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Published online 6 March 2009

Nucleic Acids Research, 2009, Vol. 37, No. 9 ***2771–2778***
doi:10.1093/nar/gkp146

Long 3′-UTRs target wild-type mRNAs for nonsense-mediated mRNA decay in *Saccharomyces cerevisiae*

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Received December 6, 2008; Revised February 18, 2009; Accepted February 19, 2009

ABSTRACT

The nonsense-mediated mRNA decay (NMD) pathway, present in most eukaryotic cells, is a specialized pathway that leads to the recognition and rapid degradation of mRNAs with premature termination codons and, importantly, some wild-type mRNAs. Earlier studies demonstrated that aberrant mRNAs with artificially extended 3′-untranslated regions (3′-UTRs) are degraded by NMD. However, the extent to which wild-type mRNAs with long 3′-UTRs are degraded by NMD is not known. We used a global approach to identify wild-type mRNAs in *Saccharomyces cerevisiae* that have longer than expected 3′-UTRs, and of these mRNAs tested, 91% were degraded by NMD. We demonstrate for the first time that replacement of the natural, long 3′-UTR from wild-type *PGA1* mRNA, which encodes a protein that is important for cell wall biosynthesis, with a short 3′-UTR renders it immune to NMD. The natural *PGA1* 3′-UTR is sufficient to target a NMD insensitive mRNA for decay by the NMD pathway. Finally, we show that *nmd* mutants are sensitive to Calcofluor White, which suggests that the regulation of *PGA1* and other cell wall biosynthesis proteins by NMD is physiologically significant.

INTRODUCTION

Nonsense-mediated decay (NMD) is a highly conserved mechanism for recognizing and rapidly degrading mRNAs with premature termination codons. It has an important protective role because it prevents the synthesis of potentially deleterious truncated proteins and is responsible for making many mutations recessive including many human disease mutations. More recently it has been recognized that NMD has a second, important role in the regulation of wild-type gene expression. Wild-type mRNAs that are regulated by the NMD pathway have been identified by global expression profiling in the yeast *Saccharomyces cerevisiae*, *Drosophila melanogaster* and humans. When NMD is inactivated 5–10% of the yeast transcriptome is affected (1–3). NMD affects a similar percentage of transcripts in the *Drosophila* and human transcriptome (4,6). Thus, NMD also serves an important cellular function in regulation of gene expression.

Studies in *S. cerevisiae*, *Caenorhabditis elegans*, the plant *Nicotiana*, and cell lines from *Drosophila* and humans suggest that termination codons are recognized as premature when positioned too far upstream of the poly(A) tail (7–13). mRNAs transcribed from genes containing mutations that generate aberrant extended 3′-untranslated regions (3′-UTRs) are degraded by NMD (7,11). Also synthetic mRNAs terminating translation at normal termination codons with extended 3′-UTRs are also degraded by NMD (7,11). *In vitro*, translation termination at stop codons followed by long 3′-UTRs is biochemically distinct from termination at stop codons followed by a normal 3′-UTR (7). The aberrant translation termination on mRNAs with long 3′-UTRs depends on Upf1p and can be rescued by tethering poly(A)-binding protein close to the stop codon. Collectively, these data are the basis for the faux 3′-UTR model, which predicts that these mRNAs are degraded by NMD because the stop codon is not in the correct context for the translating ribosomes to interact with 3′-UTR bound proteins leading to an aberrant translation termination and NMD (7). If the faux 3′-UTR model is correct, then natural cellular (wild-type) mRNAs with longer than normal 3′-UTRs should be targets for NMD by virtue of their long 3′-UTRs. However, the extent to which wild-type mRNAs with long 3′-UTRs are degraded by NMD is not known.

Consistent with the faux 3′-UTR model, most *S. cerevisiae* 3′-UTRs tend to be short. They typically range in size from 50 to 200 nt, with a median length of 121 nt (14). *S. cerevisiae* has a small but significant number of mRNAs with longer 3′-UTRs. We hypothesized that these transcripts are a novel class of wild-type

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mRNAs that are substrates for the NMD pathway, thus limiting their longevity in cells.

Here, we have identified a subset of wild-type *S. cerevisiae* mRNAs that have longer than expected 3′-UTRs. We demonstrate for the first time, to our knowledge, that wild-type *S. cerevisiae* mRNAs with long 3′-UTRs are strongly enriched for degradation by the NMD pathway. Many of these mRNAs are previously undetected wild-type substrates for the NMD pathway. Further we show that the natural long 3′-UTR of the *PGA1* mRNA is a NMD targeting mechanism because replacement of the 3′-UTR of a NMD-insensitive mRNA with the *PGA1* 3′-UTR is sufficient to target this hybrid mRNA for Upf1p-dependent decay. *PGA1* encodes an enzyme that is involved in glycosylphosphatidylinositol (GPI) anchor synthesis. Mutants lacking a functional NMD pathway are sensitive to the cell wall disruptor Calcofluor White suggesting that the NMD regulation of mRNAs encoding proteins involved in GPI-anchored cell wall protein biosynthesis is physiologically significant.

