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An internal open reading frame triggers nonsense-mediated decay of the yeast *SPT10* mRNA

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Yeast cells containing a temperature-sensitive mutation in the *PRT1* gene were found to selectively stabilize mRNAs harboring early nonsense codons. The similarities between the mRNA decay phenotypes of *prt1-1* cells and those lacking the nonsense-mediated mRNA decay (NMD) factor Upf1p led us to determine whether both types of mutations cause the accumulation of the same mRNAs. Differential display analysis and mRNA half-life measurements demonstrated that the *HHF2* mRNA increased in abundance in *prt1-1* and *upf1*Δ cells, but did not manifest a change in decay rate. In both mutant strains this increase was attributable to stabilization of the *SPT10* transcript, an mRNA encoding a transcriptional regulator of *HHF2*. Analyses of chimeric mRNAs used to identify the *cis*-acting basis for NMD of the *SPT10* mRNA indicated that ribosomes scan beyond its initiator AUG and initiate at the next downstream AUG, resulting in premature translation termination. By searching a yeast database for transcripts with sequence features similar to those of the *SPT10* mRNA, other transcripts that decay by the NMD pathway were identified. Our results demonstrate that mRNAs undergoing leaky scanning are a new class of endogenous NMD substrate, and suggest the existence of a novel cellular regulatory circuit.

Keywords: leaky scanning/mRNA degradation/mRNA surveillance

Introduction

Cells contain several mechanisms aimed at maintaining a high level of accuracy in the flow of genetic information, including proofreading systems that ensure the integrity of RNA synthesis and processing (Chin and Pyle, 1995; Jeon and Agarwal, 1996), tRNA aminoacylation (Freist *et al*., 1996) and peptide elongation (Yarus, 1992). However, these mechanisms are not flawless and cannot compensate for errors arising from mutations within an organism's genome. As a consequence, some mRNAs lack complete open reading frames (ORFs). Another surveillance process, nonsense-mediated mRNA decay (NMD), ensures that such transcripts do not accumulate. Shown to operate in a broad spectrum of eukaryotic organisms, this pathway triggers the accelerated decay of mRNAs containing premature in-frame termination codons (Losson and Lacroute, 1979; Leeds *et al.*, 1991; Peltz *et al*., 1993, 1994; Pulak and Anderson, 1993; Whitfield *et al*., 1994; Maquat, 1995; Caponigro and Parker, 1996; Jacobson and Peltz, 1996; Ruiz-Echevarria *et al*., 1996).

The existence of a cellular pathway capable of assessing the translational capacity of individual transcripts raises the question of whether the substrates of this pathway are restricted to 'aberrant' mRNAs. In addition to mRNAs with premature termination codons, substrates of this pathway include intron-containing RNAs that have escaped from the nucleus to the cytoplasm, mRNAs with extended 3′-UTRs, and a subset of transcripts harboring upstream open reading frames (uORFs) (Pinto *et al*., 1992; He *et al*., 1993; Pulak and Anderson, 1993; Peltz *et al*., 1994; Cui *et al*., 1995; Oliveira and McCarthy, 1995; Muhlrad and Parker, 1999; F.Sherman, personal communication). In yeast, the factors and sequences that promote NMD have been characterized extensively. Rapid degradation of nonsense-containing yeast mRNAs requires the *trans*-acting factors encoded by the *UPF1*, *NMD2/UPF2* and *UPF3* genes (Leeds *et al*., 1991, 1992; Peltz *et al*., 1993; Cui *et al*., 1995; He and Jacobson, 1995; Lee and Culbertson 1995; He *et al*., 1997), as well as a *cis*-acting sequence 3′ to the premature termination codon, termed a downstream element (DSE; Peltz *et al*., 1993, 1994; Zhang *et al*., 1995; Ruiz-Echevarria *et al*., 1996).

The phenomenon of NMD also illustrates how the pathways of mRNA decay and translation can be interrelated. Other examples of such interrelationships include experiments demonstrating that: (i) inhibition of translational elongation, by drugs or mutations, can block decapping and stabilize most mRNAs (Peltz *et al*., 1992; Beelman and Parker, 1994); (ii) *cis*-acting sequences that modulate mRNA decay can be located within protein coding regions and require ongoing translation elongation for their function (Wisdom and Lee, 1991; Heaton *et al*., 1992; Herrick and Jacobson, 1992; Caponigro *et al*., 1993; Hennigan and Jacobson, 1996); (iii) shortening or removal of the poly(A) tail and 5′ cap, mRNA appendages with specific roles in translational initiation, comprise rate-limiting steps in the decay of several mRNAs (Munroe and Jacobson, 1990; Muhlrad and Parker, 1994; Tarun and Sachs, 1995; Jacobson, 1996); and (iv) factors involved in mRNA decay are polysome-associated (Atkin *et al*., 1995, 1997; Mangus and Jacobson, 1999).

The connections between mRNA decay and translation led us to identify *PRT1*, the gene encoding the p90 subunit of initiation factor eIF3 (Naranda *et al*., 1994; Danaie *et al*., 1995), as an additional regulator of NMD. We have utilized cells harboring an allele of this gene (*prt1-1*), or an allele of *UPF1* (*upf1*Δ), to characterize the spectrum of endogenous substrates of this pathway. Using differential display analysis, mRNAs whose abundance increased in the mutants, but not in the parental wild-type strains, were identified. Further analysis indicated that the increased

levels of these mRNAs were attributable to either their stabilization or to increases in their transcription. The *HHF2* mRNA was in the latter category and the basis for its increased transcription was investigated. We found that the half-life of the *SPT10* mRNA, which encodes a transcriptional regulator of the *HHF2* gene (Dollard *et al*., 1994), is increased in both the *prt1-1* and *upf1*Δ strains. Further characterization of the *SPT10* transcript indicated that this mRNA becomes a substrate for the NMD pathway as a consequence of premature translational termination resulting from leaky scanning of the ribosome past the translation initiation codon and subsequent initiation at a downstream, out-of-frame AUG. This atypical mode of translation and turnover may well define a novel cellular regulatory circuit.

Results

Nonsense-containing transcripts are stabilized in prt1-1 cells

The importance of specific translation events in the decay of individual mRNAs has been explored in experiments analyzing the consequences of their inhibition (see Introduction). To address the role of translation initiation in mRNA decay, we measured mRNA half-lives in a set of mutant strains that block translation initiation at various steps and to varying degrees. Our initial experiments analyzed the consequences of a temperature-sensitive mutation in *PRT1*. The *prt1-1* allele is attributable to a single amino acid substitution (S518F) that confers rapid inhibition of growth and translation at 37°C, as well as a defect in the interaction of the ternary complex (eIF2-GTP-tRNA$_i^{Met}$) with the 40S ribosomal subunit *in vitro* (Hartwell and McLaughlin, 1969; Feinberg *et al*., 1982; Keierleber *et al*., 1986; Evans *et al*., 1995). After cells were incubated at 37°C for 15 min, protein synthesis decreased rapidly by 95–97% in the *prt1-1* mutant, while it increased moderately in wild-type cells over the same time course (Figure 1A). The initiation defect associated with the *prt1-1* mutation was confirmed by analyzing its effects on cellular polysome profiles. Post-mitochondrial extracts from wild-type cells exhibited a normal distribution of ribosomes, whereas *prt1-1* extracts manifested a drastic reduction in polysomes and an ~2-fold increase in monosomes (Figure 1B).

