1996-07-01

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Et al.

MOLECULAR AND CELLULAR BIOLOGY, July 1996, p. 3833–3843 Vol. 16, No. 7
0270-7306/96/$04.00+0

Functional Mapping of the Translation-Dependent Instability Element of Yeast *MATα1* mRNA

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Received 8 February 1996/Returned for modification 19 March 1996/Accepted 4 April 1996

The determinants of mRNA stability include specific *cis*-acting destabilizing sequences located within mRNA coding and noncoding regions. We have developed an approach for mapping coding-region instability sequences in unstable yeast mRNAs that exploits the link between mRNA translation and turnover and the dependence of nonsense-mediated mRNA decay on the activity of the *UPF1* gene product. This approach, which involves the systematic insertion of in-frame translational termination codons into the coding sequence of a gene of interest in a *upf1Δ* strain, differs significantly from conventional methods for mapping *cis*-acting elements in that it causes minimal perturbations to overall mRNA structure. Using the previously characterized *MATα1* mRNA as a model, we have accurately localized its 65-nucleotide instability element (IE) within the protein coding region. Termination of translation 5′ to this element stabilized the *MATα1* mRNA two- to threefold relative to wild-type transcripts. Translation through the element was sufficient to restore an unstable decay phenotype, while internal termination resulted in different extents of mRNA stabilization dependent on the precise location of ribosome stalling. Detailed mutagenesis of the element's rare-codon/AU-rich sequence boundary revealed that the destabilizing activity of the *MATα1* IE is observed when the terminal codon of the element's rare-codon interval is translated. This region of stability transition corresponds precisely to a *MATα1* IE sequence previously shown to be complementary to 18S rRNA. Deletion of three nucleotides 3′ to this sequence shifted the stability boundary one codon 5′ to its wild-type location. Conversely, constructs containing an additional three nucleotides at this same location shifted the transition downstream by an equivalent sequence distance. Our results suggest a model in which the triggering of *MATα1* mRNA destabilization results from establishment of an interaction between translating ribosomes and a downstream sequence element. Furthermore, our data provide direct molecular evidence for a relationship between mRNA turnover and mRNA translation.

mRNA turnover is a regulated process that is essential to the course of gene expression and dependent on specific *cis*-acting sequences and *trans*-acting factors (28, 40, 44, 45). In *Saccharomyces cerevisiae*, as in mammalian cells, one major class of sequences that regulates mRNA decay rates also promotes poly(A) shortening, a rate-limiting event for the turnover of many mRNAs (for reviews, see references 7, 10, 28, 49). Conventional mapping of such instability elements (IEs) involves the construction of chimeric genes, composed of segments encoding both stable and unstable mRNAs, and the analysis of in vivo decay rates of the resulting chimeric mRNAs. This approach in *S. cerevisiae*, combined with deletion and mutational analyses, has successfully localized instability determinants to the coding regions of the *MATα1* (6, 39), *HIS3* (22), *STE3* (19), *SPO13* (56), and *RPL2* (48) mRNAs, the 3′ untranslated regions of the *STE3* (19) and *MFA2* (38) mRNAs, and the 5′ untranslated regions of the *PPR1* (47) and *SDH2* (9) mRNAs.

Here, we report a new method for mapping coding region IEs in inherently unstable mRNAs. Development of the new approach was made possible by the identification of gene products required for nonsense-mediated mRNA decay (11, 17, 31, 32, 33, 41, 43) and prior demonstration of an intimate link between mRNA decay and translation. The latter is exemplified by the location of some IEs to mRNA coding regions (6, 19, 22, 54), the accelerated degradation of mRNAs promoted by premature translational termination (16, 34, 41), the association of *trans*-acting degradation factors with ribosomes (2, 4, 8, 46), and the stabilization of mRNAs that occurs when translation is inhibited (3, 5, 23, 28, 42, 49, 57). Of particular relevance to the present study were earlier experiments which showed that normally unstable chimeric *PGK1-MATα1* and *ACT1-MATα1* mRNAs were stabilized when an in-frame translational termination codon was inserted at the junction of the sequences from the respective stable and unstable mRNA components of the chimeras (39). Since *MATα1* IE activity required its translation, we hypothesized that translation-dependent IEs could, in general, be mapped by insertion of in-frame nonsense codons. Those inserted 5′ to the element should stabilize the transcript, while downstream codons should be inconsequential to mRNA half-life. To circumvent activation of the nonsense-mediated mRNA decay pathway, half-lives of allelic transcripts could be measured in a strain deficient for Upf1p, a *trans*-acting factor essential for activity of this decay pathway (32, 33, 41).

We have used the *MATα1* gene as a model to test the feasibility of nonsense codon mapping since the precise coding sequence location of its 65-nucleotide (nt) IE has been defined (6, 39). In addition to mapping the element, this method has allowed us to identify a boundary for the translation dependence of element function, thus providing insight into the role of the IE as a destabilizer of the *MATα1* transcript. Application of the new mapping protocol to other genes should facilitate localization of potential coding-region IEs and thereby

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TABLE 1. Plasmids used in this study

Plasmid	Description
pGAL*MATα1*	*MATα1* gene fused to *GAL1* promoter on yeast centromeric plasmid
pGAL*MATUAG18*	Same as pGAL*MATα1* except with nonsense codon at position 18
pGAL*MATUAG52*	Contains nonsense codon at position 52
pGAL*MATUAG67*	Contains nonsense codon at position 67
pGAL*MATUAG76*	Contains nonsense codon at position 76
pGAL*MATUAG77*	Contains nonsense codon at position 77
pGAL*MATUAG78*	Contains nonsense codon at position 78
pGAL*MATUGA79*	Contains nonsense codon at position 79
pGAL*MATUAG80*	Contains nonsense codon at position 80
pGAL*MATUAG81*	Contains nonsense codon at position 81
pGAL*MATUAG82*	Contains nonsense codon at position 82
pGAL*MATUAG83*	Contains nonsense codon at position 83
pGAL*MATUAG84*	Contains nonsense codon at position 84
pGAL*MATUAG85*	Contains nonsense codon at position 85
pGAL*MATUAG139*	Contains nonsense codon at position 139
pGAL*MATΔ82*	Same as pGAL*MATα1* except with deletion of codon 82
pGAL*MATUAG78Δ*	Same as pGAL*MATUAG78* except with deletion of codon 82
pGAL*MATUGA79Δ*	Same as pGAL*MATUGA79* except with deletion of codon 82
pGAL*MATUAG80Δ*	Same as pGAL*MATUAG80* except with deletion of codon 82
pGAL*MATUAG81Δ*	Same as pGAL*MATUAG81* except with deletion of codon 82
pGAL*MATi82*	Same as pGAL*MATα1* except with insertion of extra codon 82
pGAL*MATUAG78i*	Same as pGAL*MATUAG78* except with insertion of extra codon 82
pGAL*MATUGA79i*	Same as pGAL*MATUGA79* except with insertion of extra codon 82
pGAL*MATUAG80i*	Same as pGAL*MATUAG80* except with insertion of extra codon 82
pGAL*MATUAG81i*	Same as pGAL*MATUAG81* except with insertion of extra codon 82