MATERIALS AND METHODS

Yeast strains and media

The *S. cerevisiae* strains used in this study are W303a (*MAT**a** ade2-1 ura3-11 his3-11,15 trp1-1 leu2-3,112 can1-100*), AAY320 [*MAT**a** ade2-1 ura3-11 his3-11,15 trp1-1 leu2-3,112 can1-100 upf1-Δ2 (URA3)*], AAY334 (*MAT**a** ura3-1 his3-11,15 trp1-1 leu2-3,112 rpb1-1*) and AAY335 (*MAT**a** ura3-1 his3-11,15 trp1-1 leu2-3,112 rpb1-1 upf1-Δ2 (URA3)*]. Unless otherwise stated, *S. cerevisiae* strains were grown and maintained using standard techniques. Susceptibility of wild-type yeast cells and *upf1Δ* mutant to Calcofluor White was done as described in Ram and Klis (15).

3′-RACE analysis

Yeast total RNA was extracted by the hot phenol method from yeast cells harvested at mid-log phase (16). Total RNA used for 3′-RACE (3′-rapid amplification of cDNA ends) was also used for quantitative northern blot analysis. 5 μg of total RNA was reverse transcribed using SuperScript™ II RT (Invitrogen Corporation, Carlsbad, CA, USA). The cDNA was PCR amplified using the Abridged Universal Amplification Primer (AUAP) provided with the 3′-RACE kit and the one primer specific to the mRNA as described in Supplementary Figure 1. The primary PCR reactions were used as templates for nested PCR reactions as described in the 3′-RACE user manual. The secondary PCR reactions were then run on 1.5% agarose gels.

Quantitative northern blot analysis

mRNA steady-state levels and half-lives were measured as described by Kebaara *et al.* (17). Oligolabeled DNA probes were used to probe the northern blots. DNA probes were generated using primer sets for amplifying yeast open reading frames (ORFs) based on the sequences available from Saccharomyces Genome Database (SGD).

RESULTS

Identification of wild-type mRNAs with longer than expected 3′-UTRs

Primary mRNA sequence was predicted by combining information from genome-wide analysis of mRNA length, global identification of transcription start sites, ORF lengths and prediction of 3′-end processing sites (Figure 1). *Saccharomyces cerevisiae* mRNA lengths were determined genome-wide by Hurowitz and Brown (18). mRNA lengths were compared with ORF lengths as annotated in the SGD (January 2003). They found that mRNA lengths closely approximated the ORF

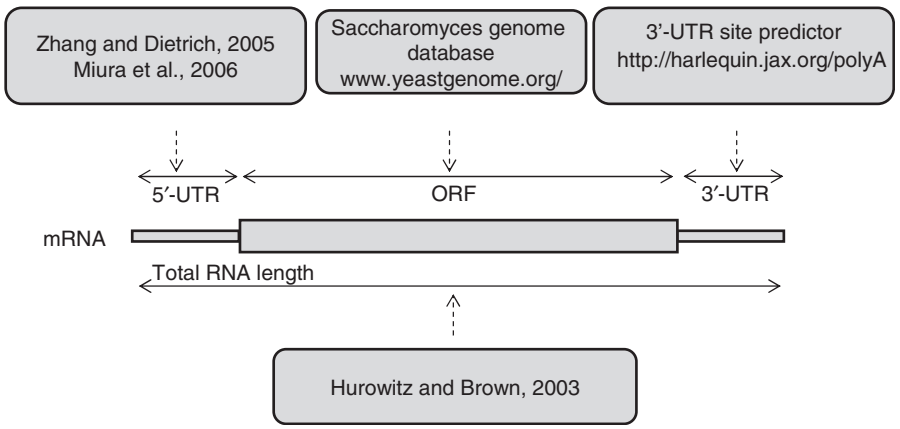

Figure 1. Strategy used to predict the primary sequence of *S. cerevisiae* mRNAs. The 5′-UTR length was determined from the location of the transcription start site [Zhang and Dietrich, 2005 (20); http://data.cgt.duke.edu/5sage.php; Miura *et al.*, 2006 (19); http://yeast.utgenome.org/]. The ORF lengths were obtained from the SGD (www.yeastgenome.org/). The mRNA 3′-processing Site Predictor [Graber *et al.*, 2002 (21); http://harlequin.jax.org/polyA/] was used to predict the lengths of the 3′-UTRs. The 3′-UTR lengths were determined experimentally by 3′-RACE. Virtual northern transcript lengths were from a genome-wide study measuring transcript lengths [Hurowitz and Brown, 2003 (18); http://microarray-pubs.stanford.edu/vnorth/].

length plus 256 nt for the combined 5′-UTR and 3′-UTR lengths. A total of 159 transcripts were categorized as transcripts greater than maximum length (18). These transcripts were predicted to have either an extended 5′-UTR and/or 3′-UTR.