To determine whether *prt1* mutations might also affect mRNA decay rates, we measured the half-lives of two classes of cellular mRNAs after shifting cultures of mutant or wild-type cells to non-permissive growth conditions. Cells were incubated at 37°C for 0 or 15 min, transcription was then inhibited by addition of the drug thiolutin (Jimenez *et al*., 1973; Tipper, 1973; Herrick *et al*., 1990), and aliquots of the respective cells were removed at various times after transcriptional inhibition for RNA isolation and Northern blot analysis. The blots were probed with DNAs complementary to mRNAs that were either

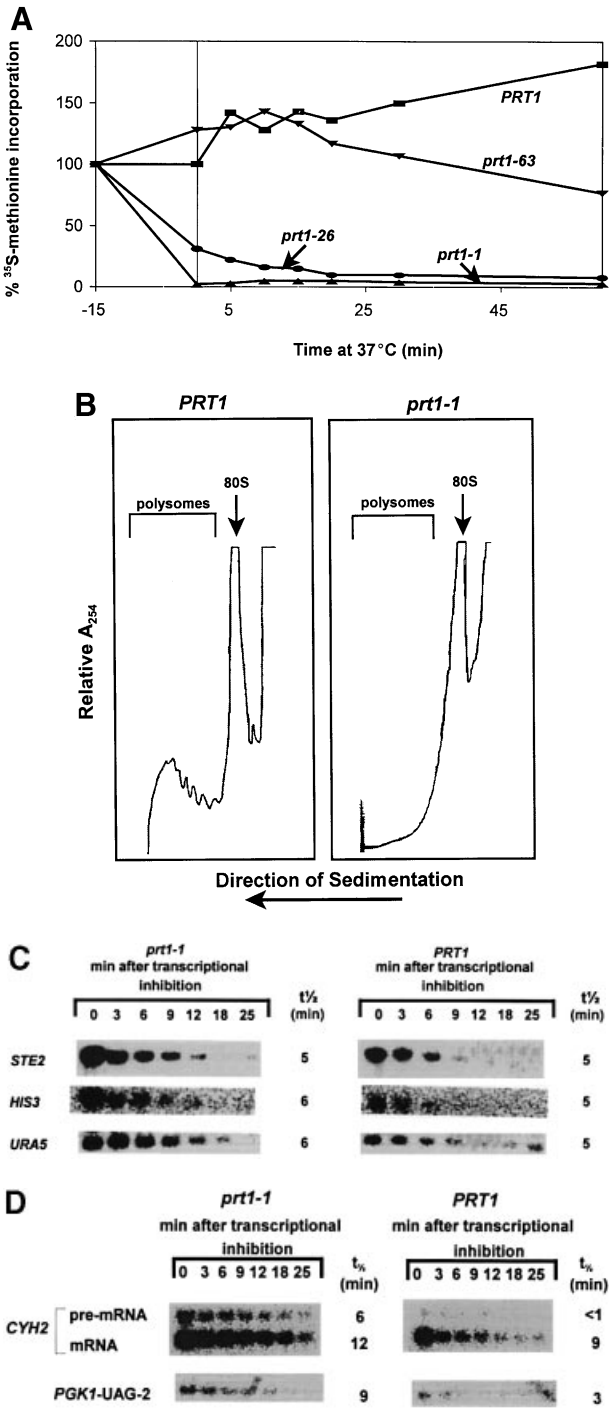

Fig. 1. Cells harboring the *prt1-1* allele have impaired translation activity and stabilize nonsense-containing mRNAs. (**A**) Translation rates of wild-type, *prt1-1*, *prt1-26* and *prt1-63* cells at 37°C. Cells from strains TP11B-4-1 (*prt1-1*), TDE-16A (*prt1-26*), TC3-212-3 (*prt1-63*) and TP11B-4-1 harboring plasmid pRS316-*PRT1* (*PRT1*) were used to determine translation rates. The data are presented as the percentage of amino acids incorporated at a given time point compared with t_0 (for the same cells). ■, *PRT1*; ▲, *prt1-1*; ●, *prt1-26*; ▼, *prt1-63*. (**B**) Polysome profiles of mutant (*prt1-1*) and wild-type cells. Post-mitochondrial extracts were prepared from *prt1-1* and *PRT1* cells (*prt1-1* cells harboring plasmid pRS316-*PRT1*) and fractionated on sucrose gradients. (**C**) Decay of inherently unstable mRNAs in wild-type and *prt1-1* cells after a shift to 37°C. Cells were incubated at 37°C for 15 min, after which time transcription was inhibited by the addition of thiolutin. Aliquots of cells were removed for RNA isolation at different times thereafter and the decay rates of the *STE2*, *HIS3* and *URA5* transcripts were monitored by Northern blotting. (**D**) Decay of nonsense-containing mRNAs in wild-type and *prt1-1* cells after a shift to 37°C. Cultures of wild-type or *prt1-1* cells were treated as in (C). Decay rates of the *CYH2* mRNA and pre-mRNA, as well as the nonsense-containing *PGK1*-UAG-2 transcript, were determined by Northern blotting.

constitutively unstable (inherently unstable mRNAs) or unstable as a consequence of premature translational termination.

Consistent with earlier studies showing that inhibition of translation initiation *in cis* does not lead to mRNA stabilization (Vega Laso *et al*., 1993; Beelman and Parker, 1994; Sagliocco *et al*., 1994), there was no significant effect of the *prt1-1* mutation on the decay rates of inherently unstable mRNAs. After a 15 min shift to 37°C, half-lives of the *STE2*, *HIS3* and *URA5* mRNAs were comparable in *prt1-1* and wild-type cells (Figure 1C). The lack of effect applied to other mRNAs having a broad range of decay rates and was not dependent on the time of incubation at 37°C (Table I).

In contrast to the results with 'normal' mRNAs, the data of Figure 1D demonstrate that the *prt1-1* mutation engenders a stabilization of nonsense-containing mRNAs. This effect was evident with two different mRNAs: the *CYH2* pre-mRNA, a naturally occurring substrate (He *et al*., 1993), and the *PGK1*-UAG-2 mRNA, the product of a *PGK1* allele harboring a nonsense mutation at nucleotide 361 (Peltz *et al*., 1993). In *prt1-1* cells, the former RNA was stabilized ~6-fold (wild type, $t_{1/2}$ = <1 min; *prt1-1*, $t_{1/2}$ = 6 min) and the latter RNA was stabilized ~3-fold (wild type, $t_{1/2}$ = 3 min; *prt1-1*, $t_{1/2}$ = 9 min; see Figure 1D and Table I).

To test whether the stabilizing effects of the *prt1-1* mutation on nonsense-containing mRNAs were limited to legitimate substrates, we measured the half-life of the *PGK1*-UAG-6 mRNA, a transcript whose 'late' nonsense mutation at nucleotide 1449 fails to render it a substrate for NMD (Peltz *et al*., 1993). Experiments similar to those of Figure 1D showed that the half-life of this mRNA was indistinguishable in the mutant and wild-type cells ($t_{1/2}$ = >35 min; Table I).

Similar analyses were conducted with cells harboring other alleles of *PRT1* (*prt1-26* and *prt1-63*), as well as mutations in the genes encoding the p16 subunit of eIF3 (*sui1-1*), the α subunit of eIF2 (*sui2-1*), the eIF2 kinase (*GCN2^c*) and the cap-binding protein eIF4E (*cdc33–42*) (Altmann *et al*., 1989; Castilho-Valavicus *et al*., 1990; Wek *et al*., 1990; Evans *et al*., 1995). Although most of these mutations have substantial inhibitory effects on translation initiation (e.g. see Figure 1A; Hanic-Joyce *et al*., 1987; Altmann *et al*., 1989; Linz *et al*., 1997; and data not shown), none except *sui1-1* had any significant effect on the decay rates of inherently unstable or nonsense-containing mRNAs. Cells harboring the *sui1-1* mutation showed only a very modest stabilization of the *CYH2* pre-mRNA and mRNA (data not shown).

Table I. Half-lives of mRNAs measured in *prt1-1*and *PRT1* strains at 37°C

Strain	*prt1-1*	*PRT1*	*prt1-1*
Time[a] (min)	15	15	0
mRNA		Half-life (min)	
CYH2 pre-mRNA	6	<1	3
CYH2 mRNA	12	9	9
PGK1-UAG-2	9	3	ND
PGK1-UAG-6	>35	>35	ND
CDC4	7	ND	7
MFA2	5	5	ND
STE2	5	5	ND
URA5	6	5	5
GCN4	14	ND	14
HIS3	6	5	7
LEU2	10	ND	14
PAB1	6	ND	9
ACT1	30	ND	30

[a]Indicates the time cells were incubated at 37°C before inhibiting transcription with thiolutin. Following the drug treatment, decay rates were measured at 37°C.
ND, not determined.