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5′ to 3′)
MATUAG18	AAG CAT CCA AAT CAT AGA GAA ACA CAG CGG
MATUAG52	TCA AGA AAG ATA TCT AGA TTC CTG TTC CTT
MATUAG67	ATA AAA TCC AAA TTT AGA GGA TAG CGT CTG
MATUAG76	CTG GAA GTC AAA ATT AGC AGT TTC GAC AGT
MATUAG77	GAA GTC AAA ATA CTT AGT TTC GAC AGT TCA
MATUAG78	GTC AAA ATA CTC AGT AGC GAC AGT TCA ATA
MATUGA79	AAA ATA CTC AGT TTT GAC AGT TCA ATA AGA
MATUAG80	ATA CTC AGT TTC GAT AGT TCA ATA AGA CAT
MATUAG81	CTC AGT TTC GAC AGT AGA ATA AGA CAT CTA
MATUAG82	AGT TTC GAC AGT TCT AGA AGA CAT CTA TAA
MATUAG83	TTC GAC AGT TCA ATT AGA CAT CTA TAA AAT
MATUAG84	GAC AGT TCA ATA AGT AGT CTA TAA AAT CTT
MATUAG85	AGT TCA ATA AGA CAT AGA TAA AAT CTT CAA
MATUAG139	ACT ACT TCG CGC AAT AGT ATA ATT TTA TAA
MATΔ82	CAG TTT CGA CAG TTC AAG ACA TCT ATA AAA
MATUAG78Δ	CAG TAG CGA CAG TTC AAG ACA TCT ATA AAA
MATUGA79Δ	CAG TTT TGA CAG TTC AAG ACA TCT ATA AAA
MATUAG80Δ	CAG TTT CGA TAG TTC AAG ACA TCT ATA AAA T
MATUAG81Δ	CAG TTT CGA CAG TAG AAG ACA TCT ATA AAA T
MATi82	CAG TTT CGA CAG TTC AAT AAT AAG ACA TCT ATA
MATUAG78i	CAG TAG CGA CAG TTC AAT AAT AAG ACA TCT ATC
MATUGA79i	CAG TTT TGA CAG TTC AAT AAT AAG ACA TCT ATA
MATUAG80i	CAG TTT CGA TAG TTC AAT AAT AAG ACA TCT ATA
MATUAG81i	CAG TTT CGA CAG TAG AAT AAT AAG ACA TCT ATA
MATAH01	TTG AAG ATT TTA TAG ATG TC
MATAH02	GCG AGC AGA GAA GAC AAG AC
RP-18	CAA GAG CAA GAC GAT GGG GAG

provide further understanding of the *cis*-acting determinants of mRNA stability.

MATERIALS AND METHODS

Yeast strains and plasmids. The yeast strains used in this study were yRP582 (*MAT***a** *rpb1-1 ura3-52 leu2-3,112*; provided by C. Decker and R. Parker, University of Arizona, Tucson) and yAH01, which is isogenic to yRP582 except that it contains a disruption of *UPF1* (*upf1*Δ). Disruption of the chromosomal *UPF1* gene in yAH01 was accomplished by using a plasmid-borne *upf1*::*LEU2* allele constructed by standard techniques (35) from pRS314*UPF1*. The latter contained the *UPF1* gene on a 4.2-kb *Eco*RI-*Bam*HI DNA fragment (33) subcloned into the yeast shuttle vector pRS314 (55). pRS314*UPF1* was digested with *Bgl*II to remove a 1.6-kb sequence from the *UPF1* gene, and a *LEU2* gene from plasmid pJJ250 was cloned into the *Bgl*II sites of the resulting plasmid (29). To facilitate cloning of the *LEU2* marker, pJJ250 was digested with *Hin*dIII, which cuts once, 3′ of *LEU2*. The 5′ overhangs were filled in with Klenow enzyme (Boehringer Mannheim Biochemicals), and a *Bgl*II linker (New England Biolabs) was ligated. This DNA was digested with *Bgl*II and *Bam*HI, and the *LEU2* containing fragment was then ligated to the plasmid carrying the partial deletion of the *UPF1* gene. Transformation of yRP582 with a 4.4-kb *Xho*I-*Bam*HI fragment from pRS314*upf1*::*LEU2* was followed by Southern analysis of genomic DNA to check for *UPF1* disruption. Disruption was also monitored by Northern (RNA) blotting to detect stabilization of the nonsense-containing *CYH2* premRNA and hence the presence of a nonsense decay phenotype (reference 18 and data not shown).

Table 1 lists plasmids containing wild-type and allelic *MATα1* genes which differ by the presence or absence of a single in-frame translational termination codon and/or the deletion or insertion of an AAT triplet at codon 82. Plasmids containing an in-frame translational termination codon in the *MATα1* sequence are numbered according to the codon changed. The plasmids are all derived from pGAL*MATα1* (provided by G. Caponigro and R. Parker), in which a *GAL1* promoter fused to a *MATα1* gene was ligated to *Eco*RI-*Hin*dIII-cut pSEH.BX (a derivative of pRIP1H [39] in which *Xho*I, *Hin*dIII, and *Bgl*II sites were replaced by *Pvu*I, *Nhe*I, and *Cla*I sites, respectively). They were transformed into strains yRP582 and yAH01 by using a modification of the lithium acetate method of yeast transformation (53), and synthetic medium lacking uracil was used for their selection and maintenance.

Site-directed mutagenesis. The insertion of in-frame nonsense codons into the *MATα1* coding sequence was performed by using a modification of the procedure described by Kunkel et al. (30). A *Bam*HI-*Hin*dIII fragment harboring the *MATα1* gene from pGAL*MATα1* was cloned into the polylinker of pBluescriptII KS+ phagemid (Stratagene), and this construct transformed into *Escherichia coli* CJ236 (*dut-1 ung-1*). R408 helper phage (Promega) was used for synthesis of single-stranded phagemid DNA and was infected into cells at a ratio of five phage per cell. Approximately 400 ng of uracil-containing single-stranded DNA isolated from infected cells was used per in vitro mutagenesis reaction performed with a commercially available mutagenesis kit (Bio-Rad). Second-strand DNA synthesis was primed by a mutagenic oligonucleotide complementary to the sequence being mutated except for a 1- to 3-nt mismatch, depending on the oligonucleotide used (Table 2). All oligonucleotides were either purchased from

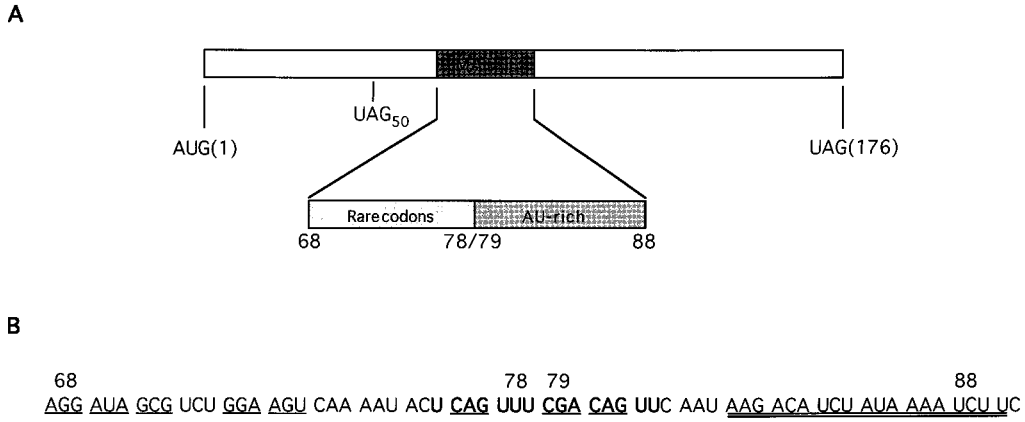

FIG. 1. Schematic of the *S. cerevisiae MATα1* coding region. (A) Locations of the 65-nt IE and the normal sites of translation initiation [AUG(1)] and termination [UAG(176)]. The two parts of the element, a 5′ 33-nt sequence enriched for rare codons and a 3′ 32-nt AU-rich sequence (6), are indicated. UAG_{50} depicts the location, relative to the IE, of the in-frame translational termination codon inserted at *PGK1-MATα1* and *ACT1-MATα1* hybrid fusion junctions generating stabilized chimeric transcripts (39). (B) Primary sequence of the 65-nt element. Codons 78 and 79 define the 3′- and 5′-most codons of the rare codon and AU-rich sequences, respectively (6). Rare codons are indicated by a single underline, and double underlines mark the 19-nt sequence within the AU-rich span postulated to be a specific protein binding site (6). Sequences with complementarity to 18S rRNA (45) are indicated in boldface. All numerals refer to codon numbers.

Operon Technologies, Inc., or synthesized on an Oligo 1000 DNA synthesizer (Beckman). Replicative-form DNAs were transformed into *E. coli* MV1190, which selects for the mutated, non-uracil-containing DNA strand. Plasmids were sequenced over the region being mutated by the dideoxy-chain terminator method (52), using a Sequenase version 2.0 DNA sequencing kit (USB/Amersham) and the sequencing primers *MATAH01*, *MATAH02*, and *RP-18* (Table 2). Those with desired termination codons were digested to remove the allelic *MATα1* DNAs, and the *Bam*HI-*Hin*dIII fragments were subcloned into the original yeast vector downstream of the *GAL1* promoter.

