
Translational competence of ribosomes released from a premature termination codon is modulated by NMD factors

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ABSTRACT

In addition to their well-documented roles in the promotion of nonsense-mediated mRNA decay (NMD), yeast Upf proteins (Upf1, Upf2/Nmd2, and Upf3) also manifest translational regulatory functions, at least *in vitro*, including roles in premature translation termination and subsequent reinitiation. Here, we find that all *upf1*Δ strains also fail to reinitiate translation after encountering a premature termination codon (PTC) *in vivo*, a result that led us to seek a unifying mechanism for all of these translation phenomena. Comparisons of the *in vitro* translational activities of wild-type (WT) and *upf1*Δ extracts were utilized to test for a Upf1 role in post-termination ribosome reutilization. Relative to WT extracts, non-nucleated extracts lacking Upf1 had approximately twofold decreased activity for the translation of synthetic *CAN1/LUC* mRNA, a defect paralleled by fewer ribosomes per mRNA and reduced efficiency of the 60S joining step at initiation. These deficiencies could be complemented by purified FLAG-Upf1, or 60S subunits, and appeared to reflect diminished cycling of ribosomes from endogenous PTC-containing mRNAs to exogenously added synthetic mRNA in the same extracts. This hypothesis was tested, and supported, by experiments in which nucleated WT or *upf1*Δ extracts were first challenged with high concentrations of synthetic mRNAs that were templates for either normal or premature translation termination and then assayed for their capacity to translate a normal mRNA. Our results indicate that Upf1 plays a key role in a mechanism coupling termination and ribosome release at a PTC to subsequent ribosome reutilization for another round of translation initiation.

Keywords: yeast; NMD; translation initiation; translation termination; ribosome reutilization

INTRODUCTION

The fidelity of protein synthesis is dependent on a functional translational apparatus and on the integrity of mRNA coding sequences. A premature termination codon (PTC) can disrupt mRNA integrity, generating an abbreviated open reading frame (ORF) that can give rise to a potentially toxic polypeptide fragment (Pulak and Anderson 1993). To obviate the accumulation of such polypeptide fragments, the offending mRNAs are recognized and degraded by the nonsense-mediated mRNA decay (NMD) pathway (Jacobson and Izaurralde 2007; Isken and Maquat 2008). Quality control by NMD requires the activities of the highly conserved *UPF1*, *UPF2/NMD2*, and *UPF3* gene products in all eukaryotic cells, as well as those of the *SMG1-9* genes in

metazoans (Jacobson and Izaurralde 2007; Isken and Maquat 2008; Yamashita et al. 2009). Of the three Upf proteins, Upf1 appears to serve as the central regulator of the NMD pathway, with Upf2/Nmd2 and Upf3 regulating Upf1 function (Page et al. 1999; Maderazo et al. 2000).

While a requirement for the Upf proteins in promoting the rapid decay of nonsense mRNAs is well established, other functions for these proteins, and the possible connections between these functions and the promotion of mRNA decay, are only beginning to be understood. Notably, several experimental approaches have indicated that the Upfs have additional activities in translation. A possible role for these proteins in translation reinitiation emerged from analyses of premature termination in yeast cell-free extracts (Amrani et al. 2004). These studies demonstrated that a significant fraction of ribosomes terminating translation at PTCs *in vitro* reinitiated at proximal AUG codons, and that reinitiation was Upf1-dependent even though NMD was inactive in the cell-free lysates. Although these data suggest a positive role for the Upfs in regulating translation reinitiation, other data imply a translational repression function. Studies in

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.1987710>.

yeast using *CUP1* nonsense reporter constructs (Muhlrad and Parker 1994, 1999; Sheth and Parker 2006) and in mammalian cells using dual luciferase reporters (Isken et al. 2008) suggest that Upf1 association with a nonsense-containing mRNA represses its translation and that this event may be an obligatory precursor to subsequent mRNA decay. Additional experiments in yeast indicate that the Upf factors may also affect termination fidelity since nonsense codon read-through is enhanced when any of the three factors is inactive or absent (Weng et al. 1996a,b, 1998; Maderazo et al. 2000; Wang et al. 2001). Although the latter observation has been made with numerous nonsense alleles and with numerous Upf-deficient strains (Weng et al. 1996a,b, 1998; Maderazo et al. 2000; Wang et al. 2001), recent experiments demonstrate that the apparent loss of termination fidelity is an indirect consequence of the stabilization of the NMD-sensitive *ALR1* mRNA and the resulting increase of intracellular Mg^{2+} levels (Johansson and Jacobson 2010). Finally, there is also evidence that Upf regulation of translation may not be limited to nonsense-containing mRNAs. The same study that implicated a Upf1 role in translation reinitiation in vitro also showed that the absence of Upf1 leads to decreased translation of a wild-type (WT) *CAN1/LUC* fusion mRNA (Amrani et al. 2004). Moreover, Upf1 appears to be required for optimal translation of some normal yeast transcripts in vivo (Muhlrad and Parker 1999), and a hyperphosphorylated mutant of Upf1 that is impaired for NMD leads to decreased translation of a wild-type *LUC* reporter mRNA in rabbit reticulocyte lysates (Isken et al. 2008).

Collectively, the effects of Upf deficiency on the translation of normal and PTC-containing mRNAs are indicative of an important but poorly understood function of the Upf proteins. Accordingly, we have explored further the role of the Upf factors in translation, both in vivo and in vitro. To do so, we first used chimeric *PGK1/LUC* nonsense alleles to test whether the Upf proteins played a role in post-termination reinitiation activity in intact cells. Having obtained experimental support for such a role, we then exploited the yeast cell-free translation system to elucidate the translational defects arising from NMD inactivation. These analyses revealed that extracts prepared from *upf1Δ* cells manifested a Upf1-complementable deficiency in apparent initiation activity. Additional experimentation strongly suggested that Upf1 is required in vitro for efficient termination and release of ribosomes at a PTC and their subsequent reutilization for additional rounds of translation.