Utilization of differential display analysis to identify mRNAs that accumulate in prt1-1 and upf1Δ strains

Since mutations in *UPF1*, *NMD2/UPF2* or *UPF3* also cause selective stabilization of nonsense-containing mRNAs, we considered the possibility that the *PRT1* gene product was another component of this set of post-transcriptional regulators. To assess this possibility, we used mRNA differential display analysis (Liang and Pardee, 1995) to determine whether cells harboring *prt1-1* or *upf1*Δ mutations promoted stabilization of the same set of mRNAs. These experiments showed that the *HHF2*, *RPL13a*, *LYS14* and *ACO1* mRNAs, as well as the intron-containing pre-mRNA encoded by the ribosomal protein gene *CRY2*, were differentially expressed in both *prt1-1* and *upf1*Δ cells (Figures 2 and 3; and data not shown). In addition, *upf1*Δ cells accumulated the *DFR1* mRNA and *prt1-1* cells accumulated the *YAP1* mRNA (data not shown). The significance of the RNAs detected in this screen was underscored by the finding that the *CRY2* pre-mRNA, previously shown to be stabilized in *upf1*Δ cells (F.He and A.Jacobson, unpublished data; Zhen *et al*., 1995), was also stabilized in *prt1-1* cells (Figure 2).

The LYS14 and HHF2 mRNAs are differentially expressed in prt1-1 and upf1Δ cells, but are not stabilized

To determine whether the increased level of the *HHF2* mRNA resulted from mRNA stabilization, we isolated RNA from *prt1-1*, *upf1*Δ and wild-type cells after inhibiting transcription, and measured mRNA decay rates. Figure 3A and B shows that the *HHF2* mRNA is more abundant in *prt1-1* and *upf1*Δ cells compared with their wild-type counterparts, but its half-life is not increased. This indicated that the change in abundance was attributable to an increase in synthesis. A similar experiment, performed to monitor the half-life of the *LYS14* mRNA in the mutant and wild-type strains, gave comparable results (Figure 3C and data not shown). These experiments demonstrate that the increased levels of *HHF2* and *LYS14*

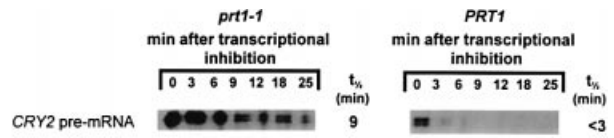

Fig. 2. The *CRY2* pre-mRNA is stabilized in the *prt1-1* mutant strain. Cultures of wild-type or *prt1-1* cells were grown at 24°C, shifted to 37°C and treated with thiolutin as in Figure 1. RNA was isolated at various times after the transcription block and analyzed for *CRY2* pre-mRNA abundance using an *in vitro* transcribed anti-sense probe.

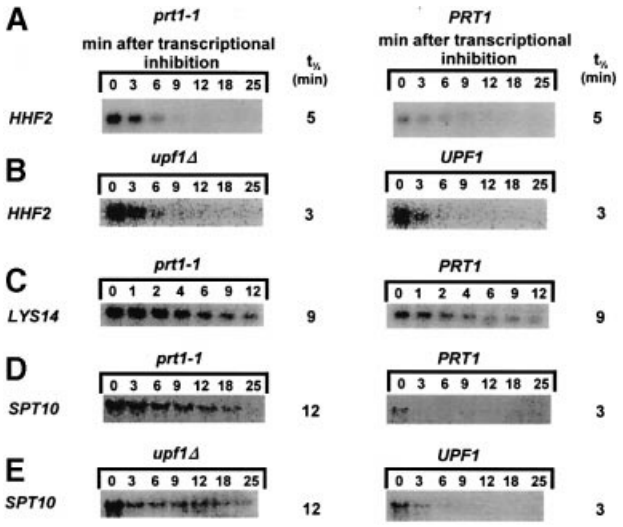

Fig. 3. The increased abundance of the *HHF2* mRNA is attributable to stabilization of the mRNA encoding its transcriptional regulator. Aliquots of RNA isolated from the indicated strains were used for Northern analysis. (**A**) In *prt1-1* cells the abundance of the *HHF2* mRNA is increased but its half-life is not. Cells from *prt1-1* (TP11B-4-1) and *PRT1* (Sc252) strains were shifted to 37°C for 15 min prior to the addition of thiolutin. At various times after transcription was inhibited, aliquots of cells were removed for RNA extraction and analyzed by Northern blotting. (**B**) In *upf1*Δ cells the abundance of the *HHF2* mRNA is increased but its half-life is not. Aliquots of RNA isolated from *upf1*Δ (AH01) and *UPF1* (RP582) cells at various times after transcription was inhibited by a shift to 37°C were analyzed by Northern blotting using the same probe as in (A). (**C**) In *prt1-1* cells the abundance of the *LYS14* mRNA is increased, but its half-life is not. Decay of the *LYS14* mRNA was measured in *prt1-1* and *PRT1* cells treated as in (A), using a fragment of the *LYS14* gene as a probe. Note the time course used. (**D**) The *SPT10* mRNA is stabilized in *prt1-1* cells. Decay of the *SPT10* mRNA was measured in *prt1-1* and *PRT1* cells treated as in (A), using a fragment of the *SPT10* gene as a probe. (**E**) The *SPT10* mRNA is stabilized in *upf1*Δ cells. Decay of the *SPT10* mRNA was measured in *upf1*Δ and *UPF1* cells treated as in (B), using the same probe as in (D).

mRNAs are the result of increased transcription, not an increase in mRNA half-life.

The half-life of the SPT10 mRNA, encoding a transcriptional regulator of HHF2, is increased in the prt1-1 and upf1Δ strains

Since the experiments of Figure 3A and B indicated that increases in the level of *HHF2* mRNA were attributable to changes in mRNA synthesis, we attempted to identify a transcriptional regulator of *HHF2* that would perhaps be a substrate for the NMD pathway. *HHF2* encodes a copy of histone H4, so we analyzed the mRNA decay rates of three known transcriptional regulators of histone genes, *HIR1*, *HIR2* and *SPT10* (Osley and Lycan, 1987; Natsoulis *et al*., 1991, 1994; Sherwood *et al*., 1993; Dollard *et al*., 1994). Figure 3D and E shows Northern blots probed for the *SPT10* mRNA after transcriptional inhibition. The half-life of the *SPT10* mRNA is increased 3- to 4-fold in both the *prt1-1* and *upf1*Δ mutant strains, although the half-lives of the *HIR1* and *HIR2* transcripts were unaffected (data not shown). Thus, the identification of *HHF2* in the differential display screen appears to be the result of increasing the half-life of the mRNA encoded by its transcriptional regulator. We observed previously a similar relationship between a subset of the *URA* mRNAs