For construction of *MATα1* alleles having a deletion or extra copy of codon 82 (AAT), the *MATα1* sequences of the nonsense-containing alleles *MATUAG78*, *MATUGA79*, *MATUAG80*, and *MATUAG81* were subcloned into the vector M13mp19 (36). Recombinant phage were plaqued on *E. coli* JM109, and after verification of the presence of an insert, phage stocks were prepared. These phage were used to infect *E. coli* CJ236, and the uracil-containing single-stranded DNA was isolated. Mutagenesis was performed with the appropriate oligonucleotides (Table 2) essentially as described for the nonsense codon insertion experiments.

mRNA synthesis and decay measurements. Transcriptional pulse-chase experiments were performed essentially as described by Decker and Parker (12) except that galactose inductions were performed for 30 min and medium containing 4% glucose was preheated to 59°C. Cells harvested both prior to induction and at various times with respect to inhibition of transcription were concentrated, frozen rapidly on dry ice, and stored at −70°C. Total cellular RNA was extracted by the hot phenol method, and mRNA decay rates were measured as described elsewhere (18). RNA fractionated by electrophoresis was transferred to Zeta probe membranes (Bio-Rad), and blots were probed to detect *MATα1* and *STE2* transcripts. Fragments of the *MATα1* (0.5-kb *Bam*HI-*Hinc*II fragment) and *STE2* (2.3-kb *Sal*I fragment) genes were labeled by a random priming procedure (15), using a kit from Boehringer Mannheim Biochemicals. Northern blots were quantitated by using a Betascope blot analyzer. Half-life values were obtained by expressing data as the $\log_{10}$ of the percentage of each mRNA remaining versus time at 36°C. All values represent the average ±1 standard deviation of at least two half-life determinations, using two independently isolated transformants.

Analysis of a second *MATα1* transcript of lower molecular weight. To determine the nature of a potential *MATα1* decay intermediate, DNA fragments with specificity for the 5′ and 3′ termini of the full-length transcript were generated. A 0.5-kb *Bam*HI-*Hinc*II fragment spanning the *MATα1* coding sequence, with the exception of the 3′-terminal 70 nt, was digested with *Hae*III, yielding a 161-bp 5′ complementary fragment. Digestion of the 0.5-kb DNA with *Msp*I generated a 3′ complementary sequence of 136 bp. Digests were electrophoresed in 1.5% agarose, and DNA fragments of interest were purified by using ultrafree-MC filter units (Millipore Corp., Bedford, Mass.) and labeled by the random priming protocol.

Purification and fractionation of poly(A)$^+$ RNA were performed by using oligo(dT)-cellulose columns as previously described (27). Oligo(dT)-cellulose, type 3, was purchased from Collaborative Research Inc., Lexington, Mass.

RESULTS

In-frame translational termination codons map the *MATα1* IE to its coding sequence location. The *MATα1* mRNA is inherently unstable, having a half-life of approximately 5 min (23, 39, 45). Previous analyses of chimeric genes identified *MATα1* coding sequences conferring mRNA-destabilizing activity (39), and subsequent studies defined a 65-nt IE spanning nt 201 to 266 as the minimal sequence capable of destabilizing an mRNA (6). The element, whose location within the *MATα1* coding region is depicted in Fig. 1A, is bipartite. The first 33 nt are highly enriched for rare codons, while the following 32 nt are predominantly AU rich. Both sequences are necessary for element activity (6) and are shown in Fig. 1B. To establish the feasibility of our new approach to mapping coding region instability elements, we first determined the effect that inserting in-frame nonsense codons at positions 18, 52, and 139 had on *MATα1* mRNA half-life (Fig. 2A). The first two nonsense codons precede the IE, whereas that at position 139 follows the IE. Transcription of allelic *MATα1* mRNAs was regulated by fusing all constructs to a *GAL1* promoter. Following a 30-min induction in medium containing galactose, transcription was terminated by the combined effects of glucose repression and thermal inactivation of RNA polymerase II, and mRNA half-lives were measured in both *UPF1* and *upf1Δ* isogenic strains. In all experiments, inhibition of transcription was monitored by following decay of the unstable *STE2* mRNA, whose half-life of 5 to 6 min is comparable to that of the wild-type *MATα1* mRNA (23).

In the *upf1Δ* strain, in which nonsense-mediated mRNA decay is blocked, nonsense codons at positions 18 and 52 stabilized the *MATα1* transcript to half-lives of ~13.8 and ~12.5 min, respectively (Fig. 2A and B). However, if ribosomes were allowed to translate to codon 139, the mRNA remained unstable, with a wild-type half-life of ~5.2 min (Fig. 2A and B). In all strains, the *STE2* mRNA had a half-life of ~5 to 6 min (Fig. 2A and C), hence ruling out the possibility that extended half-lives for the *MATUAG18* and *MATUAG52* alleles were a consequence of incomplete inhibition of transcription. These initial results demonstrated the feasibility of nonsense codon insertion as a method to localize an IE within the coding sequence and were in agreement with a previous observation that an in-frame translational termination codon 5′ to the IE stabilizes the encoded mRNA (39).

In the *UPF1* background, the allelic *MATα1* transcripts were very unstable, with half-lives of ~1.0 min, except for

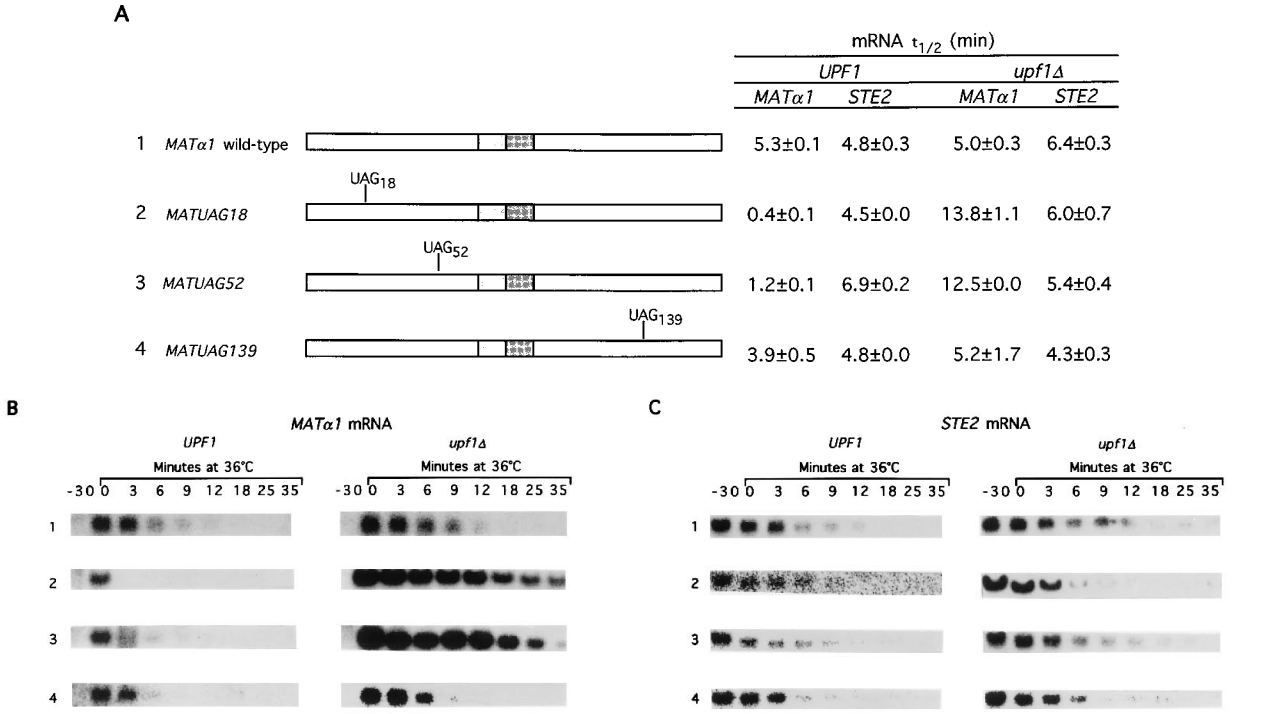

FIG. 2. Effects of early and late nonsense codons on the stability of the *MATα1* mRNA. (A) Schematics and mRNA half-lives for the wild-type *MATα1* gene and three nonsense alleles. The locations of single in-frame translational termination codons relative to the bipartite IE (shaded regions) are shown. *MATα1* alleles are named according to the nucleotide triplet changed to a nonsense codon. mRNA half-lives were obtained for the *STE2* transcripts and the wild-type and allelic *MATα1* mRNAs in *UPF1* and *upf1*Δ strains as described in Materials and Methods. (B) Northern blots depicting decay of wild-type and allelic *MATα1* mRNAs in *UPF1* and *upf1*Δ strains. Blots are numbered according to the *MATα1* alleles shown in panel A. Cells were grown to early log phase (optical density at 600 nm of ~0.3) and induced for expression of *MATα1* from a *GAL1* promoter by addition of 2% galactose. Following a 30-min induction, transcription was terminated rapidly by simultaneously shifting cells to 36°C and adding glucose to a final concentration of 2%. Cells were harvested for RNA isolation prior to induction (−30) and at the indicated times with respect to shifting to 36°C. (C) Northern blots depicting decay of the *STE2* mRNA in all strains in which *MATα1* mRNA half-lives were determined. Blot numbers indicate those strains containing the correspondingly labeled *MATα1* allele.