RESULTS

Mutations in a *PGK1/LUC* reporter attenuate its reinitiation activity

We have previously shown that, in vitro, yeast ribosomes will reinitiate translation subsequent to premature termination, provided that Upf1 is present in the translation

reaction (Amrani et al. 2004). This observation indicated that premature termination was inefficient and suggested that Upf1 might influence the extent to which prematurely terminating ribosomes remain associated with an mRNA (Amrani et al. 2004). Here, we used three approaches to understand the significance of Upf1-dependent translational reinitiation in vitro. First, we tested for reinitiation in vivo and evaluated its dependence on NMD factors. We then used a cell-free translation system to identify the steps in translation initiation that are affected in the absence of Upf1 activity. Finally, to understand the link between a factor involved in translation termination and its resulting initiation phenotype, we devised an in vitro assay to assess the abilities of ribosomes undergoing premature versus normal termination events to be reutilized in subsequent rounds of translation.

To determine whether Upf1 and other NMD factors affect post-termination reinitiation at a PTC in vivo, we constructed a reinitiation reporter and evaluated its expression in wild-type yeast strains and in strains devoid of NMD function. The reporter, a chimeric *PGK1/LUC* gene (Fig. 1A, construct 1) that encodes an mRNA subject to NMD (Fig. 1B), consists of the following: (1) the *PGK1* 5'-untranslated region (UTR); (2) the first 5% of the *PGK1* open reading frame (ORF) followed by a UAG stop codon; (3) an 82-nucleotide (nt) *PGK1* coding region sequence element whose first AUG codon, designated +19 AUG (relative to the U of the UAG), is out-of-frame with the upstream *PGK1* ORF; (4) the *LUC* ORF lacking its normal start codon, fused in-frame with the +19 AUG; and (5) the 3'-UTR of *PGK1*. Luciferase assays, performed on extracts of wild-type yeast strains bearing this construct, yielded activity that was assigned a value of 100% for purposes of comparison with subsequent experiments (Fig. 1C). In this, and all other assays, luciferase activity was normalized to both total protein content of the sample, as well as to the level of the construct-specific mRNA. The derived values were thus expressed as relative light units (RLUs)/ μ g of protein per unit of mRNA.

Our analyses indicated that the luciferase activity derived from the reporter represented reinitiation from the +19 AUG codon downstream from the partial *PGK1* ORF or from one additional in-frame AUG located five codons downstream from the +19 AUG. First, we measured luciferase activity from cells containing construct 2 (Fig. 1A), in which the +19 AUG and the additional downstream AUG codon were deleted, and observed a fivefold decrease in luciferase expression relative to construct 1 (Fig. 1C). (The residual luciferase activity appears to derive from an in-frame AUG codon 82 nt into the *LUC* sequence [Fig. 2, construct 6].) Second, in a separate construct, the *LUC* ORF was placed out-of-frame with respect to the +19 AUG, as well as out-of-frame with the *PGK1* ORF (Fig. 1A, construct 3). This construct yielded almost no detectable luciferase activity (Fig. 1C), indicating that the *LUC* ORF has to

