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

AIDAN N. HENNIGAN AND ALLAN JACOBSON*

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655-0122

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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 the 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-independent 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...
TABLE 1. Plasmids used in this study

| Plasmid          | Description                                                                 |
|------------------|-----------------------------------------------------------------------------|
| pGALMATAl       | MAT1 gene fused to GAL1 promoter on yeast centromeric plasmid               |
| pGALMATUA1G18    | Same as pGALMATAl except with nonsense codon at position 18                |
| pGALMATUA1G252   | Contains nonsense codon at position 52                                     |
| pGALMATUA1G67    | Contains nonsense codon at position 67                                      |
| pGALMATUA1G76    | Contains nonsense codon at position 76                                      |
| pGALMATUA1G77    | Contains nonsense codon at position 77                                      |
| pGALMATUA1G78    | Contains nonsense codon at position 78                                      |
| pGALMATUA1G79    | Contains nonsense codon at position 79                                      |
| pGALMATUA1G80    | Contains nonsense codon at position 80                                      |
| pGALMATUA1G81    | Contains nonsense codon at position 81                                      |
| pGALMATUA1G82    | Same as pGALMATAl except with deletion of codon 82                         |
| pGALMATUA1G87A   | Same as pGALMATUA1G78 except with deletion of codon 82                     |
| pGALMATUA1G79A   | Same as pGALMATUA1G79 except with deletion of codon 82                     |
| pGALMATUA1G80A   | Same as pGALMATUA1G80 except with deletion of codon 82                     |
| pGALMATUA1G81A   | Same as pGALMATUA1G81 except with deletion of codon 82                     |
| pGALMATUA1G82    | Same as pGALMATAl except with insertion of extra codon 82                 |
| pGALMATUA1G87I   | Same as pGALMATUA1G78 except with insertion of extra codon 82              |
| pGALMATUA1G79I   | Same as pGALMATUA1G79 except with insertion of extra codon 82              |
| pGALMATUA1G80I   | Same as pGALMATUA1G80 except with insertion of extra codon 82              |
| pGALMATUA1G81I   | Same as pGALMATUA1G81 except with insertion of extra codon 82              |

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 (MATa npk1-1ura3-52 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 (upf1Δ). 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 EcoRI-BamHI DNA fragment (33) subcloned into the yeast shuttle vector pRS314 (55). pRS314/UPF1 was digested with BglII to remove a 1.6-kb sequence from the UPF1 gene, and a LEU2 gene from plasmid pJ250 was cloned into the BglII sites of the resulting plasmid (29). To facilitate cloning of the LEU2 marker, pJ250 was digested with HindIII, which cuts once, 3'- of LEU2. The 5'- overhangs were filled in with Klenow enzyme (Boehringer Mannheim Biochemicals), and a BglII linker (New England Bio- labs) was ligated. This DNA was digested with BglII and BamHI, and the LEU2 containing fragment was then ligated to the plasmid carrying the partial deletion of the UPF1 gene. Transformation of pRS582 with a 4.4-kb XhoI-BamHI 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 pre-mRNA and hence the presence of a nonsense decay phenotype (reference 18 and data not shown).

Table 1 lists plasmids containing wild-type and allelic MATAl 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 MATAl sequence are numbered according to the codon changed. The plasmids are all derived from pGALMATAl (provided by G. Caponigro and A. Parker), in which a GAL1 promoter fused to a MATAl gene was ligated to EcoRI-HindIII cut pSHEH.BX (a derivative of pRIPIH [30] in which XhoI, HindIII, and BglII sites were replaced by NotI, NcoI, and Clal 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 MATAl coding sequence was performed by using a modification of the procedure described by Kunkel et al. (30). A BamHI-HindIII fragment harboring the MATAl gene from pGALMATAl was cloned into the polylinker of pBluescriptII KS+ phagemid (Stratagene), and this construct transformed into Escherichia coli CJ236 (Δ lam−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 (upf1Δ; R. Parker), in which a 3-nt mismatch, depending on the oligonucleotide used (Table 2). All oligonucleotides were either purchased from

