MOLECULAR AND CELLULAR BIOLOGY, Feb. 1995, p. 809–823 Vol. 15, No. 2
0270-7306/95/$04.00+0


# Characterization of *cis*-Acting Sequences and Decay Intermediates Involved in Nonsense-Mediated mRNA Turnover

KEVIN W. HAGAN,[1] MARIA J. RUIZ-ECHEVARRIA,[1] YONG QUAN,[2] AND STUART W. PELTZ[1,2]*

*Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School,[1] and The Graduate Program in Microbiology and Molecular Genetics, Rutgers University,[2] Piscataway, New Jersey 08854*

Received 27 July 1994/Returned for modification 2 September 1994/Accepted 14 October 1994

**Several lines of evidence indicate that the processes of mRNA turnover and translation are intimately linked and that understanding this relationship is critical to elucidating the mechanism of mRNA decay. One clear example of this relationship is the observation that nonsense mutations can accelerate the decay of mRNAs in a process that we term nonsense-mediated mRNA decay. The experiments described here demonstrate that in the yeast *Saccharomyces cerevisiae* premature translational termination within the initial two-thirds of the *PGK1* coding region accelerates decay of that transcript regardless of which of the stop codons is used. Nonsense mutations within the last quarter of the coding region have no effect on *PGK1* mRNA decay. The sequences required for nonsense-mediated mRNA decay include a termination codon and specific sequences 3′ to the nonsense mutation. Translation of two-thirds of the *PGK1* coding region inactivates the nonsense-mediated mRNA decay pathway. This observation explains why carboxyl-terminal nonsense mutations are resistant to accelerated decay. Characterization of the decay of nonsense-containing *HIS4* transcripts yielded results mirroring those described above, suggesting that the sequence requirements described for the *PGK1* transcript are likely to be a general characteristic of this decay pathway. In addition, an analysis of the decay intermediates of nonsense-containing mRNAs indicates that nonsense-mediated mRNA decay flows through a pathway similar to that described for a class of wild-type transcripts. The initial cleavage event occurs near the 5′ terminus of the nonsense-containing transcript and is followed by 5′→3′ exonucleolytic digestion. A model for nonsense-mediated mRNA decay based on these results is discussed.**

The rates at which mRNAs decay play an important role in regulation of gene expression. The validity of this statement stems from two observations: (i) mRNA half-lives can vary from each other by as much as 100-fold (24, 47, 51, 54, 55), and this variation determines, in part, the cytoplasmic abundances of these RNAs, and (ii) the rate of decay of an mRNA can be modulated, and its half-life can vary depending on the cell type or the environmental situation of the cell (e.g., hormonal situation, stress, cell cycle, etc.) (2, 47, 54). The goal of our work is to understand the cellular mechanisms that determine the stabilities of mRNAs.

We are studying mRNA decay in the yeast *Saccharomyces cerevisiae*. Our results, as well as work from other laboratories, strongly indicate that mRNA turnover and translation are intimately linked processes and that understanding this relationship is critical in elucidating the mechanism of mRNA decay (1, 7, 8, 11, 19, 21, 31, 45, 47, 49–51, 65). The role of translation in determining mRNA decay rates is not indirect, and for some instability elements these sequences must be actively translated in order for them to function (1, 8, 11, 19, 21, 31, 45, 48, 57, 65). For example, *cis*-acting sequences that require translation in order to promote rapid mRNA turnover have been identified in the protein-coding regions as well as in 3′-untranslated regions (3′-UTR) of mRNAs (1, 7, 8, 11, 19, 21, 25, 31, 45, 48, 49, 57, 60).

One clear example of the relationship between translation and mRNA decay is the observation that nonsense mutations in a gene can reduce the abundance of the mRNA transcribed from that gene in a process that we term nonsense-mediated mRNA decay (50). Reduced mRNA levels or decreased stabilities of nonsense-containing transcripts have been observed in both prokaryotes and eukaryotes (4–6, 9, 10, 14, 18, 20, 33, 35–37, 39, 43, 46, 48, 52, 64). In the yeast *S. cerevisiae*, a nonsense-containing *URA3* mRNA is stabilized in a strain containing an amber suppressor tRNA, indicating that mRNA destabilization is linked to premature translational termination (36).

We have been characterizing the *cis*-acting sequences required to accelerate the decay of nonsense-containing mRNAs. The *PGK1* transcript, normally a stable, abundant mRNA (half-life = 60 min), was used as our model substrate to investigate this process (27, 48). Previous results (48) have demonstrated that (i) amber mutations located within the amino-terminal two-thirds of the protein-coding region accelerate the rate of decay of the *PGK1* transcript up to 12-fold, whereas amber mutations located within the carboxyl-terminal one-third of the protein-coding region have no effect on decay; (ii) specific sequences 3′ to the nonsense mutation, which we have defined as downstream elements, are required for nonsense-mediated mRNA decay; and (iii) the *PGK1* transcript contains a stabilizer element that, when translated, inactivates the nonsense-mediated mRNA decay pathway. The stabilizer element in the *PGK1* gene is positioned within the protein-coding region such that it constitutes the boundary between nonsense mutations which do or do not affect mRNA decay.

*trans*-acting factors involved in nonsense-mediated mRNA decay have been identified in studies undertaken to investigate a class of yeast non-tRNA nonsense suppressors (13, 34). Mutations in the *UPF* genes were isolated as allosuppressors of a *his4* frameshift allele (13, 34). Subsequent studies demon-

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, 675 Hoes Ln., Piscataway, NJ 08854. Phone: (908) 235-4790. Fax: (908) 235-5223. Electronic mail address: Peltz@RWJA.UMDNJ.EDU.

strated that the *upf1* and *upf3* alleles (and, as more recently identified, *upf2*) lead to the selective stabilization of nonsense-containing mRNAs without affecting the rates of decay of most other mRNAs (12, 22, 33, 34, 48, 52). The *UPF1* and *UPF2* genes have been cloned and sequenced (12, 17, 34).

In this report we have focused on three aspects of the nonsense-mediated mRNA decay pathway. We have characterized the *cis*-acting elements required for nonsense-mediated decay, including determination of whether the type of nonsense codon affects the activity of the nonsense-mediated mRNA decay pathway and whether the downstream elements identified in amber-containing *PGK1* transcripts are also functional when inserted downstream of the other stop codons. We also asked whether the resistance to decay of carboxyl-terminal nonsense mutations is independent of the type of nonsense codon. The second aspect examined was the question of how global are the conclusions drawn about the nonsense-mediated mRNA decay pathway with the *PGK1* transcript as the model substrate. We have characterized the decay of the *HIS4* transcript synthesized from nonsense-containing *his4* alleles. The third aspect we have addressed concerns the identification of the important cleavage events involved in nonsense-mediated mRNA decay. Using a yeast strain in which a major 5′→3′ exoribonuclease, encoded by *XRN1*, has been deleted, we found that decay intermediates of this pathway can be identified. On the basis of these results, a model that describes the mechanism of nonsense-mediated mRNA decay is discussed.

## MATERIALS AND METHODS

**Yeast strains, growth conditions, and transformation procedures.** The yeast strains used in this study are RY262 (*MAT*α *his4-519 ura3-52 rpb1-1*) (48), SWP154 (*MAT*α *upf1*::*ura3 ura3-52 rpb1-1 his4-38 leu2-1*) (48), PLY36 (*MAT*α *his4-38 met14 SUF1-1 upf1-2 ura3-52*) (32, 34), JD5ts (*MAT*α *his4-38 leu2-1 trp1-Δ1 ura3-52 rpb 1-1*) (generously provided by Allan Jacobson, University of Massachusetts Medical Center, Worcester), YW5ts (*MAT*α *his4-38 leu2-1 trp1-Δ1 ura3-52 rpb1-1 upf1*::*HISG*) (a derivative of RY262), YQ5ts (*MAT*α *his4-38 leu2-1 trp1-Δ1 ura3-52 rpb1-1 his4*::*TRP1*) (prepared for this study), YL5ts (*MAT*α *his4-38 leu2-1 trp1-Δ1 ura3-52 rpb1-1 upf1*::*hisG his4*::*TRP1*) (prepared for this study), BJ5464 (*MAT*α *can1 GAL his3-Δ200 leu2Δ1 pep4*::*HIS3 prb1Δ1.6R trp1 ura3-52*) (generously provided by Arlen Johnson, Dana-Farber Cancer Institute, Boston, Mass.), and BJ5464XRN$^-$ (*MAT*α *can1 GAL his3-Δ200 leu2Δ1 pep4*::*HIS3 prb1Δ1.6R xrn1*::*LEU2 trp1 ura3-52*) (also supplied to us by Arlen Johnson). Yeast media were prepared as described previously (53). Cells were cultured on media lacking uracil or lacking tryptophan and uracil to select and maintain the plasmids used in these studies. Cells lacking plasmids were grown nonselectively on yeast extract-peptone-dextrose (YPD) medium. Cells harboring the *rpb1-1* allele were grown at 24°C; all other yeast cells were grown at 30°C. Yeast transformations were performed with a modification (58) of the lithium acetate protocol (29).

Yeast strains YQ5ts and YL5ts, harboring a disruption of the *HIS4* gene, were constructed by transforming a 3.9-kb *Sst*I-*Bst*EII fragment of pUC18HIS4KO harboring the *HIS4* gene disruption (*his4*::*TRP1*; see below and Table 1). Recombinants were selected on medium lacking tryptophan, and disruption of the *HIS4* gene was confirmed by blot analysis of genomic DNA cleaved with *Sst*I-*Bgl*II and probed with a 2.5-kb *Sst*I-*Bgl*II *HIS4* DNA fragment.

**Materials.** Enzymes were obtained from Boehringer Mannheim, Gibco-BRL, or New England Biolabs. *Taq* polymerase was generously provided by Steven Brill, Rutgers University. Radioactive nucleotides were purchased from Amersham or New England Nuclear. The amber and ochre SMURFT linkers were purchased from Pharmacia.

**Oligodeoxyribonucleotides.** The oligonucleotides used in these studies were obtained from the Center for Advanced Biotechnology and Medicine DNA Synthesis Network Laboratory. The oligodeoxynucleotides used in these studies are as follows (the numbers associated with the oligodeoxyribonucleotides are used below in descriptions of the plasmids): 1, 5′-CGATAGTAATATTTATATATTTATATTTTTAAAATATTTATTTATTTATTTATTTAAGAT-3′; 2, 5′-TTAAGTTAACTTAA-3′; 3, 5′-TGAGTGAGTGAGATATCTCACTCACTCA-3′; 4, 5′-AATAGATCTATTCTGGACCATTGTCCA-3′; 5, 5′-TTTAGTTAACTTAAGACTTCATCATTGCTGATGCTT-3′; 6, 5′-TTTAGATATCTCACTCACTCAGACTTCATCATTGCTGATGCTT-3′; 7, 5′-CTTCAAGTCCAAATCTTGGACAGAC-3′; and 8, 5′-ACGTGACCTCTGTGCTTTCTAGTCT-3′ (see below for explanation of underlining).

**mRNA decay measurements and RNA preparation and analysis.** The mRNA decay rates for the various *PGK1* alleles transformed into strains harboring the temperature-sensitive allele of the RNA polymerase gene (*rpb1-1*) were determined as described previously (12, 48). The half-lives of the nonsense-containing *HIS4* transcripts were determined as before (12, 48) in *UPF1*$^+$ and *upf1*Δ strains (YQ5ts and YL5ts, respectively) harboring the temperature-sensitive RNA polymerase allele (*rpb1-1*) in which the *HIS4* gene was disrupted by the *TRP1* gene. Thus, no endogenous *HIS4* transcript is synthesized. The mRNA decay rates in strains BJ5464 and BJ5464XRN$^-$ were determined by RNA blotting analyses of RNAs isolated at different times after inhibition of transcription with the antifungal agent thiolutin (26, 30, 63). Cells were grown to mid-log phase (optical density at 600 nm, 0.7 to 1.0) at 30°C, 100-ml aliquots of cells were harvested and resuspended in 18 ml of fresh medium containing 10 μg of thiolutin per ml, and 2-ml aliquots were removed at various times after thiolutin addition. Half-life measurements were carried out at both 24 and 30°C. Cell pellets were obtained by rapid centrifugation and aspiration of excess culture medium and were frozen in dry ice. RNA extraction, RNA blotting, and hybridization were performed as described previously (48, 62). The results of these experiments were quantitated by using a Bio-Rad model G-250 Molecular Imager or a Bio-Rad model GS-670 Imaging Densitometer.

**Preparation of radioactive probes.** DNA probes were labelled to high specific activity with [α-$^{32}$P]dCTP (16) or by 5′ end labelling of single-stranded oligodeoxynucleotides with [γ-$^{32}$P]ATP (56). Oligonucleotide 1 (see "Oligodeoxyribonucleotides" above) was used to monitor the decay of *PGK1* alleles used in this study. A 0.6-kb *Eco*RI-*Hin*dIII fragment from pGEM-42-CYH2 was used to prepare a radioactive probe to monitor the decay of the *CYH2* mRNA. A 0.5-kb *Bam*HI-*Hpa*I fragment from pBluescript KS− (U3) was used to prepare probes for the U3 small nuclear RNA (42). A 4.0 kb *Sst*I-*Sph*I fragment from pUC18-HIS4 was used to monitor the decay of the *HIS4* transcript (49).

**Plasmid constructions.** The plasmid constructions are described below and are summarized in Table 1. The plasmids have been named according to the plasmid backbone (i.e., pUC or pRIP), which yeast gene was used (i.e., *HIS4* or *PGK1*), whether sequences from the genes were deleted (Δ) or inserted (IN), and the type of nonsense codon that was inserted (i.e., UAA, UAG, or UGA). The tag in the 3′-UTR is denoted by (-AU). *PGK1* alleles with almost twice the amount of protein-coding region are denoted by 2x. The restriction site in which the nonsense codon was inserted is also indicated.

PCRs (30 cycles) were performed in a total volume of 100 μl, and reaction mixtures contained 125 ng of DNA template, 50 pmol of DNA primers, and 5 U of *Taq* DNA polymerase. The samples were subsequently extracted once with chloroform and once with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated in ethanol, resuspended in a suitable amount of Tris-EDTA (pH 7.5), and cleaved with the appropriate restriction endonucleases. Digests were heat inactivated at 65°C for 15 min and ligated with the plasmids of interest.