MATUAG139 whose half-life of ~4.0 min more closely resembled the wild-type mRNA half-life (Fig. 2A and B). The highly unstable nature of the mRNAs with early nonsense codons is indicative of activation of the nonsense-mediated mRNA decay pathway (41, 43, 45, 46).

Termination of translation within the IE yields mRNAs of different stabilities dependent on the extent of ribosome translocation. To develop nonsense codon insertion as a general approach for mapping coding region IEs, we sought to understand the consequences on mRNA stability of inhibiting translation within an IE. We therefore inserted in-frame translational terminators at codons 67, 79, and 85 of the *MATα1* gene (Fig. 3A) and measured the resulting mRNA half-lives. *MATUAG67* terminates translation immediately 5′ to the IE; *MATUGA79* changes the first codon in the 3′ 32-nt AU-rich sequence to a terminator, such that ribosomes translate only the rare-codon segment of the IE; and *MATUAG85* terminates translation after ribosomes progress through 60% of the AU-rich sequence, equivalent to 80% of the entire element. Ribosome stalling at codons 67 and 79 yielded transcripts with half-lives of 9.8 and 9.4 min, respectively, in the *upf1*Δ strain, indicating an approximately twofold stabilization relative to wild-type mRNA decay (Fig. 3). However, translation to codon 85 yielded an mRNA half-life of ~4 min, indicating that ribosome progression had been sufficient to promote normal decay of the *MATα1* transcript (Fig. 3). mRNA stabilization arising

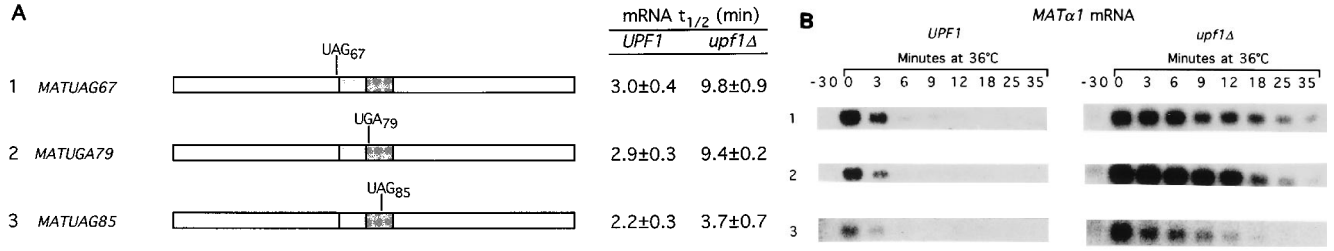

FIG. 3. Effects of inhibiting translation within and immediately before the IE. (A) Locations of single in-frame translational termination codons within the *MATα1* coding region and summary of the half-life data obtained for the *MATα1* mRNA in *UPF1* and *upf1*Δ strains. *MATUAG67* terminates translation at the codon adjacent to the IE's 5′-most codon. *MATUGA79* changes the first codon in the mapped AU-rich sequence, hence allowing ribosomes to translate the rare-codon sequence. *MATUAG85* allows translation of approximately 80% of the entire IE. (B) Northern blots showing decay of the *MATα1* transcripts depicted in panel A in *UPF1* and *upf1*Δ strains. Blots are numbered according to the allele present. RNA pulse-chase experiments were performed as described in the legend to Fig. 2.

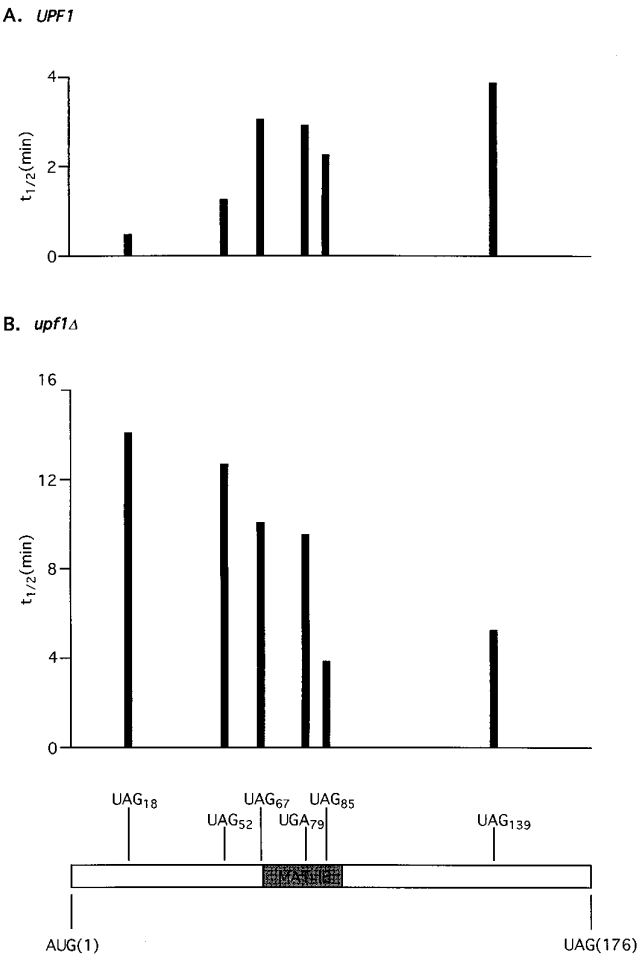

FIG. 4. Summary of the consequences of premature translational termination on the decay of the *MATα1* mRNA. Shown are positions of the *MATα1* nonsense mutations analyzed in the experiments of Fig. 2 and 3 and the decay rates of the respective transcripts in *UPF1* and *upf1*Δ strains.

from partial translation of the element, as in the case of translating only the rare-codon sequence in *MATUGA79*, is consistent with a previous report in which deletion of the 3′ AU-rich portion of the element stabilized the transcript (6) and indicates that both segments of the 65-nt element are necessary for mRNA instability.

Half-life values for these three transcripts in the *UPF1* genetic background reflected the overall conclusion gained from the *MATUAG18* and *MATUAG52* alleles. While the transcripts of the current alleles were found to be slightly more stable (mRNA half-lives of ~2 to 3 min) than those with more 5′-proximal nonsense codons (compare Fig. 3A with Fig. 2A), they were still significantly less stable than the wild-type transcript (Fig. 3A), reflecting at least partial activation of the nonsense-mediated mRNA decay pathway. Moreover, the half-life of the *STE2* mRNA, measured in all strains, did not fluctuate significantly (data not shown), indicating that the differences observed in the decay of the respective *MATα1* mRNAs were not attributable to variations in the degree of transcriptional inhibition.

Destabilization of the *MATα1* transcript is mediated by translation of a two-codon interval of the IE's AU-rich sequence. Half-lives of the six *MATα1* transcripts analyzed in the experiments of Fig. 2 and 3 are summarized in Fig. 4. Most striking is the abrupt nature of the transition to slower mRNA decay rates in the *UPF1* strain (Fig. 4A) and the transition to more rapid mRNA decay in the *upf1*Δ strain (Fig. 4B). The former phenomenon has been observed previously for the *PGK1*, *CYC1*, and *HIS4* mRNAs (16, 32, 41, 59) and may reflect the existence of *cis*-acting sequences capable of inactivating nonsense-mediated mRNA decay (41, 43). The sudden, twofold decrease in mRNA stability that occurred as ribosomes traversed the IE in the *upf1*Δ strain (Fig. 4B) was unanticipated, however, and we were interested in mapping more precisely this stability transition. To this end, constructs with single, in-frame UAG translational termination codons at positions 76, 77, 78, 80, 81, 82, 83, and 84 were constructed by oligonucleotide site-directed mutagenesis (Fig. 5A). This set of constructs, together with *MATUGA79* and *MATUAG85* (Fig. 3A), allows ribosomes translating the respective mRNAs to progress through the IE in increments of one codon. The effects of such ribosome progression on mRNA decay rates were determined in the *UPF1* and *upf1*Δ isogenic strains.

