buffer XD containing 150 mM KCl), 50 ml of buffer XF (buffer XD containing 250 mM KCl), 50 ml of buffer XG (buffer XD containing 500 mM KCl), and 50 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 dithiothreitol, 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 as a protein standard.

Determination of the decay complex. A two-hybrid system was used to detect the interactions among Upf1p, Upf2p, and Upf3p (12). The 3.6-kb *Nco*I-*Bam*HI fragments from the wild-type *FLAG-UPF1* gene or its mutant alleles were subcloned into *Nco*I-*Bam*HI sites of the pACT2 and pAS2 plasmids (pACT2-UPF1 and pAS2-UPF1) obtained from S. J. Elledge, Baylor College of Medicine. To construct pACT2-UPF2, the 5′ fragment of the *UPF2* gene was amplified by using oligodeoxynucleotides 39 and 40, digested with *Nco*I and *Hin*dIII, and ligated together with a *Hin*dIII-*Xho*I *UPF2* fragment and *Nco*I-*Xho*I-digested pACT2. To construct the pAS2-UPF2 fusion plasmid, pACT2-UPF2 was digested with *Xho*I, blunt ended with Klenow enzyme, and digested with *Nco*I. The *Nco*I-*Xho*I *UPF2* fragment was isolated and inserted into *Nco*I-*Sma*I sites of the pAS2 plasmid. To prepare pAS2-UPF3 and pACT2-UPF3 fusions, the *UPF3* gene was amplified by using oligodeoxynucleotides 41 and 42 from a plasmid containing a genomic fragment harboring the *UPF3* gene (29), digested with *Nco*I-*Bam*HI, and inserted into *Nco*I-*Bam*HI sites of pAS2 and pACT2 plasmids. Various combinations of plasmids were then transformed into yeast strain Y190. Filter and liquid assays were used to monitor β-galactosidase activity and were carried out as described before (30). In liquid assay, cells were grown to mid-log phase, extracts were prepared by vortexing cells with glass beads, and 75 μg of total protein was used in β-galactosidase assays.

RESULTS

Deletion analysis of the *UPF1* gene. To identify regions in Upf1p that are important for its activity in modulating nonsense suppression and mRNA turnover, eight *upf1* alleles with in-frame deletions in the coding region of the *UPF1* gene were constructed (Fig. 1A) and analyzed in a *upf1*Δ strain harboring *tyr7-1* and *leu2-2* nonsense alleles. The *FLAG-UPF1* allele, which consists of an epitope tag located at the amino terminus of the *UPF1* protein-coding region, was utilized to construct the mutant *upf1* alleles. The FLAG epitope allowed detection and subsequent purification of the wild-type and mutant forms of Upf1p from yeast cells (8, 28). Cells harboring the *FLAG-UPF1* allele had the same mRNA turnover and nonsense suppression phenotypes as cells harboring the wild-type *UPF1* gene (8, 28). The effects of these deletion alleles on either a low-copy-number (YCp lac22, centromere-based) or a high-copy-number (YEp lac112, 2μm-based) plasmid were assayed in a *upf1*Δ strain for the ability of Upf1p to function in the nonsense-mediated mRNA decay pathway. The abundance of the *CYH2* precursor was determined. The inefficiently spliced *CYH2* precursor, which contains an intron near the 5′ end, is a naturally occurring substrate for nonsense-mediated mRNA decay and has been demonstrated to be a reliable indicator of the activity of this decay pathway (5, 14, 17). The status of the nonsense-mediated mRNA decay activity in cells can be easily determined by comparing the ratio of the abundance of the *CYH2* precursor to that of the *CYH2* mRNA on an RNA blot.

Seven deletions (*upf1Δ2* to *upf1Δ8*) (Fig. 1B and C) within the protein-coding region of the *UPF1* gene resulted in accumulation of the *CYH2* precursor regardless of whether the *upf1* alleles were present on high- or low-copy-number plasmids. Deletions that removed the putative ATPase and helicase motifs, along with other regions that are not conserved among helicase superfamily I, inactivated the mRNA decay function of Upf1p (Fig. 1B and C). However, the abundance of the *CYH2* precursor was low in strains containing the *upf1Δ1* allele present on a high-copy-number plasmid (Fig. 1B). The *upf1Δ1* allele removed the entire amino-terminal cysteine- and histidine-rich region (and its putative zinc fingers) (Fig. 1A). The decay activity of the *upf1Δ1* allele was not unique to the *CYH2* precursor, since the abundances of the transcripts synthesized from nonsense-containing *PGK1*, *leu2-2*, and *tyr7-1* alleles were also low in strains harboring either the wild-type *UPF1* gene or the *upf1Δ1* allele inserted into a high-copy-number plasmid, while the RNA levels of these transcripts were high in a *upf1*Δ strain or in strains containing the other *upf1* deletion alleles (Fig. 1D and 2 and data not shown).

The nonsense suppression phenotype of the *upf1Δ1* to *upf1Δ8* alleles inserted into high- or low-copy-number plasmids was determined in a *upf1*Δ strain harboring *leu2-2* and *tyr7-1* nonsense alleles by replica plating these cells in medium lacking leucine and tyrosine (-Leu-Tyr) and monitoring growth. Strains harboring the *upf1Δ2* to *upf1Δ8* alleles, in which the Upf1p mRNA decay activity was abolished, grew on this medium (data not shown). Unexpectedly, however, the yeast strain harboring the *upf1Δ1* allele (Fig. 2A) present on a high- or low-copy-number plasmid also grew on -Leu-Tyr medium, demonstrating suppression of the *leu2-2* and *tyr7-1* nonsense alleles (Fig. 2B). Shown for comparison is the growth phenotype in cells harboring the DE572AA allele inserted into high- or low-copy-number plasmids (Fig. 2B). This *upf1* mutant resulted in inactivation of the nonsense-mediated mRNA decay pathway but did not allow growth on this medium when present on a high-copy-number plasmid (38). Thus, deletion of the cysteine- and histidine-rich region of the *UPF1* gene suggests a new class of *upf1* alleles with a phenotype opposite to that of a subset of mutations in the helicase region with the phenotypes described above; i.e., this class is functional in nonsense-mediated mRNA decay but is inactive in preventing suppression at a premature stop codon.

Specific mutations in the cysteine- and histidine-rich region of Upf1p result in inactivation of its function in preventing suppression at nonsense codons. The results described above suggest that the cysteine- and histidine-rich region of Upf1p may be important for preventing nonsense suppression. To further understand the function of this region, site-specific mutations in this region were constructed. Fifteen cysteine and histidine residues occur within the 91-amino-acid span encompassing the amino terminus of Upf1p (Fig. 3A). Several cysteine and histidine residues were replaced by either serine or arginine residues (Fig. 3A). The mutant *upf1* alleles were subcloned into either high- or low-copy-number plasmids and transformed into a *upf1*Δ yeast strain.

The expression levels of the mutant proteins were determined by Western blotting (immunoblotting) analysis and compared with that of wild-type Upf1p by using a monoclonal antibody directed to the FLAG epitope as described previously (8, 38). A 95-kDa nonspecific protein cross-reacted with the anti-FLAG antibody in the *upf1*Δ strain (8) (Figs. 3B and C). The expression levels of the mutant forms of Upf1p were approximately equivalent to that of wild-type Upf1p when the *UPF1* alleles were expressed from a high-copy-number plasmid (Fig. 3C) but were slightly lower when the alleles were expressed from a low-copy-number plasmid (Fig. 3B). Taken together, these results indicate that the wild-type and mutant forms of Upf1p were efficiently expressed in yeast cells.