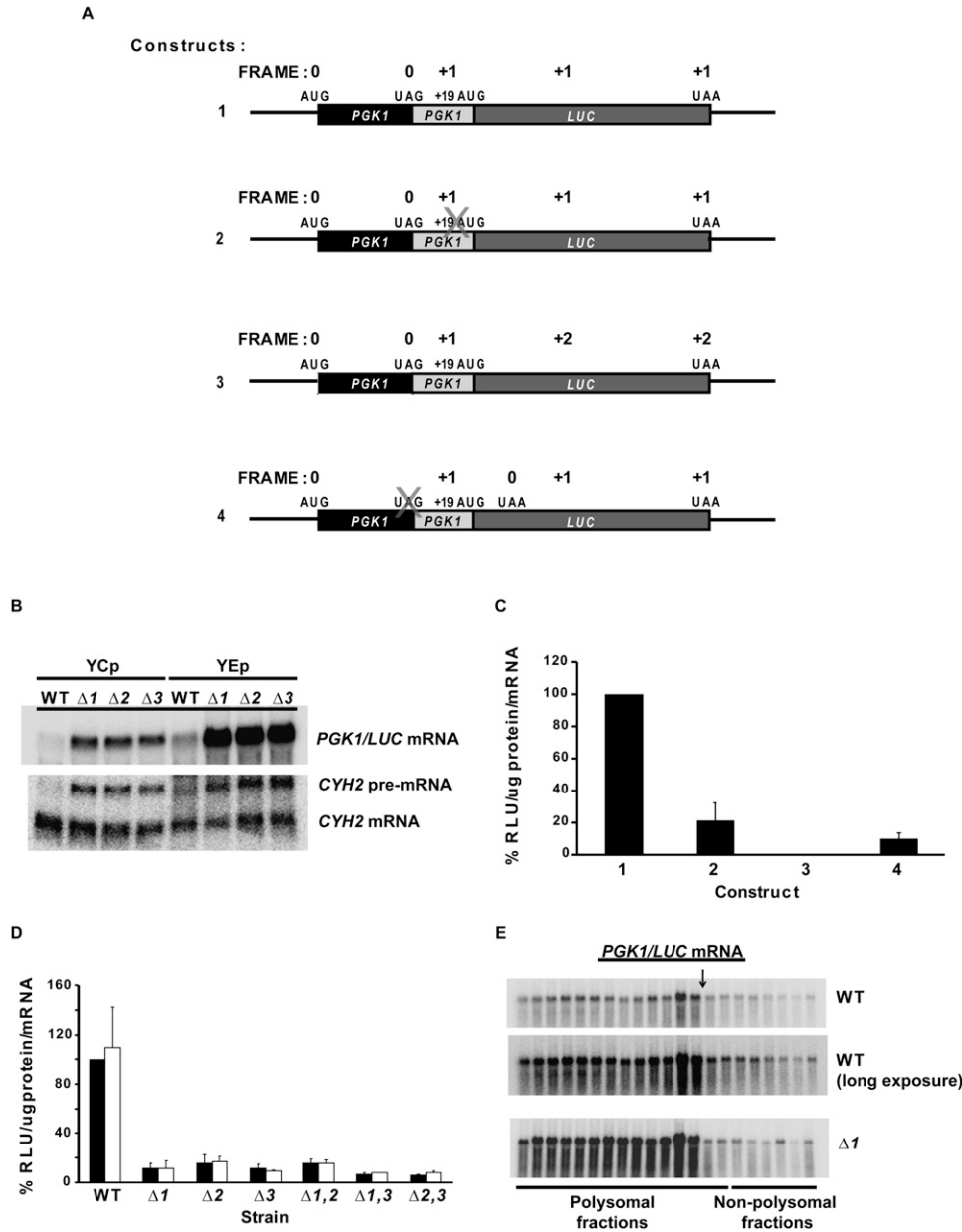


FIGURE 1. Reinitiation activity is reduced in strains bearing mutations in the NMD pathway. (A) Schematic representation of *PGK1/LUC* chimeric constructs 1–4. Start and stop codons are indicated, as are their reading frames and that of the *LUC* coding region, relative to the *PGK1* start codon. (X) Indicates deletion or mutation of a particular feature; (+19 AUG) denotes the first of two in-frame *PGK1* AUGs downstream from the PTC. Details of the constructs are given in Table 2. (B) Northern analysis of the *PGK1/LUC* mRNA expressed from either a low-copy (YCp) or high-copy (YEp) vector in wild-type (WT) cells or in isogenic cells harboring deletions of *UPF1*, *UPF2/NMD2*, or *UPF3*. Blots were stripped and reprobed for *CYH2* transcripts as an internal control for NMD function (*CYH2* pre-mRNA) and loading (*CYH2* mRNA). (C) Relative luciferase activities of constructs 1–4 normalized for protein content and mRNA levels in the WT strain HFY114. The luciferase activity of construct 1 was assigned a value of 100%. (D) Reinitiation activity. Construct 1 was inserted into either a low-copy (YCp) or a high-copy (YEp) vector and transformed into the indicated strains. Relative luciferase activities, normalized for protein content and mRNA levels, are shown for YCp-bearing strains (black boxes) and for YEp-bearing strains (white boxes). The luciferase activity of construct 1 on a YCp plasmid in a WT strain was assigned a value of 100%. (Δ1) *upf1Δ*; (Δ2) *nmd2Δ*; (Δ3) *upf3Δ*; and combinations thereof. Vertical bars represent standard error of the mean. (E) Polysomal distribution of the *PGK1/LUC* reporter mRNA in WT and *upf1Δ* cells. (Top panel) WT cells; (middle panel) WT cells, long exposure; (bottom panel) *upf1Δ* cells. Note that the top and bottom panels were exposed for the same length of time. Polysomal and non-polysomal fractions are indicated, as is the position of the 80S peak (vertical arrow).

be in-frame with the +19 AUG to produce functional luciferase protein. Finally, deletion of the *PGK1* stop codon (Fig. 1A, construct 4), which allows translation until a

termination codon 75 nt into the *LUC* coding region, also resulted in a substantial decrease in luciferase activity (Fig. 1C). These results strongly implicate the +19 AUG codon

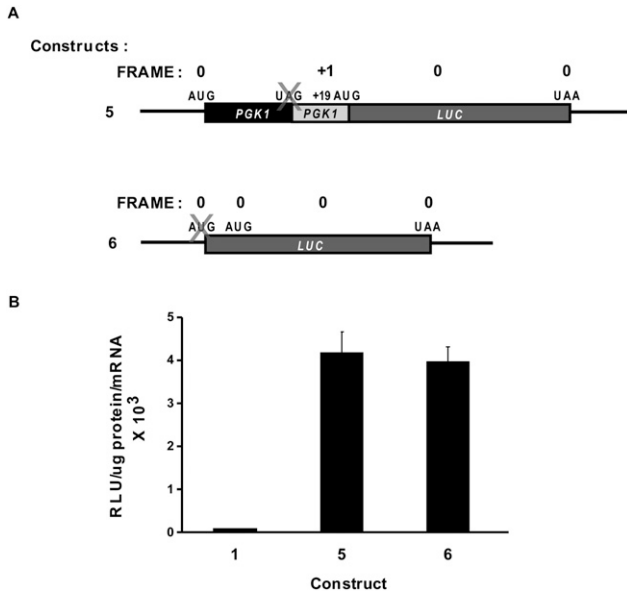


FIGURE 2. Determination of ribosomal initiation efficiency and proportion of ribosomes that reinitiate downstream from the *PGK1* stop codon. (A) Schematic representations of chimeric *PGK1/LUC* constructs 5 and 6. Labeling of start and stop codons and reading frame is as depicted in Figure 1, with “X” indicating deletion or mutation of a particular feature. (B) Luciferase activities of constructs 5 and 6 in the wild-type strain HFY114. Activity is expressed as RLU/microgram of protein per unit of mRNA. The activity of construct 1 (Fig. 1) is shown for purposes of comparison. Vertical bars represent standard error of the mean.

in the expression of luciferase from a translational reinitiation event in the *PGK1/LUC* mRNA derived from construct 1.

