

Conventional 3' end formation is not required for NMD substrate recognition in *Saccharomyces cerevisiae*

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ABSTRACT

The recognition and rapid degradation of mRNAs with premature translation termination codons by the nonsense-mediated pathway of mRNA decay is an important RNA quality control system in eukaryotes. In mammals, the efficient recognition of these mRNAs is dependent upon exon junction complex proteins deposited on the RNA during pre-mRNA splicing. In yeast, splicing does not play a role in recognition of mRNAs that terminate translation prematurely, raising the possibility that proteins deposited during alternative pre-mRNA processing events such as 3' end formation might contribute to the distinction between normal and premature translation termination. We have utilized mRNAs with a 3' poly(A) tail generated by ribozyme cleavage to demonstrate that the normal process of 3' end cleavage and polyadenylation is not required for mRNA stability or the detection of a premature stop codon. Thus, in yeast, the distinction between normal and premature translation termination events is independent of both splicing and conventional 3' end formation.

Keywords: nonsense-mediated mRNA decay; 3' end formation; mRNA degradation; translation termination; poly(A) binding protein

INTRODUCTION

The degradation of mRNA is a potent control point in the regulation of gene expression. Indeed, the expeditious degradation of mRNAs that have not completed nuclear pre-mRNA processing or that fail to encode a functional polypeptide represents a robust quality control response conserved from yeast to mammals aimed, in part, to prevent the production of deleterious protein products. One such class of substrates for regulated degradation includes mRNAs that harbor premature termination codons (PTCs) and which are rapidly eliminated through the nonsense-mediated pathway of mRNA decay (NMD) (for review, see Baker and Parker 2004; Maquat 2004).

A critical step in the targeting of NMD substrates for rapid down-regulation is proficient recognition of the mRNA as "aberrant." In mammals, NMD is primarily triggered when translation termination occurs at least 50–55 nucleotides (nt) upstream of the last exon–exon junction. The affect of the exon–exon junction is mediated by the exon junction

complex (EJC), which is a multiprotein assembly deposited during pre-mRNA splicing 20–24 nt upstream of the exon–exon junction and includes UPF3, a protein required for NMD (Le Hir et al. 2000, 2001; Kim et al. 2001). Premature translation termination coupled with the failure to dissociate downstream EJC proteins from the mRNA is thought to lead to bridging interactions between a Upf1p-containing complex formed at the premature termination codon and proteins associated with the EJC (Behm-Ansmant and Izaurralde 2006; Kashima et al. 2006). Subsequent recruitment of RNA degradation enzymes ultimately triggers rapid decay of the mRNA. The efficient recognition of mammalian NMD substrates is, therefore, intimately coupled to nuclear pre-mRNA processing events through exon definition and the deposition of the EJC.

In the budding yeast, *Saccharomyces cerevisiae*, recognition of PTC-containing mRNA substrates by NMD is independent of mRNA splicing and the deposition of an EJC. One unifying theme for most NMD substrates is, however, the alteration in the normal spatial relationship between the translation termination codon and downstream *cis*-acting sequences and the mRNA binding proteins (mRNPs) that bind to these sequences. Considering these observations, two models have been suggested to rationalize NMD substrate recognition in yeast (Hilleren and Parker 1999; Jacobson and Peltz 2000; Baker and

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Parker 2004). One model, analogous to that implicated in mammalian NMD, suggests that the coding region harbors a protein mark that, if not removed by an elongating ribosome, remains on the mRNA and subsequently acts negatively to trigger NMD. An alternative model proposes that a proper translation termination event is associated with interactions that involve one or more proteins bound proximally to the native stop codon, and that NMD is triggered when termination occurs in the absence of these stabilizing interactions. Despite significant progress in understanding NMD in many model systems, it remains unresolved how the position of a translation termination codon relative to other features of the mRNA leads to the manifestation of NMD in yeast.

In the absence of a requirement for pre-mRNA splicing in the recognition of NMD substrates in yeast, it has been suggested that alternative pre-mRNA processing events, such as 5' capping or 3' end formation, may define the mRNP domains associated with a proper mRNA translation termination event (Hilleren and Parker 1999). 3' end formation is particularly appealing as the process results in a distinct mRNP architecture that serves important roles in mRNA nucleocytoplasmic transport and translation (Brodsky and Silver 2000; Hammell et al. 2002). Moreover, Upf3p is a nucleocytoplasmic shuttling protein whose export from the nucleus is required for NMD in yeast (Shirley et al. 2002), consistent with the formation of a nuclear mRNP that acts to facilitate cytoplasmic mRNA surveillance.