**(i) Preparation of nonsense-containing *PGK1* alleles.** Preparation of the plasmid pUC9PGK(-AU) was described previously. This plasmid harbors a wild-type *PGK1* allele in which a tag was inserted into the 3′-UTR at the *Cla*I site (48). The nucleotide positions cited for the *PGK1* gene are derived from the EMBL database (accession number M17195). Nonsense-containing *PGK1* premature nonsense alleles were constructed by insertion of linkers bearing either UAA or UGA stop codons in all three reading frames into various restriction endonuclease sites within the *PGK1* coding region (see Fig. 1B for a depiction of the restriction sites). The sequences of the linkers encoding the UAA and UGA stop codons (in all reading frames) are given in "Oligodeoxyribonucleotides" above. Oligonucleotide 2 (see "Oligodeoxyribonucleotides") encodes the UAA-containing linker as well as an *Hpa*I site (underlined), while oligonucleotide 3 encodes the UGA-containing linker harboring an *Eco*RV site (underlined). The oligonucleotides for the linkers were hybridized as before and inserted into the *Asp* 718, *Bgl*II, *Hin*cII, and *Xba*I sites in the *PGK1* coding region (48). Briefly, the plasmid pUC9PGK(-AU) was digested to completion with *Asp* 718 or *Bgl*II, the termini were blunt ended with the Klenow fragment of DNA polymerase I, and the linkers were ligated to the cleaved plasmid (10:1 molar excess of linker to plasmid). The products were digested with the restriction endonuclease specific for each linker. Plasmid DNAs were purified by electrophoresis on agarose gels, ligated again, and transformed into *Escherichia coli* DH5α. Multiple *Hin*cII and *Xba*I sites within the *PGK1* gene necessitated partial digestion of pUC9PGK (-AU) with these enzymes, blunt ending of *Xba*I digestion products, and isolation of singly cut plasmid from agarose gels. Ligation of the nonsense-containing linker was done as described above. Linker insertion was confirmed by DNA restriction digest analysis and DNA sequencing. The *Bam*HI-*Hin*dIII fragments containing the mutant *PGK1* alleles were subcloned into the centromere-based plasmid pRIP1 (45). The resultant *PGK1* alleles are depicted in Fig. 1 and are listed in Table 1. The location of the nonsense codon in each construction is expressed as the percentage of the protein-coding region traversed by the ribosome prior to encountering a nonsense codon (see Fig. 1).

**(ii) Preparation of nonsense-containing mini-*PGK1* alleles.** A *PGK1* allele with a UAA stop codon at 5.6% of the *PGK1* protein-coding sequence and with the region between 5.6 and 92.6% of the *PGK1* protein-coding sequence deleted was constructed by digesting pUC9PGK(-AU)H2(3)UAA and pUC9PGK (-AU)BglIIUAA with *Hpa*I and *Hin*dIII. The resultant 3.5-kb fragment from the former plasmid and the 0.4-kb fragment from the latter plasmid were isolated and ligated together to create pUC9PGKΔ1UAA. A similar plasmid containing a UGA stop codon at 5.6% of the *PGK1* protein-coding sequence and with between 5.6 and 92.6% of the *PGK1* protein-coding sequence deleted was con-

TABLE 1. Plasmids used in this study

| Plasmid(s) | Description |
|---|---|
| pRIPPGK(-AU) | *PGK1* gene with a DNA tag inserted into the 3′-UTR |
| pRIPPGK(-AU)H2(3)UAA and pRIPPGK (-AU)H2(3)UGA | Linker containing ochre or opal codons inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] |
| pRIPPGK(-AU)AspUAA and pRIPPGK(-AU)AspUGA | Linker containing ochre or opal codons inserted at 39% of the *PGK1* coding region (*Asp* 718 site, 789 bp) |
| pRIPPGK(-AU)H2(2)UAA and pRIPPGK (-AU)H2(2)UGA | Linker containing ochre or opal codons inserted at 55% of the *PGK1* coding region [*Hin*cII(2) site, 979 bp] |
| pRIPPGK(-AU)H2(1)UAA and pRIPPGK (-AU)H2(1)UGA | Linker containing ochre or opal codons inserted at 67.7% of the *PGK1* coding region [*Hin*cII(1) site, 1,138 bp] |
| pRIPPGK(-AU)XbaUAA and pRIPPGK(-AU)XbaUGA | Linker containing ochre or opal codons inserted at 76.2% of the *PGK1* coding region (*Xba*I site, 1,244 bp) |
| pRIPPGK(-AU)BglUAA and pRIPPGK(-AU)BglUGA | Linker containing ochre or opal codons inserted at 92.6% of the *PGK1* coding region [*Bgl*II(3) site, 1,449 bp] |
| pRIPPGK(-AU)Δ1UAA and pRIPPGK(-AU)Δ1UGA | Linker containing ochre or opal codons inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] followed by a deletion of between 5.6 and 92.6% of the *PGK1* coding region |
| pRIPPGK2xPGK(-AU)UAA and pRIPPGK2xPGK(-AU)UGA | Linker containing ochre or opal codons inserted at 92.6% of the *PGK1* coding region [*Bgl*II(3) site, 1,449 bp] followed by between 5.6 and 92.6% of the *PGK1* coding region |
| pRIPPGK(-AU)BglUAAIN1 and pRIPPGK (-AU)BglUGAIN1 | Linker containing ochre or opal codons inserted at 92.6% of the *PGK1* coding region [*Bgl*II(3) site, 1,449 bp] followed by the complete downstream element (*PGK1* nt 1138 to 1243) |
| pRIPPGK(-AU)Δ1UAGIN1, pRIPPGK(-AU)Δ1UAAIN1, and pRIPPGK(-AU)Δ1UGAIN1 | Linker containing amber, ochre, or opal codons inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] with a deletion of between 5.6 and 92.6% of the *PGK1* coding region followed by the complete downstream element (*PGK1* nt 1138 to 1243) |
| pRIPHIS4 | *HIS4* wild-type gene |
| pRIPHIS4NUGA | Linker containing opal codons inserted at 9.9% of the *HIS4* coding region (*Nhe*I site, 1,570 bp) |
| pRIPHIS4SUGA | Linker containing opal codons inserted at 19.6% of the *HIS4* coding region (*Sal*I site, 1,801 bp) |
| pRIPHIS4BsUGA | Linker containing opal codons inserted at 38.8% of the *HIS4* coding region [*Bst*XI(2) site, 2,262 bp] |
| pRIPHIS4BgUGA | Linker containing opal codons inserted at 70.4% of the *HIS4* coding region (*Bgl*II site, 3,020 bp) |
| pRIPHIS4HUGA | Linker containing opal codons inserted at 79.0% of the *HIS4* coding region (*Hpa*I site, 3,228 bp) |
| pRIPHIS4XUGA | Linker containing opal codons inserted at 97.0% of the *HIS4* coding region (*Xba*I site, 3,660 bp) |
| pRIPHIS4XUGAIN1 | Linker containing opal codons inserted at 97.0% of the *HIS4* coding region (*Xba*I site, 3,660 bp) followed by the complete downstream element (*PGK1* nt 1138 to 1243) |
| pRIPHIS4ΔUAA | Linker containing ochre codons inserted at 9.9% of the *HIS4* coding region [*Nhe*I site, 1,570 bp) with a deletion of between 9.9 and 97.0% of the *HIS4* coding region |
| pRIPHIS4ΔUAAIN1 | Linker containing ochre codons inserted at 9.9% of the *HIS4* coding region (*Nhe*I site, 1,570 bp) with a deletion of between 9.9 and 97.0% of the *HIS4* coding region followed by the complete downstream element (*PGK1* nt 1138 to 1243) |
| pUC18HIS4KO | *HIS4* allele in which between 19.6 and 70.4% of the *HIS4* coding region is replaced with a 1.6-kb *Sal*I-*Bgl*II fragment harboring the *TRP1* gene from plasmid pG-1 |

structed by digesting pUC9PGK(-AU)H2(3)UGA and pUC9PGK(-AU)BglII UGA with *Eco*RV and *Hin*dIII and ligating together the fragments analogous to those described above to create pUC9PGKΔ1UGA. The structure of each construct was confirmed by DNA restriction digest analysis and DNA sequencing. The *Bam*HI-*Hin*dIII fragments containing the mutant *PGK1* alleles were subcloned into the centromere-based plasmid pRIP1 to create pRIPPGK (-AU)Δ1UAA and pRIPPGK(-AU)Δ1UGA. The resultant *PGK1* alleles are depicted in Fig. 2 and are listed in Table 1. We refer to these as nonsense-containing mini-*PGK1* alleles.

**(iii) Preparation of *PGK1* alleles containing almost twice the amount of protein coding sequence as in wild-type *PGK1*.** A *PGK1* allele in which sequences 3′ of a UAA nonsense mutation inserted at 92% of the *PGK1* protein-coding region were replaced with sequences from the *PGK1* gene that contained from 5.6% of the *PGK1* protein-coding region to the end of the *PGK1* gene was constructed by cleaving pUC9PGK(-AU)H2(3)UAA and pUC9PGK(-AU)BglIIUAA with *Hpa*I and *Hin*dIII. The resultant 1.4-kb fragment of the former plasmid and 4.5-kb fragment of the latter plasmid were ligated together to create pUC9PGK(-AU)UAA. An analogous *PGK1* double allele containing a UGA stop codon was constructed by digesting pUC9PGK(-AU)H2(3)UGA and pUC9PGK(-AU)BglIIUGA with *Eco*RV and *Hin*dIII and ligating together the fragments analogous to those described above to create pUC92xPGK(-AU)UGA. The structure of each construct was confirmed by DNA restriction digest analysis and DNA sequencing. The *Bam*HI-*Hin*dIII fragments containing the mutant *PGK1* alleles were subcloned into the centromere-based plasmid pRIP1 to create pRIP2xPGK(-AU)UAA and pRIP2xPGK(-AU)UGA. The resultant *PGK1* alleles are depicted in Fig. 3 and are listed in Table 1.

**(iv) Preparation of mini-*PGK1* alleles harboring a downstream element 3′ of a nonsense codon.** UAA- and UGA-containing mini-*PGK1* alleles harboring a downstream element identified previously in the *PGK1* gene (48) (see Fig. 2) were constructed as follows. Oligonucleotide 4 (*PGK1* nucleotides [nt] 1224 to 1243 followed by a *Bgl*II site) (see "Oligodeoxyribonucleotides" above) and oligonucleotide 5 (*PGK1* nt 1138 to 1159 preceded by an *Hpa*I site) were used as primers in a PCR to synthesize a DNA fragment containing the downstream element (*PGK1* nt 1138 to 1243) synthesized by PCR. This DNA fragment was cleaved with *Hpa*I and *Bgl*II and ligated to a 3.8-kb *Hpa*I-*Bgl*II fragment of pUC9PGK(-AU)H2(3)UAA, resulting in plasmid pUC9PGKΔ1UAAIN1. Similarly, oligonucleotide 4 and oligonucleotide 6 (see "Oligodeoxyribonucleotide" above), consisting of *PGK1* sequences between nt 1138 and 1159 preceded by an *Eco*RV site, were used to generate PCR products which were cleaved with *Eco*RV and *Bgl*II and ligated to a 3.8-kb *Eco*RV-*Bgl*II fragment of pUC9PGK (-AU)H2(3)UGA to construct pUC9PGK(-AU)Δ1UGAIN1. The *Bam*HI-*Hin*dIII fragments containing the mutant *PGK1* alleles were subcloned into the plasmid pRIP1, resulting in pRIPPGK(-AU)Δ1UAAIN1 and pRIPPGK(-AU)

Δ1UGAIN1. The resultant *PGK1* alleles are depicted in Fig. 2 and are listed in Table 1.

**(v) Preparation of nonsense-containing *HIS4* alleles.** *HIS4* alleles with premature nonsense codons were constructed by insertion of a linker bearing UGA stop codons in all three reading frames (oligonucleotide 3; see "Oligodeoxyribonucleotides" above) into six restriction sites (in different constructs) within the *HIS4* protein-coding region (the locations of the restriction sites are depicted in Fig. 4A; the location of the nonsense codon in each construction is expressed as the percentage of the protein-coding region traversed by the ribosome prior to encountering a nonsense codon). The nucleotide positions cited for the *HIS4* gene are derived from its EMBL database entry (accession number J01331). The plasmid pUC18HIS4, containing the wild-type *HIS4* gene, was cleaved with one of the restriction enzymes shown in Fig. 4A, and the UGA stop linker was ligated into the blunt-ended plasmid as described for the *PGK1* nonsense-containing alleles. Stop codons were inserted into the following restriction endonuclease sites: *Nhe*I (bp 1570; 9.9% of the *HIS4* coding region), *Sal*I (bp 1801; 19.6% of the *HIS4* coding region), *Bst*XI(2) (bp 2262; 38.8% of the *HIS4* coding region), *Bgl*II (bp 3020; 70.4% of the *HIS4* coding region), *Hpa*I (bp 3228; 79% of the *HIS4* coding region), and *Xba*I (bp 3660; 97% of the *HIS4* coding region). Linker insertion was confirmed by DNA restriction digest analysis and DNA sequencing. The *Sst*I-*Sph*I fragments containing the mutant *HIS4* alleles were subcloned onto the centromere-based plasmid pRIP1. The resultant *HIS4* alleles are listed in Table 1.

**(vi) Preparation of nonsense-containing mini-*HIS4* alleles.** A *HIS4* allele that contained a deletion of between 9.9 and 97% of the *HIS4* protein-coding region was constructed by cleaving pUC18HIS4 with *Nhe*I and *Xba*I and ligating the resultant 4.5-kb fragment with a linker containing UAA stop codons (oligonucleotide 2; see "Oligodeoxyribonucleotides" above) in all three reading frames as described above, creating the plasmid pUC18HIS4ΔUAA. The construct was confirmed by DNA restriction analysis and DNA sequencing. The *Sst*I-*Sph*I fragment containing the nonsense-containing mini-*HIS4* allele was subcloned into the centromere-based plasmid pRIP to form the plasmid pRIPHIS4ΔUAA. The resultant mini-*HIS4* allele is depicted in Fig. 4B and is listed in Table 1.

