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Identification and Characterization of a Sequence Motif Involved in Nonsense-Mediated mRNA Decay

SHUANG ZHANG,[1,2] MARIA J. RUIZ-ECHEVARRIA,[1] YONG QUAN,[1,2] 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

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In both prokaryotes and eukaryotes, nonsense mutations in a gene can enhance the decay rate or reduce the abundance of the mRNA transcribed from that gene, and we call this process nonsense-mediated mRNA decay. We have been investigating the *cis*-acting sequences involved in this decay pathway. Previous experiments have demonstrated that, in addition to a nonsense codon, specific sequences 3′ of a nonsense mutation, which have been defined as downstream elements, are required for mRNA destabilization. The results presented here identify a sequence motif (TGYYGATGYYYYY, where Y stands for either T or C) that can predict regions in genes that, when positioned 3′ of a nonsense codon, promote rapid decay of its mRNA. Sequences harboring two copies of the motif from five regions in the *PGK1*, *ADE3*, and *HIS4* genes were able to function as downstream elements. In addition, four copies of this motif can function as an independent downstream element. The sequences flanking the motif played a more significant role in modulating its activity when fewer copies of the sequence motif were present. Our results indicate the sequences 5′ of the motif can modulate its activity by maintaining a certain distance between the sequence motif and the termination codon. We also suggest that the sequences 3′ of the motif modulate the activity of the downstream element by forming RNA secondary structures. Consistent with this view, a stem-loop structure positioned 3′ of the sequence motif can enhance the activity of the downstream element. This sequence motif is one of the few elements that have been identified that can predict regions in genes that can be involved in mRNA turnover. The role of these sequences in mRNA decay is discussed.

The rates at which mRNAs decay play an important role in regulating gene expression. mRNA half-lives can vary from each other by as much as 100-fold (28, 55, 59, 62, 64), and this variation determines, in part, the cytoplasmic abundance of these RNAs. In addition, the decay rate 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., hormones, stress, cell cycle, etc. [3, 55, 62]). The goal of our work is to understand the cellular mechanisms that determine the stability of mRNAs.

We are studying mRNA decay in the yeast *Saccharomyces cerevisiae*. Our results, as well as work from other laboratories, strongly indicate that the processes of mRNA turnover and translation are intimately linked and that understanding of this relationship is critical in elucidating the mechanism of mRNA decay (2, 5, 9, 11, 15, 21, 23, 29–31, 41, 53, 55–59, 76). The role of translation in determination of mRNA decay rates is direct, and, for certain instability elements identified in protein coding regions and 3′ untranslated regions (3′-UTRs), translation of the protein coding region must occur in order for them to function (2, 11, 15, 21, 23, 29, 41, 53, 60, 66, 76).

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 we term nonsense-mediated mRNA decay (55, 56, 58). Reduced mRNA levels or decreased stabilities of nonsense-containing transcripts have been observed in both prokaryotes and eukaryotes (6–8, 13, 14, 18, 20, 22, 42, 44–47, 51, 54, 56, 71, 75). For example, 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 (45).

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 (17, 42, 43). Mutations in the *UPF* genes were isolated as allosuppressors of a *his4* frameshift allele (17, 43). Subsequent studies demonstrated that strains harboring the *upf1* and *upf3* alleles, and as shown in more recent studies also *upf2*, lead to the selective stabilization of nonsense-containing mRNAs without affecting the decay rates of most other mRNAs (16, 26, 42, 43, 56). The *UPF1* and *UPF2* genes have been cloned and sequenced (16, 25, 43).

We have been characterizing the *cis*-acting sequences required to accelerate the decay of nonsense-containing mRNAs. The PGK1 transcript, normally a stable and abundant mRNA, was used as our model substrate to investigate this process (24, 56). Previous results have demonstrated the following (24, 56): (i) nonsense codons, independent of the type, located within the amino-terminal two-thirds of the protein-coding region accelerate the decay rate of the PGK1 transcript up to 12-fold, whereas nonsense 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; (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-

* 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 La., Piscataway, NJ 08854. Phone: (908) 235-4790. Fax: (908) 235-5223. Electronic mail address: Peltz@RWJA.UMDNJ.EDU.

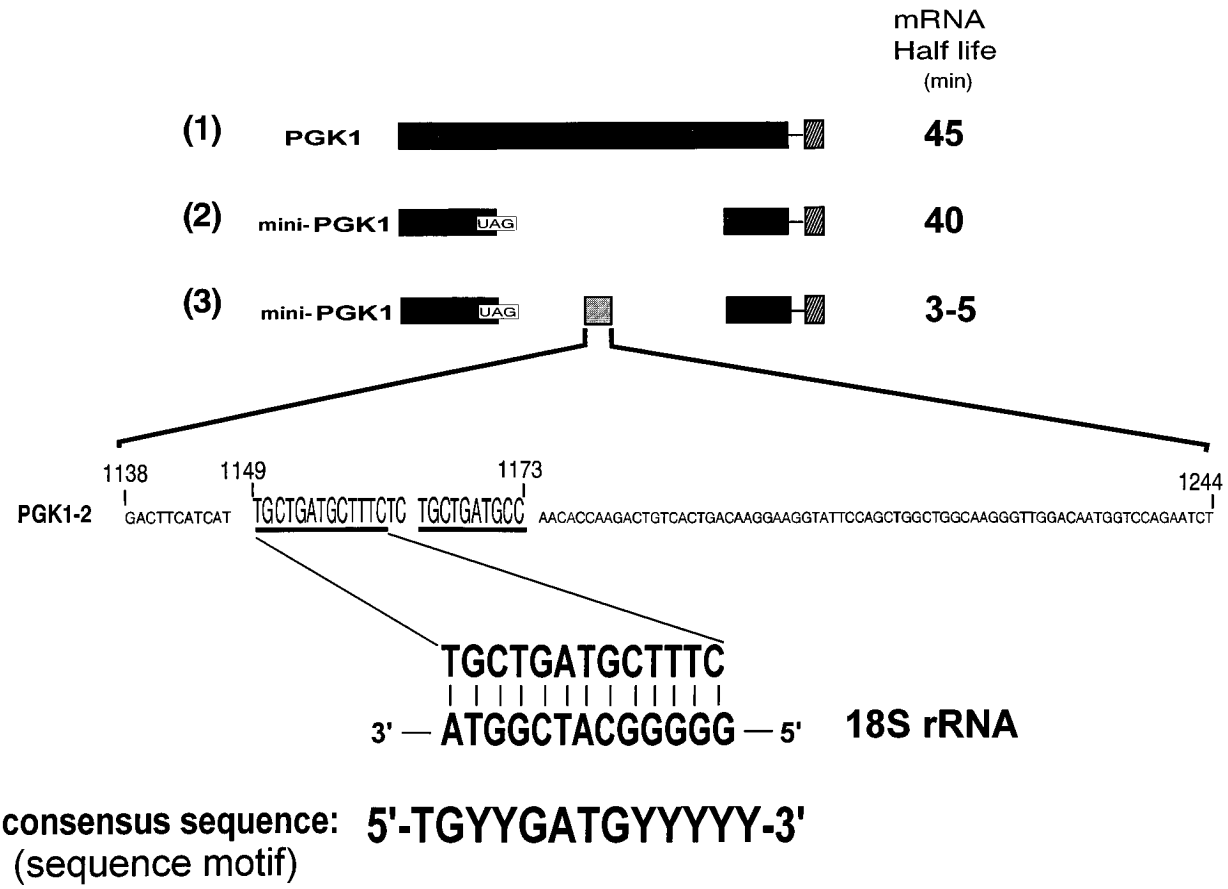

FIG. 1. Identification of genes harboring the sequence motif. Schematic representations of the wild-type *PGK1* gene (construct 1) and of mini-*PGK1* nonsense alleles are shown. Mini-*PGK1* nonsense alleles containing a 5′-proximal nonsense mutation in which the sequence between 5.6 and 92.6% of the *PGK1* protein-coding region has been deleted that either lack (construct 2) or contain (construct 3) a downstream element are shown. The tag in the 3′-UTR is indicated as a thick hatched box. 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. The downstream element is represented by a shaded thick bar. mRNA decay rates of these *PGK1* alleles were determined previously (24, 56) and are shown to the right of the depictions of the genes. The sequences from the *PGK1* gene (nt 1138 to 1244) harboring a previously identified downstream element (56) are displayed. The region of the downstream element that is complementary to the 18S rRNA is underlined, and both this sequence and its complementary rRNA sequence are enlarged below the downstream element. The consensus sequence (or sequence motif) used in a computer search to identify sequences in other genes that harbored two copies of this motif is shown.

coding region such that it constitutes the boundary between nonsense mutations which do or do not affect mRNA decay. More recent results have demonstrated that the characteristics of this decay pathway are not unique to the PGK1 mRNA, and the results described above have been basically recapitulated for the HIS4 mRNA (24).

The importance of sequences downstream of a stop codon in the activation of the nonsense-mediated mRNA decay pathway was suggested when a *PGK1* nonsense allele (which we have called the mini-*PGK1* allele) in which most of the *PGK1* protein-coding region downstream of an early nonsense mutation was deleted resulted in a 10-fold stabilization of its mRNA. Experiments that inserted short regions of the deleted DNA back into the mini-*PGK1* nonsense allele demonstrated that a 106-nucleotide (nt) sequence, when positioned 3′ of the nonsense codon, can promote rapid decay of its mRNA, and we have called this sequence the downstream element (reference 56, and see Fig. 1). Nucleotides essential to the destabilizing function of the 106-nt downstream element were defined by deletion analysis. The 5′-proximal 34-nt sequence of this element was shown to be necessary for nonsense-mediated mRNA decay, and approximately 80 nt of 5′-proximal sequence was necessary to function as an independent element (56).