We predicted the primary mRNA sequence for 116 of the 159 transcripts categorized as greater than maximum length. These 116 mRNAs correspond to the mRNAs with verified ORFs. The 5′-UTR lengths were determined from the location of the transcription start sites, when available (19,20) and the ORF lengths were obtained from the SGD (Figure 1). The mRNA 3′-processing site predictor, a tool generated by Graber *et al.* [http:/harlequin.jax.org/polyA/; (21)] was used to identify probable cleavage and polyadenylation sites. Since most normal *S. cerevisiae* mRNAs have 3′-UTRs that range in size from 50 to 200 nt, we selected 350 nt as an arbitrary cutoff. Any transcript with a predicted 3′-UTR longer than 350 nt was considered to have a long 3′-UTR. The cleavage and polyadenylation site closest to the stop codon was chosen for most mRNAs, except when there was a much stronger site predicted downstream. Of the 116 mRNAs, 56 were predicted to have long 3′-UTRs (Supplementary Table S1).

We experimentally tested these predictions for 11 mRNAs, 6 of which are shown in Table 1. We saw a strong correlation between the length of the 3′-UTR and the expected length based on the total mRNA length (18) less than the ORF and 5′-UTR lengths. The functional classes of genes encoding mRNAs with longer than expected UTRs are significantly enriched for genes involved in the regulatory control of cellular processes, especially transcription, signal transduction, cell-cycle control and metabolism (18). Among these are transcripts that encode protein components of the kinetochore, proteins involved in telomere maintenance and cell wall biogenesis. The *nmd* mutants have altered kinetochore function, shorter telomeres and altered telomeric silencing (1,22,23).

Wild-type mRNAs with long 3′-UTRs are strongly enriched for degradation by the NMD pathway

If the presence of a long 3′-UTR on a wild-type mRNAs is a targeting mechanism for NMD, we expect wild-type mRNAs with long 3′-UTRs to be enriched for mRNAs that are degraded by the NMD pathway. mRNAs that are degraded by the NMD pathway accumulate to higher levels and have a longer half-life in mutants lacking a functional NMD pathway (*upf1Δ* strains) relative to isogenic *UPF1* strains [Figure 2; (17)]. To test our hypothesis, we randomly selected 11 mRNAs with 3′-UTRs >350 nt and measured their abundance in wild-type and *upf1Δ* mutants. Ten of these mRNAs accumulated to higher levels in an *upf1Δ* mutant (Table 2). Half-lives were determined for 5 of the 10 mRNAs that accumulated in an *upf1Δ* mutant relative to an isogenic *UPF1* strain. All had longer half-lives in the *upf1Δ* mutant than the isogenic *UPF1* strain (Figure 2 and Table 2). Thus, there is a strong correlation between long 3′-UTRs and decay by the NMD pathway.

PGA1, *MPA43*, *DON1* and *SSY5* mRNAs were previously characterized as direct substrates for the NMD pathway (1,27). *PGA1* mRNA had a longer half-life in an *upf1Δ* strain than an *UPF1* strain, and it was down-regulated upon NMD reactivation (1,27). In the NMD reactivation system, a factor required for NMD *(UPF2)* is under the control of a regulated promoter. Yeast cells with this system have an inactive NMD pathway unless the cells are grown in inducing conditions. Upon NMD reactivation, transcripts that are NMD substrates are rapidly degraded (27). *MPA43, DON1* and *SSY5* mRNAs were associated with Upf1p (27). Upf1p preferentially associates with mRNAs that are direct NMD substrates (27). Our results are consistent with these results with the exception of *SSY5* mRNA. In our system, *SSY5* mRNA was degraded at the same rate in *upf1Δ* and *UPF1* strains.

A long 3′-UTR is sufficient to target wild-type *PGA1* mRNA for NMD

A role for long 3′-UTRs in targeting wild-type mRNAs for NMD predicts that the replacement of the 3′-UTR of a NMD-insensitive mRNA with the long 3′-UTR of a wild-type mRNA should make the mRNA sensitive to NMD. This prediction was tested by replacing the 3′-UTR of the NMD-insensitive *miniPGK1* mRNA with the *PGA1* 3′-UTR [(28); Figure 3]. Wild-type *PGA1* mRNA has a ~750-nt 3′-UTR and is degraded by the NMD pathway (Figure 2). The *mini-PGK1* was selected because it is not degraded by the NMD pathway and it has been used as a reporter to study the *cis*-requirements for NMD [(28); Figure 3A]. The 3′-UTR of the hybrid *miniPGK1* with

Table 1. Experimental validation of primary mRNA structure of representative mRNAs

Standard name	Systematic name	5′-UTR length (nt)	ORF length (nt)	3′-UTR length (nt)		Transcript length (nt)	
				Predicted	3′-RACE	Virtual northern	northern
PGA1	YNL158W	41, 59	597	675	750	1459	1400
ENT4	YLL038C	NA	744	1838	600[a]	2520	2500
MPA43	YNL249C	25, 98, 172	1629	570	300, 600	2263	2300
SPC24	YMR117C	NA	642	1890	1550	2303	2350
SSY5	YJL156C	85	2064	428	420-500	2700	3000
	YPR174C	13, 14, 21	666	1601	1600	2280	2370

[a]The 3′-RACE results correspond to a weak cleavage and polyadenylation signal located ~600-nt downstream of the *ENT4* stop codon.
NA, not available.