**(vii) Preparation of a mini-*HIS4* allele containing a downstream element 3′ of the nonsense codon.** The mini-*HIS4* allele containing a downstream element from the *PGK1* gene was prepared by cleaving plasmid pUC18HIS4ΔUAA with *Hpa*I and blunt end ligating the *PGK1* downstream element 3′ of the nonsense codon (48) (*PGK1* nt 1139 to 1243), resulting in plasmid pUC18HIS4ΔUAAIN1. The DNA fragment containing the downstream element was generated by a PCR with oligonucleotides 4 and 5 as primers (see "Oligodeoxyribonucleotides" above). The structure of this allele was confirmed by DNA restriction analysis and DNA sequencing. The *Sst*I-*Sph*I fragment containing this *HIS4* allele was subcloned into the centromere-based plasmid pRIP to form pRIPHIS4Δ UAAIN1. The resultant *HIS4* allele is depicted in Fig. 4B, and the plasmid is listed in Table 1.

**(viii) Preparation of a *HIS4* allele harboring a *PGK1* downstream element 3′ of a carboxyl-terminal nonsense mutation.** A *HIS4* allele harboring a UGA stop codon at 97% of the *HIS4* protein-coding region in which a downstream element from the *PGK1* gene was inserted was constructed by cleaving pUC18HIS4X UGA with *Eco*RV-*Sph*I and cleaving pUC18HIS4ΔUAAIN1 with *Hpa*I-*Sph*I. The resultant 5.8-kb fragment from the former plasmid and 1.0-kb fragment from the latter plasmid were isolated and ligated together to create pUC18 HIS4XUGAIN1. The structure of this construct was confirmed by DNA restriction digest analysis and DNA sequencing. The *Sst*I-*Sph*I fragment containing the mutant *HIS4* allele was subcloned into the centromere-based plasmid pRIP to create pRIPHIS4XUGAIN1. The resultant *HIS4* allele is depicted in Fig. 4C and is listed in Table 1.

**(ix) Construction of a plasmid harboring a disruption of the *HIS4* gene.** A plasmid in which between 19.6 and 70.4% of the *HIS4* coding region is replaced with the *TRP1* gene was constructed by first cleaving pUC18HIS4 with *Sal*I and *Bgl*II. The resultant 5.4-kb fragment was ligated with a 1.6-kb *Sal*I-*Bgl*II fragment harboring the *TRP1* gene from plasmid pG-1, forming plasmid pUC18HIS4KO. The structure of this construct was confirmed by DNA restriction analysis, and the plasmid harboring this allele is listed in Table 1.

**Mapping 5′ and 3′ termini of mini-*PGK1* alleles containing the downstream element. (i) Primer extension analysis.** A primer complementary to a region 61 nt from the 5′ end of the wild-type *PGK1* mRNA (oligonucleotide 7; see "Oligodeoxyribonucleotides" above) was hybridized to RNAs isolated from strains harboring plasmids containing mini-*PGK1* alleles with the downstream element, and primer extension analysis was performed as described previously (3). Oligonucleotide 7 was also used as a primer for dideoxy chain termination sequencing (56) in order to generate a ladder for size comparisons of primer extension products. Oligonucleotide 8 was used for primer extension analysis of the *CYH2* transcript and is complementary to nt 342 to 366 of the *CYH2* gene, 50 nt from the 5′ end of the RNA.

**(ii) RNase H mapping.** Site-specific cleavage of poly(A) was performed by using a variation of published procedures (38). Twenty micrograms of RNA was heated to 70°C for 10 min and chilled on ice with the addition of $H_2O$ to a total volume of 17 μl. One microliter of 20 mM EDTA (pH 8.0) and 1 μl of 0.5-μg/μl oligo(dT) were added. The reaction mixtures were incubated for 30 min at room temperature, 1 μl of 1 M KCl was added, and after a 10-minute incubation at room temperature, 20 μl of RNase H reaction mix (56 mM $MgCl_2$, 40 mM Tris Cl [pH 8.0], 20 μg of RNase H per ml) was added to the mixtures. The samples were incubated at 37°C for 30 min, phenol-chloroform-isoamyl alcohol extracted, and chloroform-isoamyl alcohol extracted, and the RNAs were precipitated with ethanol. The RNAs were visualized by RNA blotting analysis as described above.

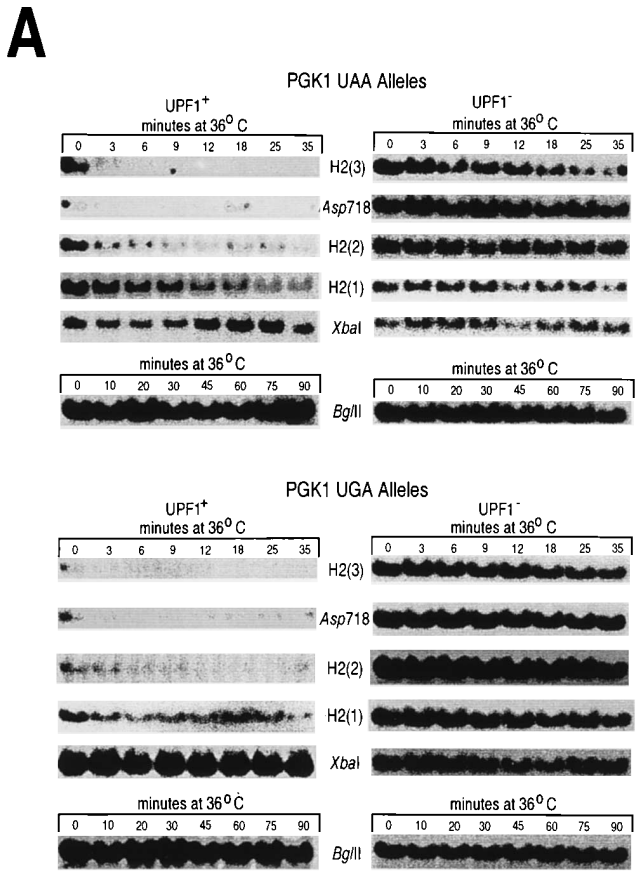
**(iii) S1 nuclease protection.** S1 analysis was performed by using as a probe a 3′-end-labelled double-stranded DNA fragment obtained after digestion of plasmid pUC9PGK(-AU) with *Bgl*II and *Hin*dIII. This fragment is complementary to the *PGK1* sequence from position 1448 to 1823 and contains the 64-nt AU-rich region inserted at bp 1561 of the *PGK1* gene. The AU-rich region is complementary to the RNA synthesized from the plasmid-borne *PGK1* alleles of interest but not to the wild-type *PGK1* mRNA. Total RNA (75 μg) was added to $3 \times 10^4$ cpm of the probe in 50 μl of hybridization buffer {40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] [pH 6.3], 0.4 M NaCl, 1 mM EDTA [pH 6.5], 70% formamide}, heated for 10 min at 85°C, and hybridized overnight at the appropriate temperature. After addition of 450 μl of S1 buffer (280 mM NaCl, 30 mM Na acetate [pH 4.6], 4.5 mM $ZnSO_4$, 20 mg of carrier DNA per ml), the hybrids were digested with 100 U of S1 nuclease for 30 min at 30°C. The reaction was stopped by addition of 113 μl of S1 nuclease stop buffer (2.5 M $NH_4$ acetate, 50 mM EDTA [pH 8.0]), and the digestion products were phenol extracted, precipitated, and separated on a 6% polyacrylamide gel containing 6 M urea. A sequencing reaction was used to determine the sizes of the fragments.

## RESULTS

**Destabilization of the *PGK1* transcript is dependent on the position of a nonsense mutation within the *PGK1* protein-coding region but is independent of the type of nonsense codon.** The *PGK1* gene was chosen in order to study the effects of nonsense mutations on the decay of mRNAs because it encodes an abundant, stable mRNA (23, 26, 45, 48). The location of a nonsense mutation in the *PGK1* gene is expressed in terms of the percentage of the coding region that is translated prior to the ribosome reading the nonsense codon. As described previously, in order to distinguish the nonsense-containing transcript from the endogenous *PGK1* mRNA, a DNA tag was inserted into the 3′-UTR of the *PGK1* gene (48).

The relationship between the location of a UAA or UGA nonsense mutation and the half-life of the *PGK1* mRNA was investigated by inserting a linker harboring either stop codon into restriction sites at various positions in the *PGK1* gene (Fig. 1B). The mutant alleles were transferred to yeast centromere plasmids and transformed into yeast cells harboring the *rpb1-1* temperature-sensitive allele of the RNA polymerase II gene (44). The rates of decay of the wild-type and mutant *PGK1* mRNAs were determined by RNA blotting analyses of RNAs isolated at different times after transcription was inhibited by shifting the culture to the nonpermissive temperature (36°C). The results of these experiments are shown in Fig. 1A and summarized in Fig. 1B. The presence of nonsense codons within the amino-terminal two-thirds of the *PGK1* coding sequence accelerated the rate of decay of the mutant mRNAs, while *PGK1* alleles containing nonsense mutations in the last quarter of the *PGK1* transcript were unaffected (Fig. 1). As internal controls, the rates of decay of unrelated, stable RNAs encoded by the *CYH2* and U3 genes were determined and shown to have the same half-life in all cells regardless of the nature of their respective *PGK1* alleles (data not shown). Thus, the mRNA decay rates for the UAA and UGA nonsense-containing *PGK1* alleles mirrored the results previously observed for the decay of the amber-containing *PGK1* transcripts (48).

The product of the yeast *UPF1* gene is required for the rapid turnover of nonsense-containing mRNAs (22, 33, 34, 48, 50–52). Either the *upf1-2* mutation or deletion of the *UPF1* gene from the yeast genome (*upf1*Δ) will selectively stabilize mRNAs containing early nonsense mutations, while the rates of decay of most other mRNAs are unaffected (33, 48, 50–52). Previous work has demonstrated that *PGK1* amber-containing mRNAs were stabilized to wild-type *PGK1* levels in a *upf1*Δ



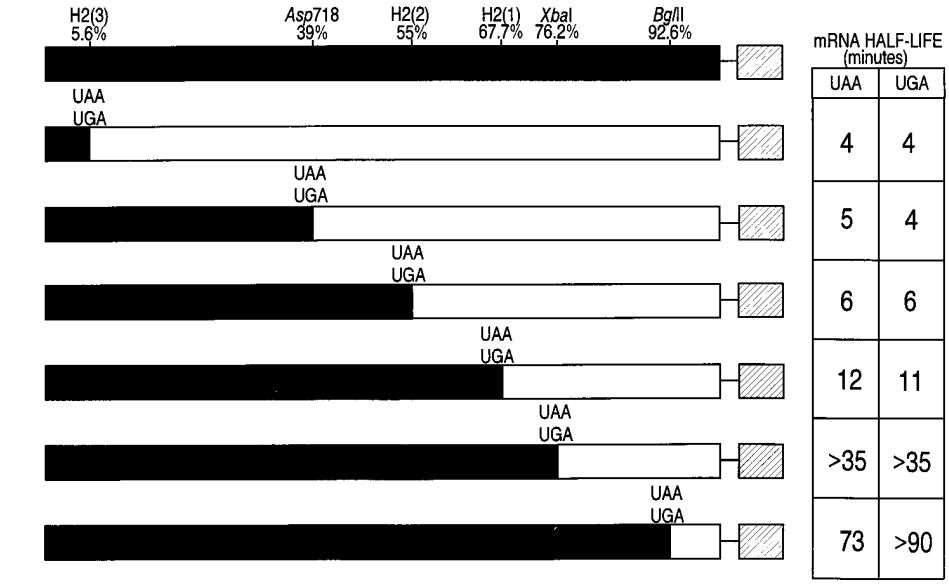




FIG. 1. RNA blot analysis of *PGK1* alleles containing premature nonsense codons. Linkers with UAA or UGA nonsense codons in all three reading frames were inserted individually into six restriction sites located in the protein-coding region of the *PGK1* gene. For simplicity, the location of each nonsense codon within the *PGK1* gene is presented as a percentage of the *PGK1* coding sequence translated by the ribosome prior to encountering a stop codon. mRNA turnover rates for the nonsense-containing *PGK1* alleles were determined as described in the text. (A) mRNA decay rates for *PGK1* containing premature UAA and UGA codons were measured in strains RY262 (*UPF1*$^+$) and SWP154 (*upf1*Δ). The coding region location and restriction sites used to insert the stop codon for each *PGK1* allele are indicated: *Hin*cII(3) [H2(3)] site, stop codon located at 5.6% of the *PGK1* coding region (codon 23); *Asp* 718 site, stop codon located at 39% of the *PGK1* coding region (codon 164); H2(2) site, stop codon located at 55% of the *PGK1* coding region (codon 229); H2(1) site, stop codon located at 67.7% of the *PGK1* coding sequence (codon 282); *Xba*I site, stop codon located at 76.2% of the *PGK1* coding sequence (codon 317); and *Bgl*II site, stop codon located at 92.6% of the *PGK1* coding sequence (codon 385). The time course of the decay measurements for *PGK1* alleles harboring the nonsense codon in the *Bgl*II site were for 90 min, while the other time courses were for 35 min. (B) Summary of the mRNA decay rate measurements for *PGK1* alleles in *UPF1*$^+$ and *upf1*Δ strains. A diagram of the various nonsense-containing *PGK1* alleles is shown beside the summary of the results. The *PGK1* protein-coding region is represented as a rectangle. The region of the protein-coding region that was translated prior to termination of translation is filled in; the coding sequence 3′ of the termination codon is unfilled. Noncoding sequences are represented by thin lines, and the tag in the 3′-UTR is indicated as hatched boxes. The wild-type *PGK1* allele with the relevant restriction enzyme sites is depicted at the top.