We have suggested that the role of the downstream element may be to promote a ribosomal pause or to interact with a ribosome-associated factor that is scanning 3′ of the termination codon (24, 56, 58). Within the 34-nt sequence that was identified by deletion analysis as necessary for activity are two ATG codons that are contained within nine identical nucleotides (Fig. 1). Deletion of the two ATG codons within the 9-nt repeat motif inactivated the downstream element and stabilized the mRNA (56). Furthermore, an analysis of possible complementary sequences showed that the 9-nt sequence encompassing the two ATG codons is complementary to sequences in yeast 18S rRNA (references 56 and 58 and Fig. 1). On the basis of these observations, we suggested that the region complementary to the 18S rRNA may be an important component of the downstream element, and we define this complementary region as the sequence motif (Fig. 1).

The results described in this paper attempt to define the role of the sequences in the downstream element. We demonstrate that the sequence motif can be utilized to predict regions in other genes that can function as downstream elements in the

TABLE 1. List of oligodeoxynucleotides used in this study

Oligodeoxynucleotide	Sequence
1	5′-CGATAGTAATATTTATATATTTATATTTTTAAAATATTTATTTATTTATTTATTTAAGAT-3′
2	5′-AATAGATCTCAAGTTAACACCAACAAGGA-3′
3	5′-AGTCGCTAGCTAGGTTTTGGTTATTGCCGATGA-3′
4	5′-AATAGATCTCAACAAGAAACCGGCAGCAC-3′
5	5′-AGTCGCTAGCTAGCTCACAGAGCTCACTCTTCT-3′
6	5′-AGTCGCTAGCTAGACACGAATTGAGCTCTTTGGC-3′
7	5′-AATAGATCTGCGGTACCGAAGGCATCGT-3′
8	5′-AATAGATCTCGAAACCGACCATAGAAGAG-3′
9	5′-AGTCGCTAGCTAGCTTTGGCTGATGTTTACATC-3′
10	5′-AGTCGCTAGCTAGCATCAACGATGCCTTCGGT-3′
11	5′-GATCCTGGCTGATGTTTACATCAACGATGCCA-3′
12	5′-GATGTGGCATCGTTGATGTAAACATCAGCCAG-3′
13	5′-AATAGATCTGAACTTTTGAACATCTTC-3′
14	5′-AGTCGCTAGCTAGCGTTACCACATCGAA-3′
15	5′-CTAAGATCTGGACGTAATTTGATTGCTTC-3′
16	5′-AGTCGCTAGCTACCGTGGTAATTGATGTTGG-3′
17	5′-GATCGAATTCCCAAGATGGGAATTC-3′
18	5′-GATCGAATTCCCATCTTGGGAATTC-3′
19	5′-TTTAGTTAACTTAAACACGAATTGAGCTCTTTGGCT-3′
20	5′-TTTAGATATCACACGAATTGAGCTCTTTGGCT-3′
21	5′-ATCTTCAAAGTTGTCTGT-3′
22	5′-TTTAGTTAACTTAAACACGAATTGAGCTCTTTGGCT-3′
23	5′-TTTAGATATCACAGAATTGAGCTCTTTGGCT-3′

nonsense-mediated mRNA decay pathway. In addition, we demonstrate that multiple copies of the sequence motif can function as an independent downstream element and that the regions flanking the sequence motif can modulate its activity in the nonsense-mediated mRNA decay pathway.

MATERIALS AND METHODS

Yeast strains, growth conditions, and transformation procedures. The yeast strain used in this study was RY262 (*MAT*α *his4-519 ura3-52 rpb1-1* [47]). RY262$^-$ (*MAT*α *his4-519 ura3-52 UPF1*::*hisG rpb1-1*) lacks the *UPF1* gene and was also used to determine mRNA decay rates (24). The *Escherichia coli* DH5α strain was used to amplify plasmid DNA. Yeast media were prepared as described elsewhere (61). Cells were cultured on media lacking uracil to select and maintain the plasmids used in these studies. Cells lacking plasmids were grown nonselectively on YPD medium (4). Cells harboring the *rpb1-1* allele were grown at 24°C. Yeast transformations were performed according to the lithium acetate protocol (38) as modified by Schiestl and Gietz (67).

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. With the exception of the amber and ochre SMURFT linkers (Pharmacia), the oligonucleotides used in these studies were obtained from the Center for Advanced Biotechnology and Medicine DNA Synthesis Network Laboratory (Table 1).

mRNA decay measurements and RNA preparation and analysis. The mRNA decay rates of the various *PGK1* alleles transformed into strains harboring the temperature-sensitive allele of RNA polymerase (*rpb1-1*) were determined as described previously (16, 56). The results of these experiments were quantitated with a Bio-Rad model G-250 molecular imager or a Bio-Rad model GS-670 imaging densitometer. The mRNA abundances were often normalized by use of CYH2 or U3 RNA (50).

Preparation of radioactive probes. DNA probes were labelled to high specific activity with [α-^{32}P]dCTP (19) or by 5′-end labelling of single-stranded oligodeoxynucleotides with [γ-^{32}P]ATP (65). Oligonucleotide 1 (Table 1) was used to monitor the decay of PGK1 mRNAs 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 (24, 72).

Plasmid constructions. PCR (35 cycles) were performed in a 100-μl total volume, and the reaction mixtures contained 25 ng of DNA template, 30 pmol of DNA primers, and 5 U of *Taq* DNA polymerase. The samples were subsequently extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform, 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.

Preparation of mini-*PGK1* alleles containing downstream elements from the *PGK1*, *HIS4*, and *ADE3* genes. The mini-*PGK1* alleles containing a downstream element identified from either the *PGK1*, the *HIS4*, or the *ADE3* gene were prepared by cleaving plasmid pUC9PGK1(-AU)H2(3)UAG (the construction of this plasmid is described in reference 56) with *Nhe*I and *Bgl*II, isolating a 3.5-kb DNA fragment, and ligating it with a DNA fragment synthesized by PCR containing one of the downstream elements shown in Fig. 2. The DNA fragment containing PGK1-1L (PGK1-1, the full-length downstream element [Fig. 2]), was generated by PCR with the plasmid pUC9PGK1(-AU), harboring the *PGK1* gene (32), as the template and with oligonucleotides 4 and 6 as the primers (Table 1). Similarly, oligonucleotides 5 and 4 were used as primers in the PCR described above to prepare a DNA fragment containing the downstream element PGK1-1S (lacking two copies of the sequence motif [Fig. 3A, item 3]). The DNA fragments were cleaved with *Nhe*I and *Bgl*II and were inserted into the mini-*PGK1* gene as described above. The putative downstream element from the *HIS4* gene was synthesized by PCR with oligonucleotides 2 and 3 as primers (Table 1), and the plasmid pUC18-HIS4 (57), harboring the *HIS4* gene, was used as the template. The DNA fragment was cleaved with *Nhe*I and *Bgl*II and was inserted into the mini-*PGK1* gene as described above. The putative downstream element from the *ADE3* gene was synthesized by PCR with oligonucleotides 15 and 16 as primers (Table 1), and the plasmid pLK25 (70), harboring the *ADE3* gene, was used as the template. The DNA fragment was cleaved with *Nhe*I and *Bgl*II and was inserted into the mini-*PGK1* gene as described above. The putative downstream element from the *PGK1* gene (Fig. 2, PGK1-0) was synthesized by PCR with oligonucleotides 13 and 14 as primers (Table 1), and the plasmid pUC9-PGK1(-AU) (56), harboring the *PGK1* gene, was used as the template. The DNA fragment was cleaved with *Nhe*I and *Bgl*II and was inserted into the mini-*PGK1* gene as described above. The structure of each allele was confirmed by DNA sequencing. The *Bam*HI-*Hin*dIII fragments containing the *PGK1* alleles were subcloned into the centromere-based plasmid pRIP1 to form pRIPPGK1(-AU)UAGSZ-1L, pRIPPGK1(-AU)UAGSZ-1S, pRIPPGK1(-AU)UAGSZHIS4-1, pRIPPGK1(-AU)UAGSZADE3-1, and pRIPPGK1(-AU)UAGSZ-0. The resultant *PGK1* alleles are depicted in Fig. 3A and 4, and descriptions of the plasmids are given in Table 2.

UAA- and UGA-containing mini-*PGK1* alleles harboring the PGK1-1 downstream element were prepared by cleaving plasmids pUC9PGK1(-AU)UAA and pUC9PGK1(-AU)UGA (the construction of these plasmids is described in reference 24) with *Hpa*I and *Eco*RV, respectively, and with *Bgl*II, isolating a 3.5-kb DNA fragment. The DNA fragments containing the downstream elements were synthesized by PCR. Oligonucleotides 22 and 4 were used to prepare the UAA-containing PGK1-1L downstream element, and oligonucleotides 23 and 6 were used to synthesize the UGA-containing PGK1-1L downstream element. These DNA fragments, generated by PCR, were cleaved with *Hpa*I and *Eco*RV, respectively, and both DNA fragments were cleaved with *Bgl*II. The DNA fragments were ligated with the vector described above. The structures of these alleles were confirmed by DNA sequencing. The *Bam*HI-*Hin*dIII fragments containing the *PGK1* allele of interest were subcloned into the centromere-based plasmid pRIP1 to form pRIPPGK1(-AU)UAAMR-1L and pRIPPGK1(-AU)UGAMR-1L, respectively. Schematic representations of these genes are shown in Fig. 3B and are listed in Table 2.