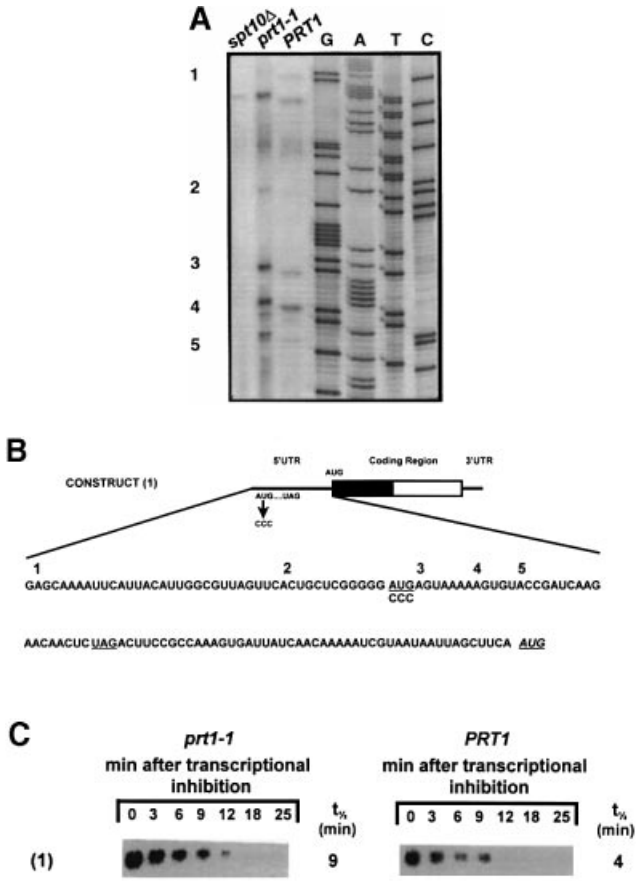

Fig. 4. The uORF present in the *SPT10* transcript does not promote NMD. (**A**) Primer extension analysis of the 5′ end of *SPT10* mRNA. RNA was prepared from *spt10*Δ (strain GNX197-8B), *prt1-1* and *PRT1* cells (see legend to Figure 3) harboring an *SPT10/PGK1* chimeric gene (construct 3; see Figure 5A), and used for primer extension with an oligonucleotide complementary to the *SPT10* sense strand at positions +30 to –5 (Table VI, oligonucleotide 4). (**B**) Schematic indicating the *SPT10/PGK1* chimeric mRNA used for primer extension analysis. The AUG of the *SPT10/PGK1* chimera used in (A) was mutated to CCC to produce construct 1 and is indicated on the sequence of the 5′ end of *SPT10* mRNA. Single underlining identifies the start and stop codons of the potential uORF; underlining and italics identifies the start codon of the principal ORF. The primer extension stop sites detected in (A) are numbered above the sequence. The black box represents *SPT10* sequence and the open box represents *PGK1* sequence. The thin lines represent the 5′- and 3′-UTRs. (**C**) Mutating the AUG of the uORF does not affect the half-life of the *SPT10* mRNA in *prt1-1* cells. RNA prepared from *prt1-1*and *PRT1* cells (see legend to Figure 3 for strains) harboring a plasmid carrying a mutation of the uORF AUG (construct 1) was analyzed by Northern blotting as in Figure 3. The probe used was the *SPT10* portion of construct 1 (endogenous *SPT10* mRNA is considerably larger than the chimeric mRNA and is not present on the segment of the blot presented). The number to the left of the RNA blots indicates the construct shown schematically in (B).

and their transcriptional regulator, *PPR1* (Leeds *et al*., 1991; Peltz *et al*., 1994).

Sequences within the 5′ segment of the SPT10 mRNA promote NMD

Since previous work has shown that the stop codon present in a uORF can be the source of the premature termination codon that triggers NMD (Pinto *et al*., 1992; Cui *et al*., 1995; Oliveira and McCarthy, 1995), we used primer extension analysis to determine whether a putative uORF identified by sequence analysis (Figure 4B) was localized

Table II. Half-lives of the *SPT10–PGK1* chimeric mRNAs

mRNA	Half-life (min)			
	prt1-1	*PRT1*	*upf1*Δ	*UPF1*
SPT10 (endogenous)	12	3	12	3
construct 1 (uORF mutation)	9	4	9	<3
construct 2 (5′-UTR + 73 nt)	18	12	12	9
construct 3 (5′-UTR + 94 nt)	12	3	12	3
construct 2-1 (UAG insertion)	12	3	9	3
construct 3-2 (AUG mutation)	12	12	12	9

within the boundaries of the 5′ end of the *SPT10* mRNA (Figure 4A). As shown in Figure 4A, the *SPT10* mRNA has several transcriptional start sites, two of which include the putative uORF. These transcription start sites are specific to the *SPT10* transcript, since they are not present in RNA isolated from control *spt10*Δ cells (Figure 4A, lane 1). To determine whether translation of the uORF was responsible for NMD of the *SPT10* mRNA, the only AUG codon of the uORF was mutated to CCC and the half-life of this mutant mRNA (construct 1; Figure 4B) was measured. (We chose to mutate the AUG and not the putative termination codon of the uORF because a second, in-frame terminator is present within the 5′-UTR). This change had no significant effect on the decay rate of the *SPT10* mRNA in wild-type, *upf1*Δ or *prt1-1* cells (Figure 4C and Table II), indicating that, in spite of the low abundance of transcripts 1 and 2, recognition of a termination codon within the uORF was not the stimulus for rapid mRNA decay.

To localize the 3′ boundary of *SPT10* sequences required for NMD, we prepared chimeras between *SPT10* and a 3′ segment of the well characterized *PGK1* gene. The fragment of *PGK1* includes the last 8% of the coding region and the 3′-UTR, and lacks a DSE (Peltz *et al*., 1993). Constructs 2 and 3 of this series contain the 5′-UTR and first 73 or 94 bp of *SPT10* coding sequence fused to the *PGK1* segment (Figure 5A). The half-lives of each of the encoded mRNAs were determined in wild-type and mutant cells by Northern blot analysis after transcriptional inhibition, and the results are shown in Figure 5B and C. The blots in both panels demonstrate that the chimera including *SPT10* sequences extending 94 nt into the coding region (construct 3) encodes an unstable mRNA whose half-life is increased 4-fold in *prt1-1* or *upf1*Δ mutant cells. The mRNA with 21 fewer nucleotides of *SPT10* sequence (encoded by construct 2) is only slightly destabilized in wild-type cells and, in mutant cells, has a half-life that is either comparable to wild type (*upf1*Δ), or slightly higher (*prt1-1*). These results indicate that sequences sufficient to promote NMD are present within the 5′-UTR and the first 94 nt of the coding region of the *SPT10* mRNA.

Aberrant translation initiation of the SPT10 mRNA triggers NMD

The experiments of Figure 5 indicated that the region sufficient to promote rapid decay of the *SPT10* mRNA is located within the boundaries of its 5′-UTR and first 94 nt of coding region. Since the experiments of Figure 4 showed that the uORF present in the *SPT10* 5′-UTR does

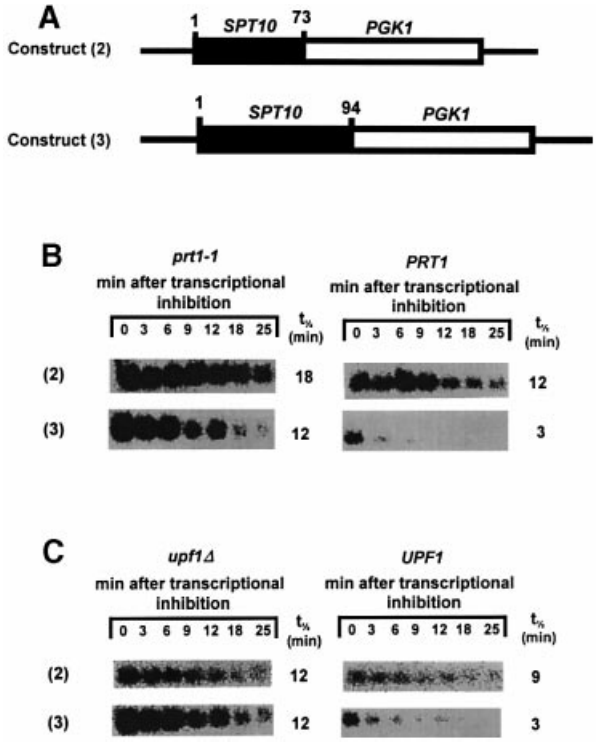

Fig. 5. The 5′-UTR and first 94 nt of coding region are sufficient to promote NMD of the *SPT10* mRNA. (**A**) *SPT10/PGK1* chimeric constructs. Construct 2 includes the 5′-UTR and the first 73 bp of *SPT10* fused to *PGK1* at its *Bgl*II site. Construct 3 is identical to construct 2 except that it includes an additional 21 bp of *SPT10* coding region sequence. The open boxes represent *PGK1* sequence and the black boxes represent *SPT10* sequence. Horizontal lines repesent the 5′- and 3′-UTRs. (**B**) Northern blots using RNA prepared from *prt1-1* and *PRT1* cells (see legend to Figure 3) harboring the chimeric genes illustrated in (A). The blots were probed with the *SPT10* portion of the chimeric gene. (**C**) Northern blots using RNA prepared from *upf1*Δ and *UPF1* cells (see legend to Figure 3). The blots were probed with the same fragment of the *SPT10* gene used in (B). The numbers to the left of the blots in (B) and (C) correspond to the constructs shown schematically in (A).