The nonsense suppression phenotype in cells containing the *leu2-2* and *tyr7-1* alleles and harboring *upf1* alleles with mutations in the cysteine- and histidine-rich region were monitored by growth on -Leu-Tyr medium. The results from these experiments are shown in Fig. 4 and summarized in Table 2. Three

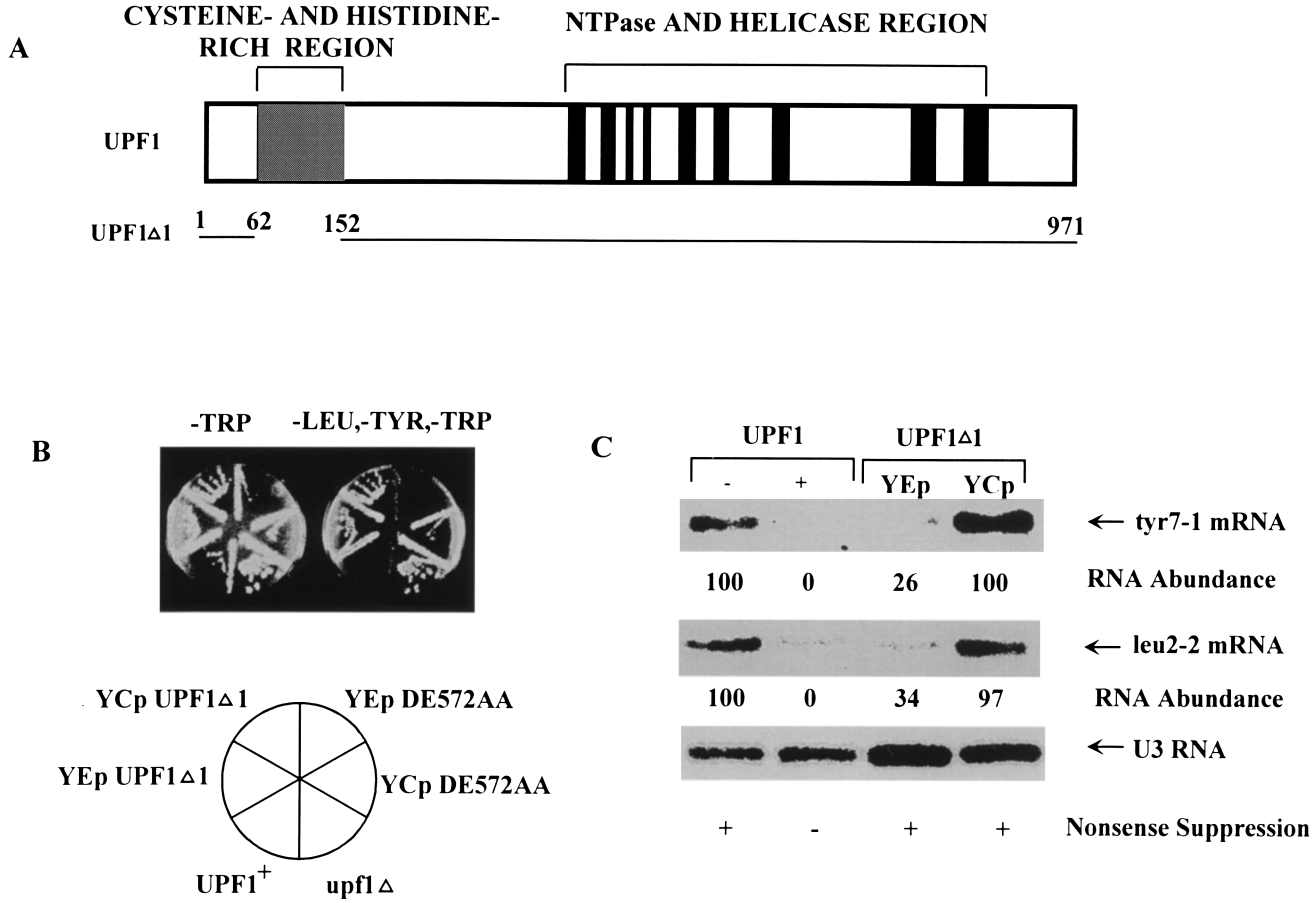

FIG. 2. Yeast cells harboring the *upf1Δ1* allele do not accumulate the *leu2-2* and *tyr7-1* mRNAs but allow nonsense suppression of these alleles. (A) Schematic representation of the *UPF1* gene and the *upf1Δ1* allele, which harbors an in-frame deletion of the cysteine- and histidine-rich region of Upf1p. The numbers indicate the 5′ and 3′ amino acids that demarcate the deleted region, and the absence of the line represents the region deleted. NTPase, nucleoside triphosphatase. (B) Growth characteristics of yeast cells harboring the *upf1Δ1* allele on -Leu-Tyr medium. For comparison, the growth characteristics of the yeast strain harboring the DE572AA *upf1* allele were also monitored (38). A photograph of the plates and a schematic representation of the plate indicating the *upf1* allele that each of the cells harbored are shown. (C) The *leu2-2* and *tyr7-1* mRNA levels in yeast cells harboring the *upf1Δ1* allele were determined by Northern (RNA) blotting and normalized to the concentration of U3 RNA. The relative mRNA abundances in cells harboring *upf1Δ1* alleles were obtained as described in Materials and Methods and are shown below the autoradiograph.

classes of *upf1* mutants with mutations in the cysteine- and histidine-rich region were identified. One class of mutations, which includes the C72S and H110R *upf1* alleles, when expressed in both single-copy and multicopy plasmids did not allow growth on -Leu-Tyr medium, indicating that these mutations did not affect the nonsense suppression activity of Upf1p (Fig. 4; Table 2). The second class of mutations are those that when expressed from single-copy plasmid allowed growth on -Leu-Tyr medium (C65S, C84S, and C148S *upf1* alleles) (Fig. 4; Table 2), indicating suppression of the *leu2-2* and *tyr7-1* nonsense alleles. When these mutants were overexpressed from a high-copy-number plasmid, however, cells did not grow on -Leu-Tyr medium (Fig. 4; Table 2). The third class of *upf1* alleles, which includes H94R, H98R, C122S, and C125S, results in nonsense suppression of the *leu2-2* and *tyr7-1* alleles when these mutants are expressed from both high- and low-copy-number plasmids, although cells harboring H94R and C125S *upf1* alleles inserted into a high-copy-number plasmid grew at a lower rate (Fig. 4; Table 2).

To relate the nonsense suppression phenotypes of strains harboring the *upf1* alleles with their effect on nonsense-mediated mRNA decay, the abundances of nonsense-containing mRNAs were determined by RNA blotting analysis of a *upf1Δ* strain harboring the various *upf1* alleles. The results demonstrated that the abundance of the *CYH2* precursor in cells harboring the C72S and H110R *upf1* alleles (class I), which did not affect the nonsense suppression phenotype, also did not alter the mRNA abundance of the *CYH2* precursor in these cells (Fig. 5A; Table 2). Yeast strains harboring class III *upf1* alleles (H94R, H98R, C122S, and C125S), which allowed nonsense suppression of *tyr7-1* and *leu2-2* alleles, accumulated the *CYH2* precursor (Fig. 5A; Table 2). Interestingly, strains harboring class II *UPF1* alleles (C65S, C84S, and C148S) (Table 2) exhibited low levels of *CYH2* precursor regardless of whether the mutant *upf1* alleles were expressed in low or high copy numbers (Fig. 5A). Nonsense suppression was observed only when these *upf1* alleles were expressed from a low-copy-number plasmid (Fig. 4; Table 2). The abundances of the nonsense-containing *PGK1*, as well as the *leu2-2* and *tyr7-1*, mRNAs, whose transcripts are degraded by the nonsense-mediated mRNA decay pathway (Fig. 1D and 2C), were also low in strains harboring class II cysteine- and histidine-rich region *upf1* alleles (Figs. 5B and C; Table 2). Half-life measurements of the *CYH2* precursor and the nonsense-containing *leu2-2*, *tyr7-1*, and *PGK1* mRNAs demonstrated that the steady-state levels of these RNAs were a consequence of the rate at which