In accordance with a role for 3' end formation in the detection of NMD substrates, mRNAs that terminate translation at a natural stop codon but harbor extended 3' UTRs are efficiently recognized as substrates for NMD (Pulak and Anderson 1993; Muhlrud and Parker 1999). For example, the yeast *cyc1-512* mRNA is deleted for sequences important for natural 3' end cleavage and polyadenylation and, as a result, cryptic downstream cleavage sites are used at varying efficiencies to generate the mRNA 3' terminus. The targeting of this mRNA to NMD suggests that the shift in position of the 3'-terminal mRNP domain structure relative to the natural stop codon fails to support proper translation termination and is sufficient for recognition of the mRNA as a substrate for NMD. Furthermore, this observation is in accord with the model that pre-mRNA processing events contribute to the formation of discrete mRNP domains and that an appropriate association between the terminating ribosome and the mRNP deposited by 3' end formation is important in the discrimination of proper from improper translation termination events and the downstream fate of the mRNA.

RESULTS AND DISCUSSION

To test if conventional 3' end formation is required for NMD, we utilized mRNA reporter constructs previously described by Dower and colleagues (Dower et al. 2004).

Specifically, self-cleaving hammerhead ribozyme elements inserted immediately downstream from the translation termination codon replace mRNA cleavage and polyadenylation signals on a plasmid-borne *GFP* and promote the generation of mRNA with 3' ends independent of the cleavage and polyadenylation machinery (*GFP-Rz* mRNA). One construct, *GFP-A75Rz*, harbors an additional 75-residue stretch of DNA-encoded adenosine residues immediately upstream of the ribozyme element. The *GFP-A75Rz* mRNA reporter is, therefore, polyadenylated independent of the normal process of 3' cleavage and polyadenylation. Considering that *GFP-A75Rz* mRNA is efficiently exported from the nucleus and robustly translated (Dower et al. 2004), this reporter is ideal to examine if the nuclear process of 3' end formation is required for discrimination between normal and premature translation termination and the triggering of NMD. As a control, we also examined the reporter mRNA, *GFP-pA*, which harbors the yeast *TDH3* 3' UTR containing native cleavage and polyadenylation signals. It is of note that Dower and colleagues reported that the ribozyme-cleaved *GFP-Rz* mRNA is properly processed at its 3' terminus and is unadenylated, confirming that the ribozyme is active in vivo, and that a poly(A) tail normally added to nascent messages by poly(A) polymerase is absent from the ribozyme-cleaved mRNAs. Moreover, in contrast to the poly(A)⁺ *GFP-A75Rz* mRNA, *GFP-Rz* mRNA failed to be exported from the nucleus, accumulated at, or near, the site of transcription, and was not translated to levels detectable by Western blot analysis (Dower et al. 2004).

Analysis of *GFP-pA*, *GFP-Rz*, and *GFP-A75Rz* reporter mRNAs in wild-type cells revealed significant differences in steady state mRNA levels (Fig. 1A, lanes 1,5,9). Specifically, the ribozyme-cleaved *GFP-Rz* mRNA was reduced in abundance threefold in comparison to *GFP* mRNA generated by conventional 3' end formation (*GFP-pA* mRNA). The reduced levels of *GFP-Rz* mRNA is consistent with previous observations (Dower et al. 2004) and may be a consequence of either a reduction in mRNA expression due to retention of the mRNA species near its site of synthesis, or an enhanced rate of degradation that occurs to mRNAs retained in the nucleus (Das et al. 2003; Kuai et al. 2005). In contrast, the steady state levels of *GFP-pA* and *GFP-A75Rz* mRNA were strikingly similar. To determine that steady state mRNA levels accurately correspond to the stabilities of the *GFP* mRNAs, the mRNA half-lives were determined by transcriptional shut-off of the *GAL1* promoter upstream of the plasmid-borne *GFP* (Decker and Parker 1993). The *GFP-pA* and *GFP-A75Rz* mRNAs were relatively stable and exhibited similar half-lives of 8 ± 1 and 13 ± 2 min, respectively (Fig. 2A). The relative abundance of the three *GFP* reporter steady state mRNA levels, moreover, did not change significantly in the absence of the essential NMD factor, *UPF1* (Fig. 1A, lanes 2,6,10), indicating that the *GFP* mRNAs are not substrates for the