not stimulate NMD, we focused on the coding region. Close examination of the *SPT10* coding region sequence shown in the experiments of Figure 5 to be sufficient for triggering NMD (construct 3), indicated that this segment of mRNA harbored an AUG and a downstream UAG that are both in the +1 reading frame (Figure 6A). This, coupled with the poor sequence context of the first AUG (a U in the –3 position; Baim and Sherman, 1988; Cigan *et al*., 1988; Yun *et al*., 1996), suggested that a ribosome may occasionally scan past the first AUG, initiate at the AUG in the +1 reading frame, and then encounter a premature termination signal 15 codons downstream. Consistent with the notion that the ribosome can scan and initiate downstream of the first AUG is the observation that a frameshift mutation introduced 43 nt downstream of the initiation codon shifts the reading frame of the +1 AUG into the wild-type frame and produces a protein with partial function (Natsoulis *et al*., 1994). Based on these observations, we tested the leaky scanning model by preparing another construct in which the +1 AUG was mutated to CCC to prevent its use as an initiation codon (Figure 6A, construct 3-2). (The next available AUG is in the normal *SPT10* ORF, and its use as an initiation

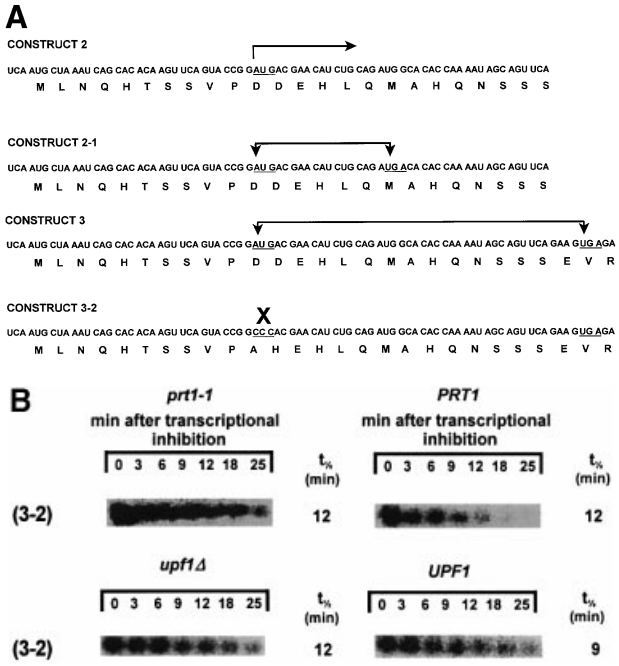

Fig. 6. Mutation of the out-of-frame AUG in the *SPT10* mRNA stabilizes the mutant transcript. (**A**) *SPT10* sequences present in chimeric constructs. *SPT10* sequences of constructs 2 and 3, used in Figure 5, are shown. In addition, the figure illustrates the mutations introduced into these constructs to test the leaky scanning hypothesis (constructs 2-1 and 3-2), including the introduction of an out-of-frame UGA (construct 2-1) and mutation of the out-of-frame AUG to CCC (construct 3-2). (**B**) Northern blots using RNA prepared from the *prt1-1*, *PRT1*, *upf1*Δ and *UPF1* strains (see legend to Figure 3) harboring construct 3-2 on a centromere plasmid. The probe used was the *STP10* portion of the chimeric gene.

codon would simply produce a protein lacking the first 16 amino acids; see Figure 6A). Construct 3-2 was transformed into the *prt1-1* and *upf1*Δ strains, and their respective wild-type parent strains, and decay rates of the construct 3-2 mRNA were determined (Figure 6B). Consistent with the 'leaky scanning' model, the half-life of the construct 3-2 mRNA was increased 3- to 4-fold in the wild-type strains (compare with Figure 5A and B), but not significantly affected by the *prt1-1* and *upf1*Δ mutations. These results indicate that the AUG present in the +1 reading frame is necessary to promote NMD.

To test the leaky scanning model further, we prepared another chimeric construct. The results shown in Figure 5 demonstrated that the construct 2 mRNA was stabilized in the wild-type strains, suggesting that a component necessary to promote NMD was missing from this construct. Close inspection of this mRNA (Figure 6A, construct 2) revealed that, although the +1 AUG is present, the chimera lacks the premature termination codon in the +1 reading frame (termination of the +1 reading frame in this mRNA occurs further downstream, 12 nt into the *PGK1* 3′-UTR). Therefore, construct 2 was mutated to introduce a premature termination codon in the +1 reading frame six codons downstream of the +1 AUG (Figure 6A, construct 2-1). The construct 2-1 mRNA now has a half-life of 3 min in wild-type strains, i.e. the point mutation caused a 4-fold decrease in the stability of the construct 2 mRNA (Table II and data not shown). Together, the results from the experiments of Figures 4, 5 and 6 strongly suggest that leaky scanning triggers NMD of the *SPT10* mRNA.

Table III. Genes identified in the TransTerm database whose transcripts are candidates for NMD by leaky scanning

UBP7	*BUD3*	*YCR079w*
REV7	*YPR204w*	*YCR026c*
STE50	*YIL041w*	*YCR030c*
PFK26	*YFR017c*	*YHL050c*
FAA3	*YFR013w*	*YCL012w*
RAD18	*YCR068w*	*YCR007c*

Identification of other genes whose transcripts decay via the NMD pathway

To identify other mRNAs that may be substrates for the NMD pathway due to leaky scanning, we assessed initiation codon context. Characterization of the *CYC1* and *CYC7* mRNAs demonstrated that an A or a G in the –3 position relative to the AUG yielded better translation initiation than a C or a U in this position (Baim and Sherman, 1988; Yun *et al.*, 1996). For the *HIS4* gene, it was shown that A or C at position –3 yielded the greatest levels of translation (Cigan *et al.*, 1988). Interestingly, the *SPT10* mRNA has a U in the –3 position (see Figure 6A). To identify genes whose transcripts are in a suboptimal translation initiation context, we searched a database that contained sequences for approximately one-tenth of the known yeast genes (TransTerm database; Dalphin *et al.*, 1996). Of the 550 sequences in the database, ~80 have a suboptimal initiation context nucleotide at the –3 position. The sequence of each of these genes was retrieved and analyzed further to determine whether the encoded mRNAs could be candidates for NMD by leaky scanning. The criteria used for screening included: (i) the presence of a U or C in the –3 position relative to the initiation codon (suboptimal sequence context); (ii) the presence of a downstream AUG that is out-of-frame with the initiator AUG, in an optimal sequence context, and within a distance of 90 nt; and (iii) the occurrence of a premature termination codon in the same frame as the out-of-frame AUG. Eighteen mRNAs that satisfied these criteria were identified and are listed in Table III. Preliminary experiments demonstrated that three of these mRNAs were sufficiently abundant to be detected by routine Northern blotting (the *UBP7*, *REV7* and *STE50* mRNAs), and they were chosen for further analysis.

Figure 7A presents the sequence of the 5′ end of the coding region of the *UBP7* mRNA. The initial AUG is present in a suboptimal sequence context, having U in the –3 position. The next AUG is in the +1 reading frame and, with an A in the –3 position, is present in an optimal context. This segment of the *UBP7* mRNA also contains a UAA termination codon in the +1 reading frame. Consistent with the leaky scanning hypothesis, the half-life of the *UBP7* mRNA was increased at least 2- to 3-fold in the *prt1-1* and *upf1*Δ strains ($t_{1/2}$ = >> 25 min) relative to its decay rate in the respective wild-type strains ($t_{1/2}$ = 18–20 min; Figure 7C).