**TABLE 2. Oligonucleotides used in this study**

| Name          | Sequence (5’ to 3’) |
|---------------|--------------------|
| MATUAG18      | AAG CAT CCA AAT CAT AGA GAA |
| MATUAG252     | TCA AGA AGA ATA TCT AGA TTC TCG |
| MATUAG67      | ATAA AAA TCC AAA TTT AGA GGA TAG |
| MATUAG76      | CTT GAA GTG AAA ATT AGC AGT TTC |
| MATUAG77      | GAC AGT |
| MATUAG78      |AGT TCA |
| MATUAG80      | ATT AGT TCT GTA GAT AGA CAT ATA |
| MATUAG80I     | ATC AGT TCT GTA GAT AGA AGA CAT |
| MATUAG81      | CTC AGT TCT GTA GAC AGA ATA AGA CAT CT |
| MATUBA82     | GTT GAC AGT TCT AGA CAT AGA CAT |
| MATUBA83     | TTA AAA |
| MATUBA84     | GAG AGT TCA ATA AGT AGT CTA TAA |
| MATUBA85     | AGT TCA ATA AGA CAT AGA TAA CAT CTA |
| MATUBA86     | ACT TCT CGC AAT AGT ATA TTT TAA |
| MATUBA87     | CAT TTA CGA CAT TAA |
| MATUBA88     | CAG TAG CGA TCT AAG ACA TCT |
| MATUBA89     | ATA AAA |
| MATUBA90     | CAG TTT CGA TAG TCT AAG ACA TCT |
| MATUBA91     | ATA AAA |
| MATUBA92     | CAG TTT CGA TCT AAC AAG ACA TCT |
| MATUBA93     | ACA TCT ATA |
| MATUBA94     | CAG TTT CGA TCT AAC AAG ACA TCT |
| MATUBA95     | ACA TCT ATA |
| MATUBA96     | CAG TTT CGA TCT AAC AAG ACA TCT |
| MATUBA97     | ACA TCT ATA |
| MATUBA98     | CAG TTT CGA TCT AAC AAG ACA TCT |
| MATUBA99     | ATA AAA |
| MATUBA100     | TIG AAG ATT TTA TAG ATC |
| MATUBA101     | GCC AGC AGA GAA GAC GAG AC |
| RP-18         | CAA CAG GAC GAT GGG GAG |
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/American) and the sequencing primers MATAH01, MATAH02, and RP-18 (Table 2). Those with desired termination codons were digested to remove the allelic MATA1 DNAs, and the BamHI-HindIII fragments were subcloned into the vector M13mp19 (36). Recombinant phage were plated on E. coli IM09, 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 fractionation by electrophoresis was transferred to Zeta probemembranes (Bio-Rad), and blotswere probed todetect hybridfusionjunctions generatingstabilized 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. Rare codons are indicated by a single underline, and double underlinesmark the 19-nt sequence within the AU-rich span postulated to be a specific protein binding site (6). Sequences with complementarity to 18S RNA (45) are indicated in boldface. All numerals refer to codon numbers.

RESULTS

In-frame translational termination codons map the MATA1 IE to its coding sequence location. The MATA1 mRNA is inherently unstable, having a half-life of approximately 5 min (23, 39, 45). Previous analyses of chimeric genes identified MATA1 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 MATA1 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 MATA1 mRNA half-life (Fig. 2A). The first two nonsense codons precede the IE, whereas that at position 139 follows the IE. Transcription of allelic MATA1 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 MATA1 mRNA (23).

In the upf1Δ strain, in which nonsense-mediated mRNA decay is blocked, nonsense codons at positions 18 and 52 stabilized the MATA1 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 MATAUG18 and MATAUG52 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 MATA1 transcripts were very unstable, with half-lives of ~1.0 min, except for
MATUAG139 whose half-life of ~4.0 min more closely resembled the wild-type MATa1 mRNA. 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 MATa1 gene (Fig. 3A) and measured the resulting mRNA half-lives.

**FIG. 2.** Effects of early and late nonsense codons on the stability of the MATa1 mRNA. (A) Schematics and mRNA half-lives for the wild-type MATa1 gene and three nonsense alleles. The locations of single in-frame translational termination codons relative to the bipartite IE (shaded regions) are shown. MATa1 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 MATa1 mRNAs in UPF1 and upf1Δ strains as described in Materials and Methods. (B) Northern blots depicting decay of wild-type and allelic MATa1 mRNAs in UPF1 and upf1Δ strains. Blots are numbered according to the MATa1 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 MATa1 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 MATa1 mRNA half-lives were determined. Blot numbers indicate those strains containing the correspondingly labeled MATa1 allele.

**FIG. 3.** Effects of inhibiting translation within and immediately before the IE. (A) Location of single in-frame translational termination codons with in the MATa1 coding region and summary of the half-life data obtained for the MATa1 mRNA in UPF1 and upf1Δ strains. MATUAG67 terminates translation immediately 5’ to the IE; MATUAG79 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 upf1Δ 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 MATa1 transcript (Fig. 3). mRNA stabilization arising from the 60% AU-rich sequence is evident in these data.

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**Notes:**

- MATUAG67 terminates translation immediately 5’ to the IE.
- MATUAG79 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.
- Northern blots showing decay of the MATa1 transcripts depicted in panel A in UPF1 and upf1Δ strains. Blots are numbered according to the allele present. RNA pulse-chase experiments were performed as described in the legend to Fig. 2.
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 upf1Δ 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 upf1Δ 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 MATUAG79 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 upf1Δ isogenic strains.

In the upf1Δ 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 MATUAG79 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 and 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, MATUAG79, 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...
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 MATUGA79 and MATUGA85 (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 upf1Δ 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 MATUGA84 construct and marked with an asterisk (blot 8) is the 5' MATα1 species (see text and Fig. 8).

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 upf1Δ strains. Successive constructs, together with the alleles MATUGA79 and MATUGA85 (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 upf1Δ 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 MATUGA84 construct and marked with an asterisk (blot 8) is the 5' MATα1 species (see text and Fig. 8).