FIG. 2. The sequences of the downstream elements from the *PGK1*, *HIS4*, and *ADE3* genes. A computer search identified regions in other genes that contained two copies of the putative sequence motif identified previously in the *PGK1* gene (see Table 3). Shown in this figure are five sequences from the *PGK1*, *HIS4*, and *ADE3* genes, which harbor two copies of the putative sequence motif (underlined sequence) that were tested to determine whether they could promote nonsense-mediated mRNA decay. The numbers above the sequence indicate the 5′ and 3′ boundaries of the DNA fragments that were utilized to determine whether they function as downstream elements.

Preparation of mini-*PGK1* alleles containing multiple copies of the sequence motif. The plasmid pRIPPGK(-AU)UAGSZ-1Δ3, which harbors the PGK1-1 downstream element in which the 3′ flanking sequences were deleted (see Fig. 6), was cleaved with *Bgl*II and ligated with a double-stranded oligonucleotide containing two copies of the sequence motif. The double-stranded oligonucleotide was prepared by annealing oligonucleotides 11 and 12 (Table 1), encoding *Bgl*II sites at both ends of the fragment. The order of nucleotides in these alleles was confirmed by sequencing. *PGK1* alleles containing the double-stranded oligonucleotide in both orientations were identified. pRIPPGK1(-AU)UAGSZ-1Δ3_SM contains the DNA fragment harboring the sequence motifs in correct orientation, while pRIPPGK1(-AU)UAGSZ-1Δ3_rSM harbors the DNA fragment containing the sequence motifs in the reverse orientation. Schematic representations of the genes are shown in Fig. 5 and are listed in Table 2.

Preparation of mini-*PGK1* alleles harboring deletions of the PGK1-1 downstream element. The amber-containing mini-*PGK1* alleles harboring deletions of the PGK1-1 downstream element were constructed as follows. The DNA fragments harboring deletions of the PGK1-1 downstream element (Fig. 2) were prepared by PCR. The 5′ primers for the reactions encoded an *Nhe*I site, while the 3′ primers encoded a *Bgl*II site. The particular oligonucleotides used for each construction are described in Table 2. The DNA fragments were cleaved with *Nhe*I and *Bgl*II and ligated to a 3.5-kb *Nhe*I-*Bgl*II fragment isolated from pUC9PGK(-AU)H2(3)UAG. The structures of these alleles were confirmed by DNA sequencing. The *Bam*HI-*Hin*dIII fragments containing these *PGK1* alleles were subcloned into the centromere-based plasmid pRIP1 to form plasmids pRIPPGK1(-AU)UAGSZ-1Δ1 to pRIPPGK1(-AU)UAGSZ-1Δ8 and are depicted in Fig. 6 and listed in Table 2.

Preparation of mini-*PGK1* alleles harboring deletions of the PGK1-1 downstream element with a stem-loop structure 3′ of the downstream element. The constructs pRIPPGK1(-AU)UAGSZ-1Δ2, pRIPPGK1(-AU)UAGSZ-1Δ3, pRIPPGK1(-AU)UAGSZ-1Δ5, pRIPPGK1(-AU)UAGSZ-1Δ7, and pRIPPGK1(-AU)UAGSZ-1Δ8 (Table 2) were cleaved with *Bgl*II and ligated with a double-stranded DNA fragment harboring a stem-loop structure containing compatible *Bgl*II ends. The DNA fragment harboring the stem-loop structure was prepared by annealing oligonucleotides 17 and 18 (Table 1). The structures of these alleles were confirmed by DNA sequencing, and they resulted in plasmids pRIPPGK1(-AU)UAGSZ-1Δ2_SL, pRIPPGK1(-AU)UAGSZ-1Δ3_SL, pRIPPGK1(-AU)UAGSZ-1Δ5_SL, pRIPPGK1(-AU)UAGSZ-1Δ7_SL, and pRIPPGK1(-AU)UAGSZ-1Δ8_SL. A schematic representation of these plasmids is shown in Fig. 7B and summarized in Table 2.

RESULTS

Identification of DNA fragments containing the sequence motifs from the *PGK1*, *HIS4*, and *ADE3* genes that can function as downstream elements. Nonsense-mediated mRNA decay requires both a nonsense mutation and a specific downstream element 3′ of the nonsense codon. A downstream element was identified previously in the *PGK1* gene, and the nucleotides essential to the destabilizing function of the downstream element were defined by deletion analysis (56). The 5′-proximal 34-nt sequence of this element was shown to be necessary for nonsense-mediated mRNA decay and to harbor two ATG codons which are contained within nine identical nucleotides (Fig. 1 and reference 56). An analysis of possible complementary sequences showed that this motif is complementary to sequences in yeast 18S rRNA and led us to the hypothesis that mRNA-rRNA interactions are important for nonsense-mediated mRNA decay (Fig. 1 and references 56, 58, and 59). We define sequences that are complementary to this region of the 18S rRNA as the sequence motif (Fig. 1).

To identify sequences in other genes that contain two copies of the putative sequence motif as found in the original downstream element, a computer analysis was performed. The computer search parameters allowed matches in sequences that have G · C or G · U base pairs in the complementary region (Table 3). The distance between the sequence motifs was allowed to be no greater than 25 nt. Similar searches that allowed for one or two mismatches within the motif were also executed. We demanded that the sequences identified by the computer search contain the AT(G/T/C) sequence within the two motifs. Previous results demonstrated that deletion of the two ATG codons inactivated the downstream element (56), and preliminary results indicate that this G nucleotide can be changed to T or C without loss of activity of the downstream element (59a). The yeast genes in GenBank were searched for two copies of the motif as described above (approximately 2,000 genes), and a summary of the computer analysis is given in Table 3.

The computer search identified many genes that contained two copies of the sequences complementary to the region of the 18S rRNA (Table 3). Interestingly, two additional regions in the *PGK1* gene containing two copies of the putative motif were identified (Table 3, PGK1-1 and PGK1-0; Fig. 2). Previous results suggested that the *PGK1* gene harbored redundant downstream elements (56). Less restrictive computer searches identified a larger number of genes harboring the sequence motif. For example, 369 genes in the yeast database that harbored at least one perfect copy of the sequence motif were identified, and more than 75% of the yeast genes harbor at least one copy of an imperfect sequence motif containing a single change (data not shown). A computer search to identify two imperfect copies of the sequence motif (two mistakes at any position) demonstrated that 100 yeast genes out of approximately 2,000 contain these imperfect motifs (data not shown).