Figure 7A also shows the sequence of the 5′ end of the *REV7* mRNA, another transcript that satisfied our criteria for NMD by leaky scanning. Like the *UBP7* mRNA, the

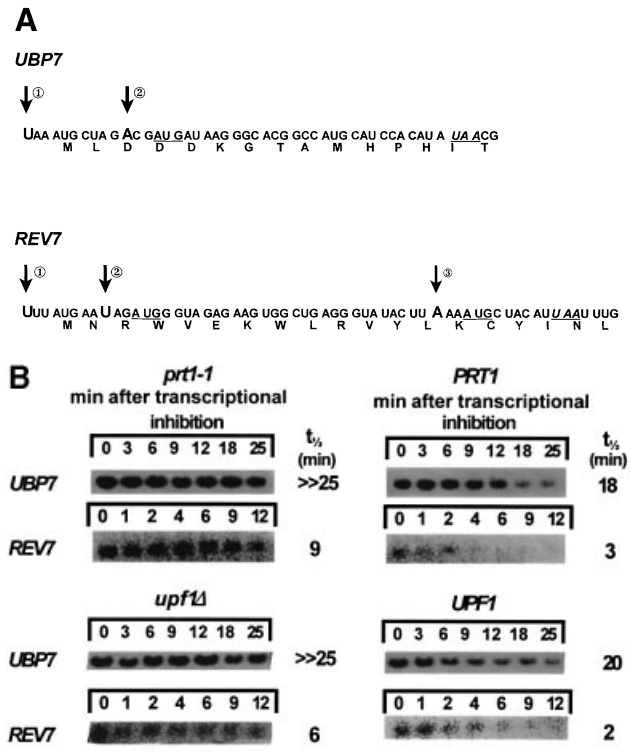

Fig. 7. The *UBP7* and *REV7* mRNAs are substrates for the NMD pathway. (**A**) The sequence of the *UBP7* mRNA indicating the –3 position of the initiator AUG (①) and the –3 position of the first out-of-frame AUG in an optimal context (②). The AUG codon that serves as the potential initiation codon if leaky scanning occurs is underlined. The termination codon encountered if initiation begins from the downstream AUG is shown underlined and in italics. Also shown is the sequence of the *REV7* mRNA indicating the –3 position of the initiator AUG (①), the –3 position of the out-of-frame AUG in a suboptimal context (②) and the –3 position of the second out-of-frame AUG, which is in an optimal sequence context (③). The AUG codons that serve as potential initiation codons if leaky scanning occurs are underlined. The termination codon encountered if initiation begins from either downstream AUG is shown underlined and in italics. (**B**) Northern blots using RNA prepared from the pairs of *prt1-1* and *PRT1*, and *upf1*Δ and *UPF1* cells. See legend to Figure 3 for strains. The probes used were fragments of the *UBP7* and *REV7* genes. Note the different time points in the respective blots.

initiation codon of *REV7 is* present in a suboptimal context. Furthermore, there are two AUG codons downstream in the +2 reading frame, one of which is present in a suboptimal context (U at –3) and the other in an optimal context (A at –3). A termination codon is also present in the +2 reading frame. Decay rates of the *REV7* mRNA in mutant and wild-type cells were consistent with NMD by leaky scanning since the half-life of this mRNA was also stabilized 3-fold in the *prt1-1* and *upf1*Δ cells (Figure 7B). Similarly, the half-life of the *STE50* mRNA, a third candidate for NMD by leaky scanning, was increased 2-fold in both mutant strains (data not shown). We had shown previously that the half-life of most wild-type mRNAs is unaffected by mutation of the *UPF1* gene (Leeds *et al.*, 1991). Inspection of those genes indicate that they are not candidates for leaky scanning based on the criteria used here, and the half-lives measured in *UPF1* and *upf1*Δ strains are consistent with this interpretation. Collectively, these data strongly suggest that leaky scanning can trigger NMD.

Discussion

Cells harboring the prt1-1 allele stabilize nonsense-containing transcripts

The documented interrelationships of mRNA decay and translation (see Introduction) prompted us to analyze mRNA turnover rates in a set of yeast mutants harboring mutations that affected the activity of specific translation initiation factors. These experiments demonstrated that one mutation, *prt1-1*, led to a substantial and selective stabilization of nonsense-containing mRNAs. All other mutations analyzed (affecting the activity of eIF2, eIF3 or eIF4E) had little or no effect on the decay rates of any mRNA studied. One explanation for the discrepancies between the different mutants is that they reflect the different extent to which translation has been inhibited. At 37°C, cells harboring the *prt1-1* allele have <5% of their normal translation activity and polyribosomes (Figure 1). Cells harboring other *prt1* alleles, or other initiation factor mutations, all show less drastic effects on translation activity (Figure 1 and data not shown; Hanic-Joyce *et al*., 1987; Altmann *et al*., 1989; Yoon and Donahue, 1992; Linz *et al*., 1997). Moreover, *prt1-1* cells incubated at the semi-permissive temperature of 32°C show no effects on mRNA decay rates even though translation rates are reduced by 50% (data not shown). These data suggest that, unlike reductions in translational elongation, where even modest effects promote some degree of mRNA stabilization (Peltz *et al*., 1992; Zuk and Jacobson, 1998), decreases in translation initiation may need to be very severe in order to affect turnover rates of nonsense-containing mRNAs.The effects in initiation-compromised cells also differ from those in which elongation has been inhibited because, in the latter, all classes of mRNA are stabilized (Peltz *et al*., 1992; Zuk and Jacobson, 1998), presumably because of occlusion of the 5′ cap by stalled ribosomes (Beelman and Parker, 1994).

A role for eIF3 in nonsense-mediated mRNA decay?

The substrate specificity of the mRNA stabilizing effects of the *prt1-1* mutation indicates that the decay of nonsense-containing mRNAs, but not inherently unstable mRNAs, requires either a minimal amount of ongoing translation and/or the specific function of the *PRT1* protein. This conclusion is supported in part by earlier studies which demonstrated that inhibition of yeast mRNA translation *in cis*, using 5′-UTR insertions of stable stem–loop structures, did not stabilize inherently unstable mRNAs (Beelman and Parker, 1994; Sagliocco *et al*., 1994; Oliveira and McCarthy, 1995; Linz *et al*., 1997). It is unlikely, however, that the stabilizing effects of the *prt1-1* mutation are attributable to a requirement for continued synthesis of an unstable protein, since translation rates were also severely reduced in *prt1-26* cells (Figure 1) but mRNA decay rates were unaffected. A role for ongoing translation in the decay of nonsense-containing transcripts is suggested by experiments showing that their decay: (i) occurs on polysomes (He *et al*., 1993; Zhang *et al*., 1997); (ii) can be reversed by the expression of suppressor tRNAs (Losson and Lacroute, 1979); and (iii) requires the activity of a set of factors shown also to be involved in the

regulation of translational termination (Czaplinski *et al*., 1998).

eIF3 has been shown to stabilize the 43S preinitiation complex, promote binding of the 43S preinitiation complex to the mRNA and enhance dissociation of 40S and 60S ribosomal subunits (Trachsel and Staehelin, 1979; Feinberg *et al*., 1982; reviewed in Merrick, 1992). Of these functions, the one that may be linked to events occurring at premature translational termination is the dissociation of ribosomal subunits, i.e. eIF3 may initiate its dissociation function by binding to a terminating ribosome. In this respect, it is of interest that recent studies have demonstrated that: (i) the *UPF1* protein interacts with, and presumably regulates, the polypeptide release factors Sup35p and Sup45p (Czaplinski *et al*., 1998; A.Maderazo, F.He, D.Mangus and A.Jacobson, manuscript submitted); and (ii) *Escherichia coli* IF3 may promote ribosome dissociation at translation termination by removing deacylated tRNA from the partial P site of the 30S subunit (Karimi *et al*., 1999). Thus, the comparable consequences that *upf1* and *prt1* mutations have for nonsense-containing mRNAs may be indicative of disruptive effects on related events at translational termination. This interpretation of *PRT1* function suggests that mRNA decay phenotypes ought to be obtained with mutations in genes encoding other eIF3 subunits, such as *SUI1*. While we detected only a modest stabilizing effect on nonsense-containing mRNAs in cells harboring the *sui1-1* mutation (data not shown), we note with interest that cells containing the *mof2-1* allele of *SUI1* do appear to stabilize this class of mRNAs (Cui *et al*., 1999).

The effects of the *prt1-1* mutation on the decay of specific yeast mRNAs have also been examined previously. Barnes *et al*. (1993) showed that *SSA1* and *SSA2* heat-shock mRNAs were destabilized in *prt1-1* cells, and Cereghino *et al*. (1995) showed that *SDH1* and *SDH2* mRNAs suffered a similar fate. These mRNAs thus appear to require translation to maintain their stability, a phenomenon analogous to that uncovered in experiments characterizing the effects of blocking translation of yeast mRNAs *in cis* (Muhlrad *et al*., 1995) or *in trans* (Schwartz and Parker, 1999). These results, and those of Barnes *et al*. (1993) and Cereghino *et al*. (1995), suggest that, for some mRNAs, interruption of mRNA translation initiation at a very early step prevents formation of an mRNP structure that is capable of minimizing the rate of decapping (Muhlrad *et al*., 1995; Schwartz and Parker, 1999).