Constructs containing either a deletion (MATΔ82) or an insertion (MAT882) 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 upf1Δ 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 upf1Δ 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 MATUGA85 transcripts by 2.7 and 1.9 min, respectively, and had only minor effects on the half-lives of the MATUGA78 and MATUGA81 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-
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 pGALMATUAG84 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 UPF1 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...
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 \textit{MATa1} deletion experiments (6). Termination of translation 5’ to the IE was found to stabilize the \textit{MATa1} 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 \textit{MATa1} mRNA with apparent destabilizing activity (39), and since bona fide stable mRNAs in \textit{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 \textit{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

| \textbf{MATa1 wild-type:} | 5’ CAG UUU CGA CAG UUC AAG | 5.3±0.1 | 5.0±0.3 |
| \textbf{MATa82:} | 5’ CAG UUU CGA CAG UUC AAG | 4.9±0.1 | 4.7±0.4 |
| \textbf{MATUA6782:} | 5’ CAG UUG AAG CAG UUC AAG | 4.2±0.3 | 9.6±0.2 |
| \textbf{MATUA679:} | 5’ CAG UUU CGA CAG UUC AAG | 3.8±0.7 | 6.7±0.6 |
| \textbf{MATUA680:} | 5’ CAG UUU CGA UAG UUC AAG | 2.6±0.2 | 5.7±0.8 |
| \textbf{MATUA681:} | 5’ CAG UUU CGA CAG UAG AAG | 3.1±0.1 | 5.1±0.2 |
| \textbf{MAT82:} | 5’ CAG UUU CGA CUCUAAU AAG | 4.2±0.5 | 4.5±0.6 |
| \textbf{MATUA6782:} | 5’ CAG UUG AAG CAG UUC AAG | 3.6±0.9 | 9.8±2.1 |
| \textbf{MATUA679:} | 5’ CAG UUU CGA CUCUAAU AAG | 4.3±1.9 | 9.9±0.4 |
| \textbf{MATUA680:} | 5’ CAG UUU CGA UAG UUC AAG | 2.4±0.1 | 9.1±1.5 |
| \textbf{MATUA681:} | 5’ CAG UUU CGA CAG UAG AAU AAG | 2.7±0.0 | 6.1±0.7 |

**FIG. 7.** Single-codon insertions or deletions shift the position of the \textit{MATa1} mRNA stability transition region. (A) mRNA sequences and half-lives of \textit{MATa1} 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 \textit{MAT82} or \textit{MAT Δ82}) or nonsense-containing mRNAs. In-frame translational termination codons are depicted in boldface. Half-lives obtained for the \textit{MATa1} deletion/insertion transcripts in \textit{UPF1} and \textit{upf1Δ} strains were determined as in Fig. 2. (B to D) Bar plots illustrating the changes in the stability of nonsense-containing \textit{MATa1} mRNAs in the \textit{upf1Δ} 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.
and the downstream TATG, may account for the observed elements, i.e., the distances between the inserted TAG codons subtledifferences between the newly created 39 end formation sizes that this sequence acts as an internal 39 TATG...TTT stretch spanning codons 96 to 106. We hypoth-
ent with this interpretation is the observation that the codon creates a complete 39 9 introduction of an upstream TAG translation termination signal in the mRNA 39 AT-rich sequence motifs have been proposed to constitute a processing event is allele-specific. A variety of predominantly full-length mRNAs (Fig. 8C) suggest that the efficiency of such a possibility, at least for the PGK1 mRNA, have been reported previously (19, 41).
cis-acting determinants of 39 end formation and mRNA stability. Several MATa1 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 MATa1, identified a tripartite consensus sequence for mRNA 3’ end processing, TAG...TAGT/TATGT...TTT (60). Analysis of the MATa1 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 MATa1 nonsense-containing alleles, since the introduction of an upstream TAG translation termination codon creates a complete 3’ end formation element. Consis-
tent with this interpretation is the observation that the MATUAGA79 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. Termi-
nation of transcription in the vicinity of the proposed element would produce a transcript of approximately 370 nt, in agree-
ment with our estimated size of the 5’ MATa1 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’ MATa1 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’ MATa1 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 transla-
tional 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 MATa1 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 MATa1 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 MATa1 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. Experi-
ments using one-codon deletions or insertions (Fig. 7B to D)
Interestingly, the stability transition sequence also coincides with a translational pause on the rRNA base-pairing interaction was previously proposed to in- 
terpolate to 18S rRNA (Fig. 1B). A hypothetical mRNA- 
data, linking a two-fold change in potential 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 conjunc- 
tion 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 transloca-
tion, 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. Al-
ternatively, 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. Caponi-
gro 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 (UAG384) promoted mRNA stabilization. These results suggest that nonsense 
codon position simply determines whether the rare codon seg-
ment 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 fac-
tors and thus demand different positionings of the ribosome for its role in mRNA destabilization. These alternatives are currently being tested.

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