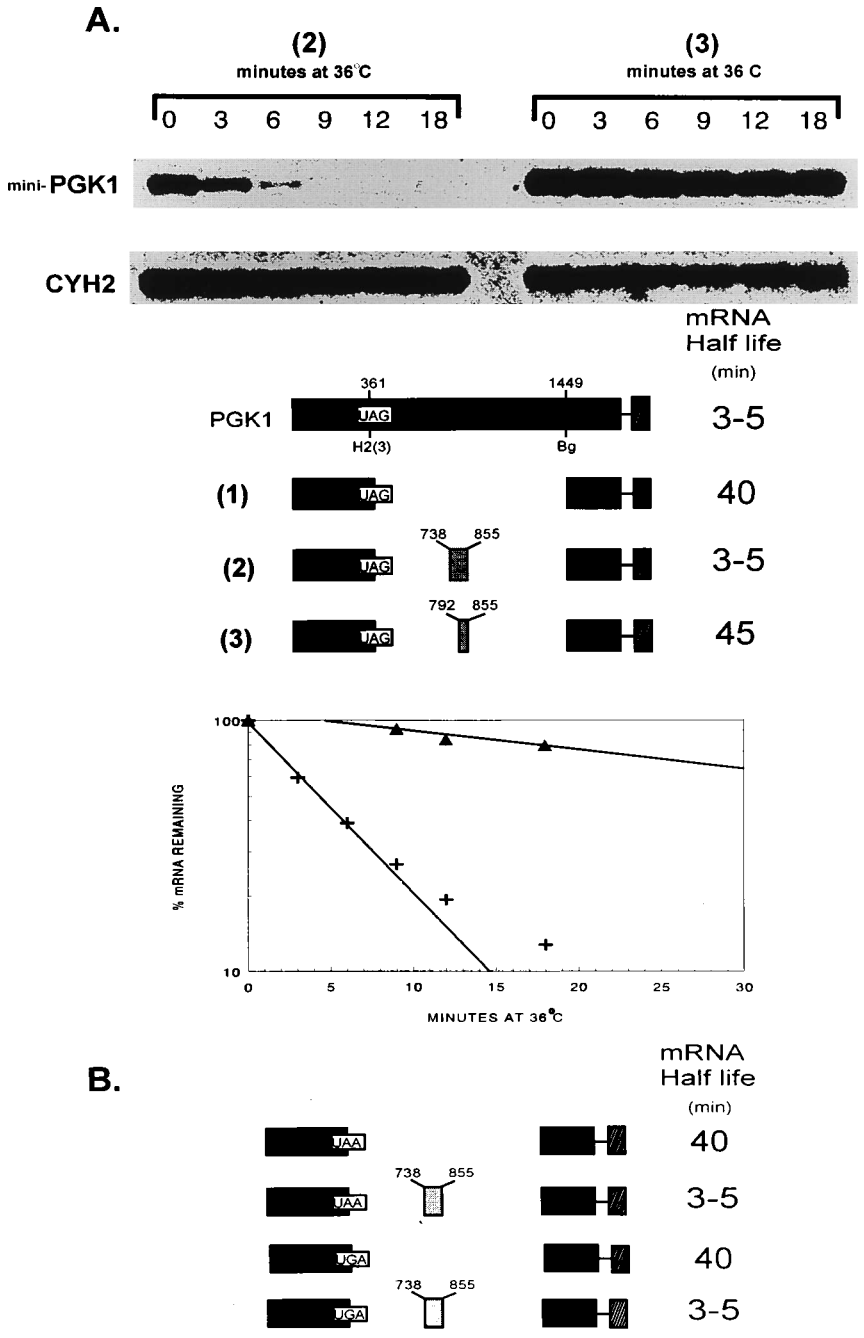

FIG. 3. A sequence from the *PGK1* gene identified from the computer search is an active downstream element. (A) The putative PGK1-1 downstream element from the *PGK1* gene (Fig. 2) either containing (construct 2) or lacking (construct 3) two copies of the sequence motif (Fig. 2, the PGK1-1 downstream element) was inserted into the mini-*PGK1* gene lacking any downstream elements (described in Fig. 1, construct 2) downstream of a 5′-proximal amber mutation. The downstream element is represented by a shaded thick bar. The tag in the 3′-UTR is indicated as a thick hatched box. The mRNA decay rates of these *PGK1* alleles were determined in strain RY262 as described in Materials and Methods. The RNA blots for these experiments are shown above the schematic representations of the *PGK1* alleles. The parenthesized numbers above the blots correspond to the *PGK1* alleles shown schematically below. As a control, the half-life of the CYH2 transcript is shown. *PGK1* allele 2 contains the downstream element harboring the sequence motifs [plasmid pRIPPGK1(-AU)UAGSZ-1L], while *PGK1* allele 3 lacks the sequence motifs [plasmid pRIPPGK1(-AU)UAGSZ-1S]. Also shown is the 5′-proximal nonsense-containing *PGK1* allele (allele 1) in which the mRNA half-life was determined previously (56). A graphical representation of the results is shown below the diagram of the genes. A semilog plot of the percent of the mRNA remaining versus time after transcription was inhibited is shown. +, measurements from the mini-*PGK1* allele harboring the PGK1-1 downstream element containing the sequence motif (construct 2); ▲, measurements from the mini-*PGK1* allele harboring the PGK1-1 in which the sequence motif was deleted (construct 3). (B) The downstream element (Fig. 2, PGK1-1), represented by a shaded thick bar, was inserted into a mini-*PGK1* allele 3′ of either a UAA or UGA nonsense mutation [plasmids pRIPPGK1 (-AU)UAAMR-1L and pRIPPGK1(-AU)UGAMR-1L, respectively (Table 2)]. mRNA decay rates of these *PGK1* alleles were determined as for panel A. Schematic representations of the *PGK1* alleles are the same as described above, and a summary of the data is shown.

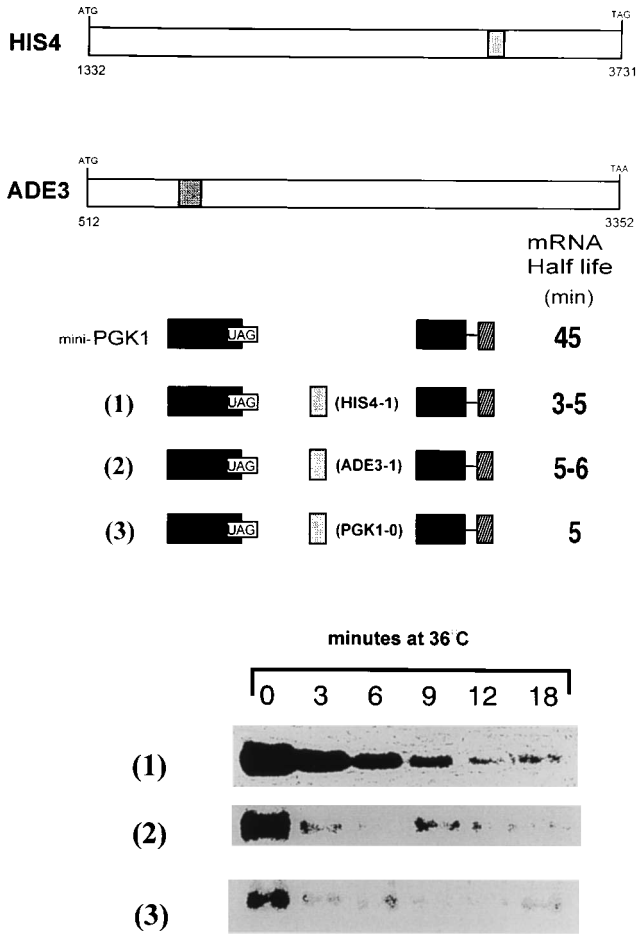

FIG. 4. Sequences from the *PGK1*, *HIS4*, and *ADE3* genes identified from the computer search are active as downstream elements. Regions from the *HIS4*, *ADE3*, and *PGK1* genes (Fig. 2, *PGK1-0*, *ADE3*, and *HIS4*) were inserted into the mini-*PGK1* gene downstream of a 5′-proximal amber mutation [Table 2, plasmids pRIPPGK1(-AU)UAGSZHIS4-1, pRIPPGK1(-AU)UAGSZADE3-1, and pRIPPGK1(-AU)UAGSZ-0, respectively; see Materials and Methods for a description of how these plasmids were constructed]. The downstream element is represented by a shaded thick bar. The tag in the 3′-UTR is indicated as a thick hatched box. mRNA decay rates of these *PGK1* alleles were determined in strain RY262 as described in Materials and Methods, and the results were normalized to the concentration of the U3 RNA. The RNA blots for these experiments are shown below the schematic representations of the *PGK1* alleles. Shown above the diagram of the mini-*PGK1* alleles are schematic representations of the *HIS4* and *ADE3* genes. The shaded regions of the representations are the locations of the putative downstream elements in these genes that were tested for activity. The numbers in these representations indicate the locations of the translation start and stop sites within their respective genes.

To determine whether sequences containing the putative motifs can function as a downstream element, a DNA fragment from the *PGK1* gene identified in the computer search that either contained or lacked two copies of the sequence motif was inserted 3′ of the amber codon in the mini-*PGK1* allele (Fig. 3A, constructs 2 and 3; see Fig. 2 for the sequence of the downstream element [PGK1-1] inserted into the mini-*PGK1* gene). The mini-*PGK1* gene was constructed from a *PGK1* allele harboring an amino-terminal amber mutation in which most of the protein-coding region was deleted (reference 51 and Fig. 3A, construct 1). The mini-PGK1 transcript is stable unless a downstream element is inserted 3′ of the nonsense codon and therefore is an ideal substrate to analyze whether putative downstream elements are active (references 24 and 56 and Fig. 3A). The mini-*PGK1* alleles harboring the downstream elements were transferred to yeast centromere plasmids and transformed into yeast cells harboring the *rpb1-1* temperature-sensitive allele of RNA polymerase II (52). The decay rates of these mRNAs were determined by RNA blotting analyses of RNA isolated at different times after inhibition of transcription by shifting the culture to the nonpermissive temperature (36°C).

Insertion of the DNA fragment harboring the putative PGK1-1 downstream element (Fig. 2) 3′ of the amber codon (Fig. 3A) changed the half-life of the mini-PGK1 transcript from 40 min to 3 to 5 min (Fig. 3, compare construct 1 with construct 2). A 5′-proximal deletion that removed 51 nt, including the sequence motifs, stabilized the mini-PGK1 mRNA so that its decay rate was similar to that of the mini-PGK1 mRNA lacking a downstream element (Fig. 3, compare construct 3 with construct 1). The downstream element was functional independently of the type of nonsense codon, since UAA- and UGA-containing mini-PGK1 transcripts were rapidly degraded when this DNA fragment was inserted 3′ of the nonsense codon (Fig. 3B). 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 (data not shown).

The computer search identified other genes containing the motif (Table 3). To test further whether the sequence motif is a good predictor of downstream elements, DNA fragments from the *PGK1*, *HIS4*, and *ADE3* genes containing the motif (Fig. 2) were inserted 3′ of the amber mutation in the mini-*PGK1* gene (Fig. 4). The decay rates of these mRNAs were determined, and the results indicate that insertion of these DNA fragments harboring the putative downstream elements changed the half-life of the mini-PGK1 transcript from 40 min to 3 to 5 min (Fig. 4). 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 (data not shown). Collectively, these results indicate that the sequence motif shown in Fig. 1 is an excellent predictor of sequences that harbor downstream elements.

Multiple insertions of the motif can function as a downstream element. As described above, we have defined the sequence motif as sequences that are complementary to the region of the 18S rRNA shown in Fig. 1. Previous experiments characterizing the downstream element from the *PGK1* gene indicated that sequences flanking the motifs were required for complete activity of the downstream element (56). We asked whether multiple copies of the sequence motif could function as an independent downstream element. DNA fragments harboring either two or four copies of the motif from the PGK1-1 downstream element (Fig. 2) were inserted 3′ of an amber codon in the mini-*PGK1* gene (Fig. 5). As a control, the second set of motifs was also inserted in the reverse orientation. The decay rates of the mRNAs synthesized from these genes were determined, and the results of these experiments demonstrate that while two copies of the motif did not alter the decay rate of the mini-PGK1 mRNA (Fig. 5, construct 1), insertion of four copies of the motif changed the half-life of the mini-PGK1 transcript from 40 min to 5 min (Fig. 5, construct 2). The stability of the mini-PGK1 transcript in which the last two copies of the motif were inserted in the reverse orientation did not promote rapid decay of its mRNA (Fig. 5, construct 3). These results indicate that multiple copies of the motif can function as a downstream element to promote nonsense-mediated mRNA decay.