Reinitiation on nonsense-containing *PGK1/LUC* mRNA is sensitive to mutations in the NMD pathway

To assess whether NMD factors influence reinitiation events in vivo, *PGK1/LUC* construct 1, on a YCp plasmid, was transformed into isogenic yeast strains bearing deletions of *UPF1*, *UPF2/NMD2*, or *UPF3*, and combinations thereof. Although the mRNA derived from this construct is sensitive to NMD, as indicated by the observation that it is stabilized in yeast strains defective in this pathway (Fig. 1B), the expression of low-copy *PGK1/LUC* construct 1 in *upf1nmd* strains yielded a dramatic decrease in luciferase activity when compared to that in wild-type cells (Fig. 1D). It was important to determine whether this apparent decrease in luciferase activity was simply due to the increased stability of the *PGK1/LUC* mRNA and consequent increase in its steady-state level in these strains without a parallel increase in luciferase protein. To clarify this question, *PGK1/LUC* construct 1 was inserted into a high-copy YEp vector and transformed into the wild-type and *upf1nmd* mutant strains. Normalized values for luciferase activity in strains harboring the YEp construct were very similar to those in

cells harboring the construct on a YCp plasmid (Fig. 1D). Notably, the increased levels of *PGK1/LUC* mRNA in wild-type cells (Fig. 1B) did not lead to any apparent decrease in normalized values of reinitiation activity, showing that mRNA levels do not affect overall reinitiation in this reporter. Additionally, the *upf1nmd* mutant strains showed a similar reduction in luciferase activity regardless of whether there was an increase in steady-state levels of the mRNA (Fig. 1B,D). As an additional control, we used sucrose gradient analysis to evaluate the polysomal distribution of the *PGK1/LUC* mRNA in wild-type and *upf1Δ* cells. These experiments manifested indistinguishable polysomal profiles for the *PGK1/LUC* mRNA in wild-type and *upf1Δ* cells, with >90% of the mRNA on polysomes in both cases (Fig. 1E). Collectively, these results indicate that the mutation-dependent variations in reinitiation activity evident in Fig. 1D are not simply a function of increases in the abundance of *PGK1/LUC* mRNA or of decreases in its overall association with ribosomes, and that, as was seen previously in cell-free translation reactions (Amrani et al. 2004), reinitiation in vivo is decreased in the absence of the key NMD factors.

A small percentage of ribosomes reinitiate downstream from the premature stop codon

To determine the proportion of ribosomes that initiate at the normal *PGK1* AUG and are subsequently able to reinitiate, post-termination, on the *PGK1/LUC* mRNA, it was necessary to first assess the efficiency with which ribosomes initiated at the *PGK1* AUG. We made a construct in which the premature *PGK1* stop codon plus one nucleotide were deleted, resulting in a fusion ORF which begins at the *PGK1* start codon and ends at the normal *LUC* terminator (Fig. 2A, construct 5). The luciferase activity of construct 1 was found to be ~2.5% that of construct 5 (Fig. 2B), a comparison indicating that approximately one in 40 ribosomes reinitiated translation after encountering the premature *PGK1* nonsense codon.

NMD is independent of translation reinitiation

Since our data indicated that reinitiation efficiency is directly affected by the NMD factors and other experiments have shown that reinitiation can affect NMD (Zhang and Maquat 1997), it was of interest to determine whether rapid mRNA decay still occurred in the absence of reinitiation. To address this question, we introduced a strong stem-loop structure ($\Delta G = 58.3$ kcal/mol) between the *PGK1* stop codon and the *PGK1* downstream +19 AUG (Fig. 3A, construct 7). This stem-loop structure bears a 25-nt-pair stem and 4-nt loop and is preceded by a 30-nt spacer between the stop codon and the stem-loop to ensure that the structure does not interfere with termination. mRNA derived from construct 7 displayed almost no luciferase

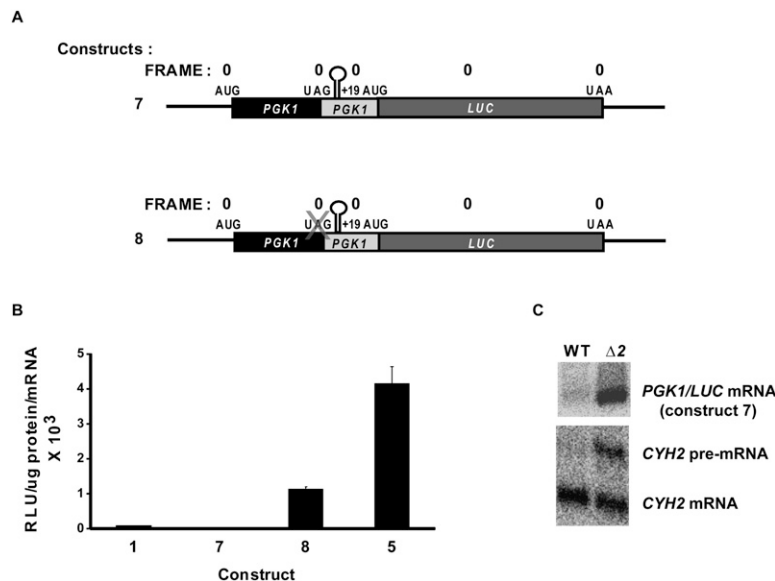


FIGURE 3. A strong stem-loop structure does not eliminate nonsense-mediated mRNA decay. (A) Schematic representations of chimeric *PGK1/LUC* constructs 7 and 8. Labeling of start and stop codons and the reading frame is as depicted in Figure 1, with “X” indicating deletion or mutation of a particular feature. (B) Luciferase activities of constructs 7 and 8 in the WT strain HFY114. Activity is expressed as RLU/microgram of protein per unit of mRNA. Activities of constructs 1 and 5 are shown for purposes of comparison. Vertical bars represent standard error of the mean. (C) Northern analysis of *PGK1/LUC* construct 7 mRNA expressed from a low-copy (YCp) vector in WT cells or in isogenic cells harboring a deletion of *UPF2/NMD2*. Blots were stripped and reprobed for *CYH2* transcripts as an internal control for NMD function (*CYH2* pre-mRNA) and loading (*CYH2* mRNA).

activity (Fig. 3B) but continued to undergo NMD since it could be stabilized in an *nmd2Δ* strain (Fig. 3C). Further insight into the potency of the stem-loop structure used in these experiments was obtained from an additional construct that contains the stem-loop but lacks the premature *PGK1* stop codon, generating an ORF that extends from the *PGK1* initiator AUG, through the stem-loop and *PGK1* downstream sequence, to the *LUC* terminator (Fig. 3A, construct 8). Luciferase activity derived from construct 8 was only 25% that of construct 5, which is identical but lacks the stem-loop (Fig. 3B). Collectively, the results of Figure 3 suggest that the strong stem-loop disrupts reinitiation (and presumably ribosome scanning) and substantially inhibits elongation, but is not sufficient to prevent NMD. Thus, we conclude that neither reinitiation nor any form of ribosome scanning beyond the termination codon is required for the promotion of NMD.