strain, regardless of the position of the amber mutation (48). We determined whether the UAA- and UGA-containing *PGK1* transcripts were also stabilized in a *upf1*Δ strain. Measurements of mRNA decay rates demonstrated that nonsense-containing *PGK1* mRNAs (harboring any of the translation stop codons) were stabilized to the rate of decay of the wild-type *PGK1* mRNA, and the extent of stabilization was position independent (Fig. 1). These results rule out the possibility that insertion of the UAA- or UGA-containing linkers created an instability element, since the half-lives of wild-type mRNAs that are inherently unstable are not altered in a *upf1*Δ strain (33, 48, 50–52). Further, these results demonstrate that the

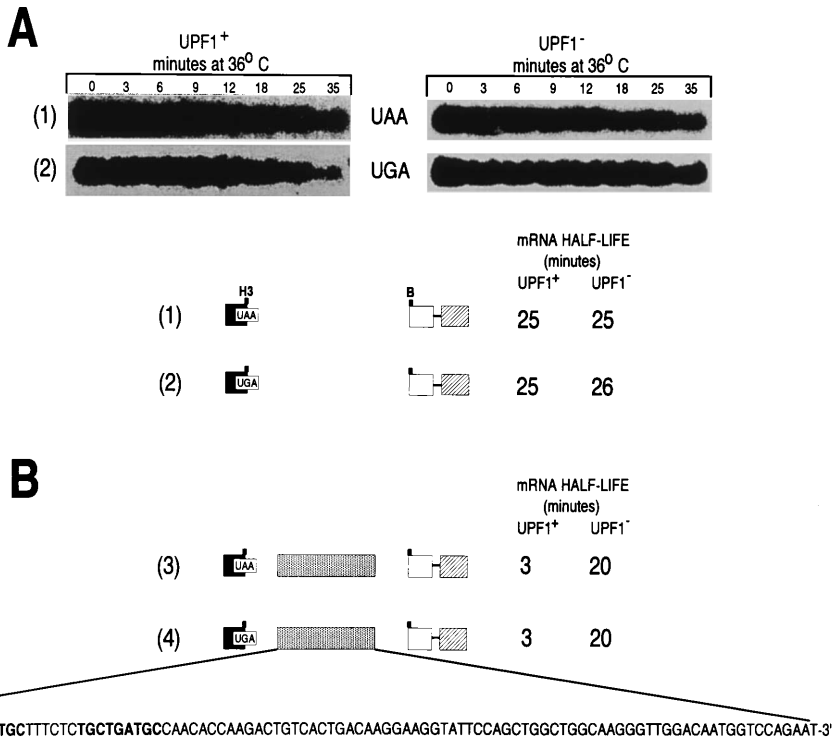



FIG. 2. Both a nonsense mutation and a downstream element are required for nonsense-mediated mRNA decay. (A) *PGK1* nonsense alleles containing a 5′-proximal nonsense mutation [either an UAA or UGA codon; located in the *Hin*cII(3) site (H3); see Fig. 1B] in which between 5.6 and 92.6% of the *PGK1* protein-coding region has been deleted. The *PGK1* coding sequence is represented by the thick bar, and the sequence that was deleted is represented by the absence of the thick bar. mRNA decay rates for these *PGK1* alleles were determined in either the RY262 (*UPF1*$^+$) or SWP154 (*upf1*Δ) strain as described in Materials and Methods. The RNA blots for these experiments are shown above the schematic representations of the *PGK1* alleles. (B) Mini-*PGK1* nonsense alleles in which between 5.6 and 92.6% of the *PGK1* protein-coding region has been replaced with a functional downstream element (*PGK* nt 1138 to 1243). The downstream element is represented by a shaded thick bar and was inserted 3′ of the nonsense mutations in the mini-*PGK1* alleles (the mini-*PGK1* allele is shown in panel A). mRNA decay rates for these *PGK1* alleles were determined in either the RY262 (*UPF1*$^+$) or SWP154 (*upf1*Δ) strain as described in Materials and Methods. Schematic representations of the *PGK1* alleles and a summary of the data are shown.

product of the *UPF1* gene functions independently of the type of nonsense codon.

**Decay of nonsense-containing *PGK1* transcripts requires both a nonsense codon and a downstream element.** Previous studies have demonstrated that premature termination of translation at an amber mutation near the amino terminus of the *PGK1* mRNA was not sufficient to destabilize its transcript. Rather, the accelerated decay of the amber-containing *PGK1* mRNA required specific sequences 3′ of a nonsense mutation. A *PGK1* allele harboring an amber codon located at 5.6% of the *PGK1* protein-coding region in which sequences between 5.6 and 92% of the protein coding region were deleted (called the mini-*PGK1* allele) stabilized this mRNA (48). Insertion of specific sequences 3′ of the amber mutation, which we have termed the downstream element, was capable of promoting nonsense-mediated mRNA decay. To determine whether UAA or UGA nonsense codons can promote accelerated decay of the *PGK1* mRNA independently of downstream sequences, mini-*PGK1* alleles were constructed in which either a UAA or UGA stop codon was located at 5.6% of the *PGK1* protein-coding region and between 5.6 and 92% of the protein-coding region was deleted [see Fig. 2A, constructs 1 and 2, for schematic representations of the deletions; the linkers containing the UAA or UGA mutations were inserted at the H2(3) site in the *PGK1* gene]. Measurements of the mRNA half-lives of UAA- and UGA-containing mini-*PGK1* alleles demonstrated that these transcripts were stabilized by this deletion (Fig. 2A). These results suggest that UAA and UGA *PGK1* nonsense-containing alleles also require sequences downstream of the nonsense codon.

We next determined whether a downstream element identified for amber-containing *PGK1* alleles also functions when positioned 3′ of the other nonsense codons. The downstream element that functioned in an amber-containing mini-*PGK1* gene was inserted distal to either the UAA or UGA codon in the mini-*PGK1* alleles described above (Fig. 2B, constructs 3 and 4). Measurements of the mRNA decay rates demonstrated that insertion of this downstream element 3′ of either a UAA or UGA nonsense mutation in a mini-*PGK1* allele decreased the half-life of this transcript from 30 min to 3 to 5 min (Fig. 2; compare construct 3 with construct 1 and construct 4 with construct 2). Further, the nonsense-containing mRNAs harboring a downstream element were stabilized in a *upf1*Δ strain, demonstrating that these mRNAs were degraded by the nonsense-mediated mRNA decay pathway (Fig. 2). Thus, we conclude that the downstream element identified previously for amber-containing *PGK1* alleles is functional when positioned 3′ to other nonsense codons.

**Nonsense mutations within the carboxyl one-third of the *PGK1* transcript are resistant to nonsense-mediated mRNA decay.** Studies presented here and elsewhere have demonstrated that when between 67 and 76.6% of the *PGK1* protein-coding sequence has been translated, the *PGK1* transcript becomes insensitive to nonsense-mediated mRNA decay (48, 50) (Fig. 1). To determine whether UAA- and UGA-containing *PGK1* alleles also become insensitive to this decay pathway, the

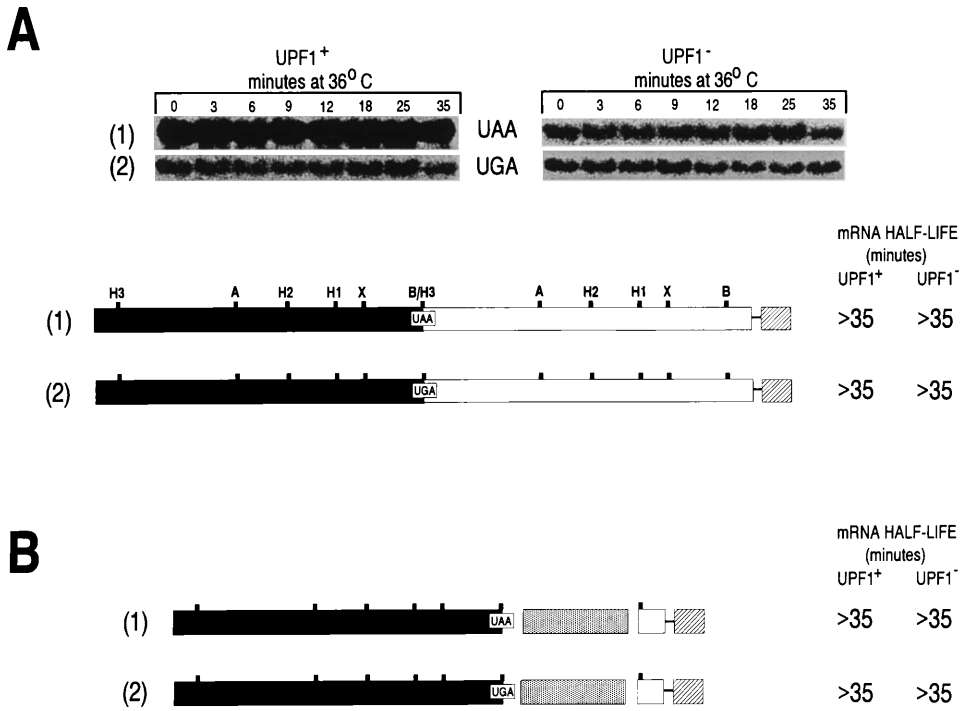



FIG. 3. Downstream elements inserted distal to 3′-proximal nonsense mutations in the *PGK1* gene do not accelerate mRNA decay. (A) *PGK1* alleles in which sequences 3′ of the nonsense mutation (UAA or UGA) inserted at 92% of the *PGK1* protein-coding region were replaced with sequences from the *PGK1* gene that contained from 5.6% of the *PGK1* protein-coding region to the end of the *PGK1* gene. The mRNA decay rates for *PGK1* alleles were determined in strain RY262 (*UPF1*$^+$) or SWP154 (*upf1*Δ). Schematic representations of the *PGK1* alleles as well as a summary of the results from the experiment are shown below the autoradiogram. (B) *PGK1* alleles in which sequences 3′ of the nonsense mutation (UAA or UGA) inserted at 92% of the *PGK1* protein-coding region were replaced with a downstream element (Fig. 2B) from the *PGK1* gene that was identified previously were constructed, and mRNA decay rates for PGK1 alleles were determined in RY262 (*UPF1*$^+$) or SWP154 (*upf1*Δ). Schematic representations of the *PGK1* alleles as well as a summary of the results from the experiment are shown. Shadings are as described in the legends to Fig. 1 and 2. Abbreviations for the restriction endonuclease cleavage sites are as follows: H3, *Hin*cII(3); A, *Asp* 718; H2, *Hin*cII(2); H1, *Hin*cII(1); X, *Xba*I; and B, *Bgl*II (Fig. 1B).

following construct was prepared: sequences 3′ of a carboxyl-terminal UAA or UGA nonsense mutation were replaced with sequences from the *PGK1* gene that contained from 5.6% of the *PGK1* protein-coding region to the end of the *PGK1* gene (Fig. 3A). These additional sequences inserted 3′ of the nonsense mutation contain downstream elements capable of promoting nonsense-mediated mRNA decay (Fig. 1). Measurements of the half-lives of these transcripts demonstrated they were unaffected by insertion of the additional sequences, since their half-lives were similar to that of wild-type *PGK1* mRNA (Fig. 3A). Similarly, if a DNA fragment containing only the downstream element characterized previously was inserted 3′ of the stop codon, the rates of decay of these nonsense-containing mRNAs were unaffected (Fig. 3B). These results indicate that 3′-proximal nonsense mutations, independent of the type of translation termination codon, are resistant to the nonsense-mediated mRNA decay pathway for reasons other than a lack of a specific downstream element.

**The characteristics of this decay pathway are not unique to the *PGK1* mRNA.** The results described here and elsewhere suggest that the ingredients necessary to promote rapid decay of nonsense-containing *PGK1* transcripts are (i) a translation termination codon (independent of the type) within the amino-terminal two-thirds of the protein coding region and (ii) a downstream sequence element. To determine whether the decay of a nonsense-containing *PGK1* mRNA was similar to the decay of mRNAs synthesized from other nonsense alleles, the sequence requirements for the degradation of nonsense-containing *HIS4* mRNAs were characterized.

To investigate the relationship between the *HIS4* mRNA decay rate and the locations of nonsense mutations in the *HIS4* protein-coding region, six *HIS4* alleles containing UGA stop codons were constructed (Fig. 4A). The half-lives of mRNAs synthesized from these alleles were determined in *UPF1*$^+$ and *upf1*Δ strains harboring the *rpb1-1* allele in which the *HIS4* gene was disrupted. In this way, interference by the endogenous *HIS4* mRNA when the turnover rates of the nonsense-containing *HIS4* transcripts were measured was avoided (see Materials and Methods). The results of these experiments indicate that nonsense mutations within the amino-terminal 80% of the protein-coding region accelerate *HIS4* mRNA decay, while a nonsense mutation near the carboxyl terminus has no effect on the mRNA decay rate (Fig. 4A). The steady-state levels of each of the nonsense-containing *HIS4* transcripts directly correspond to the half-lives of the respective nonsense-containing mRNAs (data not shown). The nonsense-containing *HIS4* mRNAs were degraded by the nonsense-mediated mRNA decay pathway, since these RNAs were stabilized to wild-type levels in a *upf1*Δ strain (Fig. 4A).