TABLE 2. Plasmids used in this study

Plasmid	Description
pRIPPGK1(-AU)	A plasmid harboring the *PGK1* gene with a DNA tag inserted into the 3′-UTR (56).
pRIPPGK1(-AU)H2(3)UAG	A plasmid harboring the *PGK1* gene containing an amber codon inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] (56).
pRIPPGK1(-AU)UAGSZ-1L, pRIPPGK1(-AU)UAGSZ-1S	Plasmids harboring a *PGK1* allele containing an amber codon inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] and harboring a deletion of the sequence between 5.6 and 92.6% of the *PGK1* coding region. The full-length PGK1-1 downstream element (nt 738–855 [Fig. 2]) [in pRIPPGK1(-AU)UAGSZ-1L] or a deleted form (nt 791–855) [in pRIPPGK1(-AU)UAGSZ-1S], lacking the sequence motifs, was inserted 3′ of the UAG codon.
pRIPPGK1(-AU)UAAMR-1L, pRIPPGK1(-AU)UGAMR-1L	Plasmids harboring a *PGK1* allele containing a UAA or UGA nonsense codon inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] and harboring a deletion of the sequence between 5.6 and 92.6% of the *PGK1* coding region. The PGK1-1 downstream element (nt 738–855 [Fig. 2]) was inserted 3′ of the termination codons.
pRIPPGK1(-AU)UAGSZ-1Δ1, pRIPPGK1(-AU)UAGSZ-1Δ2, pRIPPGK1(-AU)UAGSZ-1Δ3, pRIPPGK1(-AU)UAGSZ-1Δ4, pRIPPGK1(-AU)UAGSZ-1Δ5, pRIPPGK1(-AU)UAGSZ-1Δ6, pRIPPGK1(-AU)UAGSZ-1Δ7, pRIPPGK1(-AU)UAGSZ-1Δ8	Plasmids harboring a *PGK1* allele containing an amber codon inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] and harboring a deletion of the sequence between 5.6 and 92.6% of the *PGK1* coding region. Deletions of the PGK1-1 downstream element were synthesized by PCR and inserted 3′ of the UAG codon. The primers (Table 1) for preparation of the deleted forms of the PGK1-1 downstream elements were Δ1 (nt 752–855), oligonucleotides 9 and 4; Δ2 (nt 752–824), oligonucleotides 9 and 8; Δ3 (nt 752–791), oligonucleotides 9 and 7; Δ4 (nt 738–824), oligonucleotides 6 and 8; Δ5 (nt 738–791), oligonucleotides 6 and 7; Δ6 (nt 769–855), oligonucleotides 10 and 4; Δ7 (nt 769–824), oligonucleotides 10 and 8; and Δ8 (nt 769–791), oligonucleotides 10 and 7).
pRIPPGK1(-AU)UAGSZ-1Δ3_SM, pRIPPGK1(-AU)UAGSZ-1Δ3_rSM	A DNA fragment (oligonucleotides 11 and 12) harboring two copies of the sequence motif was inserted 3′ of the deleted downstream element found in plasmid pRIPPGK1(-AU)UAGSZ-1Δ3 in either the same orientation (_SM) or the reverse orientation (_rSM) to that found in the *PGK1* gene.
pRIPPGK1(-AU)UAGSZ-1Δ2_SL, pRIPPGK1(-AU)UAGSZ-1Δ3_SL, pRIPPGK1(-AU)UAGSZ-1Δ5_SL, pRIPPGK1(-AU)UAGSZ-1Δ7_SL, pRIPPGK1(-AU)UAGSZ-1Δ8_SL	A DNA fragment harboring a stem-loop structure (oligonucleotides 17 and 18) was inserted 3′ of several deleted forms of the PGK1-1 downstream element found in the *PGK1* alleles described in this table.
pRIPPGK1(-AU)UAGSZ-0, pRIPPGK1(-AU)UAGSZADE3-1, pRIPPGK1(-AU)UAGSZHIS4-1	Plasmids harboring the *PGK1* allele containing an amber codon inserted at 5.6% of the *PGK1* coding region [*Hin*cII(3) site, 361 bp] and harboring a deletion of the sequence between 5.6 and 92.6% of the *PGK1* coding region. The putative downstream elements from the *PGK1*, *HIS4*, and *ADE3* genes (Fig. 2) were prepared by PCR and inserted 3′ of the UAG codon.
pUC18KOM	A construct used to delete the *UPF1* gene from the chromosome (16).

Deletion analysis of a downstream element from the *PGK1* gene. Although multiple copies of the motif can function as a downstream element, sequences flanking the motif are required in downstream elements when only one or two copies of the sequence motif are present (reference 56, and see below). To address the role of the flanking sequences, the PGK1-1 downstream element (Fig. 2) was characterized further.

The sequences of the PGK1-1 downstream element (Fig. 2) required for its activity were identified by constructing 5′ and 3′ deletions of the DNA fragment harboring this element and inserting them 3′ of the amber mutation in the mini-*PGK1* gene. The half-lives of these mRNAs were determined, and the results of these experiments are summarized in Fig. 6. In the absence of the flanking sequences, one or two copies of the sequence motif 3′ of the amber codon were not sufficient to promote accelerated decay of this transcript (Fig. 5; Fig. 6, constructs 8 and 9). Inclusion of the 5′ sequences flanking the sequence motifs in the downstream element was able to partially promote the decay of the mini-PGK1 transcript (Fig. 6, compare construct 8 or 9 with construct 7 and construct 6 with constructs 4 and 5). Similarly, downstream elements containing both the motifs and the 3′ flanking sequences were also either partially or completely active in promoting the decay of the mini-PGK1 transcript (Fig. 6, compare construct 8 with constructs 1 and 5 and constructs 6 and 9 with construct 2). Interestingly, a downstream element consisting of one copy of the sequence motif and the complete 3′ flanking sequence was an active downstream element (Fig. 6, construct 2). Portions of either the 5′ or the 3′ flanking sequence, in addition to the sequence motifs, that partially restored the activity of the downstream element were able to act additively to stimulate the activity of the downstream element (Fig. 6, compare construct 7 or 5 with construct 4). Taken together, these results suggest that the sequence motifs are the most critical component of the downstream element (Fig. 6, shaded region) and that the 5′ and 3′ flanking sequences can modulate its activity.

Characterization of the role of 3′ flanking sequences in a downstream element. A comparison of the sequences flanking the motif of the three downstream elements identified in the *PGK1* gene and the two identified in the *HIS4* and *ADE3* genes did not reveal any strong sequence homologies among them (Fig. 2). Therefore, we hypothesized that there was no sequence-specific role of these flanking sequences. We postulated that a sequence motif, especially one that was not completely active, functioned more efficiently if there was a certain amount of sequence between the termination codon and the element. Results from the deletion analysis are consistent with this hypothesis (Fig. 6, compare construct 4 with construct 5 and construct 7 with construct 8). In addition, preliminary results suggest that increasing the distance between the stop codon and the sequence motif by addition of unrelated sequences between them increased the activity of the downstream element and accelerated the decay of the mini-PGK1 mRNA (78). Computer analysis of regions 3′ of the sequence motif in the downstream elements suggested that they can form RNA secondary structures (data not shown). For exam-

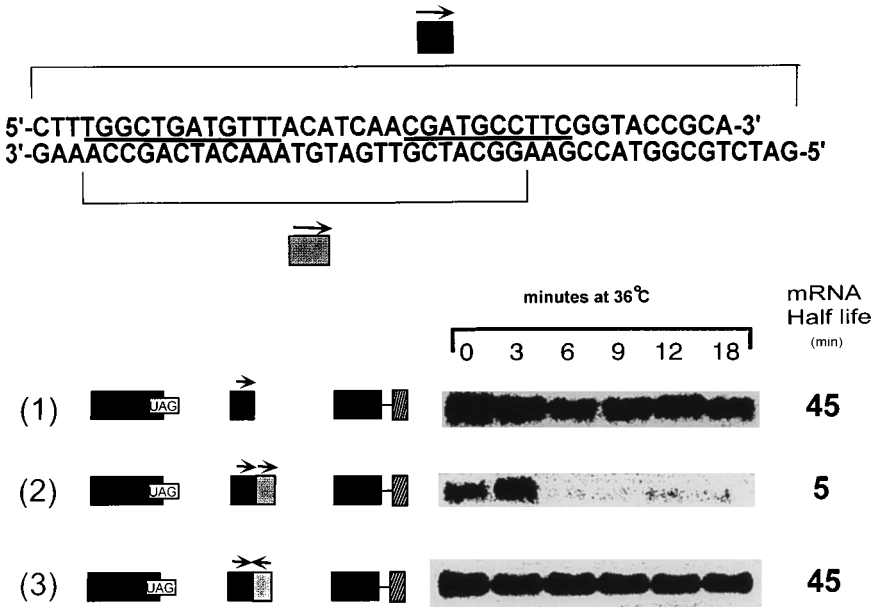

FIG. 5. Multiple copies of the sequence motif can act as a functional downstream element. A DNA fragment from the *PGK1* gene containing primarily the sequence motif from the PGK1-1 downstream element (Fig. 2) was inserted 3′ of an amber mutation in the mini-*PGK1* allele. A schematic representation of this *PGK1* allele is shown to the left of the autoradiograms [construct 1, plasmid pRIPPGK1(-AU)UAGSZ-1Δ3]. The first sequence harboring the motifs inserted in the mini-*PGK1* gene is shown above the autoradiograms and is delimited by the bracket marked by the thick black rectangle. In addition, an oligonucleotide harboring two copies of the sequence motif from the PGK1-1 downstream element was inserted into the mini-*PGK1* allele described above (construct 1) in either the same orientation [construct 2, plasmid pRIPPGK1(-AU)UAGSZ-1Δ3_SM] or the reverse orientation [construct 3, plasmid pRIPPGK1(-AU)UAGSZ-1Δ3_rSM] as found in the PGK1-1 downstream element. The sequence of the oligonucleotide is shown above the autoradiograms and is delimited by the bracket marked by a thick shaded rectangle. The arrows represent the orientation of the sequence as found in the *PGK1* gene. mRNA decay rates of these *PGK1* alleles in strain RY262 were determined as described in Materials and Methods, and the results were normalized to the concentration of the U3 RNA. The RNA blots for these experiments are shown to the right of the schematic representations of the *PGK1* alleles. A summary of the half-life measurements is shown to the right of the autoradiograms.