In the *upf1*Δ strain, half-lives of ~9 to 10 min were obtained for the allelic *MATUAG76*, *MATUAG77*, and *MATUAG78* mRNAs (Fig. 5 and 6), similar to the previously determined value for the *MATUGA79* transcript (Fig. 3A and 4B), and an intermediate half-life (~7.5 min) was obtained for the *MATUAG80* transcript (Fig. 5 and 6). In contrast, the *MATUAG81*, *MATUAG82*, *MATUAG83*, and *MATUAG84* mRNAs were unstable, with half-lives of 4 to 5 min (Fig. 5 and 6), similar to that determined for the *MATUAG85* allele (Fig. 3 and 4B). These results are summarized in a bar plot of the half-lives obtained (Fig. 6), which clearly indicates a transition in mRNA stability as translation proceeds over a two-codon interval of the IE, codons 80 and 81. The transition region is bordered by an upstream segment wherein translation termination stabilizes the mRNA and by a downstream segment in which translation termination has no effect on mRNA decay. The results demonstrate at the molecular level a clear relationship between mRNA stability and mRNA translation, supporting previous studies that have linked the two processes (6, 16, 19, 22, 23, 33, 40, 41, 44, 53). Interestingly, a second inducible *MATα1* transcript of ~370 nt was detected in RNA isolated from cells harboring the *MATUAG84* allele (indicated with an asterisk in Fig. 5B). Its characterization is described below.

Decay measurements of these allelic *MATα1* mRNAs in the *UPF1* strain yielded half-lives of ~2 to 3 min (Fig. 5A), comparable to the values obtained for the alleles *MATUAG67*, *MATUGA79*, and *MATUAG85* (Fig. 3A), whose nonsense codons are also located in the vicinity of the stability transition sequence. Half-life values on the general order of ~6 min were obtained for the *STE2* transcript in all strains in which decay of the allelic *MATα1* mRNAs was measured (data not shown), again confirming that transcription was inhibited efficiently.

Deletion or insertion of a single codon shifts the position of the *MATα1* mRNA stability transition region. The experiments of Fig. 5 and 6 demonstrated a transition in *MATα1* mRNA half-life as ribosomes translated codons 80 and 81 of the IE. One possibility was that this transition reflected a requirement for the ribosome (or a ribosome-borne factor) to interact with a downstream sequence or bound factor and that this interaction provided the signal for rapid decay of the transcript. Since it had previously been demonstrated that the 65-nt IE contains all sequence information required for the promotion of mRNA instability (6), a corollary of the previous hypothesis was that the sequences with which the ribosome interacted were probably within the 3′ portion of the IE, i.e., the AU-rich region. If so, deletion or insertion of 3 nucleotides

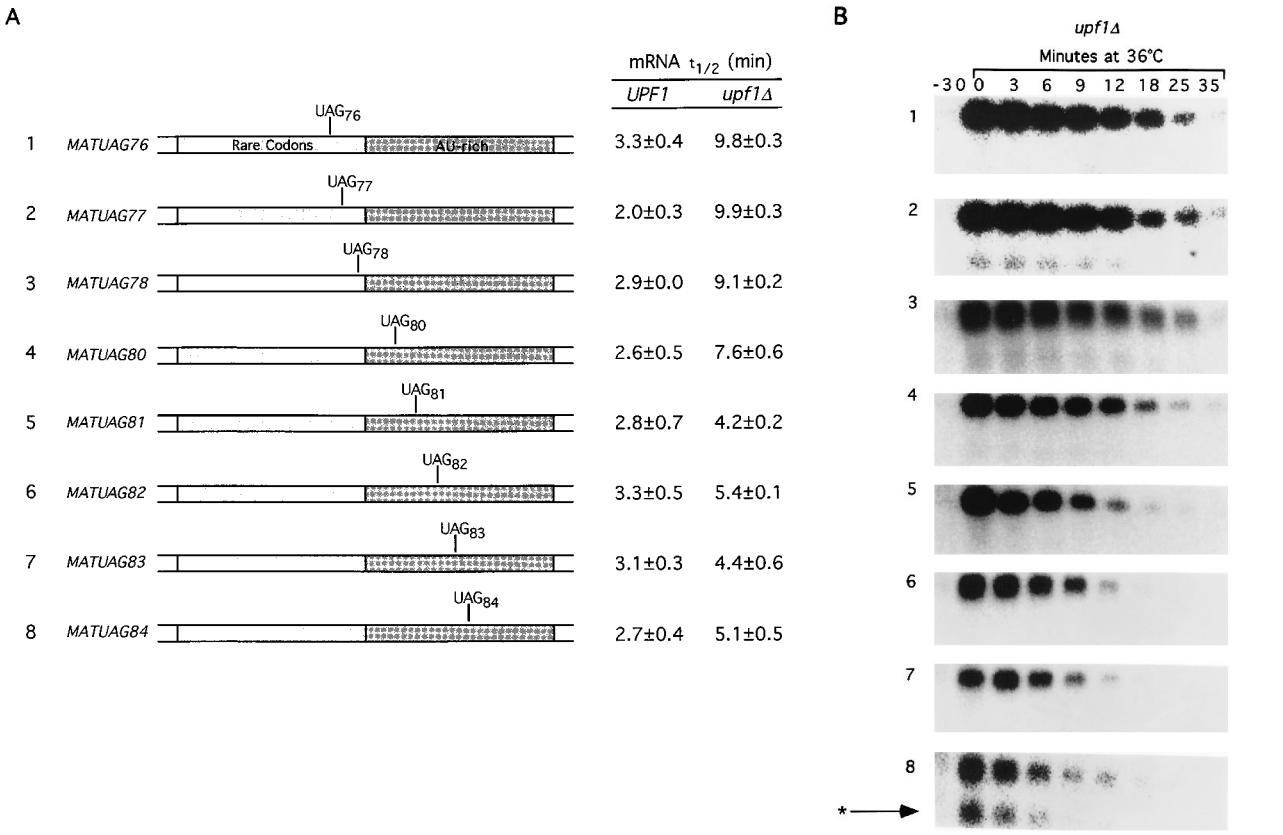

FIG. 5. Mutagenesis of the rare-codon/AU-rich sequence boundary of the *MATα1* IE. (A) Schematic of the eight allelic forms of the IE (each of which contains a single in-frame translational termination codon) and the mRNA half-lives obtained for the respective transcripts in both *UPF1* and *upf1*Δ strains. Successive constructs, together with the alleles *MATUGA79* and *MATUAG85* (Fig. 3A), allow ribosomes to translate the *MATα1* IE in one-codon increments. (B) Decay of the eight nonsense-containing *MATα1* transcripts as measured in the *upf1*Δ strain. Blot numbers correspond to the alleles shown in panel A, and RNA pulse-chase experiments were performed as described in the legend to Fig. 2. A second mRNA produced from the *MATUAG84* construct and marked with an asterisk (blot 8) is the 5′ *MATα1* species (see text and Fig. 8).

3′ to the transition should shift the stability profile in a predictable manner.

To test this model, we constructed a set of *MATα1* alleles in which nonsense mutations before, within, and after the transition were accompanied by deletion or duplication of a downstream codon. These alleles, depicted in Fig. 7A, are identical to the nonsense-containing alleles *MATUAG78*, *MATUGA79*, *MATUAG80*, and *MATUAG81* except that they also contain either a deletion or a duplication of codon 82. The choice of codon 82 for deletion or duplication was dictated by several considerations, including the following: (i) we sought to minimize disruption of the 19-nt sequence that begins at codon 82 because it is reiterated immediately downstream (with 14 of 19 nt being identical; see reference 6) and postulated to serve as a protein recognition site, possibly linked to transcript decay (6); and (ii) deletion/insertion at codon 82 also avoids those sequences specifically defining the stability transition region (codons 79 to 81) and those sequences of the IE having complementarity to 18S rRNA (Fig. 1B). It is conceivable that interference with any one of these nucleotide stretches could alter the destabilizing mechanism mediated by ribosome translocation.