NMD-deficient extracts manifest a translation defect in vitro

The in vivo results of Figures 1–3 are consistent with our earlier in vitro data (Amrani et al. 2004) and suggest that factors regulating NMD have an as-yet-undefined function that affects translation reinitiation. To characterize this role further, we returned to the analysis of yeast cell-free

translation and evaluated the ability of non-nucleated extracts prepared from WT or *upf/nmd* mutant strains to translate a synthetic *CAN1/LUC* fusion mRNA (Fig. 4A) that harbors a full-length open reading frame (ORF) and remains stable during the course of in vitro translation (Amrani et al. 2004). Figure 4B shows that, relative to WT extracts, extracts from strains lacking Upf1 activity manifested reduced luciferase expression from the *CAN1/LUC* mRNA. Similar results were observed with extracts lacking Upf2/Nmd2 or Upf3 (data not shown). Northern analyses of WT and *upf1Δ* in vitro translation reactions fractionated on sucrose gradients demonstrated that the WT extract had higher relative amounts of *CAN1/LUC* mRNA in the heavier polysome fractions, thus confirming the relatively poor translation activity of the mutant extracts and suggesting that they initiated translation inefficiently (Fig. 4C).

Further evidence for a translational deficiency in extracts from *upf/nmd* mutant strains followed from experiments that we dubbed “ribosome reutilization assays.” Here, translation of an

extract’s endogenous mRNA was allowed to proceed in the absence of any *CAN1/LUC* mRNA addition. At regular intervals (termed “time pre-*CAN1/LUC* addition” in Figs. 4–6), the translation reaction was supplemented with *CAN1/LUC* mRNA and incubated for 25 additional minutes to allow for synthesis of the luciferase fusion protein. Thus, luciferase activity obtained from the 25-min incubation with the reporter mRNA at each time point should reflect the relative functionality or availability of ribosomes and factors in the “preincubated” extracts to initiate and translate the exogenously added reporter mRNA. As shown in Figure 4D, WT extracts exhibit enhanced translatability of the reporter mRNA over time, whereas *upf1Δ* extracts (as well as *nmd2Δ* and *upf3Δ* extracts) (data not shown) fail to do so. These results suggest that, upon preincubation, WT extracts increase the availability or activity of a limiting component of the translation system, whereas the mutant extracts maintain over time a smaller and relatively unchanged pool of the functionally available component. One possible explanation for this observation, considered below, is that the increase in translation activity in WT extracts arises from translation termination and dissociation of ribosomal subunits from endogenous mRNAs since the extracts used in these assays have not undergone nuclease treatment. Thus, ribosomes in WT extracts might readily enter the pool of functional ribosomes over time, leading to an overall increase in