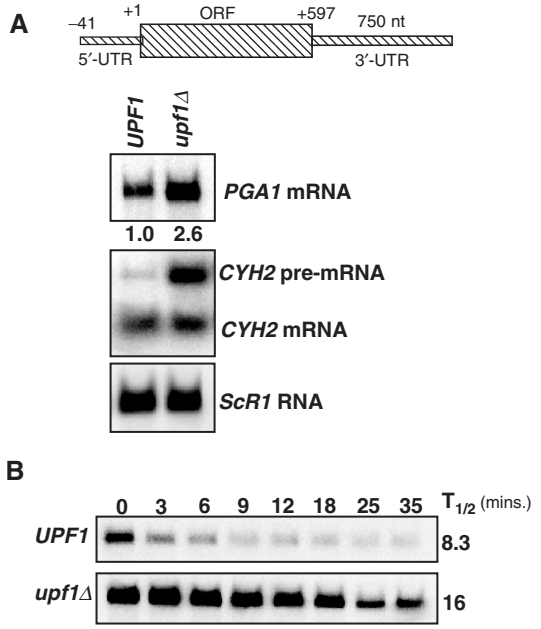

Figure 2. The *PGA1* mRNA is degraded by the NMD pathway. *PGA1* mRNA schematic and northern analysis of the *PGA1* mRNA accumulation (**A**), and half-lives (**B**) in wild-type (*UPF1*) and NMD mutant *(upf1Δ)* yeast cells. The mRNA accumulations were measured using total RNA isolated from yeast strain W303a [*UPF1* (24)] and AAY320 [*upf1-Δ2* (17)]. *CYH2* and *ScR1* are controls (25,26). *CYH2* is a control for the NMD phenotype of the yeast strains. The *CYH2* probe detects both the *CYH2* pre-mRNA and mRNA. *CYH2* pre-mRNA is inefficiently spliced. Consequently, a significant amount of the pre-mRNA is transported to the cytoplasm where it is targeted for NMD by an in-frame stop codon within the intron. In contrast, *CYH2* mRNA is not degraded by NMD. *ScR1* is an RNA polymerase III transcript that is not degraded by NMD. It was used as a loading control. mRNA half-lives were determined using total mRNA isolated from yeast strains AAY334 [*UPF1* (17)] and AAY335 [*upf1-Δ2* (17)] at time points following transcriptional arrest. The mRNA half-lives ($T_{1/2}$) are an average of at least three independent experiments.

the *PGA1* 3′-UTR (*miniPGK1-PGA1 3′-UTR* mRNA) was determined to be the same as the *PGA1* 3′-UTR using 3′-RACE (Supplementary Figure 1B). This hybrid mRNA was degraded by the NMD pathway (Figure 3B). This experiment shows that replacing the NMD-insensitive *miniPGK1 3′-UTR* with the wild-type *PGA1* mRNA 3′-UTR is sufficient to target the hybrid transcript to NMD. These results do not exclude the possibility that there exists some undetected NMD targeting elements within the long *PGA1* 3′-UTR. However, we analyzed the sequences of the long 3′-UTRs for the 10 mRNAs that are degraded by the NMD pathway using the motif-based sequence analysis Multiple EM for Motif Elicitation (MEME) tool and found no common patterns.

We also expect that replacement of the long *PGA1* 3′-UTR with a shorter 3′-UTR from an NMD insensitive mRNA should make the *PGA1* mRNA insensitive to NMD. We therefore tested whether replacing the 3′-UTR of the *PGA1* mRNA with the *ACT1* 3′-UTR would prevent decay of the *PGA1* mRNA by the NMD pathway. The *ACT1* 3′-UTR was selected because *ACT1* mRNA is not degraded by the NMD pathway [(16); Figure 4A). The 3′-UTR of the hybrid *PGA1* mRNA with the *ACT1* 3′-UTR (*PGA1-ACT1* 3′-UTR mRNA) was identical to the authentic *ACT1* 3′-UTR (Supplementary Figure 1A). The hybrid mRNA was not degraded by the NMD pathway (Figures 4B and 5). The stabilization of the *PGA1-ACT1 3′-UTR* mRNA in *UPF1* cells is not due to a protective effect of the *ACT1* 3′-UTR sequences because *PPR1-ACT1* fusion mRNA with the *ACT1* 3′-UTR sequences is still subject to NMD (Figure 4C). We previously showed that wild-type *PPR1* mRNA is targeted for decay by the NMD pathway by an element located within the 5′-UTR and the first 92 nt of the *PPR1* ORF (29).

PGA1 mRNA is immune to NMD in yeast cells expressing the *PGA1-ACT1 3′-UTR* mRNA

In contrast to the NMD-dependent decay of *PGA1* mRNA seen in most cells, *PGA1* mRNA was NMD insensitive in cells that also expressed *PGA1-ACT1 3′-UTR* from a centromeric plasmid (Figure 5A), but not in cells with extra copies of *PGA1* on a centromeric plasmid. This suggests coordinated regulation with *PGA1-ACT1 3′-UTR* mRNA. This regulation is specific to *PGA1* because *SPC24* mRNA and *CYH2* pre-mRNA were still NMD-sensitive (Fig. 5B and 5C and Table 2). *SPC24* mRNA encodes a component of the evolutionarily conserved kinetochore-associated Ndc80 complex (Ndc80p-Nuf2p-Spc24p-Spc25p), and is involved in chromosome segregation, spindle checkpoint activity and kinetochore clustering. *CYH2* encodes a component of the 60S ribosomal subunit.