Half-lives of the *STE2* mRNA and the transcripts of the *MATα1* deletion/insertion alleles were determined, as before, in both *UPF1* and *upf1*Δ strains. The short half-life of the *STE2* mRNA in all experiments (~4 to 7 min) indicated that transcription was inhibited efficiently (data not shown). Control constructs containing either a deletion (*MATΔ82*) or an insertion (*MATi82*) of codon 82 in the wild-type *MATα1* gene (Fig. 7A) were tested to establish the effects of these changes on transcript half-life. In *UPF1* and *upf1*Δ strains, both constructs produced mRNAs with a half-life of approximately 5 min, equivalent to that of wild-type *MATα1* mRNA (Fig. 7A). This result eliminates a role for this nucleotide triplet in IE-destabilizing activity.

In the *UPF1* strain, transcripts of all eight *MATα1* deletion/insertion mutants also containing a nonsense codon had half-lives of ~2.5 to 4 min (Fig. 7A), again consistent with activation of nonsense-mediated mRNA decay triggered by the respective in-frame translational termination codons. The half-lives obtained for these mutant transcripts in the *upf1*Δ strain are listed in Fig. 7A and compared in Fig. 7B to D. To establish the relationship with the parent constructs, half-lives of the four nonsense-containing mRNAs that do not have an insertion or deletion of codon 82 are illustrated in Fig. 7B. In the deletion mutants (Fig. 7C), the stability transition shifted in the 5′ direction. Compared with the original nonsense-containing transcripts, deletion of codon 82 reduced the half-lives of the *MATUGA79* and *MATUAG80* transcripts by 2.7 and 1.9 min, respectively, and had only minor effects on the half-lives of the *MATUAG78* and *MATUAG81* mRNAs (compare Fig. 7B with Fig. 7C; see also Fig. 3A, 5A, and 7A). These results support a model in which the destabilizing effects of a ribosome interaction with a downstream element have occurred three nucleo-

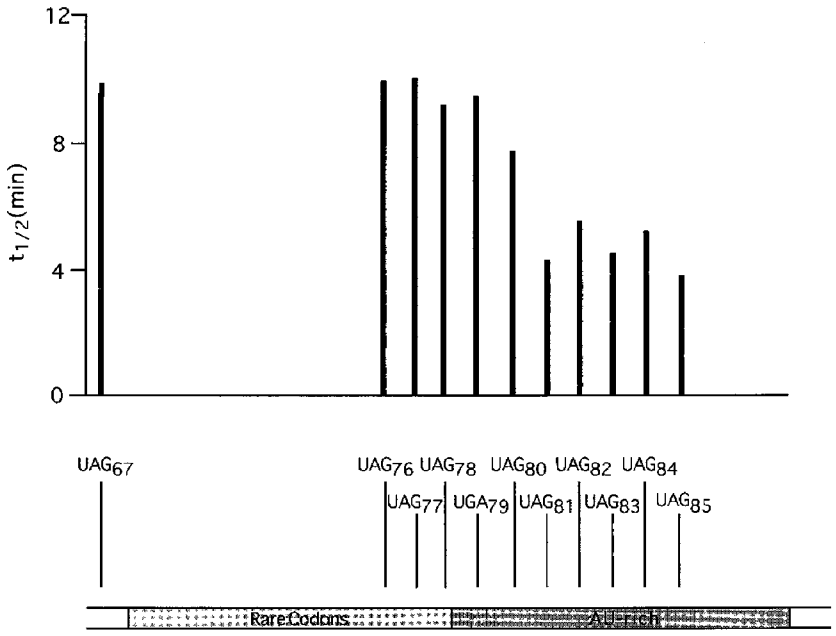

FIG. 6. Locations of in-frame translational termination codons in the *MATα1* IE versus mRNA half-life measured in the *upf1Δ* strain (summary of the data in Fig. 3 and 5). The translation-dependent stability transition is bordered by codons 79 and 81.

tides "earlier" when codon 82 is absent. Such a model of ribosome interaction with a downstream element is also supported by the results of the experiments with the codon 82 insertion mutants (Fig. 7D). The insertion of an extra codon 82 resulted in a complementary shift; i.e., the stability transition was shifted by one codon 3′ to its original location. Thus, the half-lives of the *MATUAG78* and *MATUGA79* mRNAs were largely unaffected by codon 82 duplication, but the *MATUAG80* and *MATUAG81* mRNAs increased in half-life by 1.5 and 1.9 min, respectively (compare Fig. 7B with Fig. 7D).

Characterization of the *MATUAG84* 370-nt transcript. A second inducible *MATα1* transcript was produced from the *MATUAG84* construct in both *UPF1* and *upf1Δ* strains (Fig. 5B and 8A). This mRNA, 5′ *MATα1*, has an estimated size of 370 nt and is detectable with a 161-nt probe complementary to *MATα1* 5′ sequences but not with a 136-nt probe specific for 3′ sequences (Fig. 8A). Oligo(dT)-cellulose fractionation of RNA extracted from the *upf1Δ* strain harboring pGAL*MATUAG84* resulted in retention of approximately 50% of the 5′ *MATα1* molecules, 80% of the wild-type *MATα1* mRNA, and 65% of the *STE2* mRNA (Fig. 8B). The implied presence of a poly(A) tail on the 5′ *MATα1* transcript eliminates the possibility that this mRNA is a decay intermediate produced by endonucleolytic cleavage of full-length molecules.

Interestingly, the 5′ transcript has a half-life of ~5 min in both *UPF1* and *upf1Δ* genetic backgrounds (Fig. 8A and data not shown). This contrasts to the ~2.7-min half-life of the full-length *MATUAG84* mRNA in the *UPF1* strain (Fig. 5A and 8A) and is suggestive of the 5′ transcript's resistance to nonsense-mediated decay, with decay proceeding exclusively via the inherent pathway. Closer examination of Northern blots collected during this study revealed the presence of this second *MATα1* transcript in both *UPF* genetic backgrounds of strains harboring four additional alleles, *MATUAG76*, *MATUAG77*, *MATUAG78*, and *MATUAG80* (Fig. 5B and 8C). As judged from coelectrophoresis of steady-state RNAs, the transcript has similar molecular weights in all strains (Fig. 8C). However, intracellular levels of the 5′ transcript vary for the different alleles. This is apparent from a comparison of the ratios of the 5′ transcript to full-length mRNA, which are 0.09, 0.35, 0.14, 0.28, and 1.47 in the *UPF1* background and 0.04, 0.1, 0.07, 0.08, and 0.4 in the *upf1Δ* background for the alleles *MATUAG76*, *MATUAG77*, *MATUAG78*, *MATUAG80*, and *MATUAG84*, respectively. We suspect that these 5′ transcripts arise as a consequence of premature 3′ processing events and that the variation in the levels of the respective transcripts reflects the efficiency with which such processing events occur (see Discussion).

DISCUSSION

Nonsense codon mapping of mRNA instability elements. The identification of the *cis*-acting determinants of mRNA stability has been facilitated to date by the use of chimeric genes and the analysis of deletions and other mutations. Here, we report a new method that allows localization of *cis*-acting coding-region IEs in yeast genes encoding inherently unstable mRNAs. Previous studies identified the coding-sequence location of a 65-nt IE within the *MATα1* mRNA (6, 39), and so we tested the ability of in-frame translational termination codons to map this IE in a strain inactive for nonsense-mediated mRNA decay. The localization experiments, summarized in Fig. 4B, demonstrated that insertion of nonsense codons 5′ to the IE stabilized the transcript two- to threefold, while location of a translation termination codon downstream of the IE, by allowing translation to proceed through the element, had no effect on transcript half-life. The successful localization of the *MATα1* IE by nonsense codon insertion provides a valuable tool in the search for coding-region IEs in other genes specifying inherently unstable mRNAs.