ple, a computer analysis of the sequences 3′ of the motifs in the PGK1-1 downstream element suggested that there are at least two stem-loop structures with free energies greater than 12 kcal (ca. 50 kJ) within 20 nt of these motifs. Previous results have suggested that RNA secondary structures with similar free energies can slow or halt a scanning ribosomal subunit and, when positioned 3′ to a poor translational initiation site, can improve its translational efficiency (39, 40). We hypothesized that forcing a ribosome or ribosomal subunit to pause near the sequence motifs may also enhance the functioning of that downstream element. To test this hypothesis, a DNA fragment known to form a stem-loop structure (1) was inserted 3′ of inactive or partially active downstream elements and the mRNA decay rates of these transcripts were determined (Fig. 7). Mini-*PGK1* alleles that harbored either inactive or partially active downstream elements were threefold more functional in accelerating the degradation of the mini-PGK1 transcript when the stem-loop was inserted near the sequence motif (Fig. 7, constructs 1 to 3). The stem-loop structure inserted farther downstream of the sequence motif did not increase the activity of these downstream elements (Fig. 7, constructs 4 and 5). Taken together, these results suggest that RNA secondary structures 3′ of the sequence motif can enhance its ability to promote nonsense-mediated mRNA decay.

DISCUSSION

Characterization of the sequences in a downstream element. The experiments presented here and elsewhere have demonstrated that specific sequences 3′ of a nonsense codon are required for the accelerated turnover of nonsense-containing mRNAs (24, 56). An initial analysis of the nucleotides essential for the destabilizing function of the downstream element identified previously in the *PGK1* gene demonstrated that the 5′-proximal 34-nt sequence was necessary for nonsense-mediated mRNA decay (reference 56 and Fig. 1). This region harbors two ATG codons which are bracketed by identical nucleotides that are complementary to the 18S rRNA (Fig. 1). We have defined this complementary region as the sequence motif (Fig. 1).

We hypothesized that the sequence motif is an important component of the downstream element and could be used to predict other sequences that can function in this capacity. A computer search revealed several genes containing two copies of the sequence motif (Table 3). When the computer search was performed, additional genes that allowed for one or two changes in the motif or for searching for just one copy of the sequence motif were identified. We demonstrated that five different DNA fragments from the *PGK1*, *HIS4*, and *ADE3* genes harboring two copies of the sequence motif (Fig. 2) were able to function as downstream elements when inserted 3′ of the nonsense mutation in the mini-*PGK1* gene (Fig. 3 and 4).

A downstream element from the *PGK1* gene (Fig. 2, PGK1-1) was dissected in order to identify the important regions required for its activity (Fig. 4 to 7), and a summary of these results is shown in Fig. 8A. We suggest that the region harboring the sequence motifs is the most critical component of the downstream element (Fig. 8A, domain II) and that the flanking sequences (Fig. 8A, domains I and III) can modulate its activity (Fig. 4 to 7). Four copies of the sequence motif can function independently as an active downstream element (Fig. 5). The flanking sequences, however, played a more significant role in modulating the activity of the sequence motif when fewer copies of it were present (Fig. 6 and 7). In the absence of

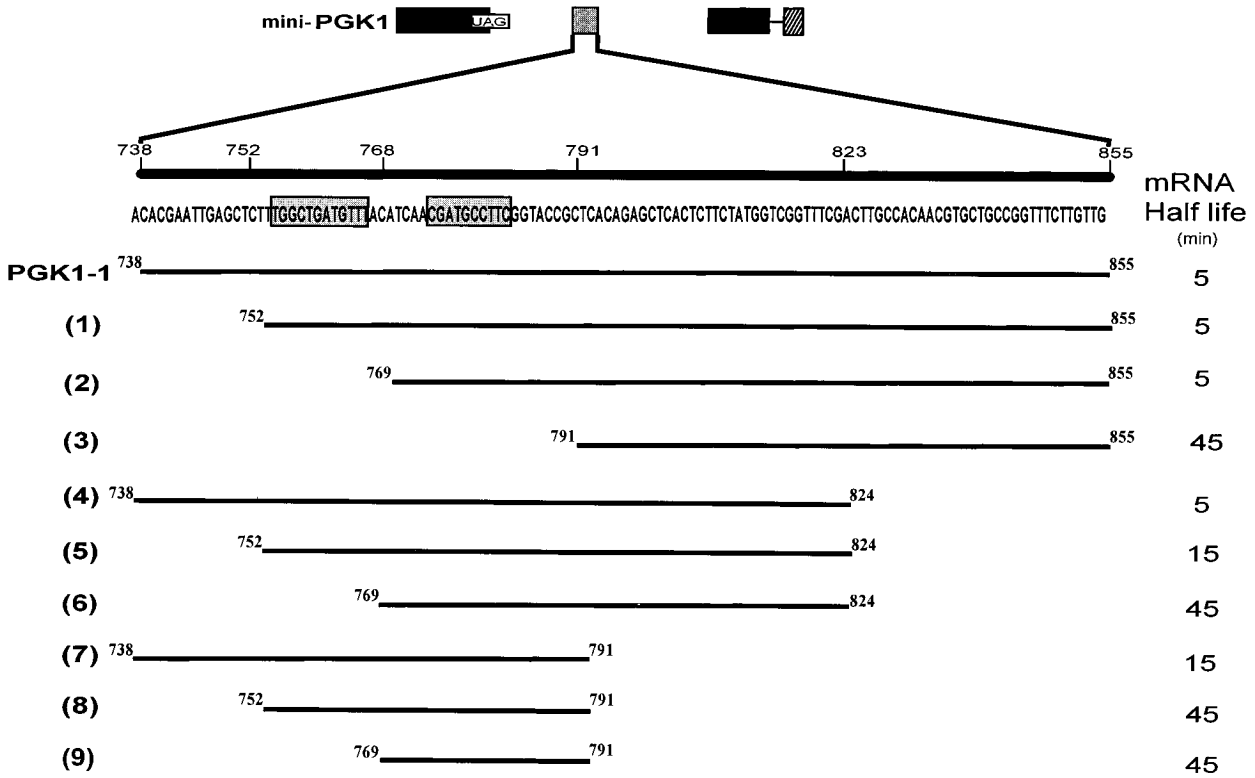

FIG. 6. Deletion analysis of the downstream element. A schematic representation of the mini-*PGK1* allele harboring the PGK1-1 downstream element is shown, and the sequence of the downstream element is shown below. The downstream element is represented by a shaded thick bar. The tag in the 3′-UTR is indicated as a thick hatched box. Within the sequences, the two motifs in this element are shaded. Below the sequence are lines representing the PGK1-1 downstream element and nine deletion mutants of this region. The downstream elements containing the various deletions were inserted into the mini-*PGK1* allele 3′ of the amber codon. Half-lives for the respective PGK1 mRNAs in strain RY262 were determined as described in Materials and Methods, and a summary of the data is shown. The plasmids harboring the mini-*PGK1* alleles with the deletions depicted in the figure are described in Table 2 and are the following: 1, pRIPPGK1(-AU)UAGSZ-1Δ1; 2, pRIPPGK1(-AU)UAGSZ-1Δ6; 3, pRIPPGK1(-AU)UAGSZ-1S; 4, pRIPPGK1(-AU)UAGSZ-1Δ4; 5, pRIPPGK1(-AU)UAGSZ-1Δ2; 6, pRIPPGK1(-AU)UAGSZ-1Δ7; 7, pRIPPGK1(-AU)UAGSZ-1Δ5; 8, pRIPPGK1(-AU)UAGSZ-1Δ3; 9, pRIPPGK1(-AU)UAGSZ-1Δ8.

3′ flanking sequences, the downstream element was less active when the sequence motif was positioned very near the termination codon, suggesting that the sequences 5′ of the motif can modulate its activity by maintaining a certain distance between the sequence motif and the termination codon (Fig. 6). Consistent with this hypothesis, preliminary results indicate that increasing the distance between the stop codon and the sequence motif by addition of unrelated sequences between them increases the activity of the downstream element (78). A downstream element, however, containing a single copy of the sequence motif and its 3′ flanking sequences can be active in promoting nonsense-mediated mRNA decay when inserted near the termination codon (Fig. 6, compare construct 6 with construct 2). We suggest that sequences 3′ of the motif form RNA secondary structures. Consistent with this view, a stem-loop structure positioned 3′ of the sequence motifs can enhance the activity of the downstream element (Fig. 7). Thus, the effects of the flanking sequences on the activity of the sequence motifs can explain why in certain downstream elements a single copy of the motif can function (Fig. 6) while other elements require two or more copies of the motif for full activity (reference 56 and Fig. 2, PGK1-2). The activity of a downstream element is a function of the quality of the sequence motif and its flanking sequences.

Previous experiments suggested that there are multiple downstream elements in the *PGK1* gene (56). Consistent with this view, two other regions in the *PGK1* gene containing two copies of the sequence motif that, when positioned 3′ of a nonsense codon, accelerate the decay of a nonsense-containing PGK1 allele have been identified (Fig. 3, 4, and 8B). A computer search identifying imperfect sequence motifs in the *PGK1* gene revealed several other putative downstream elements (Fig. 8B). Considering that nonsense mutations affect the mRNA abundance of almost all mRNAs tested in yeast and mammalian cells (55, 58), it is not surprising that there would be considerable flexibility in the sequences that can function as downstream elements. We have demonstrated that with the appropriate flanking sequences, a single copy of the motif can function in a downstream element (Fig. 6). More than 75% of the yeast genes harbor at least one copy of an imperfect sequence motif containing a single change. We are currently performing a mutagenesis analysis of the sequence motif in order to determine its precise nature.