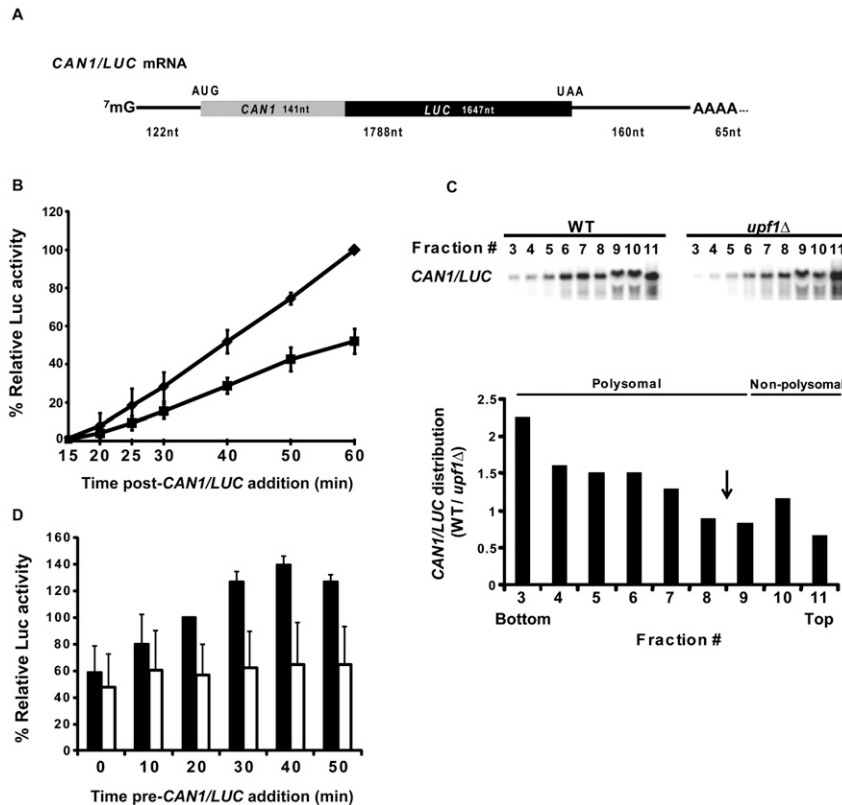


FIGURE 4. Translation of *CAN1/LUC* mRNA is decreased in *upf1Δ* extracts. (A) Schematic representation of the *CAN1/LUC* fusion mRNA. (B) Luciferase activities of WT (diamonds) and *upf1Δ* (squares) extracts prepared without micrococcal nuclease (MN) treatment and incubated with 100 ng of *CAN1/LUC* mRNA for the indicated times at 18°C. Relative luciferase activity was calculated using the WT 60-min values as 100%. (C, upper panel) Northern analysis of polysomal distribution of *CAN1/LUC* mRNA translated in vitro in WT or *upf1Δ* extracts. (Lower panel) Ratio of the polysomal distributions of the *CAN1/LUC* mRNA during in vitro translation. WT and *upf1Δ* extracts, prepared without MN treatment, were incubated with synthetic *CAN1/LUC* mRNA for 30 min at 18°C and then analyzed by fractionation on 7%–47% sucrose gradients and subsequent Northern blotting using a probe specific for the *CAN1/LUC* mRNA. The amount of *CAN1/LUC* mRNA (in arbitrary units) in each gradient fraction was determined by PhosphorImaging, and the y-axis depicts the ratios of those amounts in WT versus *upf1Δ* extracts. Sedimentation of the gradients was from right to left; the bars at the top of the panel indicate the polysomal and non-polysomal fractions. “Bottom” and “Top” refer to the regions of the gradients with the heaviest and lightest fractions, respectively. The vertical arrow depicts the position of the 80S ribosome peak. (D) Relative luciferase activities of WT (black boxes) and *upf1Δ* (white boxes) extracts treated as in B and incubated for 25 min after addition of *CAN1/LUC* mRNA at the indicated times. Relative luciferase activities were measured using WT 20-min values as 100%. Vertical bars in B and D depict standard deviation.

CAN1/LUC expression, whereas the reutilization of ribosomes that were previously engaged in translation is somehow compromised in the mutant extracts.

Upf1 affects translation in vitro

To test whether the decrease in translation of the *CAN1/LUC* reporter mRNA observed in *upf1Δ* extracts was due to the absence of Upf1 or resulted from an indirect effect of the lack of a functional NMD pathway, we assessed translation of the *CAN1/LUC* mRNA in *upf1Δ* extracts supple-

mented with either purified FLAG-Upf1 or BSA (as a control). As shown in Figure 5A, purified FLAG-Upf1 was sufficient to restore the translation activity of a *upf1Δ* extract to that approaching the activity of a WT extract. In addition, purified FLAG-Upf1 was also able to stimulate the translational capacity of a *upf1Δ* extract at different time points in a ribosome reutilization assay (Fig. 5B, cf. white boxes and gray boxes at each time point). These results indicate that Upf1 may play a direct role in enabling or enhancing the availability of functional ribosomes in vitro.

Translation initiation is compromised in *upf1Δ* extracts

To understand the nature of the translation defect in mutant extracts, we evaluated their relative efficiencies of translation initiation on the *CAN1/LUC* mRNA using a primer extension inhibition (toeprinting) assay (Dmitriev et al. 2003; Amrani et al. 2004). WT and *upf1Δ* extracts were preincubated for different lengths of time and then supplemented with *CAN1/LUC* mRNA, as in Figure 4D. After incubating the extracts with the mRNA for 25 min, cycloheximide (CHX) was added to terminate the reactions and stall elongating 80S ribosomes. Toeprinting reactions were then performed using a radiolabeled oligonucleotide that allowed visualization of a gel band corresponding to a ribosome positioned at the initiator AUG codon (Fig. 6A, upper panel). Accumulation of this toeprint was sensitive to cap analog (Fig. 6A, upper panel), indicating that it arose from initiation activity, and WT extracts routinely showed a more intense toeprint than the *upf1Δ* extracts

(Fig. 6A, upper and lower panels), reflecting better translation initiation.

Since the AUG toeprints could arise as a consequence of both de novo initiation and recycling of ribosomes on the same mRNA post-termination, we modified the assay such that CHX was added at the same time as the *CAN1/LUC* mRNA. Thus, only ribosomes undergoing de novo initiation (i.e., the first ribosome encountering the AUG codon) would contribute to the AUG toeprints. Figure 6B shows that, using this approach, only WT extracts exhibit a significant increase in the AUG toeprint within the first 20

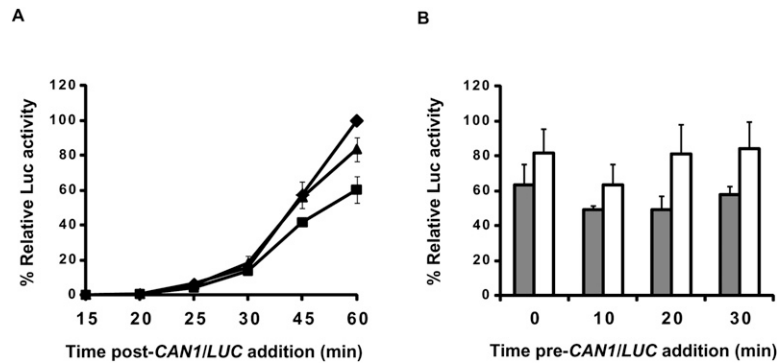


FIGURE 5. Purified FLAG-Upf1 can complement translation defects in vitro. Cell-free translation extracts were prepared from WT or *upf1Δ* cells without MN treatment and preincubated for 10 min on ice with 270–360 ng of BSA or purified FLAG-Upf1, followed by incubation at 18°C in either of two modes: (A) addition of 100 ng of *CAN1/LUC* mRNA at $t = 0$ min and sampling for luciferase activity at the indicated time points or (B) addition of 100 ng of *CAN1/LUC* mRNA at the indicated time points and translation for 25 min at 18°C prior to measurement of luciferase activity. (A) Relative luciferase values were calculated as in Figure 4B for WT extracts supplemented with BSA (diamonds), *upf1Δ* extracts supplemented with BSA (squares), or *upf1Δ* extracts supplemented with FLAG-Upf1 (triangles). (B) Relative luciferase activities of *upf1Δ* extracts supplemented with BSA (gray boxes) or FLAG-Upf1 (white boxes) normalized to the corresponding WT+BSA values at each given time point. Vertical bars in A and B depict standard deviation.