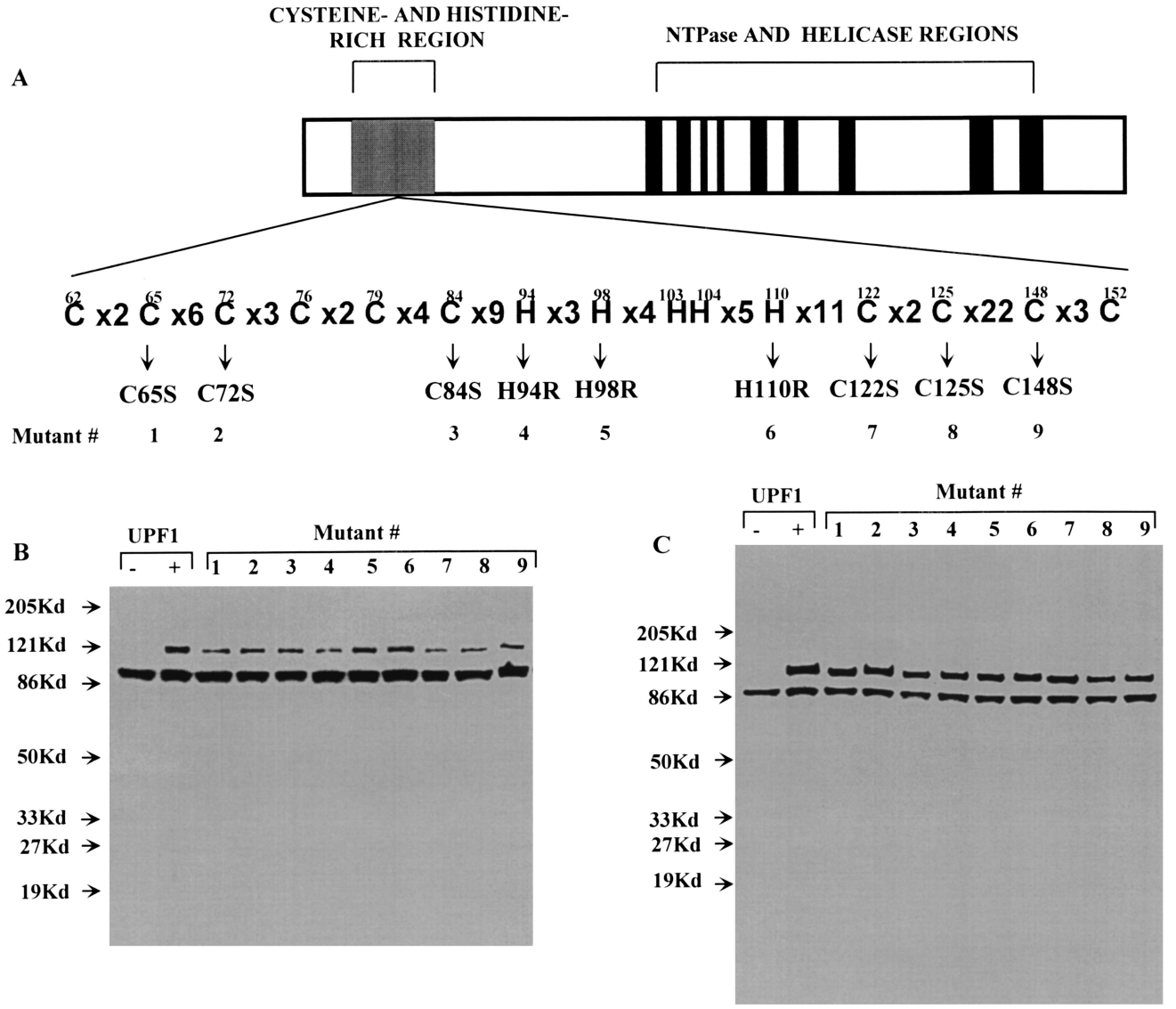

FIG. 3. Expression of mutations in the cysteine- and histidine-rich region of Upf1p. (A) Schematic representation of the *UPF1* gene and the sequence of the cysteine- and histidine-rich region. NTPase, nucleoside triphosphatase. The mutagenized amino acid residues and the nomenclature of the *upf1* alleles are indicated. (B and C) The levels of the mutant forms of Upf1p present in cells were determined by Western blot analysis of proteins extracts from PLY146 cells harboring these alleles inserted into either a low-copy-number (YCp, centromere) (B) or high-copy-number (YEp, 2μm) (C) plasmid. The positions at which molecular mass markers migrated are indicated on the left of each SDS-PAGE gel.

these transcripts decay in cells containing these mutant *upf1* alleles (data not shown).

In another study (38), we identified several mutations in the helicase region of the *UPF1* gene that resulted in accumulation of nonsense-containing mRNAs but, unlike the case for a *upf1*Δ strain, did not allow suppression of the *leu2-2* and *tyr7-1* nonsense alleles (38). From these results, Upf1p was hypothesized to modulate both mRNA decay and nonsense suppression at a premature translation termination codon. Strains containing the class II *upf1* mutations (C65S, C84S, and C148S) in the cysteine- and histidine-rich region of Upf1p and the *upf1Δ1* allele have the converse phenotype. These mutations were less active in preventing nonsense suppression without affecting the Upf1p mRNA decay function. Class III *upf1* alleles both have elevated RNA levels and allow nonsense suppression of *leu2-2* and *tyr7-1* alleles.

Site-specific mutagenesis of the *UPF1* gene. The results described above and in the accompanying paper (38) identify mutations that separate the mRNA turnover and nonsense suppression functions of the *UPF1* gene. To determine whether other mutations that separate the mRNA decay and nonsense suppression phenotypes can be identified, 11 alanine scanning mutations in which clusters of charged amino acids were changed to alanine residues throughout Upf1p were constructed (Table 3). The effects of these changes on the abundances of the nonsense-containing *leu2-2*, *tyr7-1*, and *PGK1* mRNAs and the *CYH2* precursor, as well as their abilities to suppress the *leu2-2* and *tyr7-1* nonsense alleles, were determined as described above. Results from these experiments demonstrated that none of these mutations have significantly affected either the decay or nonsense suppression activities of Upf1p (Table 3). Thus, the only mutations in Upf1p that affect

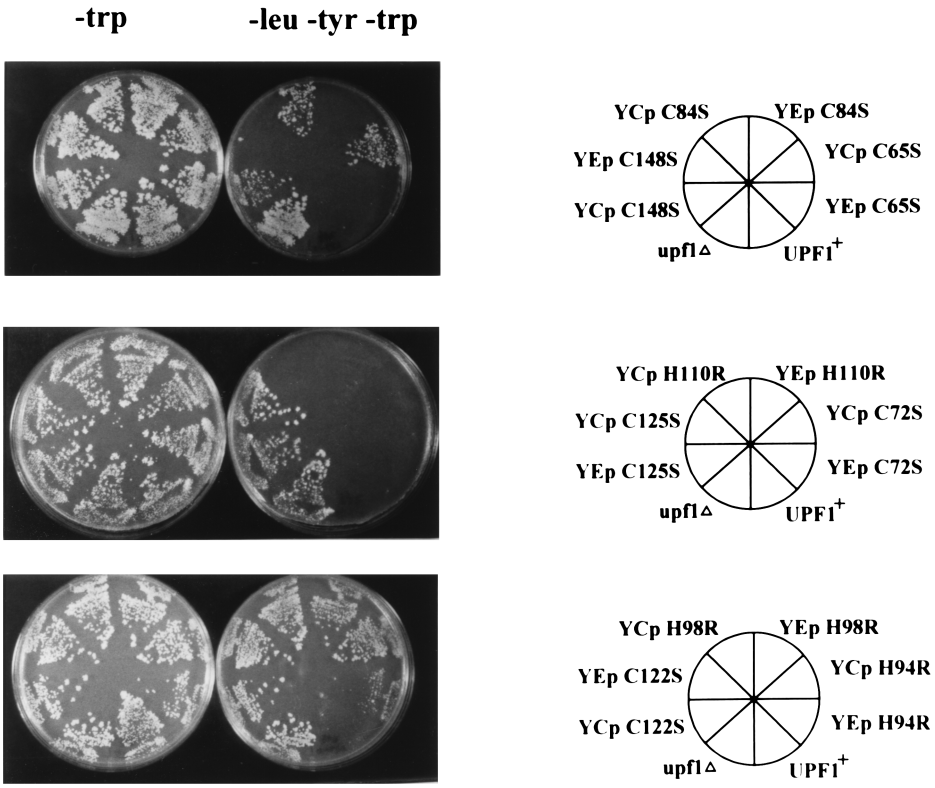

FIG. 4. Nonsense suppression phenotypes of cells harboring *upf1* alleles. The nonsense suppression phenotypes were monitored by growing cells on -Leu-Tyr medium as described in Materials and Methods. Photographs of the plates and diagrams indicating which of the *upf1* alleles are present in the cells on the plates are shown. The designations of the mutant *upf1* alleles are as described in Fig. 3.

nonsense suppression without affecting decay are located in the cysteine- and histidine-rich region, while the only mutations with converse phenotypes were located in the conserved ATPase and helicase motifs (38).

Purification and characterization of the wild-type and mutant forms of Upf1p. To relate the genetic phenotypes described above with biochemical properties of the mutant forms of Upf1p, representatives of each class of mutants were purified essentially as described previously with slight modification (8, 38) (see Materials and Methods). The ATPase activities of the wild-type and mutant forms of Upf1p were monitored as described previously (8, 38). The wild-type Upf1p hydrolyzed ATP efficiently (Fig. 6A). The C72S (class I) and C84S (class II) mutant forms of Upf1p, which have nearly wild-type mRNA decay activity, also contain ATPase activity, although slightly less than that of the wild-type Upf1p (Fig. 6A). These results were anticipated, since the ATPase activity appears to be necessary for Upf1p to function in nonsense-mediated mRNA decay (38). Interestingly, however, the C125S (class III) form of Upf1p maintained ATPase activity (Fig. 6A) even though it was unable to promote nonsense-mediated mRNA decay (Fig. 5A).

The RNA helicase activities of the wild-type and mutant forms of the Upf1p were assayed by determining the ability of

TABLE 2. Summary of genetic and biochemical properties of *UPF1* alleles in the cysteine- and histidine-rich region

Mutation class	*UPF1* allele(s)	Nonsense suppression[a] with:		Abundance of nonsense-containing mRNAs[b]	ATPase activity[c]	Helicase activity[c]	RNA:Upf1p complex[d]
		YEp	YCp				
I	C72S[e], H110R	−	−	Low	High	High	Normal
II	C65S, C84S[e], C148S	−	+	Low	High	High	Slow migration
III	H94R, H98R, C122S, C125S[e]	+	+	High	High	Low	Slow migration

[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.