The sequence motif is a good indicator of sequences that can function as downstream elements. The results presented here demonstrate that the sequence motif that we have used in our computer search is a good predictor of sequences that can function as downstream elements. Although sequences that are stability elements in mRNAs have been identified (2, 5, 9, 11, 15, 21, 23, 29, 31, 41, 53, 55–60, 66, 76), presently only two elements that can predict regions in other genes that will lead to the rapid turnover of an mRNA have been identified. One sequence motif is the AU-rich element $(AUUUA)_n$ identified in the 3′-UTRs of many lymphokines and growth factor-induc-

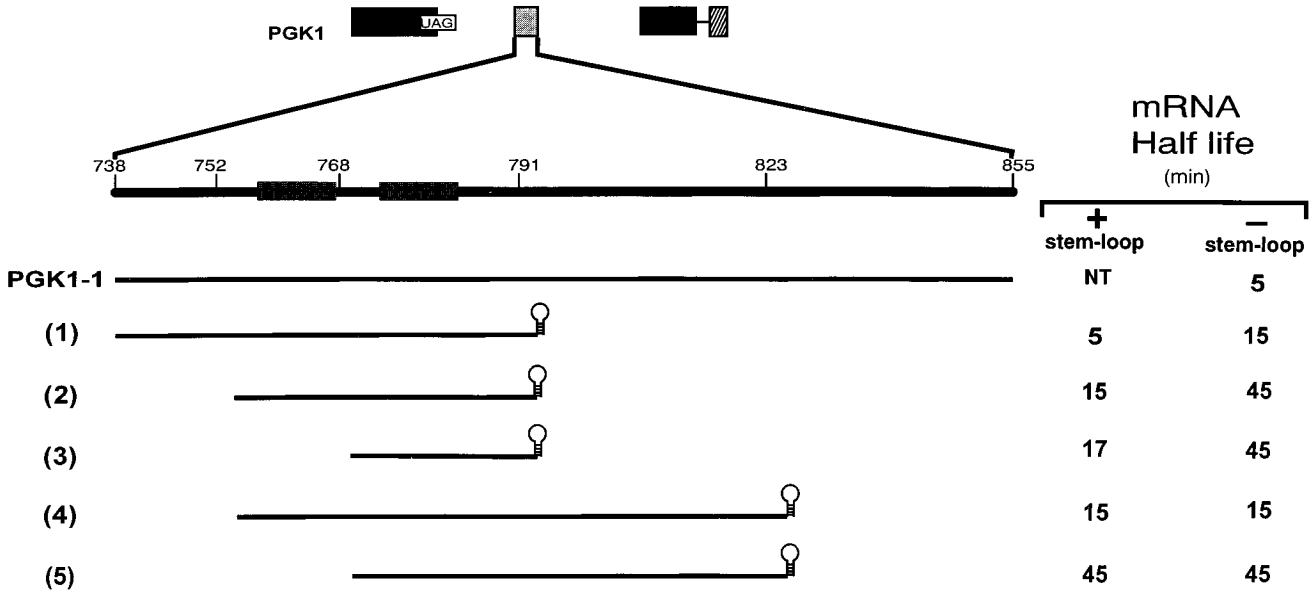

FIG. 7. Characterization of the 3′ sequences flanking the motifs in the PGK1-1 downstream element. A schematic representation of the mini-*PGK1* allele harboring the PGK1-1 downstream element is shown. The downstream element is represented by a shaded thick bar. The tag in the 3′-UTR is indicated as a thick hatched box. Below the sequence is a thick line representing the PGK1-1 downstream element (the two sequence motifs in this element are shaded), and five deletion mutants of this region in which a stem-loop structure was inserted at the 3′ boundary of the various deletion mutants as described in Materials and Methods are shown. The downstream elements containing the various deletions were inserted into the mini-*PGK1* allele 3′ of the amber codon. Half-lives for the respective PGK1 mRNAs either containing or lacking the stem-loop structure in strain RY262 were determined as described in Materials and Methods, and a summary of the data is shown. The plasmids harboring the various *PGK1* alleles are described in Table 2 and are as follows: 1, pRIPPGK1(-AU)UAGSZ-1Δ5 (without [−] stem-loop) and pRIPPGK1 (-AU)UAGSZ-1Δ5_SL (with [+] stem-loop); 2, pRIPPGK1(-AU)UAGSZ-1Δ3 (−stem-loop] and pRIPPGK1(-AU)UAGSZ-1Δ3_SL (+stem-loop); 3, pRIPPGK1 (-AU)UAGSZ-1Δ8 (−stem-loop) and pRIPPGK1(-AU)UAGSZ-1Δ8_SL (+stem-loop); 4, pRIPPGK1(-AU)UAGSZ-1Δ2 (−stem-loop] and pRIPPGK1(-AU) UAGSZ-1Δ2_SL (+stem-loop]; 5, pRIPPGK1(-AU)UAGSZ-1Δ7 (−stem-loop) and pRIPPGK1(-AU)UAGSZ-1Δ7_SL (+stem-loop].

ible mRNAs (68). The presence of this element correlates well with the rapid decay of these mRNAs. A second example is a 17-nt consensus sequence for a sequence-specific endonuclease cleavage event in the 3′-UTR of the Xlhbox2B transcript in *Xenopus* oocytes (10). On the basis of the results described above, the sequence motif described here is also a good indicator of sequences that can function as downstream elements in nonsense-mediated mRNA decay.

The sequence motif involved in nonsense-mediated mRNA decay is another example of a short RNA repeat involved in posttranscriptional regulation. Short RNA sequences that modulate both mRNA turnover and splicing have been identified (10, 27, 33, 34, 36, 37, 63, 68, 73, 74, 77). As with the results presented here, sequences flanking these motifs can influence their activity. A region in the c-*fos* 3′-UTR that is distinct from the AU-rich element and can increase the activity of either a wild-type or mutant form of the AU-rich element has been identified (12). A short RNA repeat in the alternatively spliced exon EIIIB of the rat fibronectin gene regulates its cell type expression (36). Both the cell type specificity and the degree of splicing are affected by the sequences flanking the RNA repeat (36). Thus, short RNA repeats and their flanking sequences can play significant roles in the regulation of posttranscriptional control mechanisms.

What is the role of the downstream element in nonsense-mediated mRNA decay? One possibility for the function of the downstream element may be to promote ribosome pausing, perhaps by rRNA-mRNA interactions (Fig. 1) or by promotion of an interaction with a specific factor (24, 56, 58). On the basis of this hypothesis, multiple copies of the sequence motif can function as a downstream element because a downstream element increases the potential for interaction between a ribosomal subunit or some factor and the motif. Regions flanking the sequence motif may then function by increasing the efficiency of the interaction of the ribosomal component with this sequence. For example, one copy of the sequence motif near the termination codon was able to promote nonsense-mediated mRNA decay if it contained the complete 3′ flanking sequences. We suggest that the sequences 3′ of the motif may modulate the functioning of a downstream element by promotion of stalling of a scanning ribosomal component so that it can interact with the motif. A computer analysis of the sequences 3′ of the motifs in the PGK1-1 downstream element suggests that there are at least two stem-loop structures with free energies greater than 12 kcal (ca. 50 kJ) within 20 nt of these motifs. A stem-loop structure inserted 3′ of inactive or partially active downstream elements increases the activity of this element threefold (Fig. 7). Stem-loop structures with similar free energies a short distance 3′ of a weak translation initiation site improve the translation initiation efficiency from that site (39). Thus, analogous to its role in translation initiation, an RNA secondary structure downstream of the sequence motif may cause pausing of a scanning ribosome, subunit, or other factor and allow it to interact more efficiently with the sequence motif.

An alternate role of a downstream element in nonsense-mediated mRNA decay may be to allow binding of a factor which leads to the degradation of the transcript. In this scenario, not translating these sequences unmasks the downstream element so that a factor can interact with the sequence motif. The 5′ and 3′ flanking sequences will then modulate the interaction between the factor and the element. As described above, there are several precedents demonstrating that sequences flanking an RNA binding site affect the functioning of this site.