min, whereas *upf1Δ* extracts exhibit only a minimal increase in the AUG toeprint band even at late time points, suggesting an absence of 80S ribosomes at the initiator AUG in these extracts. This result indicates further that the availability of functional ribosomes may be diminished in the absence of Upf1. To determine whether Upf1 affects an early or late step in initiation, we repeated the same assay, replacing CHX with the nonhydrolyzable GTP analog GMP-PNP, which allows 48S formation but inhibits joining of the 60S ribosomal subunit (Fig. 6C; Dmitriev et al. 2003). In this experiment, we observed that both WT and *upf1Δ* extracts exhibited strong AUG toeprint bands in the presence of GMP-PNP, indicative of the presence of 40S ribosomal subunits on the RNA, with the *upf1Δ* extracts showing a slightly diminished signal. Taken together, these results suggest that, although 40S loading is slightly impaired in *upf1Δ* extracts, Upf1 is likely to influence a late stage of translation initiation, namely, the joining of the 60S subunit to the 48S complex.

Initiation efficiency depends on prior termination events

The results depicted in Figures 4–6 implicate Upf1 in the determination of translational initiation efficiency in vitro, but

the mechanism underlying this activity and its relationship to the reinitiation defects seen in vivo (Figs. 1–3) and in vitro (Amrani et al. 2004) were unclear. The extracts used in our translation reactions are used without any prior nuclease treatment, and the ongoing translation of endogenous mRNAs includes that of a substantial number of mRNAs that are NMD substrates (He et al. 2003; Johansson et al. 2007). Since premature termination on the latter transcripts is altered in the absence of *UPF1* (Amrani et al. 2004), we considered the possibility that these altered events might influence subsequent translation initiation activity in the mutant extracts. Accordingly, we devised an assay in which the translational capabilities of ribosomes that were allowed to undergo either normal or premature termination events were tested for their ability to subsequently translate the *CAN1/LUC* reporter mRNA. To achieve

this, we first treated WT and *upf1Δ* extracts with micrococcal nuclease to eliminate the endogenous mRNAs that would act as competitors of an exogenously added translational template. With this treatment, the ability of *upf1Δ* extracts to translate the *CAN1/LUC* mRNA in vitro resembled that of WT extracts for the first 30 min of incubation (Fig. 7A) (i.e.,

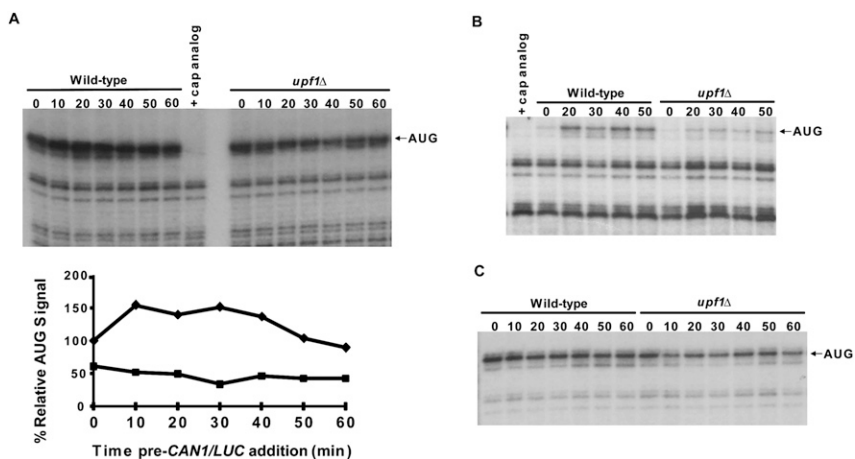


FIGURE 6. Formation of 80S toeprints is diminished in *upf1Δ* extracts. (A) Cell-free translation extracts, prepared from WT and *upf1Δ* cells without MN treatment, were incubated at 18°C. *CAN1/LUC* mRNA (100 ng) was added at the indicated time points, and reactions were further incubated for 25 min, followed by addition of 2 mM cycloheximide (CHX) for 3 min. Reactions were frozen and subsequently analyzed by toeprinting. (Upper panel) The autoradiograph and (lower panel) the densitometry of the AUG band in WT (diamonds) and *upf1Δ* (squares) reactions normalized to the WT 0-min value. (B) Translation conditions were as in A, except that 2 mM CHX was added with 100 ng of *CAN1/LUC* mRNA at each time point, followed by incubation for 25 min. Lanes in A and B in which control reactions contained 2.7 mM cap analog are indicated. (C) Translation and incubation conditions were as in B, except that CHX was replaced with 2 mM GMP-PNP.