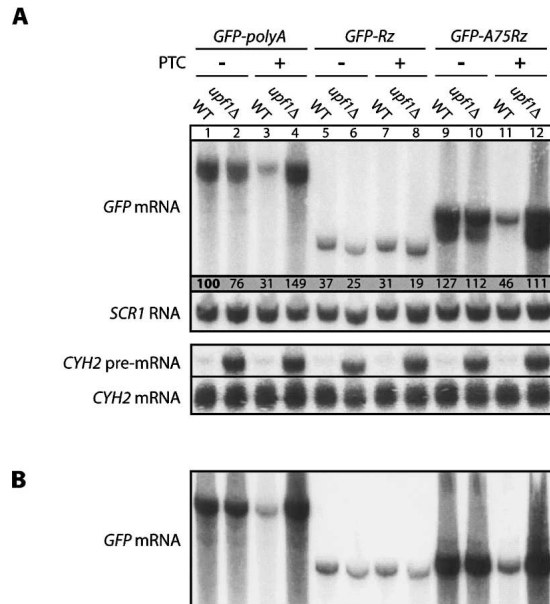


FIGURE 1. Steady state *GFP* mRNA levels in wild-type and *upf1Δ* yeast by Northern blot analysis. Untreated total cellular RNA (30 μ g) (A) or RNA hybridized to oligo(dT) and treated with RNase H (B) was separated on 6% denaturing polyacrylamide gels. *GFP* mRNA was visualized using a radiolabeled deoxyoligonucleotide complementary to the *GFP* ORF (5'-CCGGGTATCTGAAAAGCACTGAA CACCTTAAGTAAAAGTAGTGACAAG-3'). *GFP* mRNA harboring a PTC at codon 69 are indicated (+). RNA quantifications (gray box) were achieved by PhosphorImager analysis (GE Biosystems) and are based on mRNA abundance after normalization to a loading control (*SCR1* RNA) and are relative to the level (100%) of *GFP-pA* mRNA in wild-type cells. The increase in abundance of *CYH2* pre-mRNA in comparison with *CYH2* mRNA (visualized using a random primed radiolabeled cDNA) (Tharun and Parker 1999) illustrates the inhibition of NMD in yeast strains deleted for *UPF1*.

NMD pathway of decay. The increased steady state level of the NMD substrate, *CYH2* pre-mRNA, in comparison to its NMD-insensitive spliced product, *CYH2* mRNA, confirms that the NMD pathway is inhibited in *upf1Δ* strains (Fig. 1A; He et al. 1993). These findings suggest that conventional 3' end formation is not required for the stability of cytoplasmic mRNA in yeast.

To evaluate the consequence of 3' end formation by ribozyme cleavage on NMD substrate recognition, a PTC was introduced into the open reading frame (ORF) of *GFP* at codon 69 (28% into the coding region). A PTC at such an early position in the ORF is predicted to have a significant destabilizing effect if the mRNA is recognized as aberrant (Cao and Parker 2003). Furthermore, we analyzed the stability of these reporter mRNAs in both a wild-type and *upf1Δ* strain background to determine if any destabilizing effect to the mRNA by the introduction of a PTC was a consequence of the NMD pathway.

We observed that insertion of a PTC into the *GFP-pA* or *GFP-A75Rz* mRNAs reduced their steady state levels be-