Identification of a new class of substrates for the NMD pathway

mRNA differential display analysis has been used successfully to identify genes that are preferentially expressed in a wide variety of eukaryotic cells (reviewed in Liang and Pardee, 1995). We took advantage of this method to determine whether cells harboring *prt1* or *upf1* mutations stabilized comparable sets of mRNAs. As an initial test of this approach we sought to determine whether known substrates of the NMD pathway could be detected. The *CRY2* pre-mRNA, like the *CYH2*, *RP51B* and *MER2* pre-mRNAs, is a low abundance pre-mRNA, which has been shown previously to be stabilized in cells containing *upf1* mutations (He *et al*., 1993; Zhen *et al*., 1995; F.He and A.Jacobson, unpublished data). The demonstration that the *CRY2* pre-mRNA accumulated in *prt1-1* and *upf1*Δ cells validated the differential display procedure. The failure to detect the *CYH2* pre-mRNA in this screen, i.e. our routine indicator for the activity of the NMD pathway (He *et al*., 1993, 1997), was attributable to a combination of the primer sets used and the characterization of fragments <500 bp in size.

The differential display screen, and subsequent analyses presented here, demonstrate that the *SPT10* mRNA is a naturally occurring substrate for the NMD pathway and suggest that this transcript is shunted into this pathway by leaky scanning of an initiating ribosome. We base this conclusion on several observations, including: (i) an *SPT10–PGK1* chimeric transcript containing the 5′-UTR and first 73 nt of *SPT10* coding region (construct 2) was stable in *upf1*, *prt1* and wild-type strains. This construct lacked a termination codon that would be recognized as premature in the same reading frame as the +1 AUG; (ii) a related *SPT10–PGK1* chimeric transcript containing 21 additional nucleotides of *SPT10* coding sequence (construct 3) was stabilized in *prt1* and *upf1* strains, but not in wild-type cells. The additional sequence provided a stop codon in-frame with the +1 AUG that could prematurely terminate translation initiated at this AUG; (iii) mutation of the +1 AUG, such that it could not be used as a translation initiation site, stabilized the chimeric mRNA; and (iv) introduction of a stop codon into construct 2 that is in-frame with the +1 AUG created a transcript that was unstable in wild-type cells, but stabilized by the *prt1* and *upf1* mutations. Collectively, these data provide another example of the linkage of mRNA decay and translation, and suggest a model in which the ribosome occasionally scans past the initiator AUG, initiates at the next AUG, terminates at the out-of-frame nonsense codon and triggers NMD. Analyses of leaky scanning in other yeast mRNAs suggest that initiation at the second AUG can occur as frequently as initiation at the first AUG, depending on the proximity and context of the two AUG codons (Yun *et al*., 1996).

Our observations with the *SPT10* mRNA led us to consider the possibility that other yeast mRNAs behaved comparably. Using initiation codon context and availability of downstream out-of-frame initiation and termination codons as criteria, we screened the TransTerm database and identified 18 candidate mRNAs (Table III). Three of these that were sufficiently abundant for subsequent analyses (the *UBP7*, *REV7* and *STE50* mRNAs) were tested for their responses to *prt1* and *upf1* mutations, and shown to behave comparably to the *SPT10* mRNA. These results, and the fact that the TransTerm database represents only about one-tenth of all yeast mRNAs, lead us to conclude that as many as 3% of all yeast mRNAs could be subject to leaky scanning and NMD. Obviously, the reliability of this prediction may depend on the role of other sequence elements, including the relative importance of termination codon context (Grant and Hinnebusch, 1994; Bonetti *et al*., 1995) and initiation context (Cigan and Donahue, 1987; Kozak, 1997).

Leaky scanning has been recognized previously to be a mechanism by which the cell can generate multiple proteins, possibly differing in function or localization, from the same mRNA (Vivier *et al*., 1999). The data

Table IV. Yeast strains

Strain	Relevant genotype	Source
TP11B-4-1	*MAT***a** *prt1-1 ade1 leu2-3,112 ura3-52*	G.Johnston
TC3-212-3	*MAT*α *prt1-63 leu2-3,112 ura3-52*	G.Johnston
TDE-16A	*MAT***a** *prt1-26 ade2 leu2-3,112 trp1 ura3-251,373*	G.Johnston
Sc252	*MAT***a** *leu2-3,112 ura3-52 ade ile*	J.Hopper
SWP154(-)	*MAT*α *rpb1-1 his4-38 leu2-1 upf1-Δ1*	Peltz *et al*. (1993)
RY262	*MAT*α *rpb1-1 his4-519 ura3-52 UPF1*	Herrick *et al*. (1990)
AH01	*MAT***a** *leu2-3,112 ura3-52 upf1::LEU2 rpb1-1*	Hennigan and Jacobson (1996)
RP582	*MAT***a** *leu2-3,112 ura3-52 UPF1 rpb1-1*	R.Parker
GNX197-8B	*MAT***a** *his3-200 leu2-1 lys2-801 trp1-1 ura3-52 spt10::LEU2*	J.Boeke

presented here suggest that leaky scanning may also provide the cell with a novel regulatory opportunity. Any mechanism that increases the frequency of initiation at the first AUG of mRNAs destabilized by leaky scanning would enhance the amount of full-length protein produced from the transcript, as well as increase its half-life. Depending on the decay rate of the 'stabilized' form of the mRNA, such translational regulation could lead to as much as 20- to 30-fold increases in expression of selected genes. It remains to be determined whether such a mechanism is actually utilized.

Combined with earlier studies, substrates of the NMD pathway now include: (i) mRNAs that arise from genes containing nonsense mutations (Leeds *et al*., 1991; Peltz *et al*., 1993); (ii) inefficiently spliced pre-mRNAs that enter the cytoplasm with their introns intact (He *et al*., 1993); (iii) some mRNAs containing uORFs (Cui *et al*., 1995; Vilela *et al*., 1998); (iv) transcripts with extended 3′-UTRs (Pulak and Anderson, 1993; Muhlrad and Parker, 1999; F.Sherman, personal communication); and (v) some mRNAs subject to leaky scanning (this study). By inference, mRNAs that contain premature nonsense codons as a consequence of transcription or splicing errors would also be substrates. The possibility that many yeast mRNAs enter the NMD pathway by leaky scanning is in contrast to our earlier studies showing that numerous wild-type mRNAs are not substrates for this pathway (Leeds *et al*., 1991; Peltz *et al*., 1994). Analysis of the initiation codon contexts and availability of downstream initiation and termination codons in these mRNAs, including, for example, the *ACT1*, *ADE3*, *URA5* and *STE2* mRNAs, indicates that these mRNAs should not initiate decay by leaky scanning.

Materials and methods

Strains, plasmids and general methods

Yeast strains and plasmids are listed in Tables IV and V, respectively. Preparation of standard yeast media and methods for cell culture, transformation, DNA manipulation and PCR amplification were as described previously (He and Jacobson, 1995; He *et al*., 1997). *PRT1* genomic DNA used for complementation of *prt1* mutant strains was isolated by colony hybridization (Sambrook *et al*., 1989) of a *Saccharomyces cerevisiae* genomic DNA library of Sau3A partial fragments constructed in YCp50 (Rose *et al*., 1987; provided by Duane Jenness). Approximately three genomic equivalents were screened using a *PRT1* fragment (pGEM1-*PRT1*) as probe. The DNA sequence of the *PRT1* genomic clone was confirmed.

Measurement of translation rates and polysome analysis

As described previously (Peltz *et al*., 1992), cells were grown in SC-met at 24°C to an OD_{600} = 0.5–0.7. Cell cultures were shifted to 37°C by the addition of an equal volume of SC-met medium preheated to 56°C and incubated thereafter at 37°C. Aliquots of the culture (1 ml) were removed to 5 μCi of [^{35}S]Trans label (Amersham; 15 mCi/ml, 38 TBq/mmol) and incubated for 4 min at 37°C. Incorporation of labeled amino acids was monitored by trichloroacetic acid (TCA) precipitation: 0.2 ml of cold (4°C) 50% TCA was added to each aliquot, followed by heating to 70°C for 20 min, incubation on ice for 10 min and subsequent filtration through GF/C (glass fiber) filters. The filters were washed with 10 ml of 5% TCA (4°C) and 10 ml of 95% ethanol, then dried and counted in a scintillation counter. Polyribosomes were analyzed as described previously (Peltz *et al*., 1992). Cells were grown at 24°C to OD_{600} = 0.5–0.7 and then shifted to 37°C for 15 min by the addition of an equal volume of medium prewarmed to 56°C. Cells were harvested and cytoplasmic extracts were prepared and fractionated on 15–50% sucrose gradients.