The stabilization of the endogenous *PGA1* mRNA due to the expression of *PGA1ACT1 3′-UTR* mRNA is not a general effect of mRNAs producing different mRNA isoforms. The *MAK31* gene encodes two mRNA isoforms, a short form with a 3′-UTR of 200 nt and a longer form with a 3′-UTR of 920 nt. The long *MAK31* mRNA is degraded by NMD while the shorter mRNA is immune to the NMD pathway (Table 2). The expression of the short *MAK31* mRNA does not affect the stability of the long *MAK31* mRNA unlike the *PGA1* mRNA and *PGA1-ACT1 3′-UTR* mRNA.

PGA1 mRNA degradation by NMD may be physiologically significant

PGA1 encodes an essential component of GPI-mannosyltransferase II, which is involved in GPI anchor synthesis. GPI anchors are added to proteins that are to be attached to membranes (30). Pga1p, in collaboration with Gpi18p adds the second mannose residue to the GPI precursors. NMD was previously shown to regulate a set of genes coding for additional enzymes involved in the assembly of GPI-anchored cell wall proteins (1). Further, a temperature sensitive *pga1-1* mutant, which is defective in GPI anchor synthesis, was sensitive to Calcofluor White (30). Calcofluor White binds to the glucan and chitin components of yeast cell walls and interferes with cell wall integrity at higher concentrations. To examine the possibility that *nmd* mutants may have a defect in cell wall integrity, we tested whether *upf1Δ* cells are hypersensitive to Calcofluor White (Figure 6A). We found *upf1Δ* cells are much more sensitive to Calcofluor White compared

Table 2. Wild-type mRNAs with long 3′-UTRs tend to be degraded by the NMD pathway

Standard name	Systematic name	3′-UTR length (nt)	Relative mRNA accumulation (*upf1Δ*/*UPF1*)	mRNA half-life (min)	
				UPF1	*upf1Δ*
PGA1	YNL158W	750	2.6 ± 0.3	8.3 ± 3.0	16.0 ± 1.0
ENT4	YLL038C	600	2.3 ± 0.3	4.0 ± 2.6	8.7 ± 3.0
SPC24	YMR117C	1550	2.8 ± 0.1	7.3 ± 4.2	20.7 ± 3.1
DON1	YDR273W	1350	1.6 ± 0.3	7.2 ± 2.0	18 ± 3.5
GUD1	YDL238C	1300	4.2 ± 1.6	ND	ND
	YPR174C	1600	2.0 ± 0.3	ND	ND
	YKR045C	1500	2.2 ± 0.4	ND	ND
YPT35	YHR105W	1300	5.8 ± 3.3	ND	ND
MPA43	YNL249C	300	3.0 ± 0.9	6 ± 2.0	18.7 ± 4.0
		600	3.3 ± 1.3	12.8 ± 1.2	26.3 ± 3.1
MAK31	YCR020C-A	200	1.1 ± 0.2	ND	ND
		920	3.5 ± 0.6	ND	ND
SSY5	YJL156C	420–500	0.9 ± 0.2	>40	>40

The 3′-UTR length was measured by 3′-RACE. Quantitative northern blot analysis was used to measure relative mRNA accumulation in W303 [*UPF1*, (24)] and AAY320 [*upf1Δ-2*, (17)], and mRNA half-lives in AAY334 [*UPF1*, (17)] and AAY335 [*upf1-Δ2*, (17)]. The relative mRNA accumulations and mRNA half-lives are an average of at least three independent experiments.
ND, not determined.

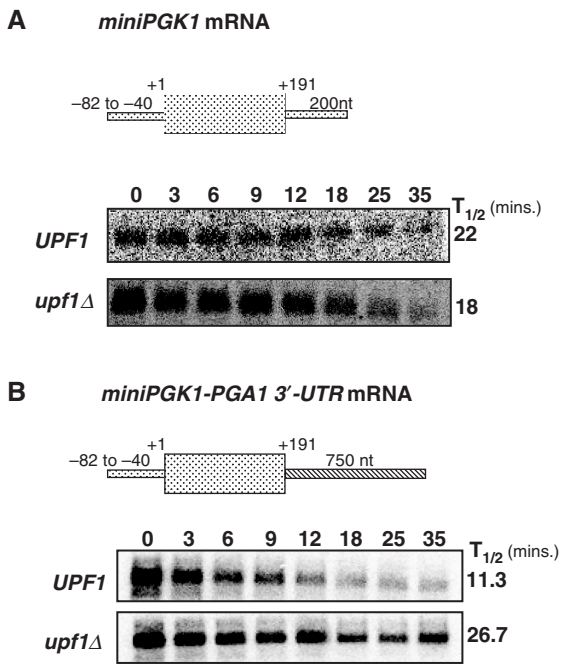

Figure 3. The long 3′-UTR of *PGA1* mRNA is sufficient to target a NMD-insensitive mRNA to NMD. The mRNA schematics and northern blot analysis of *miniPGK1* (**A**), and *miniPGK1-PGA1 3′-UTR* (**B**) mRNAs. The mRNA half-lives ($T_{1/2}$) were determined as described in Figure 2 and are shown to the right of the phosphorImages. They are an average of three independent experiments, with the exception of the half-life for the *miniPGK1* mRNA in the *upf1Δ* strain (A), which is an average of two experiments. The *miniPGK1* mRNA half-lives measured here are consistent with the half-lives determined for this mRNA by Hagan *et al.* (28).