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

[c] The assay was performed as described in Materials and Methods. Low, activity was significantly reduced compared with that of the wild-type Upf1p; high, activity was comparable to that observed with the wild-type Upf1p.

[d] A gel shift assay was used to monitor RNA:Upf1p complex formation. Normal, an RNA:Upf1p complex with the same mobility as that of the wild-type Upf1p:RNA complex was observed; slow migration, the RNA:Upfp1 complex observed had slower mobility in the assay than the complex observed with wild-type Upf1p.

[e] This mutant form of Upf1p was purified, and its biochemical properties were characterized.

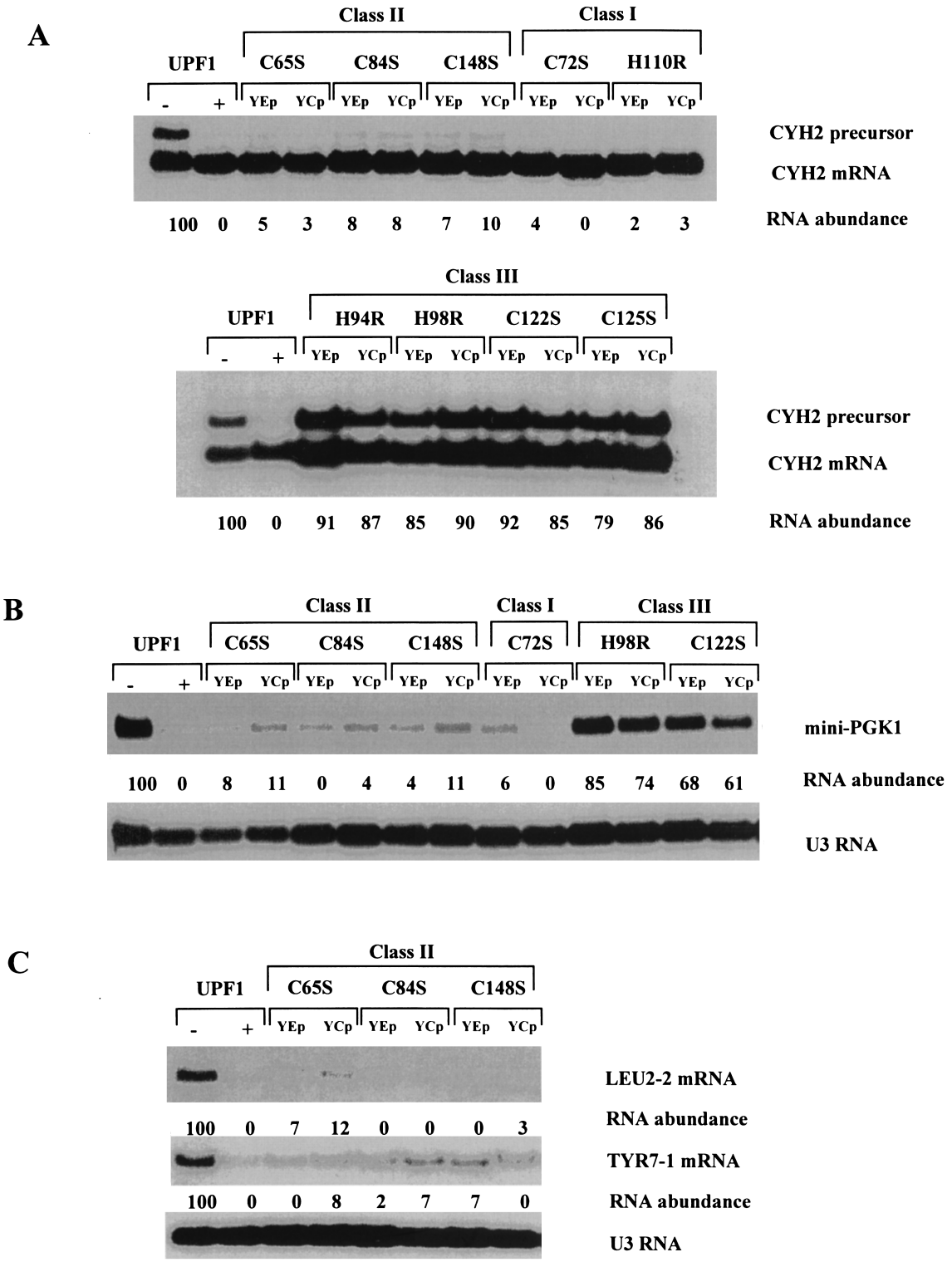

FIG. 5. Abundances of nonsense-containing mRNAs and precursor RNAs in cells harboring *upf1* alleles. Measurement of RNA abundances in cells harboring *upf1* alleles were as described in Materials and Methods. The abundance of *CYH2* precursor (A), nonsense-containing *PGK1* (mini-*PGK1*) (B), and *leu2-2* and *tyr7-1* (C) mRNAs were measured by Northern blotting and normalized to the abundance of *CYH2* mRNA (A) or U3 RNA (B and C). The relative RNA abundances were obtained as described in Materials and Methods and are shown below autoradiographs. Designations are as in Fig. 3.

TABLE 3. RNA abundance and nonsense suppression of *UPF1* alanine scanning mutants

upf1 mutant[a]	Changed amino acids	RNA abundance[b] (%)	Nonsense suppression[c]
UPF1-33	EDD	0	–
UPF1-184	EEE	13	–
UPF1-209	KD	0	–
UPF1-257	KE	2	–
UPF1-371	DKK	0	–
UPF1-608	ERK	0	–
UPF1-768	RE	7	–
UPF1-829	REK	0	–
UPF1-855	RK	0	–
UPF1-933	DDR	0	–
UPF1-959	KHE	0	–

[a] The mutants were named according to the position of the first mutagenized amino acid in Upf1p.

[b] The relative RNA abundance in cells harboring the *upf1* mutants was determined by measuring the ratio of *CYH2* precursor abundance to *CYH2* mRNA abundance. The RNA abundances of *upf1* alleles were obtained by assuming that the RNA abundance was 100% in a *upf1*Δ strain and 0% in a wild-type *UPF1* strain.

[c] Nonsense suppression phenotypes were monitored in yeast strain PLY146 in medium lacking leucine and tyrosine.

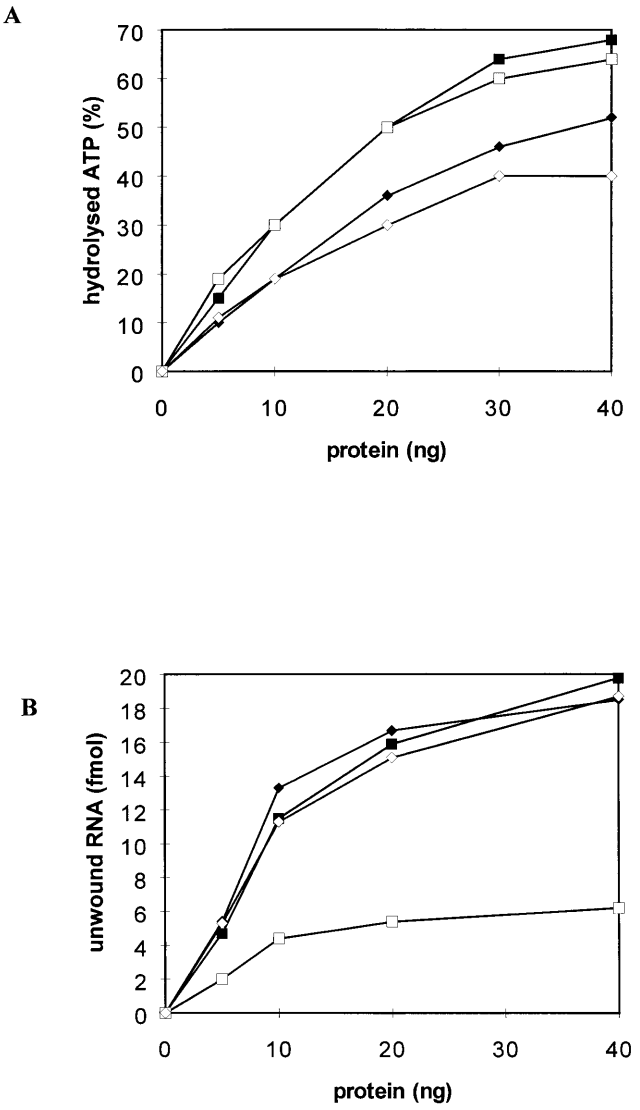

FIG. 6. ATPase and helicase activities of mutant forms of Upf1p. (A) ATPase activities of the wild-type and mutant forms of Upf1p were determined by using a charcoal assay as described in Materials and Methods. ■, wild-type Upf1p; □, C725 (class I); ◆, C845 (class II); ◇, C125S (class III). The designations of the mutants are as in Fig. 3. (B) A strand displacement assay was used to determine the relative helicase activities of the wild-type and mutant forms of Upf1p. The substrate used was prepared as described in Materials and Methods, and the helicase reactions were carried out under standard reaction conditions. ■, wild-type Upf1p; □, C125S (class III); ◆, C725 (class I); ◇, C845 (class II). The designations of the mutants are as in Fig. 3.