RNA extraction, RNA blotting, measurement of mRNA decay rates, transcript mapping and data analysis

Total RNA was isolated from 4 ml culture aliquots that were centrifuged and immediately frozen on dry ice at the indicated times as described previously (Herrick *et al*., 1990). Decay rates of individual mRNAs were determined by Northern analyses using 15 μg of RNA isolated at different times after inhibition of transcription by: (i) treatment of cells with 12 μg/ml anti-fungal agent thiolutin (for *PRT1*/*prt1-1* strain pairs; Jimenez *et al*., 1973; Tipper, 1973; generously provided by Dr Nathan Belcher, Pfizer Central Research, Pfizer, Inc., Groton, CT); or (ii) shifting *rpb1-1* temperature-sensitive cells to the non-permissive temperature (for *UPF1*/*upf1* strain pairs; Herrick *et al*., 1990). Temperature-shift experiments were performed as described (Peltz *et al*., 1993). In brief, cells were grown at 24°C to an OD_{600} of 0.5–0.7, centrifuged and resuspended in 20 ml of SC-ura medium, equilibrated to 24°C and then shifted to 37°C rapidly by the addition of 20 ml of medium prewarmed to 56°C. For *PRT1* and *prt1-1* strains, cells were shifted to the non-permissive temperature for 15 min prior to the inhibition of transcription. RNA blots, standardized for rRNA loading and integrity, were hybridized with DNA or anti-sense RNA probes specific for the genes of interest. Probes for the *ACT1*, *CYH2*, *HIS3*, *LEU2*, *MFA2*, *PAB1*, *PGK1-UAG-2*, *PGK1-UAG-6* and *STE2* mRNAs, and the *CRY2* pre-mRNA were described previously (Herrick *et al*., 1990; Leeds *et al*., 1991; Peltz *et al*. 1993; Zhen *et al*., 1995). Probes used to detect other mRNAs included: *SPT10* mRNA (full-length), a 2 kb *Sal*I–*Hin*dIII fragment from plasmid pFW217 (kindly provided by Fred Winston; Natsoulis *et al*., 1991); *CDC4* mRNA, a 3.5 kb *Sal*I fragment from plasmid SJ5141000 (kindly provided by Steve Johnson); *URA5* mRNA, a 2 kb *Eco*RI–*Kpn*I fragment excised from plasmid FL44 (kindly provided by Francois Lacroute); *GCN4* mRNA, a 3 kb *Eco*RI–*Sal*I fragment excised from plasmid p164 (kindly provided by Alan Hinnebusch); *SPT10–PGK1* chimeric mRNAs, a *Spe*I–*Bgl*II fragment containing the 5′-UTR and the first 73 or 94 bp of *SPT10* coding region excised from constructs 2 or 3; *HHF2* mRNA, a PCR fragment (~300 bp) amplified with oligonucleotides 15 and 16 (Table VI) from plasmid pCC66 (kindly provided by Fred Winston). Using genomic DNA as template, PCR was used to generate probes for the *LYS14*, *UBP7* and *REV7* mRNAs with oligonucleotides 17 and 18, 9 and 10, and 11 and 12, respectively (Table VI). Decay rates, expressed as mRNA half-lives, were determined

Table V. Plasmids

Plasmid	Description	Source
pRS316	previously described	Sikorski and Hieter (1989)
pG7P1(pGEM1-*PRT1*)	*PRT1* fragment cloned into pGEM1 vector	J.Hershey
pRS316-*PRT1*	4 kb *Hin*dIII *PRT1* fragment cloned into pRS316	this study
CRY2 pGEM-3Z	used to prepare anti-sense RNA *CRY2* probe	Zhen *et al*. (1995)
pCC66	plasmid harboring *HHF2* sequence	F.Winston
SPT10 (*Sal*I–*Hin*dIII)	plasmid harboring entire *SPT10* sequence	F.Winston
pUC9–PGK1H2(3)	previously described	Peltz *et al*. (1993)
Construct 1	oligos 5 and 6 were annealed to form a fragment of dsDNA, which was digested with *Ava*I and *Xba*I, then ligated to *Ava*I–*Xba*I digested construct 3	this study
Construct 2	*Spe*I–*Bgl*II PCR product containing the 5′-UTR and 73 bp of *SPT10* (using primers 1 and 3) was subcloned into *Spe*I–*Bgl*II digested pRS316–PGK1H2(3)	this study
Construct 2-1	ligated *Spe*I–*Bgl*II *SPT10* fragment containing a UAG mutation (using primers 1 and 8) with *Spe*I–*Bgl*II digested construct 2	this study
Construct 3	subcloned PCR fragment containing the 5′-UTR and 94 bp of *SPT10* (using primers 1 and 2) into pRS316–PGK1H2(3) plasmid	this study
Construct 3-1	ligated *Spe*I–*Pst*I *SPT10* fragment harboring CCC mutation (using primers 1 and 7) with *Spe*I–*Pst*I digested construct 3	this study

Table VI. Oligonucleotides

Number	Sequence
1	5′-AATGTCGACTACTAGTCATTTGCGGTGTTA-3′
2	5′-TATTAGATCTCCGCTGCCTCATTTCTCAC-3′
3	5′-AAAGATCTGTGCCATCTGCAGATGTTCGTCGTCATCCGG-3′
4	5′-CTTGTGTGCTGATTTAGCATTGAAGC-3′
5	5′-TCGGGGGCCCAGTAAAAAGTGTACCGATCAAGAACAACT-3′
6	5′-CTAGAGTTGTTCTTGATCGGTACACTTTTTACTGGGCCC-3′
7	5′-AAAGATCTGTGCCATCTGCAGATGTTCGTGGGCCGG-3′
8	5′-AAAGATCTGTGTCATCTGCAGATGTTCGTCGTCATCCGG-3′
9	5′-ACGAAGATATCAAGCACTACTATCCGCAGC-3′
10	5′-TTGGATCCACATTCACTAAGTTCGGAGCC-3′
11	5′-ACTACTTACCAGTCATTCAACTTGCCGC-3′
12	5′-AACAAAGATCCAAAAATGCTCTCGCCC-3′
13	5′-AGTGGTCGGTTGATGATGTGATAACTTGG-3′
14	5′-AACAATTCTAACAGCCTCTCTTGATCCCC-3′
15	5′-ACAATCGGTGGTTAAACAATCGGT-3′
16	5′-TGGGTTTCTGCGTAAATATTTGGG-3′
17	5′-GTCAGTGCGTTTACGTTTTAA-3′
18	5′-TTTGATCCACCTTAGGTGCTA-3′
19	5′-GTTGCTTAATTCCGTTGTCCCT-3′
20	5′-TACCCTTAACTTCCGAGGTTTC-3′
21	5′-AAAGCTAGCGCACACAAGTTCAGTACCGG-3′
22	5′-AAATCTAGAACCGCTGCCTCATTTCTCAC-3′

by direct counting of RNA blots using a Betagen Blot Analyzer, normalizing the data such that t_0 was equal to 100%, and plotting the data with respect to time on semi-log axes (Herrick *et al*., 1990). Individual mRNA half-lives in specific strains varied by less than ±15%. Primer extension analysis was performed according to the procedure described by Boorstein and Craig (1989). Total RNA (20 μg) was hybridized to an oligonucleotide primer complementary to the *SPT10* sense strand at positions +30 to –5 (Table VI, oligonucleotide 4). Extension reactions utilized AMV reverse transcriptase (Boehringer Mannheim). Differential display analyses utilized the RNAimage kit from GenHunter, Inc. (Nashville, TN). DNA fragments showing differential accumulation in independent RNA samples were cloned into the vector pGEM-T (Promega).

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