with wild-type yeast cells (Figure 6A). We also found that expression of the *PGA1-ACT 3′-UTR* construct in wild-type yeast cells caused the cells to be more sensitive to Calcofluor White compared with wild-type yeast cells expressing the *PGA1* mRNA from a plasmid (Figure 6B, bottom panel). Expression of the *PGA1-ACT 3′-UTR* construct had no effect on the Calcofluor White sensitivity of NMD mutant yeast cells (Figure 6B, bottom panel).

The observation that both *pga1-1* and *nmd* mutants are hypersensitive to Calcofluor White makes sense because they are both defective in GPI anchor synthesis. GPI-anchor synthesis is a multistep process. An increase or decrease in any of the enzymes involved in this multistep process leads to a defect in GPI anchor synthesis and sensitivity to Calcofluor White. Sensitivity to Calcofluor White by *nmd* mutants cannot be exclusively attributed to Pga1p because other factors required for cell wall biosynthesis have been shown to be regulated by NMD (1).

DISCUSSION

We showed that long 3′-UTRs target wild-type mRNAs for decay by the NMD pathway. Wild-type yeast mRNAs with exceptionally long 3′-UTRs are strongly enriched for degradation by the NMD pathway. Replacement of a long 3′-UTR with a shorter 3′-UTR is sufficient to prevent degradation of the wild-type *PGA1* mRNA by NMD. Further replacing the 3′-UTR of a NMD-insensitive mRNA with a long 3′-UTR is sufficient to target this hybrid mRNA for decay by the NMD pathway (Figure 3). The NMD regulation of mRNAs, including *PGA1* mRNA, that encode proteins involved in GPI-anchored cell wall protein biosynthesis, is physiologically significant because mutants lacking a functional NMD pathway are sensitive to Calcofluor White (Figure 6).

PGA1 may be regulated posttranscriptionally at multiple levels. Pga1p is one member of a group of enzymes that are responsible for GPI-anchor synthesis. Its levels likely needs to be carefully regulated to coordinate expression of the enzymes required for synthesis of GPI-anchored cell wall proteins. The NMD pathway may be responsible for maintaining *PGA1* mRNA at a low level.

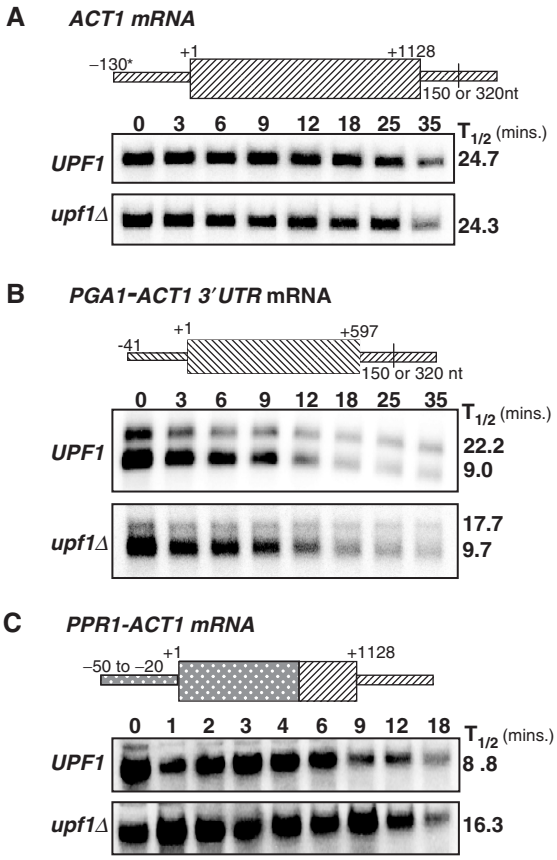

Figure 4. Replacement of the long 3′-UTR of *PGA1* mRNA with a short 3′-UTR protects this mRNA from decay by the NMD pathway. The mRNA schematics and northern blot analysis of *ACT1* (**A**), *PGA1-ACT1 3′-UTR* (**B**), and *PPR1-ACT1* (**C**) mRNAs. The mRNA half-lives were determined as described in Figure 2 and are shown to the right of the PhosphorImages.