Upf1p to displace partially duplexed RNA by using a strand displacement assay (8, 31, 37, 38). The mutant forms of Upf1p harboring C72S (class I) and C84S (class II) mutations contain helicase activity very similar to that of the wild-type Upf1p (Fig. 6B). The helicase activities of the C125S (class III) form of Upf1p, however, demonstrated a 2.5-fold reduction in helicase activity (Fig. 6B). The low level of helicase activity of this mutant Upf1p may explain why cells harboring these *upf1* alleles accumulate nonsense-containing mRNAs to higher degrees than cells harboring the C72S and C84S forms of Upf1p (Fig. 5).

The wild-type Upf1p has been shown to bind RNA (8). To determine whether the mutant forms of Upf1p have altered RNA binding properties, RNA gel shift assays were executed to monitor complex formation (38). The results demonstrated that the C72S (class I) form of Upf1p, which has wild-type decay and nonsense suppression phenotypes, bound to RNA with affinity similar to that of wild-type Upf1p (Fig. 7). Furthermore, the addition of ATP promotes release of the C72S:RNA complex, an observation which echoes that made with the wild-type Upf1p (Fig. 7). Interestingly, the Upf1p:RNA complex observed for the C84S (class II) form of Upf1p migrated more slowly than the wild-type form of Upf1p (Fig. 7). The C125S (class III) form of the Upf1p demonstrated two species of Upf1p:RNA complexes: one migrated at the same rate as the wild-type Upf1p:RNA complex, while the other migrated more slowly. Both of these mutations affected nonsense suppression.

At high concentrations of Upf1p, slowly migrating Upf1p:RNA complexes were also observed in binding reactions with wild-type Upf1p (Fig. 8A) (8). It is likely that for wild-type Upf1p, multiple Upf1p molecules bind to the RNA at high protein concentrations, forming larger complexes with reduced electrophoretic mobility. To test this hypothesis, RNAs of different lengths were added to reaction mixtures, and the mobilities of the Upf1p:RNA complexes were monitored by gel shift assays. RNAs of 98, 34, and 20 nucleotides (nt) in length were synthesized in vitro and used as substrates in the RNA binding assays. A single Upf1p:RNA band was observed in reaction mixtures containing 98-nt RNA and 50 ng of Upf1p (Fig. 8A). As the amount of Upf1p in the binding reactions was increased, protein:RNA complexes which had reduced mobilities appeared concomitantly with the reduction of the more slowly migrating complex (Fig. 8A). RNA binding reactions in which the substrate was 34 nt in length yielded a single Upf1p:RNA species even when the amount of Upf1p was increased to 300 ng (Fig. 8B). Only when the amount of Upf1p reached 600 ng or more was a complex with reduced mobility observed (Fig. 8B). A single Upf1p:RNA species was observed, however, when reaction mixtures contained the 20-nt RNA substrate even when excess Upf1p was added (Fig. 8C). These results suggest that the larger Upf1p:RNA complexes result from binding of multiple Upf1p molecules to a single RNA and that each Upf1p can bind to an RNA of approximately 17 nt or less in length.

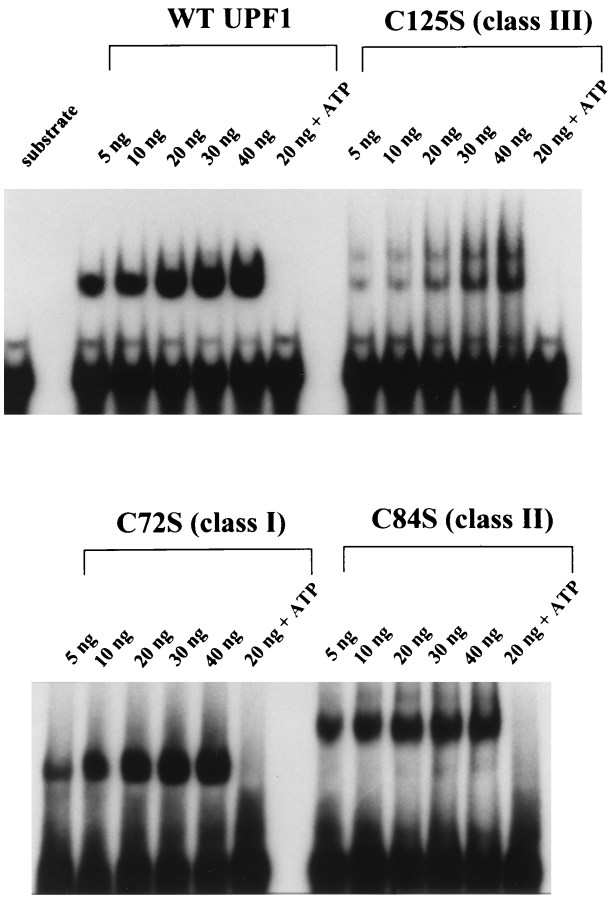

FIG. 7. Comparison of the RNA binding activities of the wild-type (WT) and mutant forms of Upf1p. RNA binding assays were performed under standard conditions with various amounts of protein (indicated on the top of each lane) as described in Materials and Methods. When indicated, 2 μl of 10 mM ATP was added after 15 min of incubation. An autoradiograph of a gel shift assay is shown. The mutant forms of the Upf1p are designated as described in the text.

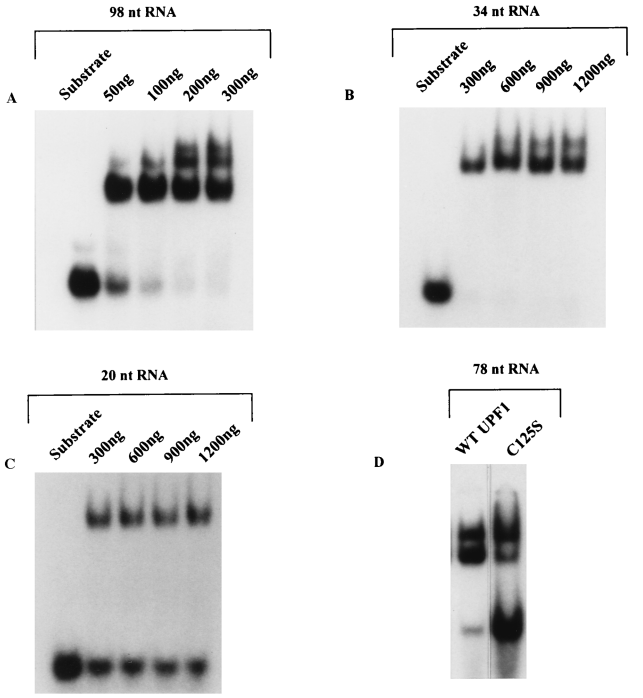

FIG. 8. Multiple Upf1p molecules can bind to an RNA substrate. RNA substrates of different lengths were used in RNA binding assays, which were carried out as described for Fig. 7. (A, B, and C) Autoradiograms of binding reactions when the RNA substrate used in the reaction was 98 nt (A), 34 nt (B), and 20 nt (C). (D) Comparison of the mobilities of the C125S:RNA complexes and wild-type Upf1p:RNA complexes. An RNA substrate 78 nt in length was used in the RNA binding assay, and 100 ng of the wild-type (WT) or C125S form of Upf1p was included in each reaction mixture. An autoradiograph of the gel shift assay is shown.

The C125S and C84S forms of Upf1p did not bind to the 20-nt RNA substrate (data not shown). The mobility of the more slowly migrating C125S:RNA complex was identical to that of the wild-type Upf1p:RNA complex, which we think contains more than one Upf1p molecule bound to the RNA substrate (Fig. 8D). Lower concentrations of the C125S form, however, were required to observe both the wild-type and more slowly migrating complexes (Fig. 7). Unlike the case for wild-type Upf1p, the appearance of the more slowly migrating complex did not accompany reduction of the faster-migrating complex (compare Fig. 7 and 8). In the case of the C84S Upf1p, the more slowly migrating complex is the only Upf1p:RNA species that can be observed (Fig. 7). These results suggest that the more slowly migrating Upf1p:RNA complex observed in wild-type Upf1p and in C125S and C84S mutant forms of Upf1p are probably different in nature. The slower-migration phenotype observed with C84S:RNA and C125S:RNA may be because dimerization or altered Upf1p structure in these mutant proteins causes a change in mobility. Further study is necessary to understand the nature of the more slowly migrating Upf1p:RNA complex observed in these mutant proteins.