Similar to the case for *PGK1*, sequences 3′ of a nonsense codon in the *HIS4* mRNA were also required to accelerate its decay. Deletion of between 9.9 and 97% of the protein-coding region 3′ of an amino-terminal nonsense codon stabilized the UAA-containing mini-*HIS4* mRNA greater than threefold (Fig. 4B). To determine whether downstream sequence elements present in the *PGK1* gene can promote decay when inserted 3′ of a nonsense mutation in the *HIS4* gene, a downstream element from the *PGK1* gene was inserted into the

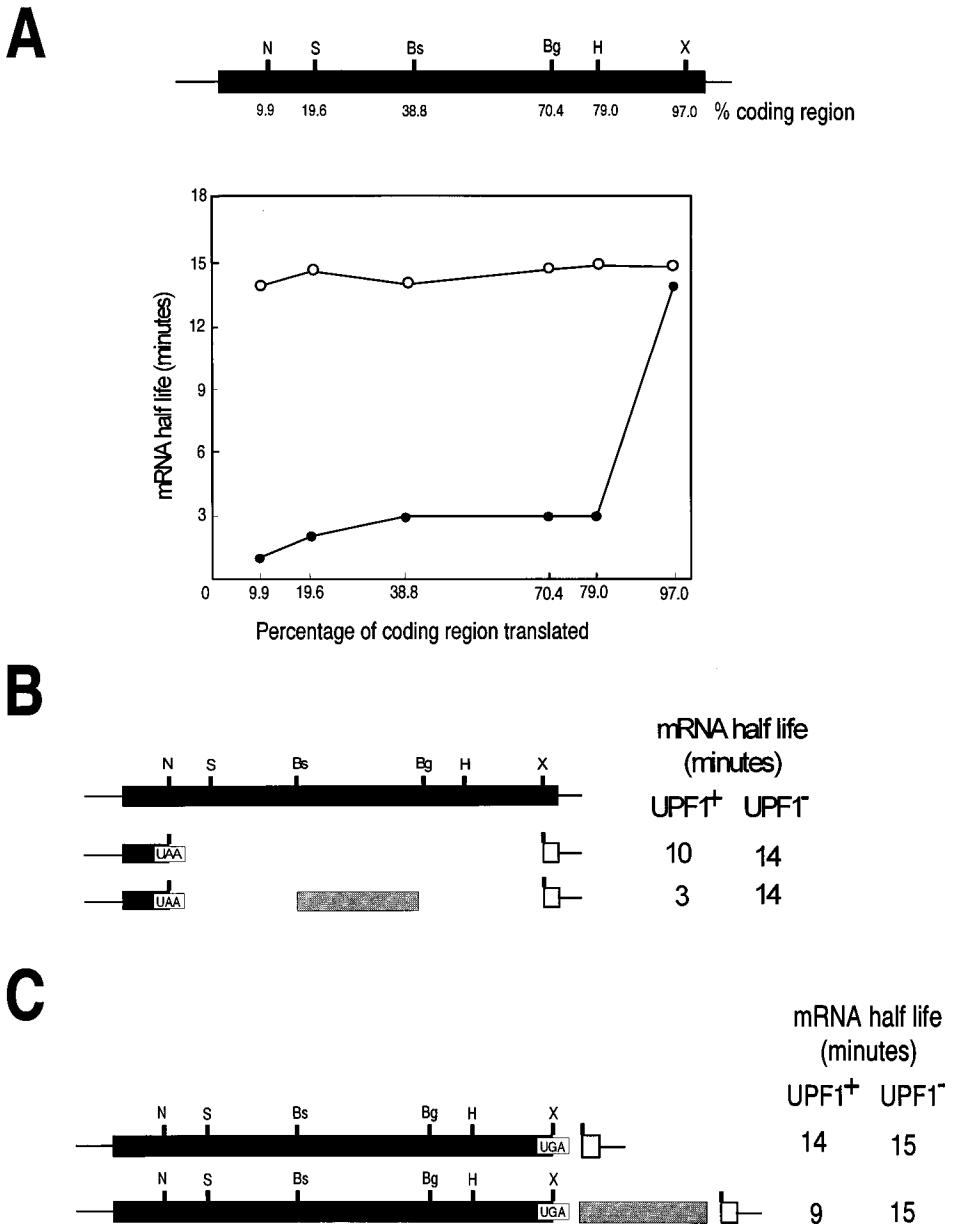



FIG. 4. Effect of UAA and UGA translation stop codons on decay of *HIS4* mRNA. (A) Diagram of the *HIS4* gene, with the protein-coding sequence represented by the thick black bar. A DNA linker containing UGA stop codons in all three reading frames was inserted into the *HIS4* coding region at the restriction sites shown (see Materials and Methods for a description of the construction of the *HIS4* alleles). The location of each nonsense mutation in the *HIS4* transcript is presented as the percentage of the *HIS4* protein-coding region that is translated before the nonsense mutation is encountered. Abbreviations: N, *Nhe*I; S, *Sal*I; Bs, *Bst*XI; Bg, *Bgl*II; H, *Hpa*II; X, *Xba*I. The half-lives of the mRNAs encoded by the *HIS4* nonsense alleles described above were determined in either *UPF1*$^+$ or *upf1*Δ cells. The results of these experiments are summarized in a graphical representation of the mRNA decay rates for the *HIS4* alleles versus the position of the nonsense codon. Filled circles, *UPF1*$^+$; unfilled circles, *upf1*Δ. (B) Two *HIS4* nonsense alleles were analyzed. The first was a *HIS4* nonsense allele containing a 5′-proximal UAA termination codon (located in the *Nhe*I site) and a deletion of the coding region spanning the segment from 9.7 to 97% (called the mini-*HIS4* allele). The second was a mini-*HIS4* allele in which a downstream element from the *PGK1* gene (see Fig. 2B) (48) was inserted 3′ of the termination codon. mRNA decay rates for these constructs were determined and are summarized. The *HIS4* coding sequence is represented by the thick black bar, and the sequence that was deleted is represented by the absence of the thick bar. The downstream element is represented by the shaded box. The *HIS4* untranslated region is represented by a thin line, and the *HIS4* coding region that is not translated is represented by an open box. (C) A *HIS4* allele was constructed in which sequences 3′ of the nonsense mutation inserted at 97% of the *HIS4* protein-coding region were replaced with a downstream element from the *PGK1* gene that was identified previously (see Fig. 2B) (48), and the half-life of this mRNA was determined in *UPF1*$^+$ and *upf1*Δ strains. A schematic representation of the *HIS4* allele as well as a summary of the results from the experiment are shown.

mini-*HIS4* gene (48) (Fig. 4B). This resulted in rapid decay of the UAA-containing mini-*HIS4* mRNA (Fig. 4B), indicating that the downstream element from the *PGK1* gene was functional in the mini-*HIS4* allele.

We wanted to determine whether, like for the *PGK1* gene, the carboxyl end of the *HIS4* protein-coding region renders a 3′-proximal nonsense mutation resistant to nonsense-mediated mRNA decay. To determine whether a *HIS4* mRNA with a 3′-proximal nonsense mutation was stable because of the lack of appropriate sequences 3′ of the stop codon, a functional

downstream element from *PGK1* was inserted 3′ of a carboxyl-terminal nonsense mutation and the decay of its mRNA was determined (Fig. 4C). The half-life of this *HIS4* mRNA was only slightly shorter than that of the wild-type *HIS4* transcript and was only mildly affected by the insertion of the downstream element (Fig. 4C). This result indicates that 3′-proximal nonsense mutations in the *HIS4* mRNA are resistant to the nonsense-mediated mRNA decay pathway for reasons other than a lack of a specific downstream element.

Taken together, these results suggest that nonsense-containing *PGK1* and *HIS4* transcripts decay by similar pathways. Nonsense-mediated mRNA decay for both transcripts requires a nonsense mutation within a specified distance of the amino termini of their protein-coding regions and a downstream sequence element. Furthermore, carboxyl-terminal nonsense mutations in these mRNAs were resistant to nonsense-mediated mRNA decay for reasons other than the lack of an appropriate downstream element.

**Deletion of the *XRN1* gene increases the abundance and stability of nonsense-containing mRNAs.** Recently it has been suggested that, for at least some mRNAs, after poly(A) shortening the initial event for mRNA decay is the removal of the 5′ cap followed by degradation of the body of the mRNA by a 5′→3′ exoribonuclease, Xrn1 (15, 28, 40). Deletion of the *XRN1* gene stabilizes several mRNAs and has been shown to increase the cytoplasmic concentration of uncapped and deadenylated transcripts (28, 40). We reasoned that an *xrn1*$^-$ strain would be useful in delineating the mechanism of nonsense-mediated mRNA decay. If the initial event is an endonucleolytic cleavage event, RNA decay intermediates that would be detectable in an *xrn1*$^-$ strain would not be detectable in a wild-type *XRN1*$^+$ strain. If, however, the initial step for nonsense-mediated mRNA decay is the cleavage of the mRNA near the 5′ termini followed by degradation of the rest of the mRNA, then in an *xrn1*$^-$ strain nearly full-length nonsense-containing transcripts should accumulate. In order to differentiate between these possibilities, the abundances of nonsense-containing mRNAs in *XRN1*$^+$ and *xrn1*$^-$ cells were determined. Wild-type and nonsense-containing *PGK1* alleles were transformed into isogenic *XRN1*$^+$ and *xrn1*$^-$ strains, and the abundances of the mRNAs of various *PGK1* alleles, *CYH2* precursor and mature RNAs, and the U3 RNA were determined by RNA blotting analyses. In the *XRN1*$^+$ strain the abundances of the UAG-, UAA-, and UGA-containing *PGK1* mRNAs were low (Fig. 5A; the nonsense mutation is located in the *Asp* 718 site [see diagram in Fig. 5A]). In an *xrn1*$^-$ strain, however, the abundances of the nonsense-containing *PGK1* mRNAs were approximately equivalent to the abundance of the wild-type *PGK1* mRNA or of the nonsense-containing *PGK1* transcript in a *upf1*$^-$ strain (Fig. 5A). Furthermore, in *xrn1*$^-$ cells the UAA-, UAG-, and UGA-containing mini-*PGK1* transcripts harboring downstream elements, which were low in abundance in wild-type *XRN1*$^+$ cells, were at least equal in abundance to these transcripts in a *upf1*$^-$ strain (Fig. 5B). The abundance of the *CYH2* precursor, whose RNA concentration is sensitive to the *UPF1* status in the cell (22) and is low in wild-type *XRN1*$^+$ cells, is also increased in *xrn1*$^-$ strains to at least the same level as detected in a *upf1*$^-$ strain (Fig. 5C). Only nearly full-length nonsense-containing or intron-containing RNAs were detectable; smaller decay intermediates were not observed (Fig. 5C and data not shown). Consistent with the increased abundances of these nonsense-containing mRNAs, the half-lives of the *CYH2* precursor and the UAA-containing mini-*PGK1* mRNA harboring a downstream element increased in an *xrn1*$^-$ strain compared with their decay rates in an *XRN1*$^+$ strain (Fig. 5D). Changes in the half-lives of the wild-type *PGK1* and *CYH2* transcripts were not detected in an *xrn1*$^-$ strain in this assay (Fig. 5D).

The observation that full-length nonsense-containing and intron-containing RNAs were present in an *xrn1*$^-$ strain indicates that the initial decay event occurs very near the 5′ termini of these mRNAs. These results suggest that degradation of nonsense-containing mRNAs may proceed in a manner analogous to a previously identified mRNA pathway for a class of wild-type mRNAs (28, 40) (see Discussion) in which cleavage of the RNA at the termini is followed by degradation of the body of these transcripts by the 5′→3′ Xrn1 exoribonuclease. The termini of these transcripts were investigated further.

The 5′ termini of the UAA- and UAG-containing mini-*PGK1* alleles harboring the downstream element and the *CYH2* RNAs were analyzed by primer extension analysis. RNAs from the *XRN1*$^+$, *xrn1*$^-$, and *upf1*$^-$ strains harboring the UAA-containing mini-*PGK1* gene were isolated. Radiolabelled DNA oligonucleotides complementary to the mini-*PGK1* mRNA and *CYH2* precursor were annealed and extended by reverse transcription. The results demonstrate that the 5′ termini of the *CYH2* and mini-*PGK1* RNAs isolated from an *xrn1*$^-$ strain harbor an additional band that was either 1 or 2 nt shorter than that for the same RNAs isolated from either wild-type or *upf1*$^-$ strains (Fig. 6; for mini-*PGK1*, compare lanes 1 [wild type] and 3 [*upf1*$^-$] with lane 2 [*xrn1*$^-$] and lanes 4 [wild type] and 6 [*upf1*$^-$] with lane 5 [*xrn1*$^-$] for *CYH2*, compare lanes 7 [wild type] and 9 [*upf1*$^-$] with lane 8 [*xrn1*$^-$]). Since an *xrn1*$^-$ strain contains both the plasmid-borne UAA-containing mini-*PGK1* allele and the endogenous *PGK1* gene as well as the spliced and unspliced forms of the *CYH2* RNA, it is not surprising to observe bands corresponding to the wild-type RNAs and RNAs with cleaved 5′ termini (Fig. 6). These results suggest that an initial cleavage event occurs near the 5′ ends of these RNAs.

The 3′ ends of these transcripts were determined by RNase H mapping and S1 analysis. To determine whether the UAA-containing mini-*PGK1* transcript contains a poly(A) tract at its 3′ terminus, RNAs from wild-type, *upf1-2*, and *xrn1*$^-$ strains were isolated, annealed with oligo(dT)$_{18}$, and treated with RNase H to degrade the poly(A) in the RNA:DNA hybrid. The RNAs were electrophoresed, and RNA blotting analysis was performed with a radiolabelled probe complementary to the mini-*PGK1* RNA. The presence or absence of a poly(A) tract was monitored by comparing the sizes of the RNAs treated with RNase H with those from untreated samples. Adenylated samples treated with RNase H become smaller and electrophorese more rapidly. In *xrn1*$^-$ strains, the mRNA of the UAA-containing mini-*PGK1* harboring the downstream element was unaffected by treatment with oligo(dT) and RNase H, indicating that this RNA was deadenylated (Fig. 7A; compare lanes 3 and 4 for the mini-*PGK1* gene). In contrast, RNAs isolated from either a wild-type or a *upf1-2* strain harbored both adenylated and deadenylated RNAs (Fig. 7A, lanes 1 and 2 and lanes 5 and 6). A similar RNase H analysis of the poly(A) tract of the *CYH2* precursor resulted in the same results obtained for the mini-*PGK1* RNA (data not shown).