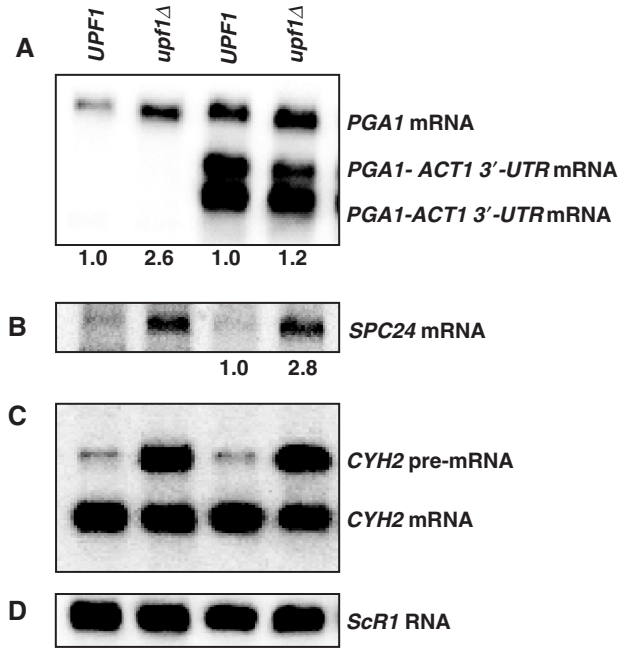

Figure 5. The *PGA1* mRNA is not degraded by the NMD pathway in yeast cells expressing the *PGA1-ACT1 3′-UTR* mRNA. Steady-state northern of the *PGA1* and *PGA1-ACT1 3′-UTR* mRNA (**A**), *SPC24* mRNA (**B**), *CYH2* pre-mRNA/mRNA (**C**), and *ScR1* (**D**), in wild-type W303a [*UPF1,* (24)] and NMD mutant AAY320 [*upf1-Δ2,* (17)] yeast cells. Relative mRNA accumulation was measured as described in Figure 2 and is shown below panel (A) for *PGA1* mRNA and (B) for *SPC24* mRNA.

Interestingly, wild-type *PGA1* mRNA becomes immune to NMD in cells that also express *PGA1-ACT1 3′-UTR*. mRNAs are protected from NMD when they are not being translated (31). The *PGA1-ACT1 3′-UTR* mRNA encodes a full length, functional Pga1p, and the mRNA is present at a high level. The effect of *PGA1-ACT1 3′-UTR* mRNA on wild-type *PGA1* mRNA suggests that wild-type *PGA1* mRNA may become stored when the amount of Pga1p protein within a cell crosses a threshold. This idea is supported by the observation that expression of *PGA1-ACT1 3′-UTR* mRNA makes wild-type yeast cells more sensitive to Calcofluor White compared with yeast cells expressing *PGA1* mRNA from a plasmid.

We predict that most wild-type yeast mRNAs with 3′-UTRs of >300 nt are degraded by the NMD pathway. *MPA43* and *MAK31* genes encode mRNAs that are alternatively processed. The short *MPA43* mRNA has a 3′-UTR of ~300 nt and the short *MAK31* mRNA has a ~200-nt 3′-UTR. The short *MPA43* mRNA is degraded by NMD, while the short *MAK31* is immune to NMD (Table 2). *MAK31* mRNA is not actively protected from NMD because the long *MAK31* mRNA (920-nt 3′-UTR) is degraded by the NMD pathway (Table 2). This suggests that 200–300 nt may be close to the minimal necessary 3′-UTR length required to target a wild-type mRNA for decay by the NMD pathway in yeast. Further, there may not be a discrete minimal 3′-UTR length required to target mRNAs for degradation by the NMD pathway. For example, the *ACT1* 320-nt 3′-UTR does not trigger degradation by the NMD pathway (Figure 4B). The lack of a discrete minimal 3′-UTR length requirement to target mRNAs for decay by the NMD pathway is not unexpected, because the secondary structure conformation of a 3′-UTR is also important. For example, the insertion of secondary structure into the 3′-UTR of an mRNA with an abnormally long 3′-UTR can suppress NMD (9).

The role of long 3′-UTRs in the degradation of wild-type mRNAs by NMD is likely evolutionarily conserved. Long 3′-UTRs target reporter mRNAs for decay by the NMD pathway in human cell lines, plants and *C. elegans* (9,10,13). *Arabidopsis* and *Nicotiana SMG-7* mRNA, which encodes a protein required for NMD, accumulation is regulated by the NMD pathway, and the *Arabidopsis SMG-7* 3′-UTR is sufficient to cause NMD-dependent regulation of a GFP reporter mRNA (10). When NMD has been inhibited by hUpf1 knockdown, 75% of the wild-type mRNAs that accumulate in human HeLa cells have 3′-UTRs that are longer than 420 nt (13). Interestingly, the functional classes of mRNAs with longer than expected UTRs in yeast and humans are similar (32). These mRNAs encode proteins involved in signal transduction, transcriptional regulation, cell-cycle control and metabolism (18,32).