Identification of the Upf decay complex. Previous studies indicated that Upf1p, Upf2p, and Upf3p are involved in the same decay pathway (5, 16). Deletion of the *UPF1*, *UPF2*, and *UPF3* genes together from the yeast chromosome has the same effect on the accumulation of nonsense-containing mRNAs as the deletion of any single *UPF* gene (5). Using the two-hybrid system, we examined whether these proteins interact with each other, possibly to form a decay complex (12). Fusion constructs between the *GAL4* DNA binding domain or activation domain and *UPF1*, *UPF2*, and *UPF3* were prepared and analyzed as described in Materials and Methods (38). Consistent with previous results (15, 16), Upf1p and Upf2p interacted with each other (Table 4, lines 1 and 2). Furthermore, Upf2p and Upf3p also demonstrated an interaction in this assay (Table 4, lines 6 and 7). The Upf2p-Upf3p interaction was direct, since similar β-galactosidase activity was observed in a *upf1*Δ strain (data not shown). The Upf2p-Upf3p and Upf1p-Upf2p interactions were observed independent of whether the *UPF2* gene was inserted into the vector containing the DNA binding domain or activation domain, although a significantly greater β-galactosidase activity was observed if the *UPF2* gene was inserted into the DNA binding domain vector. This could be a consequence of either altering the protein structures or changing the expression levels of the hybrid proteins. Upf1p-Upf1p and Upf1p-Upf3p interactions were detectable in a filter assay (data not shown) but not detectable in a liquid assay (Table 4, lines 8, 9, and 10 and data not shown), suggesting that if there are interactions between these proteins, they are very weak. Neither Upf2p-Upf2p nor Upf3p-Upf3p interaction was detected by either filter assay or liquid assay (Table 4, lines 11 and 12; data not shown).

TABLE 4. Interactions among Upf1p, Upf2p, and Upf3p[a]

Plasmids	β-Galactosidase activity	Background[b]
AS2-UPF1/ACT2-UPF2	3.38	0.25
ACT2-UPF1/AS2-UPF2	130	0.25
ACT2-C65S/AS2-UPF2	0.73	
ACT2-C122S/AS2-UPF2	0.37	
ACT2-upf1Δ1/AS2-UPF2	0.22	
AS2-UPF2/ACT2-UPF3	59	0.3
ACT2-UPF2/AS2-UPF3	1.19	0.24
AS2-UPF1/ACT2-UPF3	0.29	0.28
ACT2-UPF1/AS2-UPF3	0.3	0.24
AS2-UPF1/ACT2-UPF1	0.44	0.27
AS2-UPF2/ACT2-UPF2	0.24	0.29
AS2-UPF3/ACT2-UPF3	0.29	0.25

[a] The interactions among Upf1 proteins were determined by a two-hybrid system. The *UPF* genes were fused in frame to the plasmids AS2 (which contains the *GAL4* DNA binding domain) and ACT2 (which contains the *GAL4* activation domain). Plasmids with various combinations were transformed into yeast strain Y190, and β-galactosidase activities in cells harboring various plasmids were determined as described in Materials and Methods.

[b] The background is the average of β-galactosidase activities obtained from yeast cells harboring either one of the plasmids and a vector (pAS2 or pACT2).

To determine whether any mutants with mutations in the cysteine- and histidine-rich region of the Upf1p are altered in the ability to interact with Upf2p, the mutant *upf1* alleles were fused in frame with *GAL4*, and the interaction of mutant forms of Upf1p with Upf2p were monitored in the two-hybrid system as described above. The β-galactosidase activities in cells harboring fusions of C65S (class II), C122S (class III), or *upf1Δ1* mutant alleles along with the Upf2p-Gal4 fusion were less than 1% of what has been observed in cells harboring wild-type Upf1p, suggesting weaker interactions between the mutant forms of Upf1p and Upf2p. Interestingly, the C65S and *upf1Δ1* forms of Upf1p are active in nonsense-mediated mRNA decay (Fig. 5), although their interactions with Upf2p have been significantly reduced.

DISCUSSION

Previous studies have demonstrated that mutations in the *UPF1* gene stabilize nonsense-containing mRNAs and allow suppression of certain nonsense alleles (5, 25). We have undertaken a genetic and biochemical characterization of the *UPF1* gene and its protein product in order to understand its biochemical properties and how it functions in translation and mRNA decay. In this study and that reported in the accompanying paper (38), we have characterized approximately 40 *upf1* alleles and have identified mutations in the *UPF1* gene that separate the nonsense suppression and mRNA decay phenotypes. In addition, using the two-hybrid assay system to monitor protein-protein interactions, we have demonstrated interactions between Upf1p and Upf2p, as observed previously (15, 16), and between Upf2p and Upf3p. Mutations in the cysteine- and histidine-rich region of the *UPF1* gene abolish the Upf1-Upf2p interactions. Furthermore, mutations in the cysteine- and histidine-rich region of Upf1p cause altered Upf1p:RNA complexes. On the basis of these results, the role of the Upf protein complex in mRNA turnover and nonsense suppression is discussed below.

Deleting the amino-terminal cysteine- and histidine-rich region of Upf1p allows nonsense suppression to occur without affecting the nonsense-mediated mRNA decay pathway. Results from the previous study identified mutations in the helicase motifs of the *UPF1* gene that inactivated the nonsense-mediated mRNA decay pathway but, unlike the case for a *upf1Δ* strain, did not suppress the *leu2-2* and *tyr7-1* nonsense alleles (38). We anticipated that if Upf1p is truly a modulator of both mRNA turnover and nonsense suppression at premature stop codons, then we should also be able to identify mutations with the converse phenotype. Deletion of the entire cysteine- and histidine-rich region of Upf1p yielded such a *upf1* allele (*upf1Δ1*) (Fig. 1 and 2; Table 2). The abundances of *leu2-2* and *tyr7-1* mRNAs and the *CYH2* precursor in cells harboring the *upf1Δ1* allele on a high-copy-number plasmid were low compared with those in a *upf1Δ* strain (Fig. 1 and 2; Table 2). However, in contrast to the case for the *UPF1*$^+$ strain, cells harboring the *upf1Δ1* allele exhibited suppression of the *leu2-2* and *tyr7-1* nonsense alleles (Fig. 2B; Table 2). The *upf1Δ1* allele inserted into a centromere-based plasmid, however, was not active in promoting nonsense-mediated mRNA decay. This may be a result of perturbing the structure of Upf1p. Specific mutations in the cysteine- and histidine-rich region of Upf1p display the same phenotype as the *upf1Δ1* mutation when they are inserted into a centromere vector (Table 2).

Three classes of mutations in the cysteine- and histidine-rich region were identified. To identify important residues in the cysteine- and histidine-rich region of Upf1p required for modulating its activity, site-specific mutations in this region which change either a cysteine codon to a serine codon or a histidine codon to an arginine codon were constructed. The nonsense suppression phenotype and the abundance of nonsense-containing mRNAs were monitored in a *upf1Δ* strain harboring *leu2-2* and *tyr7-1* nonsense alleles and containing the mutant *upf1* alleles inserted into high- or low-copy-number plasmids. The results from these experiments identified three classes of *upf1* alleles (see Table 2). Class I *upf1* alleles include the C72S and H110R mutations. Yeast cells harboring these *upf1* alleles both demonstrated a low abundance of nonsense-containing mRNAs, analogous to that found in a wild-type *UPF1*$^+$ strain, and did not allow suppression of *leu2-2* and *tyr7-1* nonsense alleles (Table 2; Fig. 4 and 5). Thus, these mutations did not significantly affect the activities of Upf1p. Class III mutations include the H94R, H98R, C122S, and C125S *upf1* alleles (Table 2). Cells harboring these *upf1* alleles accumulated nonsense-containing *leu2-2*, *tyr7-1*, and *PGK1* mRNAs, as well as the *CYH2* precursor RNA. Suppression of the *leu2-2* and *tyr7-1* nonsense alleles was also observed (Fig. 4 and 5). These results indicate that the Upf1p activities in promoting mRNA decay and preventing nonsense suppression were abolished by these mutations (Table 2).

The class II *upf1* alleles are particularly interesting. Cells harboring these *upf1* mutations (C65S, C84S, and C148S) have levels of nonsense-containing *leu2-2*, *tyr7-1*, and *PGK1* mRNAs and *CYH2* precursor RNA as low as those observed in a wild-type strain (Fig. 5; Table 2). However, when present in single copy, class II *upf1* alleles allowed suppression of the *leu2-2* and *tyr7-1* nonsense alleles (Fig. 4 and 5; Table 2). On the basis of these results, we postulate that Upf1p members encoded by class II alleles are functional in promoting mRNA decay but have altered activity in preventing suppression at a premature translation termination codon.