This new mapping procedure offers certain advantages over the chimeric gene approach in that it causes minimal perturbations to overall mRNA structure, since constructs differ from

A

		mRNA $t_{1/2}$ (min)	
		UPF1	*upf1Δ*
MATα1 wild-type:	5′ CAG UUU CGA CAG UUC AAU AAG	5.3±0.1	5.0±0.3
MATΔ82:	5′ CAG UUU CGA CAG UUC AAG	4.9±0.1	4.7±0.4
MATUAG78Δ:	5′ CAG **UAG** CGA CAG UUC AAG	4.2±0.3	9.6±0.2
MATUGA79Δ:	5′ CAG UUU **UGA** CAG UUC AAG	3.8±0.7	6.7±0.6
MATUAG80Δ:	5′ CAG UUU CGA **UAG** UUC AAG	2.6±0.2	5.7±0.8
MATUAG81Δ:	5′ CAG UUU CGA CAG **UAG** AAG	3.1±0.1	5.1±0.2
MATI82:	5′ CAG UUU CGA CAG UUC AAU AAU AAG	4.2±0.5	4.5±0.6
MATUAG78I:	5′ CAG **UAG** CGA CAG UUC AAU AAU AAG	3.6±0.9	9.8±2.1
MATUGA79I:	5′ CAG UUU **UGA** CAG UUC AAU AAU AAG	4.3±1.9	9.9±0.4
MATUAG80I:	5′ CAG UUU CGA **UAG** UUC AAU AAU AAG	2.4±0.1	9.1±1.5
MATUAG81I:	5′ CAG UUU CGA CAG **UAG** AAU AAU AAG	2.7±0.0	6.1±0.7

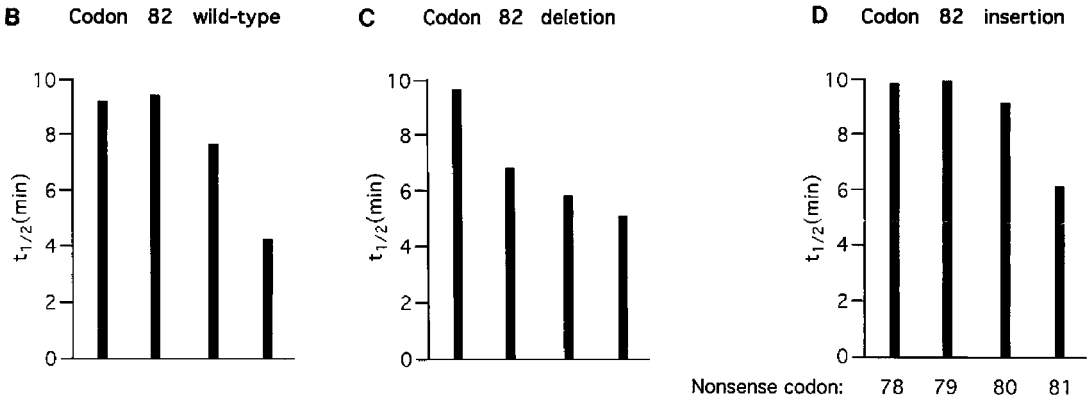

FIG. 7. Single-codon insertions or deletions shift the position of the *MATα1* mRNA stability transition region. (A) mRNA sequences and half-lives of *MATα1* deletion and insertion alleles constructed to investigate the potential for shifting the stability transition boundary. All sequences are aligned to codon 77 (CAG). Codon 82 (underlined) was deleted (Δ constructs) or an extra copy was inserted (*I* constructs) in wild-type (yielding *MATΔ82* or *MATI82*) or nonsense-containing mRNAs. In-frame translational termination codons are depicted in boldface. Half-lives obtained for the *MATα1* deletion/insertion transcripts in *UPF1* and *upf1*Δ strains were determined as in Fig. 2. (B to D) Bar plots illustrating the changes in the stability of nonsense-containing *MATα1* mRNAs in the *upf1*Δ strain resulting from deletion or insertion of codon 82. (B) Nonsense-containing mRNAs with no additional deletion or insertion present; (C) codon 82 deletion mutants; (D) codon 82 insertion mutants.

the wild-type transcript by only 1 to 3 nt. In addition, the approach is less tedious since construction of chimeric genes is not required. Our procedure does not, however, delimit the IE and requires further deletion analyses to map the 5′ and 3′ boundaries of a localized element. A potential difficulty with nonsense codon mapping may be its inability to distinguish different IEs in a gene containing multiple destabilizing sequences. Furthermore, because of the translational dependency of the mapping protocol, only those elements whose destabilizing activities require ongoing translation can be characterized.

The mRNA half-lives obtained in our studies are in agreement with those of previously reported *MATα1* deletion experiments (6). Termination of translation 5′ to the IE was found to stabilize the *MATα1* transcript two- to threefold (Fig. 2A and B, 3, and 4B), and in experiments by Caponigro et al., in which the 65-nt element was removed from the gene, the mRNA was stabilized twofold (6). In the latter studies, a threefold stabilization occurred if the AU-rich sequences immediately 3′ to the IE were also deleted (6). Furthermore, the extents of message stabilization were similar irrespective of whether the 3′ 32-nt AU-rich sequence of the element was physically removed or nonsense codons were used to block its translation (6) (Fig. 3 and 4B). Since the 65-nt IE is the only sequence element within the *MATα1* mRNA with apparent destabilizing activity (39), and since bona fide stable mRNAs in *S. cerevisiae* have half-lives as long as 60 min (23, 45), it might have been expected that mRNAs lacking this element, or its function, would have half-lives in excess of the ~14-min maximum observed here and previously (6). However, mutation of the IE of another inherently unstable mRNA, the *MFA2* mRNA, also yielded mRNAs whose maximal half-life was 14.5 min (38). This finding suggests that unstable mRNAs may have other, nondiscrete sequence features that enhance their decay rates or that stable mRNAs may contain specific sequences that promote their stability. Experiments supporting the latter

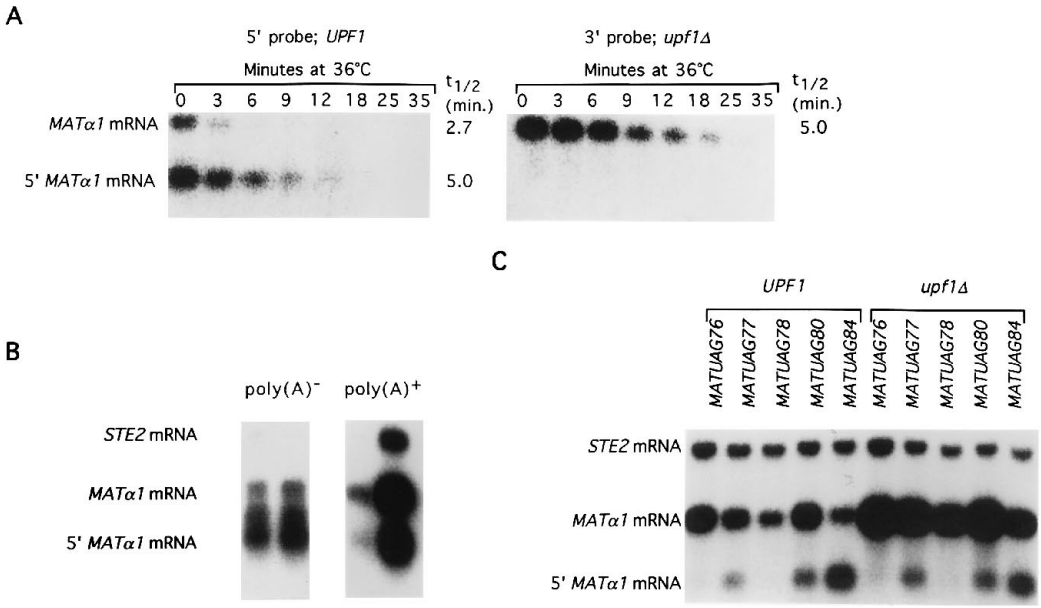

FIG. 8. Analysis of low-molecular-weight transcript of the *MATUAG84* allele. (A) An mRNA of ~370 nt is detectable by hybridization to a 161-nt probe complementary to sequences within the 5′ half of *MATα1* but not by hybridization to a 136-nt 3′ complementary probe. (B) Northern blotting of oligo(dT)-cellulose-fractionated RNA isolated from the *upf1Δ* strain harboring pGAL*MATUAG84*. (C) An mRNA of molecular weight equivalent to that of the 5′ *MATα1* transcript is detectable in four additional *MATα1* alleles expressed in both *UPF1* and *upf1Δ* strains. Variation in abundance of the full-length *MATα1* transcript reflects experimental variations in galactose induction. Hybridization to the *STE2* mRNAs serves as a control for mRNA loading efficiency. Northern blotting and mRNA half-life measurements were as described in the legend to Fig. 2 and Materials and Methods.

possibility, at least for the *PGK1* mRNA, have been reported previously (19, 41).