tween three and fourfold (Fig. 1A, cf. lanes 1 and 3, 9 and 11). Consistent with this reduction in steady state mRNA levels, we also determined that the presence of the PTC in the *GFP-A75Rz* mRNA led to a significant increase in its decay rate (half-life from 13 ± 2 min versus 3 ± 0.5 min; Fig. 2A,B). Moreover, the decreased steady state levels and the increased decay rate for the PTC-containing *GFP-A75Rz* mRNA were suppressed in the *upf1Δ* strain. (Fig. 1A, cf. lanes 3 and 4, 11 and 12; Fig. 2B, half-life = 15 ± 2 min). These observations indicate that a premature translation termination codon within *GFP-A75Rz* mRNA can trigger NMD to a similar extent as within an mRNA that undergoes normal 3' end formation. Similarly, a PTC at codon 6 of *GFP-A75Rz* mRNA was reported by Dower et al. (2004) to also lead to a reduction in steady state mRNA levels, although it was undetermined if the reduction was a consequence of NMD (i.e., dependent upon *UPF1*). The rapid decay of PTC-containing *GFP-A75Rz* mRNA dependent upon *UPF1* indicates that the process of conventional 3' end formation in yeast is not required for discrimination of normal and premature translation termination events.

The presence of a PTC within the ribozyme-cleaved reporter, *GFP-Rz* mRNA, manifested no significant affect on the steady state RNA levels (Fig. 1A, cf. lanes 5 and 7), indicating that, regardless of the presence of a PTC, this mRNA is not a substrate for NMD. This finding is consistent with the inability of a PTC to elicit NMD in

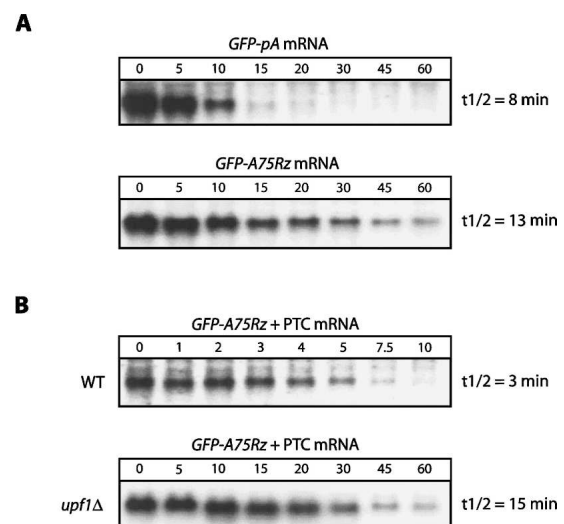


FIGURE 2. Half-life determination of *GFP* mRNA in wild-type and *upf1Δ* yeast. Cells were grown in the presence of galactose to mid-log phase, at which time glucose was added to achieve transcriptional repression of plasmid-borne *GAL1-GFP* expression, and cell aliquots were removed at various time points (specified above each lane in minutes) (Decker and Parker 1993). RNA (30 μ g) was separated on a 1.4% agarose/formaldehyde gel and transferred to nylon membrane. *GFP* mRNA was detected using the deoxyoligonucleotide listed in Figure 1. Half-life determinations were made by quantifying *GFP* mRNA levels standardized to a *SCR1* RNA loading control (not shown).

the yeast nucleus (Kuperwasser et al. 2004) and the failure of this mRNA to be efficiently translated (Dower et al. 2004), a requirement for recognition of a PTC-containing mRNA as a substrate for NMD (Belgrader et al. 1993).

During the course of our analysis, we observed a unique distribution of *GFP-A75Rz* mRNA by high resolution acrylamide gel analysis (Fig. 1A, lanes 9–12). We attribute the predominant and slower migrating species to be the nascent mRNA harboring a ribozyme-cleaved 3' terminus with a 2',3' cyclic phosphate end. We hypothesize that the modified 3'-terminal residue may be rate-limiting for its removal and impede the onset of mRNA deadenylation, consistent with the slight increase in stability of *GFP-A75Rz* mRNA as compared with *GFP-pA* mRNA, which undergoes conventional 3' end formation and harbors a typical 3' hydroxyl end (Fig. 2A). The decay of NMD substrates occurs predominantly by mRNA decapping independent of deadenylation, and it is, therefore, not anticipated that the modified 3' terminus of *GFP-A75Rz* mRNA would impact the rate of decay of this mRNA by NMD (Muhlrad and Parker 1994, Cao and Parker 2003). Consistent with this, we observe that PTC-containing *GFP-pA* and *GFP-A75Rz* are degraded prior to any significant deadenylation (Fig. 1A, lanes 3,11). A second *GFP-A75Rz* mRNA species was confirmed to represent partially deadenylated mRNA by treatment of the RNA with RNase H in the presence of oligo(dT) (Fig. 1B).