The class II *upf1* mutations that affect nonsense suppression cause formation of an altered RNA:protein complex. The RNA binding properties of the mutant forms of Upf1p were determined by gel shift assays. The C72S (class I) protein binds to RNA as efficiently as the wild-type protein and forms a similar-size complex (Fig. 7). Of interest are the class II and class III mutants, which form RNA:Upf1p complexes with reduced mobilities. The RNA:C84S Upf1p (class II) migrates more slowly

RING finger motif: C-X_2-C-X_{9-27}-C-X_{1-3}-H-X_2-C-X_2-C-X_{4-48}-C-X_2-C

Upf1p: C-X_2-C-X_{28}-H-X_3-H-X_{23}-C-X_2-C-X_{22}-C-X_3-C

FIG. 9. Comparison of the RING finger motif (top) and the cysteine- and histidine-rich region in Upf1p (bottom). C, cysteine; H, histidine; X any amino acid residue (numbers indicate the number of amino acids that can exist in these positions).

than RNA:Upf1p in a gel shift assay. The C125S Upf1p forms two species of complexes: one comigrates with the wild-type RNA:Upf1p complex, and another displays reduced mobility. The more slowly migrating complex may result from either Upf1p-Upf1p interaction or an alteration in structure of the protein which affects its electrophoretic mobility. One hypothesis is that the cysteine- and histidine-rich region is involved in modulating protein-protein interactions that affect nonsense suppression (see below).

The cysteine- and histidine-rich region of Upf1p may be a RING finger. The zinc finger motif of Upf1p is extremely cysteine and histidine rich (Fig. 3). Previous sequence analysis has suggested that the cysteine residues in this region may form a zinc finger (1, 24). Results from this study suggested that in addition to cysteine residues, some histidine residues are also important for the function of Upf1p. Since many of the cysteine and histidine residues in this region are required for the proper function of Upf1p and since the mutagenesis data do not support the existence of the traditional pattern of a zinc finger motif (9), it is possible that multiple cysteine and histidine residues form a RING finger domain (Fig. 9). A RING finger was initially observed in the human Ring1 protein (26) and was subsequently found in many other proteins, including Rad16, PML, P28, and IHV11 (13). The formation of a RING finger domain can involve eight cysteine or histidine residues. One major difference between the RING finger motif and the cysteine- and histidine-rich region of the Upf1p is the distance between the fourth histidine and the fifth cysteine (Fig. 9). The short peptide in this position of Upf1p may form an internal loop. Alternatively, it is also possible that the cysteine- and histidine-rich region of Upf1p forms a unique functional domain that has yet to be described. To understand the detailed structure of the cysteine- and histidine-rich region of the Upf1p, more detailed analysis of this region is required.

Mutations in the cysteine- and histidine-rich region weaken interactions between Upf1p and Upf2p. Using the two-hybrid system, we have confirmed the Upf1p-Upf2p interaction that has been described previously (15, 16). In addition, Upf2p and Upf3p also demonstrated interaction by this assay. On the other hand, the Upf1p-Upf1p and Upf1p-Upf3p interactions were very weak and were detectable only in filter assays after prolonged incubation. These results indicate that Upf1p, Upf2p, and Upf3p form a complex and are consistent with previous results demonstrating that *upf1*Δ *upf2*Δ or *upf1*Δ *upf3-1* strains have the same nonsense-mediated mRNA decay phenotype as a mutant with a single *UPF* mutation (5).

The Upf1p cysteine- and histidine-rich region mutants were also monitored for their ability to interact with Upf2p. The interactions of Upf2p with the C65S (class II), C122S (class III), and *upf1Δ1* forms of Upf1p were reduced to less than 1% of the wild-type Upf1p-Upf2p interaction. Cells harboring the C65S *upf1* allele were active in nonsense-mediated mRNA decay but were not active in preventing nonsense suppression of the *leu2-2* and *tyr7-1* nonsense alleles, suggesting that a strong Upf1p-Upf2p interaction may be important in preventing nonsense suppression but not absolutely necessary for nonsense-mediated mRNA decay.

Genetic analysis of the *UPF1* gene identified four groups of *upf1* alleles. In this paper and the accompanying paper we have described the genetic and biochemical characterization of a large number of *upf1* alleles. The mutant *upf1* alleles can be classified into four groups, which are summarized in Fig. 10. Group A *upf1* alleles caused phenotypes that were analogous to those of strains harboring a deletion of the *UPF1* gene; i.e., the Upf1p activities in both promoting nonsense-mediated mRNA decay and preventing nonsense suppression were abolished (Fig. 11). Group B *upf1* alleles were analogous to the wild-type *UPF1* gene and were changes that do not alter the activity of Upf1p.

Group C and D *upf1* alleles are interesting alleles because they separated the functions of Upf1p in promoting nonsense-mediated mRNA decay and preventing nonsense suppression (Fig. 11). Group C *upf1* alleles are defined as mutations that inactivated the nonsense-mediated mRNA decay pathway, leading to an increased cellular concentration of nonsense-containing mRNAs, but did not suppress the *leu2-2* and *tyr7-1* nonsense alleles. Cells harboring DE572AA, RR793AA, and TR800AA *upf1* alleles manifest the phenotype of group C *upf1* alleles (38) (Fig. 10).

Group D *upf1* alleles have the converse phenotype of group C *upf1* alleles. Cells harboring group D *upf1* alleles were active in nonsense-mediated mRNA decay but still allowed suppression of the *leu2-2* and *tyr7-1* nonsense alleles. Mutations that fall into group D are class II *upf1* alleles in the cysteine- and histidine-rich region (C65S, C84S, and C148S) as well as the *upf1Δ1* allele (Fig. 10).

Group C and group D mutations were located in distinct regions within the *UPF1* gene. All of the group C mutations were situated in the ATPase or helicase motifs, while the group D mutations were in the cysteine- and histidine-rich region of Upf1p. From the locations of these mutations, it is tempting to speculate that the cysteine- and histidine-rich region of Upf1p is involved in modulating nonsense suppression, while the ATPase and helicase regions are involved in controlling nonsense-mediated mRNA decay. Consistent with this hypothesis, the *mof4-1* allele of the *UPF1* gene, which was identified as a mutation that affects programmed −1 frameshifting efficiency, is also located within the cysteine- and histidine-rich region of Upf1p (4).

A model for the function of Upf protein complexes in nonsense-mediated mRNA decay. On the basis of these and previous results (14, 32), we present the following model for nonsense-mediated mRNA decay (Fig. 11). A nonsense mutation causes premature translational termination. We propose that a fraction of the terminating ribosomes, ribosomal subunits, or ribosome-associated factors that are involved in nonsense-mediated mRNA decay, including Upf1p, Upf2p, and upf3p, will scan the mRNA 3′ of the nonsense codon. This factor will encounter a downstream element, with which it interacts. This interaction promotes an altered ribonucleoprotein structure that renders the mRNA susceptible to cleavage very near the 5′ terminus of the RNA, leading to decapping of the nonsense-containing transcript. The uncapped nonsense-containing RNA is then a substrate for degradation by the 5′→3′ XrnI exoribonuclease (Fig. 11).

How does the Upf1p-containing complex function in preventing nonsense suppression, and how does it fit into the model described for nonsense-mediated mRNA decay? We suggest that one of the functions of the Upf complex, in combination with other factors, may be to modulate translation termination at the nonsense codon (Fig. 11). The events that occur at the translation termination codon may be critical to the subsequent steps leading to the rapid degradation of the