The 3′ terminus of the UGA-containing mini-*PGK1* mRNA harboring the downstream element was also analyzed by S1 analysis. RNAs from wild-type, *upf1*$^-$, and *xrn1*$^-$ strains were isolated and annealed with a 3′-radiolabelled DNA fragment from the mini-*PGK1* allele containing the downstream element. This fragment consists of a small part of the protein-coding region and 3′-UTR (see Fig. 7C for a schematic representation of the assay). The samples were treated with S1 nuclease, and the protected fragments were analyzed by electrophoresis followed by autoradiography. As described above,

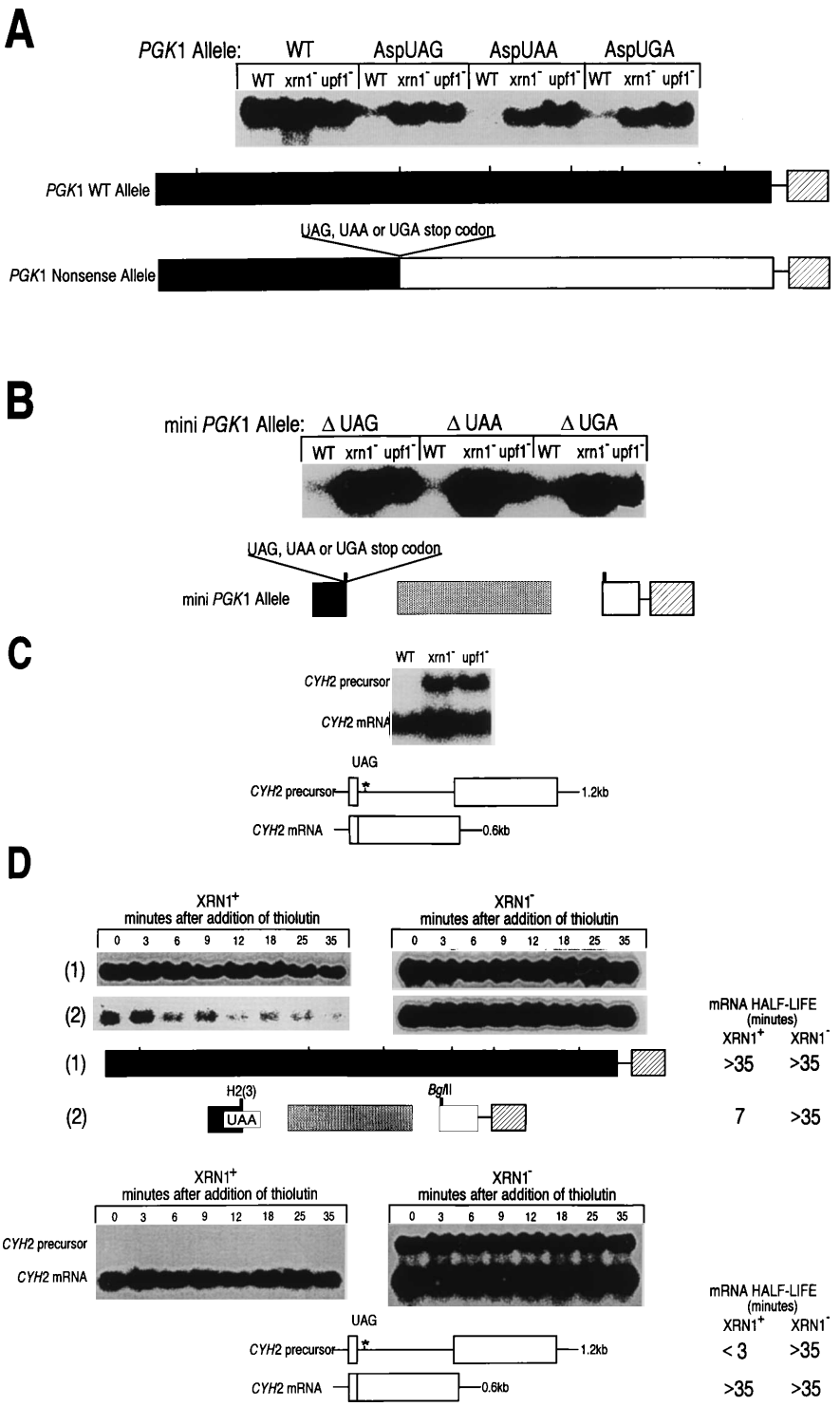



FIG. 5. *PGK1* alleles containing premature nonsense codons are stabilized in *xrn1*$^-$ strains. (A) RNAs were isolated from wild-type, *upf1*$^-$, or *xrn1*$^-$ strains harboring either the wild-type *PGK1* gene or a *PGK1* allele with a nonsense mutation located in the *Asp* 718 site (either UAG, UAA, or UGA nonsense codons; see schematic representations for the various alleles), and the abundances of these RNAs were determined by RNA blotting analysis as described in Materials and Methods. Strains: WT, wild type [BJ5464 (*XRN1*$^+$ *UPF1*$^+$)]; *xrn1*$^-$, BJ5464XRN$^-$ (*xrn1*$^-$ *UPF1*$^+$); *upf1*$^-$, PLY36 (*upf1*$^-$ *XRN1*$^+$). (B) The abundances of the transcripts of mini-*PGK1* alleles harboring downstream elements (as described for Fig. 2) were determined in the strains described for panel A. ΔUAG, mini-*PGK1* allele with a UAG codon; ΔUAA, mini-*PGK1* allele with a UAA codon; ΔUGA, mini-*PGK1* allele with a UGA codon. (C) The abundances of the *CYH2* precursor and mRNA were determined with the strains described for panel A. (D) Decay rates for the wild-type *PGK1* mRNA, the UAA-containing mini-*PGK1* mRNA harboring a downstream element, the *CYH2* precursor, and the *CYH2* mRNA were determined with wild-type (BJ5464; *XRN1*$^+$) and *xrn1*$^-$ (BJ5464XRN$^-$) strains following the addition of the transcriptional inhibitor thiolutin as described in Materials and Methods. The experiments depicted here were carried out at 22°C. Schematic representations of the wild-type *PGK1* allele (construct 1) and the UAA-containing mini-*PGK1* allele with a downstream element (construct 2) as well as the *CYH2* precursor and mRNA are shown below the autoradiograms. Shadings are as described in the legend to Fig. 2.

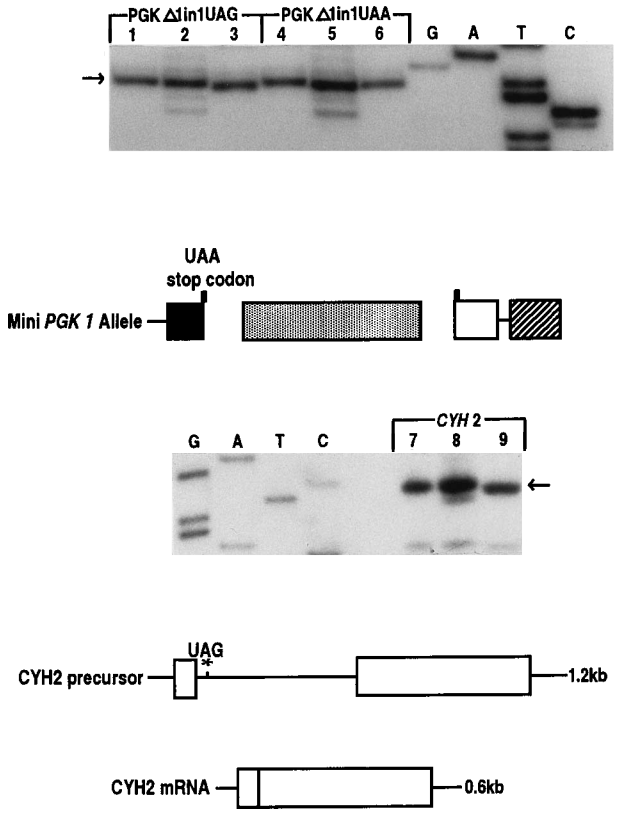



FIG. 6. Mapping of the 5′ termini of the *PGK1* transcripts with premature nonsense codons and the *CYH2* precursor in *xrn1*$^-$ strains. An oligonucleotide complementary to a region 61 nt from the 5′ end of the wild-type *PGK1* mRNA (oligonucleotide 7; see "Oligodeoxyribonucleotides" in Materials and Methods) or an oligonucleotide complementary to a region 50 nt from the 5′ end of the *CYH2* RNA in exon 1 (oligonucleotide 8) was used for primer extension analyses of RNAs from strains BJ5464 (*UPF1*$^+$), BJ5464XRN$^-$ (*xrn1*$^-$), and PLY36 (*upf1-2* [*upf1*$^-$]) containing either a UAG- or UAA-containing mini-*PGK1* allele harboring a downstream element (see the schematic representations of the *PGK1* alleles and *CYH2* RNAs below the autoradiograms). Cells were grown to mid-log phase, RNAs were isolated, and primer extension analyses were performed as described in Materials and Methods. A sequencing ladder of the wild-type *PGK1* allele (for the results of the mini-*PGK1* allele) and a standard sequencing ladder (sequence of M13mp18 with the −40 forward sequencing primer, for *CYH2* results) were run alongside the primer extension products. The major 5′ end products are indicated with arrows. Lanes 1 to 6 show the results of primer extension experiments using the *PGK1* primer; lanes 7 to 9 show the results of primer extension experiments using the *CYH2* primer. RNAs from lanes 1, 4, and 7 were isolated from wild-type (*XRN1*$^+$ *UPF1*$^+$) cells, RNAs from lanes 2, 5, and 8 were isolated from *xrn1*$^-$ cells, and RNAs from lanes 3, 6, and 9 were isolated from *upf1*$^-$ cells.

a tag was inserted into the 3′-UTR of the mini-*PGK1* allele and allowed for differentiation between the 3′ terminus of the mini-*PGK1* transcript and the 3′ terminus of the endogenous wild-type *PGK1* mRNA (Fig. 7C).

In wild-type, *xrn*$^-$, and *upf1*$^-$ strains, the 3′ termini of the wild-type and mini-*PGK1* mRNAs containing a downstream element can be detected (Fig. 7B, lanes 2 to 4, bands b and c). A large quantity of the protected fragment for the endogenous *PGK1* transcript is observed compared with that the mini-*PGK1* 3′ fragment. The DNA tag in the 3′-UTR of the mini-*PGK1* allele is AU rich, allowing for breathing of the hybrid and resulting in cleavage by the S1 nuclease, which accounts for the underrepresentation of the mini-*PGK1* mRNA (Fig. 7B, lanes 2 to 4, band c; see Fig. 7C for a diagram depicting results of the S1 analysis). These results demonstrate that the 3′ ends in wild-type and *xrn1*Δ strains are the same, indicating that the mRNA does not lose sequences upstream of the poly(A) tract. Interestingly, the mini-*PGK1* RNAs isolated from *xrn1*Δ strains also have a low concentration of shorter 3′ termini that were absent from wild-type and *upf1-2* strains (Fig. 7B, lane 3 between bands b and c). This result suggests that there is another, previously unidentified, minor decay pathway that was uncovered by monitoring the decay of nonsense-containing mRNAs in an *xrn1*$^-$ strain. Taken together, these results indicate that nonsense-containing transcripts lacking the 5′ termini and 3′ poly(A) tract were detected.

## DISCUSSION

The results presented here focus on understanding the mechanism of nonsense-mediated mRNA decay. Two aspects of this pathway were investigated. One was the characterization of the *cis*-acting sequences required for nonsense-mediated mRNA decay and determination of the generality of these results by extending these observations to an mRNA from another gene. The second aspect was the identification of the location of the cleavage sites in this pathway.

***cis*-acting sequences involved in nonsense-mediated mRNA decay.** Using the *PGK1* and *HIS4* transcripts from the yeast *S. cerevisiae* as model substrates to characterize the *cis*-acting sequences involved in nonsense-mediated mRNA decay, we have demonstrated in the results presented here that (i) a subset of nonsense mutations have a significant destabilizing effect on mRNAs, (ii) the type of nonsense codon does not modulate the activity of this decay pathway (Fig. 1), (iii) in addition to a nonsense codon, downstream sequences are required for mRNA destabilization (Fig. 2 and 4), and (iv) a nonsense codon and a downstream sequence are not sufficient for nonsense-mediated mRNA decay because the pathway is regulated by an independent, stabilizing sequence element (Fig. 3 and 4). Each point will be addressed below.

**A nonsense mutation within the first two-thirds of the *PGK1* protein-coding region and a downstream element are necessary for nonsense-mediated mRNA decay.** Our initial studies using the *PGK1* gene demonstrated that amber mutations within the amino-terminal two-thirds of the protein-coding region destabilized the *PGK1* transcript, while amber mutations inserted in the last quarter of the *PGK1* coding region had no effect on mRNA decay (48). However, since the mechanism of nonsense-mediated mRNA decay is not known, it is conceivable that the different nonsense codons, perhaps as a consequence of *trans*-acting factors involved in translational termination, have differential effects on this decay pathway. We sought to determine whether the type of nonsense codon inserted into the *PGK1* gene differentially affected the decay of this mRNA. The results demonstrate that the mRNA decay rates for UAA- and UGA-containing *PGK1* alleles were similar to those for amber-containing *PGK1* transcripts (Fig. 1). UAA- and UGA-containing *PGK1* alleles that terminate translation within the amino-terminal 67% of the protein-coding region decrease the *PGK1* mRNA half-life, while nonsense mutations inserted in the last quarter of the *PGK1* coding region have no effect on mRNA decay (Fig. 1).