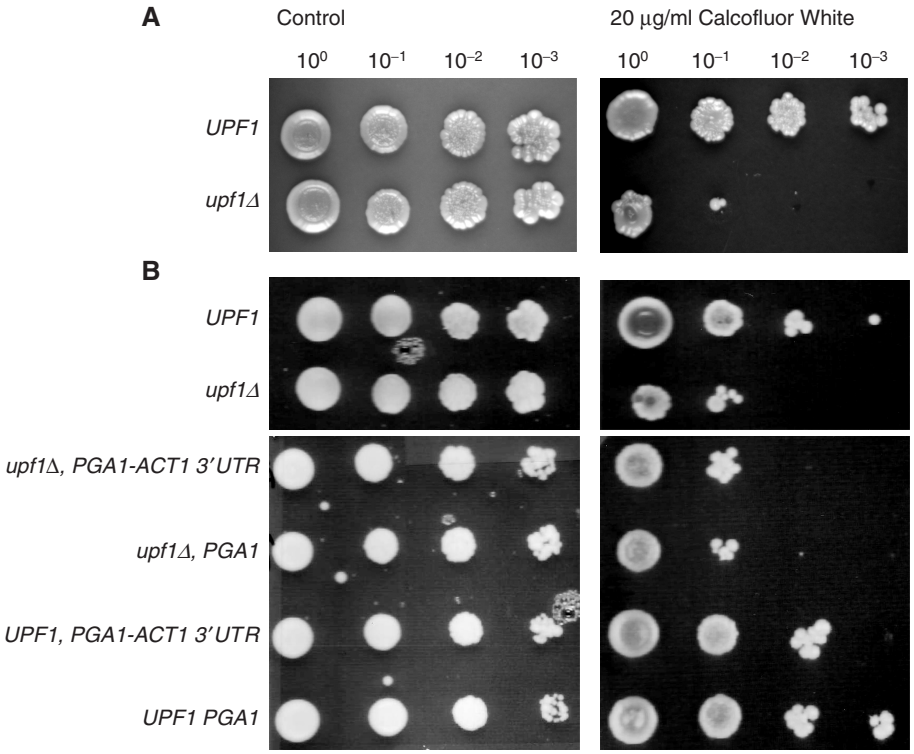

Figure 6. An *upf1Δ* mutant is hypersensitive to Calcofluor White, a disruptor of cell wall integrity. Cells were grown to mid-log phase, 10-fold serially diluted and spotted onto control medium (left) or medium supplemented with 20 μg/ml of Calcofluor White (right). W303 [*UPF1*, (24)] and AAY320 [*upf1-Δ2*, (17)] were plated on yeast extract, peptone and dextrose, pH 6.0 medium (**A**). W303 transformed with pRS315 (*UPF1*), pRS315PGA1-ACT1 3′-UTR (*UPF1, PGA1-ACT1 3′-UTR*), or pRS315PGA1 (*UPF1, PGA1*), and AAY320 *(upf1-Δ2)* transformed with pRS315 (*upf1Δ*), pRS315PGA1-ACT1 3′-UTR (*upf1Δ*, *PGA1-ACT1 3′-UTR*) or pRS315PGA1 (*PGA1 upf1Δ*) were plated on synthetic complete media lacking leucine (**B**).

The 3′-UTRs of most yeast mRNAs tend to be short and homogeneous in length, averaging 121 nt (14). Others have speculated that there is an evolutionary basis for yeast mRNAs having homogenous 3′-UTR lengths (33). We propose that NMD may provide a strong selection for short 3′-UTRs by downregulating expression of genes encoding mRNAs with long 3′-UTRs. This suggests that increase in 3′-UTR lengths are to accommodate regulatory elements that may have evolved with features to evade NMD (33).

Some mRNAs with long 3′-UTRs are not degraded by the NMD pathway suggesting that these mRNAs have evolved mechanisms to evade NMD. *SSY5* mRNAs from *S. cerevisiae* have 3′-UTRs ranging in size from 420 to 500 nt, however this mRNA is not degraded by the NMD pathway (Table 2). A significant number of human mRNAs with long 3′-UTRs are not NMD regulated (4,6). For example, the long 3′-UTRs of *Cript1* and *Tram1* mRNAs do not trigger NMD of a β-globin reporter RNA (13). Potential mechanisms that might have evolved for evasion of NMD by mRNA with long 3′-UTRs include stabilizer elements and secondary structures that bring the poly(A) tail into proximity of the stop codon. For example, yeast Pub1p binds to a stabilizer element that protects wild-type mRNAs with upstream ORFs from decay by the NMD pathway (34). Further, introduction of complementary sequences which can adopt a looped secondary structure into long 3′-UTRs of reporter mRNAs, confer protection from decay by the NMD pathway (9). The tertiary structure of the 3′-UTR could also be altered by binding of proteins to their target sites in 3′-UTRs (9). Thus, this altered 3′-UTR tertiary structure could be regulated by conditional expression of the corresponding 3′-UTR binding proteins.

NMD could regulate expression of genes that use alternative 3′-end processing. Several yeast genes have alternative 3′-end processing. For example *MAK31* and *CTR2* utilize alternative 3′-end processing sites resulting in a subpopulation of mRNAs with long 3′-UTRs [Table 2, (35)]. Thus, an expansion of our present hypothesis is that the use of alternative cleavage and polyadenylation sites resulting in a long 3′-UTR may downregulate gene expression by targeting the mRNA for rapid decay by the NMD pathway.

ACKNOWLEDGEMENTS

We thank Rachel Urhenholt for assisting with the mRNA primary sequence prediction and Dr Khalid Sayood for the MEME analysis. We thank Dr D.P. Weeks and Melanie Langford for critical reading of the manuscript.

Any opinions, findings, conclusions, or recommendations expressed in this report are ours, and do not necessarily reflect the views of the National Science Foundation.

FUNDING

National Science Foundation (MCB-0444333 to A.L.A., RIG-0642154 to B.W.K.). Funding for open access charge: MCB-0444333 and RIG-0642154.

Conflict of interest statement. None declared.

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