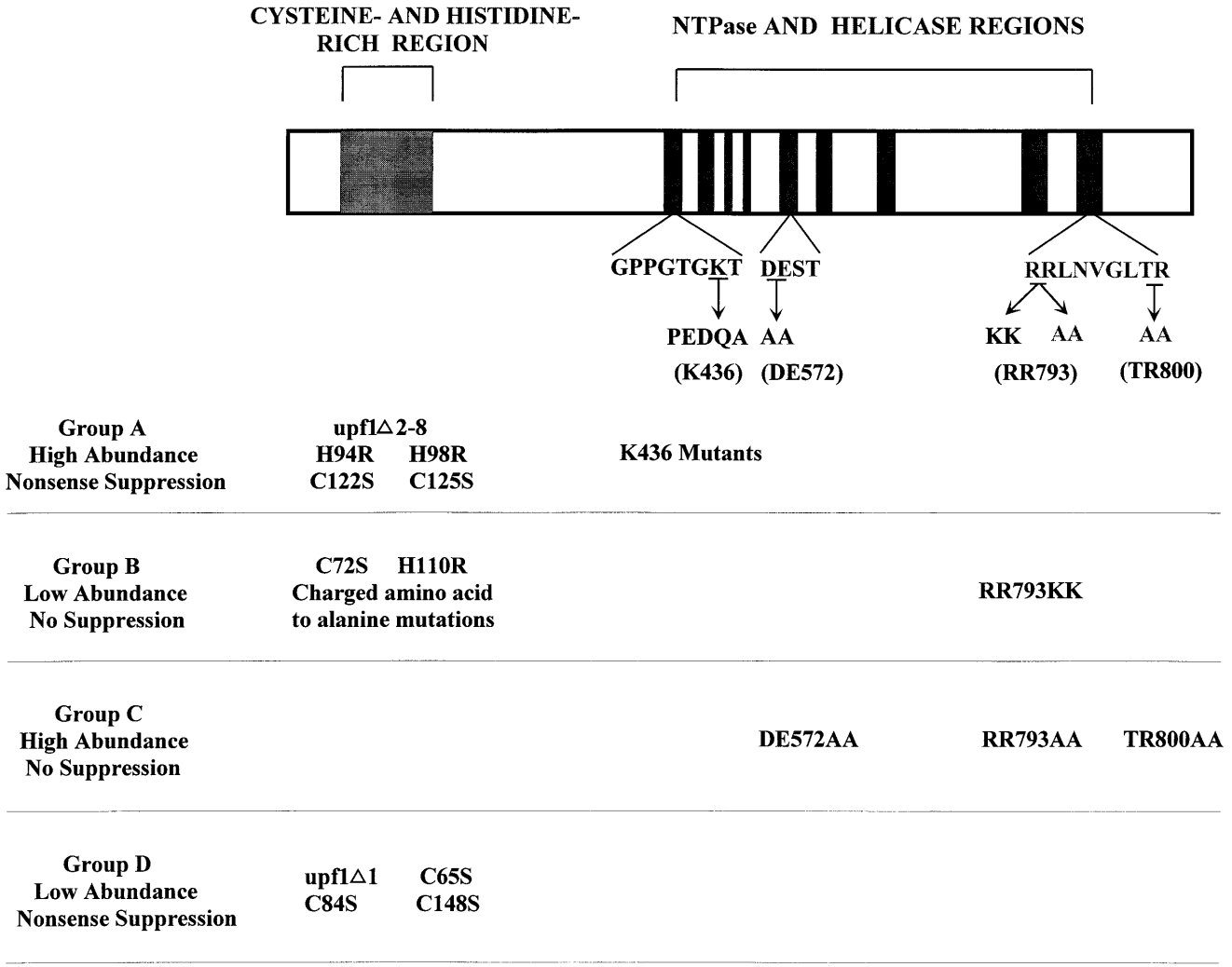

FIG. 10. Summary of the *upf1* mutants identified in this and previous studies. A schematic representation of the *UPF1* gene and the mutations that were constructed is shown. Below the gene is a description of the mutations representing the four groups, based on nonsense suppression and mRNA decay activities, of *upf1* alleles that were identified in this and the previous study (38). See the text for a full discussion of the phenotypes associated with each group of mutant *upf1* alleles. NTPase, nucleoside triphosphatase.

transcript. Previous results have demonstrated that a translation initiation and termination cycle is required in order for the nonsense-mediated mRNA decay pathway to be activated (32). Furthermore, a downstream element that is either part of the protein-coding region or upstream of a translation start site is not functional in promoting rapid decay (32). These results suggest that after translation termination, a factor must scan 3′ of the premature translation termination codon. The Upf complex may facilitate ribosomal recognition of the translation termination codon and/or unwinding of the RNA downstream of the nonsense codon, allowing the factor(s) to scan 3′ of the nonsense codon and interact with the downstream element in order to trigger nonsense-mediated mRNA decay (Fig. 11). It is conceivable that the complex required to promote degradation forms on a ribosome stalled at a translation termination codon. Alternatively, the events that occur at the termination codon influence the subsequent events, such as scanning 3′ of the stop codon, which is required in promoting degradation of the mRNA. Previous results are consistent with the hypothesis that scanning 3′ of a translation termination codon is an important part of the mechanism in promoting mRNA turnover (7). In experiments involving insertion of a strong secondary structure 5′ of the granulocyte-macrophage colony-stimulating factor AU-rich element (ARE), either before or after the normal translational terminator, the mRNA was stabilized approximately 20-fold. The same stem-loop placed 3′ to the ARE, however, had only a minor effect. In light of earlier experiments that showed that the granulocyte-macrophage colony-stimulating factor ARE was not functional when placed within coding sequences (34), these experiments suggest that translation upstream of the ARE may be important for the delivery of a ribosome-associated factor, perhaps via scanning downstream of the translation termination codon, or for ribosome-mediated changes in downstream messenger ribonucleoprotein structure. Since destabilization does not occur when the ARE is present in coding sequences, translational termination may be required to release one ribosome subunit or to shed or add a specific ribosome-associated factor.

The disparate relationship between nonsense-mediated mRNA decay and nonsense suppression of the group C and D *upf1* alleles is of special interest because it sheds light on the role of Upf1p in translation termination. Group C *upf1* alleles

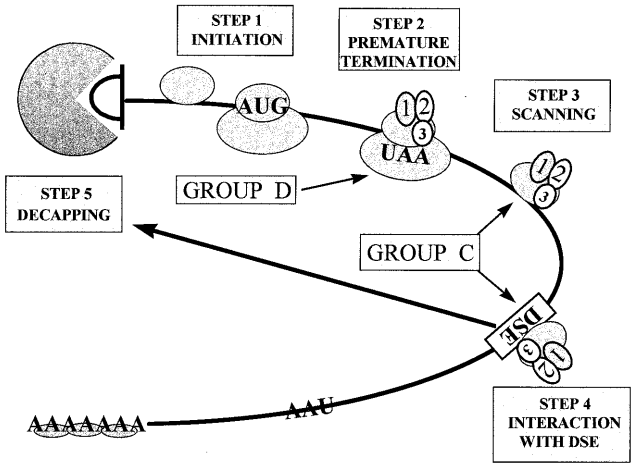

FIG. 11. Model for the mechanism by which a Upfp complex links the translation termination and mRNA decay processes. Following translation initiation (step 1), a ribosome translates mRNA until it encounters a premature translation termination codon. After translation termination (step 2), factors or ribosomal subunits scan downstream (step 3) of the nonsense codon and interact with a downstream element (DSE) (step 4). This interaction leads to decapping of the mRNA (step 5). On the basis of the results presented in this paper and the accompanying paper (38), we propose that the Upfp complex functions in nonsense-mediated mRNA decay and in modulating translation termination. One role of the Upfp complex may be to promote efficient recognition of the stop codon. When premature translation termination occurs, the Upfp complex helps the ribosome, ribosomal subunit(s), or ribosomal associated factor to scan 3′ of the termination codon and interact with a downstream element which triggers nonsense-mediated mRNA decay. Group C mutants retain the codon recognition ability of Upf1p but lose its function in nonsense-mediated mRNA decay (steps 3 and 4). Group D mutants, on the other hand, retain the decay activity of Upf1p, while its ability in stop codon recognition (step 2) is reduced. See the text for details.

have lost decay activity but still remain active in modulating translation termination of the *tyr7-1* and *leu2-2* nonsense alleles (Fig. 11). Thus, group C *upf1* alleles retain the ability to allow recognition of the nonsense codon but do not allow for recognition of the downstream element (Fig. 11). This may be a result of not allowing the appropriate factors to scan 3′ of the nonsense codon and interact with the downstream element. This scenario would explain why nonsense-containing mRNAs would be stable in cells harboring group C *upf1* alleles but nonsense suppression would not occur (Fig. 11).

Unlike group C *upf1* alleles, the group D *upf1* alleles are active in nonsense-mediated mRNA decay, but nonsense suppression is observed in cells harboring the mutations. This observation can be explained by hypothesizing that recognition of the premature translation termination codon is affected in cells harboring group D *upf1* alleles. The altered recognition would result in an increased opportunity for tRNAs to compete with translation termination factors to bind to the ribosome, resulting in suppression of nonsense alleles (Fig. 11). On the other hand, the Upfp complex containing these mutations could still mediate the ribosome-associated factor(s) scanning 3′ of the nonsense codon and promote recognition and interaction with the downstream element, accelerating decay of the mRNA (Fig. 11). Thus, in cells harboring group D *upf1* alleles, tRNAs would be anticipated to have a greater opportunity to promote nonsense suppression while the RNA is still susceptible to degradation. On the basis of the location of group D *upf1* alleles, we suggest that the amino-terminal cysteine- and histidine-rich region of the *UPF1* gene is involved in modulating translation termination.

The results presented here demonstrate a strong link between translation and mRNA decay and suggest that there is cross talk between the *trans*-acting factors involved in these processes. On the basis of the results presented here, Upf1p is a candidate for a factor that functions in both of these processes. Future experiments will entail identifying and investigating other factors interacting with Upf1p as well as developing biochemical assays to determine how these proteins function in translation termination and mRNA decay.

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