It is noteworthy that in the absence of NMD (i.e., in the *upf1Δ* genetic background) the extent of deadenylation is greater for PTC-containing *GFP-A75Rz* mRNA (Fig. 1A, cf. lanes 10 and 12; data not shown). In the absence of deadenylation-independent decapping, the difference in mRNA distribution may represent the ability of the deadenylation machinery to proceed further into the poly(A) tail of the PTC-containing *GFP-A75Rz* mRNA, since the ribosome terminates significantly upstream than when termination occurs at the native stop codon. In contrast, with the "synthetic" poly(A) tail adjacent to the termination codon, normal translation termination might serve to protect the final adenylate residues from removal, resulting in mRNA decapping and decay when the mRNA harbors an ~20-residue adenylate tail.

CONCLUSIONS

Our findings that *GFP-A75Rz* mRNA is stable indicate that conventional 3' end formation catalyzed by the cleavage and polyadenylation machinery is not obligatory for the stability of mRNA in the cytoplasm. Furthermore, this observation also indicates that, for *GFP-A75Rz* mRNA, translation termination occurs in an appropriate context to prevent mRNA destabilization, and that the mRNP composition downstream from the normal termination codon on the ribozyme-cleaved A75 tail-containing mRNA is sufficient such that mRNA destabilization does not occur.

The *UPF1*-dependent destabilization of *GFP-A75Rz* mRNA harboring a PTC indicates that premature translation termination is sufficient to trigger recognition of this mRNA as a NMD substrate and demonstrates that conventional 3' end formation is also not required for NMD substrate recognition. A direct implication of this observation is that the activity of the cleavage and polyadenylation machineries during 3' end formation does not deposit an mRNP required for the signaling of a prematurely terminating ribosome to trigger NMD. Our findings do not, however, preclude the possibility that a ribosome terminating prematurely at codon 69 of *GFP-A75Rz* mRNA fails to remove an mRNP associated with the downstream coding region that may act to negatively influence the nature of translation and the stability of the mRNA. We are not in favor of this account to explain the instability of PTC-containing *GFP-A75Rz* mRNA due to the fact that NMD can occur even when a PTC is 3' to all known negatively acting downstream sequence elements (DSE) in the yeast *PGK1* mRNA (Cao and Parker 2003). Furthermore, the mechanism for NMD substrate recognition in *S. cerevisiae* would require recognition of a heterologous DSE since the *GFP* coding region sequences originate from *Aequorea victoria*.

The observations presented here have important implications for understanding the requirements for mRNA stability of "normal" and PTC-containing mRNA in yeast. While a 3'-terminal mRNP may, in fact, act positively to protect mRNA from decay, the composition of the mRNP unlikely involves factors deposited on the mRNA during 3' end formation, per se. Rather, an obvious stabilizing factor is the poly(A) binding protein, Pab1p, shown to stabilize "normal" or PTC-containing mRNA when tethered proximal to a translation termination event in vivo (Coller et al. 1998; Amrani et al. 2004). Interestingly, Dower et al. (2004) demonstrated that the *GFP-A75Rz* mRNA associates with Pab1p in vitro, supporting the assumption that poly(A)-bound Pab1p, and not an mRNP deposited by 3' end formation, is sufficient for mRNA stabilization. Together, these observations further implicate the proximity of a translation termination event and poly(A)-bound Pab1p in the discrimination of proper from improper termination and the downstream fate of the mRNA.

Our findings may have additional significance for understanding mRNA stability and the mechanism of NMD outside of yeast. For example, exon definition is also inoperable in *Drosophila* NMD (Gatfield et al. 2003), and splicing-independent NMD has been described for Rous sarcoma virus in chicken cells (Weil and Beemon 2006). Moreover, a recent report suggests that the nonsense-mediated decay of PTC-containing immunoglobulin- μ reporter mRNA in mammalian cells is independent of an EJC (Bühler et al. 2006). Our results, together with these observations, raise the possibility that the fundamental distinction between a normal and aberrant translation

termination codon is independent of any nuclear pre-mRNA processing event, and that the role of the EJC in NMD is primarily to augment steps in NMD following the recognition of the aberrant termination codon.

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