TABLE 3. Identification of putative sequence motifs[a]

Gene[b]	Sequence[c]
Consensus	TG YYGATGYYY A T GYYGATGYYY
PGK1-0	aG gTCGATGGTCaaaaggtcaaggcttccaggaaGATGTTC
PGK1-1	TGg CTGATGTTT acA TcAaCGATGCCTTC
PGK1-2	TG CTGATGCTTTCT cT GCTGATGCC
HIS4	TG CCGATG aagA T GCCGATGTg
ADE3	Taa TTGATGTTggcaccaac tacgtT GCTGATCCTTC
RNC1	TG CaGATG T GCTGATGgT
PYC2	TG CTGATGT T GTCGATGT
BAF1	TG aCGATGa T GCTGATGCC
MCM2	G TTGATtCCTT T GTTGATGCCC
PDC5	TG CCtATGC T GCTGATGgTT
P450r	TG CTGATGT CCGATGaC
PHOSG	TG TTGATGg T GCCaATGTg
SDHZ	TG CTGATGTT T aTTGATGCCgCT
PROX	TG CTGATGT T GaTGATGaTTT
RHO4	TG CaGATG T GCTGATGgTgT
OBF1	TG aCGATG A T GCTGATGCC
HAP3	TG gCGATGCCTTCttttgtca A TGtCCGATGT
HXT3	TG CTGATGC A TTGATGC
CDC7	TG TgGATGC T GTTGATGCCT
ABF1a	TG aCGATG A T GCTGATGC
SLK1	cG CgGATGCTCC GCCGATGTTT
SSP31	cG CgGATGCTCC GCCGATGTTT
PCB	cG CCGATGTC GTTGATGTTgCC
PI3KHP	TG CTGATGCC GTTGATGTT
ERG91	G TCGATG GTaGATcCTTCT
UGA1	TG TTGATtTC T GTTGATtTTTT
GATR	TG TTGATtC T GTTGATtTTTTT
TFC1	cG TgGATGaC GTTGATGC
HIR1x	G tCCGATGTCatacc agAc aCaGATGTTGTC
ATP2	G TTtATGTTCC A GCCGATGaTTT
NADPHOR	TGg TTGATGC agt T GTCGATGCTaTT
SCB1g	Gg aTGATGTTTCg agA GCCGATGaCTTC
MSB2	G TTGATGgTT CCGATGTTTC
RPO26a	TG TCaATGTCTT cC CCaATGTTgC
ADE12	TG CTaATGCT TTGATGTT
SCS1	G TCaATG T aCCGATGCCgC
HTS1	TG CTaATGC T GCTaATGCTCT
KRE6	TG TTGATGg T GCCaATGT
CYR1	TG TCaATG GCTGATcTCTCC
ERG8	G tTCaATG GCTGATGT
SUP2	TG CCaATGTTaCC ag T GCTGATGCCTT
SPE2	G TTtATGC CCGATGTTCTT
GLU1	TG CTtATGC T GTCGATaTTgCC
HXK1	Gt TCcATG GCTGATGTgCCC
HXK2	Gt TCcATG GCCGATGTgCC
IIRU	cG TTGATGT TTGATtC
CAPk	TG tCTGATGCTC a A T TCGATGCTgCTT
SRA1	TG tCTGATGCTC a A T TCGATGCTgCTT
NIP1x	G CTGATGT agAcT CCGATGaTTCT
MDH1	G TTGATGT CTGATGaC
PFK2	G CCGATG GCaGATtTT
ACO1a	TG CTGATGCC GTTGATGTT
SUA7a	TGctTTGATGaCCTgcaa ag cTG TGATGCCCCT
PPA1	T CCGATGC TGCTGATtCCgC
GCN3	TGaaCTGATGT CTGATGCTgTT
RPA49	Ta TCGATGCC a CTGATGT
CSD2	Ta CTtATG GTTGATGCT
HAKt	Ttc TCGATGTTTTTT tttccTG CGATGCCTTC
EFT1	TG GATGTTaCTTT acA TGCCGATGCTaTCC

[a] Summary of the computer search to identify genes harboring two copies of the sequence motif. See the text for details. The DNA sequences encoding 18S rRNA and its complementary strand are as follows:

18S rRNA: 5′ 875-GGGGGCATCGGTATTC-890 3′

complementary: 3′-CCCCCGTAGCCATAAG-5′

[b] The designations and sequences of all the genes can be found in the GenBank database.

[c] Y, T or C; R, A or G. Capital letters represent nucleotides that match the motif, and lowercase letters represent nucleotides that differ from the motif.

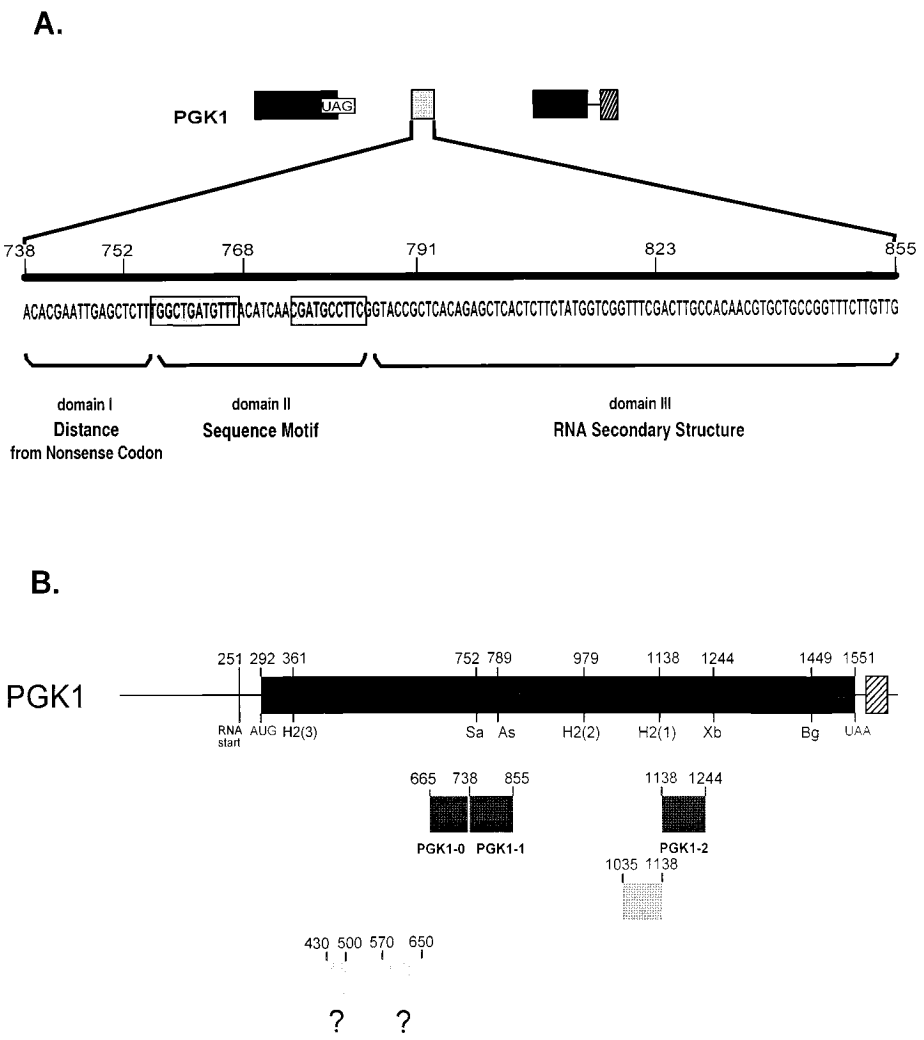

FIG. 8. A summary of the role of the sequences in a downstream element. (A) A schematic representation of the mini-*PGK1* allele harboring the PGK1-1 downstream element is shown above its sequence. The putative domains of the downstream elements are shown. The two sequence motifs in this element are shaded, are defined as domain II of the downstream element, and are thought to be critical for the activity of the downstream element. The sequences flanking domain II modulate its activity. The 5′ flanking sequences (domain I) are thought to separate the termination codon from the sequence motifs. The 3′ flanking sequences (domain III) are thought to form an RNA secondary structure which may promote pausing of ribosomes or ribosomal subunits and allow them to interact with the sequence motif. (B) A schematic representation of the *PGK1* gene containing both identified and putative downstream elements. The 5′- and 3′-UTRs of the *PGK1* gene are represented by a thin line, while its protein-coding region is represented by the thick bar. The locations of the RNA start site, the translation initiation site, various restriction sites in the coding region [H2(1) to H2(3), *Hin*cII; Sa, *Sal*I; As, *Asp*718; Xb, *Xba*I; Bg, *Bgl*II], and the translation termination sites are shown. Below the *PGK1* gene are shown regions within the protein-coding region that contain either identified or putative downstream elements. The filled-in rectangles immediately below the *PGK1* gene represent regions in the *PGK1* gene that have been demonstrated to have activity as downstream elements. The next row below has a shaded rectangle representing a region of the *PGK1* gene that contains two imperfect sequence motifs. These DNA fragments have not been tested for their activity. The third row has two stippled rectangles representing a region of the *PGK1* gene that also contains imperfect sequence motifs. The activities of these regions have also not been tested. The tag in the 3′-UTR is indicated as a thick hatched box in both panels.

A model for the mechanism of nonsense-mediated mRNA decay in *S. cerevisiae*. On the basis of previous results, we have suggested that premature translational termination leads to an altered ribonucleoprotein (RNP) structure which is a substrate for nonsense-mediated mRNA decay (24, 56). We propose that a fraction of the terminating ribosomes, ribosomal subunits, or ribosome-associated factors will scan the mRNA 3′ of the nonsense codon and interact with a downstream element. This interaction promotes an altered RNP structure that renders the mRNA susceptible to cleavage very near the termini 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. In an *xrn1*Δ strain, nearly full-length transcripts are stabilized (24, 49).

The mechanism by which nonsense-containing mRNAs are degraded appears to be similar to the decay of at least one class of wild-type mRNAs (35, 48). In this scenario, the poly(A) tract of an RNA is shortened at a particular rate until it reaches an oligo(A) form. Subsequently, the mRNA is cleaved near the 5′ end of the mRNA, removing its 5′ cap structure. The uncapped and deadenylated RNA is then degraded by the 5′→3′ Xrn1 exoribonuclease (35, 48). In the case of nonsense-mediated mRNA decay, the aberrant nonsense-containing mRNA is a substrate for decapping independently of poly(A) tail shortening (49, 69). Taken together, these results suggest

that nonsense-mediated mRNA decay is probably not a decay pathway independent from that which degrades wild-type transcripts. Rather, the decay of nonsense-containing mRNAs probably flows through the same decay pathway as that for wild-type transcripts.

Many questions concerning the mechanism of nonsense-mediated mRNA decay need to be addressed. Identifying the function of the downstream element is essential in understanding how nonsense mutations promote accelerated decay. It is important to determine whether the ribosome or ribosomal subunits bind to the downstream element or, alternatively, to identify the other factors that interact with this element. 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 and the identification of the components that interact with the downstream element.

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