The observation that a *PGK1* amber allele in which most of the *PGK1* protein-coding region downstream of an early nonsense mutation was deleted (called the mini-*PGK1* gene) resulted in stabilization of its mRNA suggested that sequences downstream of the stop codon play an important role in the activation of the nonsense-mediated mRNA decay pathway. Insertion of small regions of the deleted DNA back into the amber-containing mini-*PGK1* allele downstream of the non-

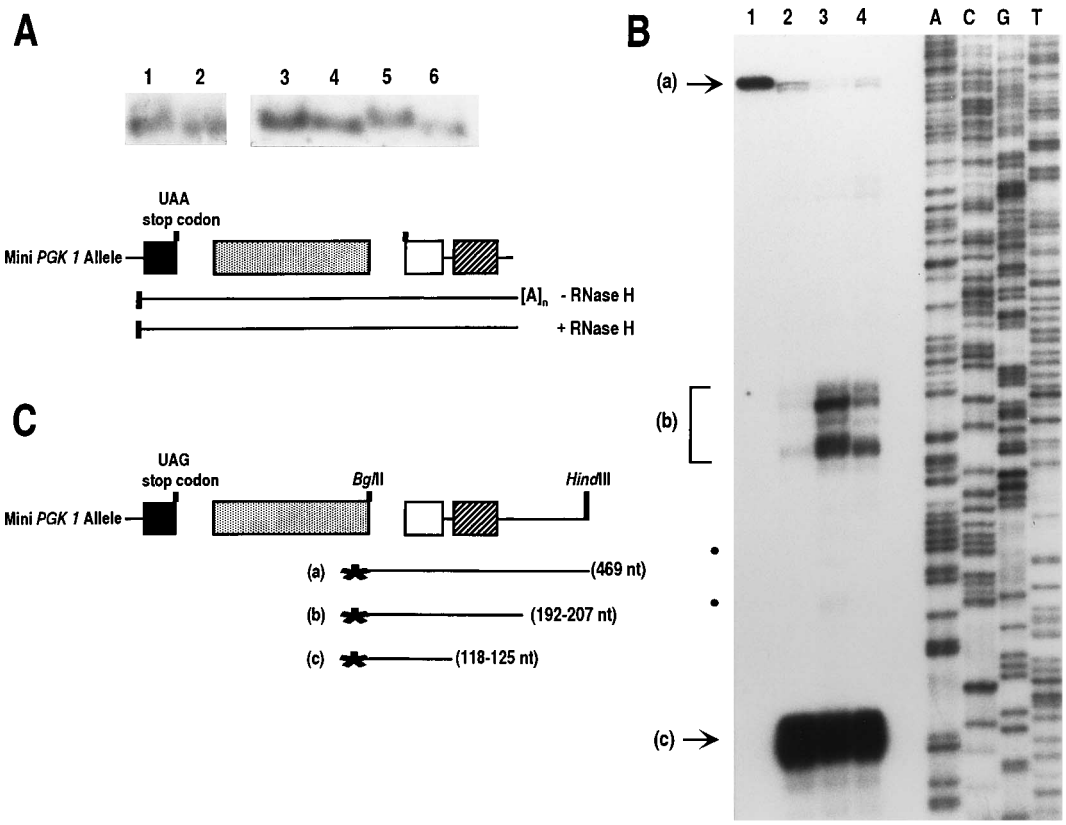



FIG. 7. Analysis of the 3′ terminus of the nonsense-containing mini-*PGK1* allele. (A) The presence or absence of the poly(A) tract was determined by RNase H mapping. RNAs from strains BJ5464 (*UPF1*$^+$), BJ5464XRN$^-$ (*xrn1*$^-$), and PLY36 (*upf1-2*) harboring the UAA-containing mini-*PGK1* allele containing a downstream element (see Fig. 2 for schematic representations of the alleles) were isolated, and RNase H mapping was performed as described in Materials and Methods. Lanes 2, 4, and 6 contain RNA treated with oligo(dT) and RNase H; lanes 1, 3, and 5 contain untreated RNA. (B) S1 analysis of the 3′ termini of the *PGK1* mRNAs. The 3′-end-labelled DNA fragment used as a probe (lane 1) was incubated with total RNA obtained from strain BJ5464 (wild type; lane 2), BJ5464XRN$^-$ (*xrn1*$^-$; lane 3), or PLY36 (*upf1*$^-$; lane 4). The lengths of the fragments protected from S1 nuclease were analyzed by denaturing acrylamide gel electrophoresis and autoradiography. The migrations of the probe (a) and the major protected fragments (b and c) are indicated on the left of the gel and are schematically represented in panel C. Note that the fragment labelled c corresponds to the S1-protected fragment obtained after hybridization of the probe with the endogenous wild-type *PGK* mRNA which lacks the tag sequence. A sequencing reaction (lanes A, C, G, and T) was run in parallel to determine the sizes of the fragments. (C) Schematic representation of the 3′ region of the UGA-containing mini-*PGK1* gene harboring a downstream element, its mRNA, the DNA probe used for S1 mapping, and the major protected fragments. The location of the probe used to identify *PGK1* mRNA and the lengths of the major S1-protected fragments are indicated. The hatched square represents the presence of a tag sequence in the 3′-UTR of the *PGK* gene. a, full-length probe; b, the protected fragment from the *Bam*HI site to the 3′ end of the from the mini-*PGK1* RNA; c, a protected fragment from the *Bam*HI site to the insertion of the DNA tag (the band that arises from the endogenous, wild-type *PGK1* transcript). The filled circles on the left of the autoradiogram in panel B indicate other minor 3′ protected fragments that are more prominent in an *xrn1*$^-$ strain than in either *upf1*$^-$ or wild-type strains.

sense codon promoted rapid decay of its mRNA (48). We have extended these observations by demonstrating that transcripts from the UAA- and UGA-containing mini-*PGK1* alleles are stabilized in the absence of a downstream element but are destabilized if a downstream element identified previously was inserted distal to the nonsense codon (Fig. 2). Taken together, these results indicate that accelerated nonsense-mediated mRNA decay requires a premature termination event and an appropriate sequence 3′ of the nonsense codon.

**The *PGK1* transcript harboring carboxyl-terminal nonsense mutations, independent of the type, is resistant to nonsense-mediated mRNA decay.** As described above, our results indicate that there are sequences within the coding region of the *PGK1* mRNA that can formally be considered stabilizer elements (Fig. 1) (52). When between 67 and 76.6% of the *PGK1* protein-coding sequence has been translated, the *PGK1* transcript becomes insensitive to nonsense-mediated decay. Sequences in the *PGK1* gene have been identified that have partial stabilizer activity when inserted upstream of nonsense codons which normally accelerate mRNA degradation. The results presented here demonstrate that the resistance of 3′-proximal nonsense mutations to accelerated mRNA decay is due to stabilizer sequences in the *PGK1* mRNA but does not depend on the type of nonsense codon present (Fig. 3).

***trans*-acting factors involved in nonsense-mediated mRNA decay.** The results reported here demonstrate that, independent of the type of nonsense codon, these *PGK1* mRNAs were stabilized to the same degree in a *upf1*Δ strain (Fig. 1). In *upf2*$^-$ and *upf3*$^-$ strains, the abundances of the nonsense-containing *PGK1* mRNAs were also increased to levels approaching that of the wild-type *PGK1* transcript (21a). Taken together, these results indicate that these *trans*-acting factors function in the nonsense-mediated mRNA decay pathway and do not discriminate between the types of nonsense codons.

**Nonsense-containing mRNAs are degraded through a common pathway.** To determine whether the *cis*-acting sequences described for the *PGK1* transcript were a general characteristic of this decay pathway, the sequence requirements for the decay of nonsense-containing *HIS4* mRNAs were investigated. Nonsense mutations (UGA codon) within the amino-terminal 80%

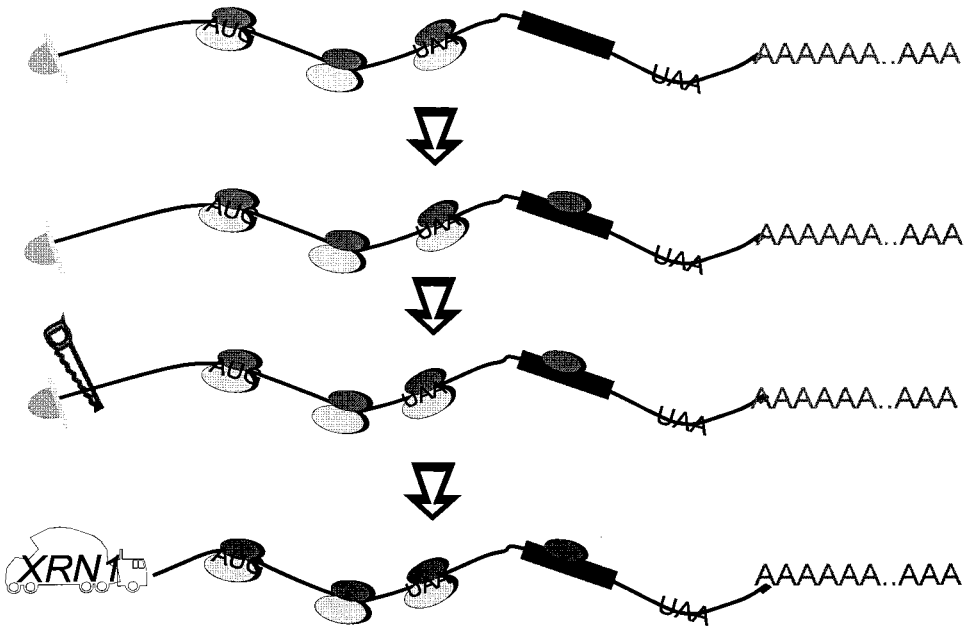



FIG. 8. A model for the decay of nonsense-containing mRNAs. The following model for the mechanism of nonsense-mediated mRNA decay is proposed. In the yeast *S. cerevisiae* the decay of nonsense-containing transcripts is a cytoplasmic event, and after translation termination at the nonsense codon, a small fraction of 40S ribosomal subunits scan downstream of the nonsense codon and encounter a sequence (called the downstream element [solid box]) with which it interacts. The interaction with the sequence downstream of the nonsense codon forms an altered RNP structure that renders the mRNA susceptible to cleavage very near the 5′ and 3′ termini of the RNA. The nonsense-containing RNA species that is cleaved near its end is then degraded by the 5′→3′ exoribonuclease.

of the *HIS4* protein-coding region destabilize the transcript (Fig. 4A). Further, analogous to the case with the *PGK1* mRNA, accelerated decay of the *HIS4* mRNA requires both a nonsense mutation and a downstream element (Fig. 4B). A nonsense-containing mini-*HIS4* mRNA lacking a downstream element is stable but can be destabilized by insertion of a downstream element identified in the *PGK1* gene (Fig. 4B). This also demonstrates that downstream elements from one gene can function when inserted within a second gene (Fig. 4B). As described below and consistent with this view, a downstream element from the *HIS4* gene has been identified and can function when inserted 3′ of a nonsense mutation in a mini-*PGK1* gene.

After most of the *HIS4* protein-coding region has been translated, the *HIS4* transcript becomes insensitive to nonsense-mediated decay (Fig. 4C). The resistance of 3′-proximal nonsense mutations to nonsense-mediated mRNA decay cannot be explained by the lack of an active downstream element or the need for a ribosome-free zone within the coding region of the *HIS4* mRNA. Rather, these results indicate that there must be sequences in the *HIS4* mRNA, similar to those found in the *PGK1* mRNA, that neutralize the destabilizing effects of any downstream nonsense mutations. This is the second example of an mRNA becoming insensitive to the nonsense-mediated mRNA decay pathway after a significant portion of the protein-coding region has been translated.

**Mutations in the *XRN1* gene stabilize the decay intermediates of nonsense-containing transcripts.** The *XRN1* gene encodes a 160-kDa 5′→3′ exoribonuclease that degrades RNAs with 5′-monophosphates but does not digest capped RNAs (32, 60, 61). Cells that are deleted of *XRN1* are viable but grow slowly, have altered rRNA processing (32, 60), and stabilize the decay of several mRNAs examined (28, 40). Furthermore, *xrn1*Δ strains contain a greater concentration of uncapped and deadenylated mRNAs, indicating that a role of the Xrn1 exoribonuclease is to rid the cells of these substrates (28, 40).

As described above, a model for the decay of at least one class of wild-type mRNAs has been proposed for the yeast *S. cerevisiae*; it consists of shortening of the poly(A) tract at a particular rate to an oligo(A) form. Subsequently, the mRNA is cleaved at its 5′ end, removing its 5′ cap structure. The uncapped and deadenylated RNA is then degraded by the 5′→3′ Xrn1 exoribonuclease (28, 40). In the absence of the Xrn1 exoribonuclease, the uncapped deadenylated transcript will be stabilized. In order to determine whether nonsense-mediated mRNA decay flows through a pathway similar to that described above, we monitored the abundance and degradation of nonsense- and intron-containing mRNAs in an *xrn1*$^-$ strain. The results indicated that the abundances of nearly full-length nonsense-containing RNAs were increased in an *xrn1*Δ strain compared with those in an *XRN1*$^+$ strain (Fig. 5), and the abundances were at least equivalent to those obtained with a *upf1*$^-$ strain. Closer inspection of the structures of the *CYH2* precursor and nonsense-containing mini-*PGK1* RNAs containing a downstream element indicated that these transcripts lacked 1 or 2 nt from the 5′ terminus and a poly(A) tract at their 3′ end (Fig. 6 and 7). These results suggest that the decay event is not an endonucleolytic cleavage located near the nonsense codon but occurs near the termini of the mRNA.

Since these experiments were performed with steady-state levels of the RNAs, it is not possible to determine whether the deadenylation of the nonsense-containing mRNA occurs prior to the 5′ cleavage event. Previous results, however, have suggested that, in a transcriptional pulse-chase experiment (59), a nonsense-containing β-globin transcript is degraded prior to loss of its poly(A) tail (59). Similar results were obtained by using a transcriptional pulse-chase protocol to monitor the decay of a nonsense-containing *PGK1* transcript in an *XRN1*$^+$ strain of the yeast *S. cerevisiae* (41). Recapitulation of this experiment with an *xrn1*$^-$ strain, however, demonstrated that the nonsense-containing *PGK1* mRNA is stable (lacking 1 or 2 nt at the 5′ end) but becomes deadenylated (41). Collectively,

these results as well as the results presented here suggest that nonsense-containing mRNAs are normally cleaved at their 5′ ends and degraded prior to deadenylation. In an *xrn1*$^-$ strain, however, the uncapped transcript can be subsequently deadenylated. Furthermore, these results suggest that nonsense-mediated mRNA decay is probably not a decay pathway independent from that which degrades wild-type transcripts. Rather, nonsense-containing mRNAs may flow through the same decay pathway.

**A model for the mechanism of nonsense-mediated mRNA decay in *S. cerevisiae*.** On the basis of the results described here and elsewhere (48), we present the following model for nonsense-mediated mRNA decay (Fig. 8). A nonsense mutation causes premature translational termination resulting in an altered ribonucleoprotein (RNP) structure. We propose that a fraction of the terminating ribosomes or ribosomal subunits that are associated with factors involved in nonsense-mediated mRNA decay will then scan the mRNA 3′ of the nonsense codon. The ribosome or ribosomal subunit encounters a downstream element with which it interacts. This interaction promotes an altered RNP 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′ Xrn1 exoribonuclease (Fig. 8).