***cis*-acting determinants of 3′ end formation and mRNA stability.** Several *MATα1* alleles produced a novel transcript of approximately 370 nt (Fig. 5B and 8A and C). The presence of poly(A) tracts on these mRNAs (Fig. 8B) strongly suggests that they are not decay intermediates of the full-length transcript; rather, they appear to be products of a premature RNA processing event. The variation in the ratios of the 5′ fragments to full-length mRNA (Fig. 8C) suggests that the efficiency of such a processing event is allele specific. A variety of predominantly AT-rich sequence motifs have been proposed to constitute mRNA 3′ end formation signals in *S. cerevisiae* (1, 21, 24, 50, 51). While precise sequences vary with each gene, all require multiple sequence elements and/or specific sequence reiterations for activity (14, 20, 25, 26). A previous comparison of the 3′ UTRs of 15 yeast genes, including *MATα1*, identified a tripartite consensus sequence for mRNA 3′ end processing, TAG. . .TAGT/TATGT. . .TTT (60). Analysis of the *MATα1* coding region 3′ to the 65-nt IE revealed the presence of a TATG. . .TTT stretch spanning codons 96 to 106. We hypothesize that this sequence acts as an internal 3′ end formation signal in the *MATα1* nonsense-containing alleles, since the introduction of an upstream TAG translation termination codon creates a complete 3′ end formation element. Consistent with this interpretation is the observation that the *MATUGA79* allele does not give rise to a detectable 5′ transcript. The nonsense codon in this allele, unlike those at codons 76, 77, 78, 80, and 84, is a TGA, not a TAG. Termination of transcription in the vicinity of the proposed element would produce a transcript of approximately 370 nt, in agreement with our estimated size of the 5′ *MATα1* mRNA. The subtle differences between the newly created 3′ end formation elements, i.e., the distances between the inserted TAG codons and the downstream TATG, may account for the observed allele-specific variation in 5′ *MATα1* mRNA synthesis. Previous studies have pointed to a spatial requirement for the sequences within such elements that dictates overall element efficiency (14).

In the *UPF1* strain, the half-life of the 5′ *MATα1* transcript produced from the *MATUAG84* allele is almost twice that of the full-length transcript (Fig. 8A). Although this truncated mRNA is of sufficient length to contain its in-frame translational termination codon, it appears to be resistant to the nonsense decay pathway. This may reflect the loss of specific *cis*-acting sequences 3′ to the nonsense codon that are required for nonsense-mediated mRNA decay (16, 41, 61). Another sequence element, designated a stabilizer, appears to regulate this pathway and comprises the region of an mRNA in which nonsense codons lose the ability to promote rapid mRNA decay (41). The increase in the half-lives, in the *UPF1* strain, of *MATα1* mRNAs containing nonsense mutations at or beyond codon 67 suggests that like the *PGK1*, *CYC1*, and *HIS4* mRNAs (16, 32, 41, 59), this mRNA may contain such a stabilizer region (Fig. 4A).

Interrelationship of mRNA decay and translation. Numerous studies have pointed to an intimate link between the processes of mRNA translation and mRNA decay (6, 16, 19, 22, 23, 28, 34, 41, 42, 43, 46, 54), and results reported here support this relationship. Through a detailed mutagenesis of the *MATα1* IE's rare-codon/AU-rich sequence boundary, we have shown that ribosome progression over a two-codon region maximizes the instability phenotype of this mRNA (Fig. 5 and 6). Termination of translation 5′ to this region stabilized the *MATα1* mRNA two- to threefold, and termination within this region had intermediate stabilizing effects. We interpret these results in terms of a model in which the crucial destabilizing signal entails a physical interaction between a translating ribosome and an outlying site within the same mRNA. Experiments using one-codon deletions or insertions (Fig. 7B to D)

have shown that the translation-dependent mRNA stability transition can be predictably shifted one codon 5′ or 3′, strongly favoring an interaction between a ribosome and a downstream site. The exact nature of the downstream site with which the ribosome interacts is unknown, but it is likely that it is confined to the IE since previous studies have shown that the 65-nt element is sufficient to destabilize a heterologous gene (6). We therefore anticipate ribosome interaction with the AU-rich sequence located immediately downstream of the stability transition region. Ribosomes may recognize this sequence per se, a secondary structure within the sequence, or an RNA-protein complex.

Previous experiments point to a requirement for both the rare-codon and AU-rich sequences of the *MATα1* IE for message destabilization (6, 39). A stimulatory role for rare codons in IE activity was speculated to involve facilitation of ribosome pausing since there was no sequence specificity to the rare-codon requirement (6). The stability transition that we observe occurs at codons 80 and 81, a region of the mRNA previously assigned to the AU-rich portion of the element (6). However, codon 80 is actually the last rare codon of the IE (Fig. 1B). Hence, destabilization of the *MATα1* transcript coincides with translation of the IE's rare-codon/AU-rich sequence boundary. Interestingly, the stability transition sequence also coincides with the 3′ end of a 15-nt sequence of which 14 nt are complementary to 18S rRNA (Fig. 1B). A hypothetical mRNA-rRNA base-pairing interaction was previously proposed to induce a translational pause on the *MATα1* transcript and potentially play a role in transcript turnover (45). Our current data, linking a twofold change in *MATα1* mRNA half-life to translation of this putative 18S rRNA binding sequence, may support this conclusion.

A model for decay of the *MATα1* transcript must, therefore, account for the involvement of the entire bipartite element, the role of translating ribosomes, the observed transition in stability which is dependent on the ribosome's position on the mRNA, and our ability to move this stability boundary through mutations. We propose that during translation, ribosomes experience an appreciable decrease in their elongation rate over the IE's rare-codon sequence. This event, perhaps in conjunction with ribosome stalling mediated by mRNA-18S rRNA base-pairing interactions, may provide a sufficient time frame for interaction of the ribosome or a ribosome-bound protein with the AU-rich mRNA sequence or a protein bound to it. The possibility that the IE's AU-rich sequence can serve as a recognition site for binding of a protein involved in transcript decay has been suggested previously (6). The decay-initiating signal that results from this ribosome-mRNA interaction is likely to enhance the transcript's deadenylation rate, hence activating a deadenylation-dependent pathway of mRNA turnover in which 3′ poly(A) shortening leads to 5′ decapping and ultimately 5′-to-3′ exonucleolytic digestion of the mRNA coding sequence (7, 12, 13, 37). Removal of the rare-codon segment, by interfering with the kinetics of ribosome translocation, would prevent these ribosome-mRNA interactions and so disrupt the destabilizing activity of the IE. Similarly, nonsense codons which prevent ribosomes from reaching the IE would be expected to stabilize the transcript. What, however, is the role of nonsense codons (at position 82 and beyond) that do not lead to alterations in mRNA decay rate? One possibility is that the translational pause induced by nonsense codons is sufficient to promote decay, provided that it occurs at a site that is the appropriate distance from the AU-rich region. Alternatively, nonsense codons that allow normal decay are located sufficiently far downstream that the rare codon cluster is exposed and, hence, active. The latter possibility is consistent with a 27- to 29-nt footprint for eukaryotic ribosomes (58). A comparison of the current results with those of a previous study (6) provides some insight into these possibilities. Caponigro et al. (6) demonstrated that in the context of a *MATα1* transcript with an IE deletion, clustered rare codons 11 to 14 codons upstream of the second AU-rich region were sufficient to promote rapid decay. In the current study, however, a UAG 12 codons 5′ to the normal AU-rich region (UAG_{67}) promoted mRNA stabilization. These results suggest that nonsense codon position simply determines whether the rare codon segment is available for execution of its normal function. It is also possible that the two types of pausing event have different effects on ribosome conformation or ribosome-associated factors and thus demand different positionings of the ribosome for its role in mRNA destabilization. These alternatives are currently being tested.

ACKNOWLEDGMENTS

This work was supported by grant GM27757 to A.J. from the National Institutes of Health.

We thank Carolyn Decker, Giordano Caponigro, and Roy Parker for strains and plasmids and Andrew Bond, Feng He, Alan Maderazo, David Mangus, Ellen Welch, and Dorit Zuk for their helpful comments on the manuscript.

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