Many questions concerning the mechanism of nonsense-mediated mRNA decay need to be addressed. Future experiments will entail defining the function of the downstream element. In addition, we hypothesize that premature termination of translation results in an inappropriate RNP structure, rendering the RNA accessible to cleavage at the 5′ end of the RNA. Future experiments will include the analysis of the RNP structure of the nonsense-containing transcript.

## ACKNOWLEDGMENTS

This work was supported by a grant from the National Institutes of Health (GM48631-01) and an American Cancer Society Junior Investigator Award given to S.W.P. M.J.R-E. was supported from a grant from the Spanish Ministerio de Educación y Ciencia.

We thank Peter Leeds and Arlen Johnson for a number of yeast strains used in these studies. We thank Ying Cui, Kevin Czaplinski, Allan Jacobson, Beate Schwer, Ellen Welch, and Shuang Zhang for critical reading of the manuscript.

## REFERENCES

1. **Aharon, T., and R. J. Schneider.** 1993. Selective destabilization of short-lived mRNAs with the granulocyte-macrophage colony-stimulating factor AU-rich 3′ noncoding region is mediated by a cotranslational mechanism. Mol. Cell. Biol. **13:**1971–1980.
2. **Atwater, J. A., R. Wisdom, and I. M. Verma.** 1990. Regulated mRNA stability. Annu. Rev. Genet. **24:**519–541.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1992. Current protocols in molecular biology, vol. 2, p. 13.4.7. John Wiley and Sons, New York.
4. **Barker, G. F., and K. Beemon.** 1991. Nonsense codons within the Rous sarcoma virus *gag* gene decrease the stability of unspliced viral RNA. Mol. Cell. Biol. **11:**2760–2768.
5. **Baserga, S. J., and E. J. Benz, Jr.** 1992. β-Globin nonsense mutation: deficient accumulation of mRNA occurs despite normal cytoplasmic stability. Proc. Natl. Acad. Sci. USA **89:**2935–2939.
6. **Baumann, B., M. J. Potash, and G. Kohler.** 1985. Consequences of frameshift mutations at the immunoglobulin heavy chain locus of the mouse. EMBO J. **4:**351–359.
7. **Bernstein, P., D. Herrick, R. D. Prokipcak, and J. Ross.** 1992. Control of c-myc mRNA half-life *in vitro* by a protein capable of binding to a coding region stability determinant. Genes Dev. **6:**642–654.
8. **Caponigro, G., D. Muhlrad, and R. Parker.** 1993. A small segment of the MATα1 transcript promotes mRNA decay in *Saccharomyces cerevisiae*: a stimulatory role for rare codons. Mol. Cell. Biol. **13:**5141–5148.
9. **Cheng, J., M. Fogel-Petrovic, and L. E. Maquat.** 1990. Translation to near the distal end of the penultimate exon is required for normal levels of spliced triosephosphate isomerase mRNA. Mol. Cell. Biol. **10:**5215–5225.
10. **Cheng, J., and L. E. Maquat.** 1993. Nonsense codons can reduce the abundance of nuclear mRNA without affecting the abundance of pre-mRNA or the half-life of cytoplasmic mRNA. Mol. Cell. Biol. **13:**1892–1902.
11. **Cleveland, D. W.** 1988. Autoregulated instability of tubulin mRNAs: a novel eukaryotic regulatory mechanism. Trends Biochem. Sci. **13:**339–343.
12. **Cui, Y., K. W. Hagan, S. Zhang, and S. W. Peltz.** Identification and characterization of genes that are required for the accelerated degradation of mRNAs containing a premature translational termination codon. Genes Dev., in press.
13. **Culbertson, M. R., K. M. Underbrink, and G. R. Fink.** 1980. Frameshift suppression in *Saccharomyces cerevisiae*. II. Genetic properties of group II suppressors. Genetics **95:**833–853.
14. **Daar, I. O., and L. E. Maquat.** 1988. Premature translation termination mediates triosephosphate isomerase mRNA degradation. Mol. Cell. Biol. **8:**802–813.
15. **Decker, C. J., and R. Parker.** 1993. A turnover pathway for both stable and unstable mRNAs in yeast: evidence for a requirement for deadenylation. Genes Dev. **7:**1632–1643.
16. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13. (Addendum, **137:**266–267, 1984.)
17. **Feng, H., and A. Jacobson.** Unpublished results.
18. **Gaspar, M.-L., T. Meo, P. Bourgarel, J.-L. Guenet, and M. Tosi.** 1991. A single base deletion in the *Tfm* androgen receptor gene creates a short-lived messenger RNA that directs internal translation initiation. Proc. Natl. Acad. Sci. USA **88:**8606–8610.
19. **Gay, D. A., T. J. Yen, J. T. Y. Lau, and D. W. Cleveland.** 1987. Sequences that confer β-tubulin autoregulation through modulated mRNA stability reside within exon 1 of a β-tubulin mRNA. Cell **50:**671–679.
20. **Gozalbo, D., and S. Hohmann.** 1990. Nonsense suppressors partially revert the decrease of the mRNA level of a nonsense mutant allele in yeast. Curr. Genet. **17:**77–79.
21. **Graves, R. A., N. B. Pandey, N. Chodchoy, and W. F. Marzluff.** 1987. Translation is required for regulation of histone mRNA degradation. Cell **48:**615–626.

21a. **Hagan, K., and S. W. Peltz.** Unpublished results.

22. **He, F., S. W. Peltz, J. L. Donahue, M. Rosbash, and A. Jacobson.** 1993. Stabilization and ribosome association of unspliced pre-mRNAs in a yeast *upf1*$^-$ mutant. Proc. Natl. Acad. Sci. USA **90:**7034–7038.
23. **Heaton, B., C. Decker, D. Muhlrad, J. Donahue, A. Jacobson, and R. Parker.** 1992. Analysis of chimeric mRNAs identifies two regions within the *STE3* mRNA which promote rapid mRNA decay. Nucleic Acids Res. **20:**5365–5373.
24. **Hentze, M. W.** 1991. Determinants and regulation of cytoplasmic mRNA stability in eukaryotic cells. Biochim. Biophys. Acta **1090:**281–292.
25. **Herrick, D., and A. Jacobson.** 1992. A segment of the coding region is necessary but not sufficient for rapid decay of the *HIS3* mRNA in yeast. Gene **114:**35–41.
26. **Herrick, D., R. Parker, and A. Jacobson.** 1990. Identification and comparison of stable and unstable mRNAs in the yeast *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**2269–2284.
27. **Hitzeman, R. A., F. E. Hagie, J. S. Hayflick, C. Y. Chen, P. H. Seeburg, and R. Derynck.** 1982. The primary structure of the yeast *Saccharomyces cerevisiae* gene for 3-phosphoglycerate kinase. Nucleic Acids Res. **10:**7791–7808.
28. **Hsu, C. L., and A. Stevens.** 1993. Yeast cells lacking 5′→3′ exoribonuclease 1 contain mRNA species that are poly(A) deficient and partially lack the 5′ cap structure. Mol. Cell. Biol. **13:**4826–4835.
29. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
30. **Jimenez, A., D. J. Tipper, and J. Davies.** 1973. Mode of action of thiolutin, an inhibitor of macromolecular synthesis in *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **3:**729–738.
31. **Laird-Offringa, I. A.** 1992. What determines the instability of c-myc protooncogene mRNA? Bioessays. **14:**119–124.
32. **Larimer, F. W., and A. Stevens.** 1990. Disruption of the gene *XRN1*, coding for a 5′-3′ exoribonuclease, restricts yeast cell growth. Gene **95:**85–90.
33. **Leeds, P., S. W. Peltz, A. Jacobson, and M. R. Culbertson.** 1991. The product of the yeast *UPF1* gene is required for rapid turnover of mRNAs containing a premature translational termination codon. Genes Dev. **5:**2303–2314.
34. **Leeds, P., J. M. Wood, B.-S. Lee, and M. R. Culbertson.** 1992. Gene products that promote mRNA turnover in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**2165–2177.
35. **Lim, S.-K., C. D. Sigmund, K. W. Gross, and L. E. Maquat.** 1992. Nonsense codons in human β-globin mRNA result in the production of mRNA degradation products. Mol. Cell. Biol. **12:**1149–1161.
36. **Losson, R., and F. Lacroute.** 1979. Interference of nonsense mutations with eukaryotic messenger RNA stability. Proc. Natl. Acad. Sci. USA **76:**5134–5137.
37. **Maquat, L. E., A. J. Kinniburgh, E. A. Rachmilewitz, and J. Ross.** 1981. Unstable β-globin mRNA in mRNA-deficient β-thalassemia. Cell **27:**543–553.

38. **Mercer, J. F. B., and S. A. Wake.** 1985. An analysis of the role of metallothionein poly(A) shortening using RNA blot hybridization. Nucleic Acids Res. **13:**7929–7943.
39. **Morse, D. E., and C. Yanofsky.** 1969. Polarity and the degradation of mRNA. Nature (London) **224:**329–331.
40. **Muhlrad, D., C. J. Decker, and R. Parker.** 1994. Deadenylation of the unstable mRNA encoded by the yeast MFA2 gene leads to decapping followed by 5′→3′ digestion of the transcript. Genes Dev. **8:**855–866.
41. **Muhlrad, D., and R. Parker.** 1994. Premature translational termination triggers mRNA decapping. Nature (London) **370:**578–581.
42. **Myslinski, E., V. Segault, and C. Branlant.** 1990. An intron in the genes for U3 small nucleolar RRAs of the yeast *Saccharomyces cerevisiae*. Science **247:**1213–1216.
43. **Nilsson, G., J. G. Belasco, S. N. Cohen, and A. von Gabain.** 1987. Effect of premature termination of translation on mRNA stability depends on the site of ribosome release. Proc. Natl. Acad. Sci. USA **84:**4890–4894.
44. **Nonet, M., C. Scafe, J. Sexton, and R. Young.** 1987. Eucaryotic RNA polymerase conditional mutant that rapidly ceases mRNA synthesis. Mol. Cell. Biol. **7:**1602–1611.
45. **Parker, R., and A. Jacobson.** 1990. Translation and a forty-two nucleotide segment within the coding region of the mRNA encoded by the *MATα1* gene are involved in promoting rapid mRNA decay in yeast. Proc. Natl. Acad. Sci. USA **87:**2780–2784.
46. **Pelsy, F., and F. Lacroute.** 1984. Effect of ochre nonsense mutations on yeast *URA1* stability. Curr. Genet. **8:**277–282.
47. **Peltz, S. W., G. Brewer, P. Bernstein, and J. Ross.** 1991. Regulation of mRNA turnover in eukaryotic cells. Crit. Rev. Eukaryot. Gene Expression **1:**99–126.
48. **Peltz, S. W., A. H. Brown, and A. Jacobson.** 1993. mRNA destabilization triggered by premature translational termination depends on at least three *cis*-acting sequence elements and one *trans*-acting factor. Genes Dev. **7:**1737–1754.
49. **Peltz, S. W., J. L. Donahue, and A. Jacobson.** 1992. A mutation in tRNA nucleotidyltransferase stabilizes mRNAs in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**5778–5784.
50. **Peltz, S. W., H. Feng, E. Welch, and A. Jacobson.** 1994. Nonsense-mediated mRNA decay in yeast. Prog. Nucleic Acid Res. Mol. Biol. **47:**271–298.
51. **Peltz, S. W., and A. Jacobson.** 1993. mRNA Turnover in *Saccharomyces cerevisiae*, p. 291–328. *In* G. Brawerman and J. Belasco (ed.), Control of mRNA stability. Academic Press, New York.
52. **Peltz, S. W., C. Trotta, H. Feng, A. Brown, J. Donahue, E. Welch, and A. Jacobson.** 1993. Identification of the cis-acting sequences and trans-acting factors involved in nonsense-mediated mRNA decay, vol. H71, p. 1–10. *In* M. Tuite, J. McCarthy, A. Brown, and F. Sherman (ed.), Protein synthesis and targetting in yeast. Springer-Verlag, Berlin.
53. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
54. **Ross, J.** 1988. RNA turnover in eukaryotic cells. Mol. Biol. Med. **5:**1–14.
55. **Sachs, A. B.** 1993. Messenger RNA degradation in eukaryotes. Cell **74:**413–421.
56. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
57. **Schiavi, S. C., C. L. Wellington, A.-B. Shyu, C.-Y. A. Chen, M. E. Greenberg, and J. G. Belasco.** 1994. Multiple elements in the c-fos protein-coding region facilitate mRNA deadenylation and decay by a mechanism coupled to translation. J. Biol. Chem. **269:**3441–3448.
58. **Schiestl, R. H., and R. D. Gietz.** 1989. High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Curr. Genet. **16:**339–346.
59. **Shyu, A.-B., J. G. Belasco, and M. E. Greenberg.** 1991. Two distinct destabilizing elements in the c-*fos* message trigger deadenylation as a first step in rapid mRNA decay. Genes Dev. **5:**221–231.
60. **Stevens, A., C. L. Hsu, K. R. Isham, and F. W. Larimer.** 1991. Fragments of the internal transcribed spacer 1 of pre-rRNA accumulate in *Saccharomyces cerevisiae* lacking 5′-3′ exoribonuclease 1. J. Bacteriol. **173:**7024–7028.
61. **Stevens, A., and M. K. Maupin.** 1987. A 5′-3′ exoribonuclease of *Saccharomyces cerevisiae*: size and novel substrate specificity. Arch. Biochem. Biophys. **252:**339–347.
62. **Thomas, P. F.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77:**5201–5205.
63. **Tipper, D. J.** 1973. Inhibition of yeast ribonucleic acid polymerases by thiolutin. J. Bacteriol. **116:**245–256.
64. **Urlaub, G., P. J. Mitchell, C. J. Ciudad, and L. A. Chasin.** 1989. Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. Mol. Cell. Biol. **9:**2868–2880.
65. **Wisdom, R., and W. Lee.** 1991. The protein-coding region of c-*myc* mRNA contains a sequence that specifies rapid mRNA turnover and induction by protein synthesis inhibitors. Genes Dev. **5:**232–243.