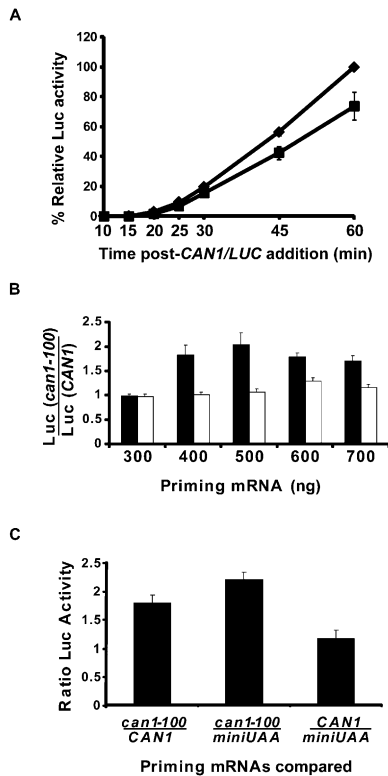


FIGURE 7. Initiation efficiency depends on prior termination events. Translation extracts prepared from WT or *upf1*Δ cells were treated with MN for 5 min at 25°C. (A) Luciferase activities of WT (diamonds) and *upf1*Δ (squares) extracts incubated with 100 ng of *CAN1/LUC* mRNA for the indicated times at 18°C. Relative luciferase activity was calculated using the WT 60-min values as 100%. (B) Treated extracts were incubated with the designated concentrations of the priming *CAN1* or *can1-100* mRNA for 7 min at 18°C, followed by addition of 300 ng of *CAN1/LUC* mRNA and incubation for an additional 25 min. Ratios of luciferase activities calculated from reactions programmed with *can1-100* and *CAN1* mRNAs are shown for WT (black boxes) and *upf1*Δ (white boxes) extracts. (C) *CAN1* or *can1-100* mRNA (500 ng of either) or 50 ng of *miniUAA* mRNA was incubated with WT extracts for 7 min, followed by addition of 300 ng of *CAN1/LUC* mRNA and further incubation for 25 min at 18°C. Ratios of luciferase activity were calculated as designated on the x-axis. Vertical bars in all panels depict standard deviation.

for the time period analyzed in subsequent assays), confirming our earlier observation that MN-treated extracts showed similar AUG toeprints of the *CAN1/LUC* transcript (Amrani et al. 2004). We then primed these extracts with excess amounts of either synthetic WT *CAN1* mRNA or its nonsense-containing counterpart (*can1-100* mRNA). We rationalized that providing an excess of the priming mRNA templates would allow most of the functional ribosomes in a given extract to be involved in translation and thereby undergo either normal or premature translation termination events. Furthermore, these terminating ribosomes might be differentially predisposed for further rounds of translation in these extracts. We tested this difference by measuring the luciferase activity generated by the subsequent addition of the *CAN1/LUC* reporter mRNA to the reactions containing

extracts translating the WT or PTC-containing mRNAs. Determining the ratio of the luciferase activities obtained from translation reactions primed with the *can1-100* versus the *CAN1* mRNA for a given extract provided a measure of the efficiency of ribosome release from the PTC and their subsequent reutilization. Figure 7B depicts the ratios of luciferase activity obtained in WT and *upf1*Δ extracts from reactions that were first primed with varying amounts of the *can1-100* or *CAN1* mRNAs. The results of this experiment indicate that WT extracts manifest as much as twofold more translation of the *CAN1/LUC* reporter mRNA when primed with high levels of the *can1-100* mRNA versus the *CAN1* mRNA, whereas the translation of the reporter mRNA in *upf1*Δ extracts is unaffected by the nature of the priming mRNA. This result suggests that in WT extracts, but not in *upf1*Δ extracts, the termination and release of ribosomes from premature stop codons generates a pool of ribosomes with greater recycling ability than ribosomes released from the normal terminator.

Since the ORF in the PTC-containing mRNA is shorter than its WT counterpart, in principle, the smaller size of the ORF (and thus greater frequency of terminating ribosomes), rather than the nature of the termination event itself, could be responsible for the increased luciferase activity in translation reactions primed with the *can1-100* mRNA in WT extracts. Hence, we repeated the assay in WT extracts using either the *can1-100* mRNA or the *miniUAA* mRNA (Amrani et al. 2004). The latter is a transcript that contains the same ORF as the *can1-100* mRNA, but which has a normal termination event because the short ORF is followed by a normal 3'-UTR (Amrani et al. 2004). A comparison of the ratios of luciferase activities obtained with the two RNAs showed that priming with the *can1-100* mRNA yielded enhanced translation of the *CAN1/LUC* mRNA relative to priming with the *miniUAA* mRNA (Fig. 7C, middle box). The experiments in Figure 7C (right box) also demonstrate that the luciferase output obtained when reactions were primed with the *miniUAA* mRNA were comparable to those primed with the *CAN1* mRNA. Taken together, these results indicate that premature termination events, not the length of the ORF, are responsible for the increased luciferase activity in this system and reflect the possibility that more ribosomes, or more active ribosomes, were released from the nonsense-containing transcript than from the WT or pseudo-WT mRNA.

To test whether Upf1 is involved in the increase of translation activity subsequent to premature termination, we primed *upf1*Δ extracts with the *CAN1* or *can1-100* mRNAs in the presence of purified FLAG-Upf1 or BSA and measured the responses of the respective extracts to *CAN1/LUC* mRNA that was added subsequently. We found that addition of purified FLAG-Upf1 was sufficient to enhance luciferase activity in *upf1*Δ extracts primed with *can1-100* mRNA, similar to that observed with WT extracts (Fig. 8A, cf. gray boxes in WT+BSA and *upf1*Δ +FLAG-Upf1). To further analyze the translation status of the *CAN1/LUC*

mRNA in reactions primed with the *can1-100* mRNA in the presence of FLAG-Upf1 or BSA, we fractionated the translation reactions on sucrose gradients and analyzed the fractions by Northern blotting for their levels of specific transcripts. These experiments showed that, in WT extracts (supplemented with BSA), the *CAN1/LUC* mRNA was distributed throughout the sucrose gradient, including the polysome and lighter fractions (Fig. 8B, left panel). In *upf1Δ* extracts (supplemented with BSA), there was significantly less *CAN1/LUC* mRNA in the polysome fractions, with most of the mRNA being confined to the sub-80S fractions. However, addition of FLAG-Upf1 to the *upf1Δ* extracts restored the polysome distribution of the *CAN1/LUC* mRNA to that seen in the WT control. Comparison of the distribution of the *CAN1/LUC* mRNA in *upf1Δ* extracts supplemented with or without FLAG-Upf1 showed up to threefold more *CAN1/LUC* mRNA in polysomal fractions of extracts that contained FLAG-Upf1 (Fig. 8C, black boxes). To determine whether the decreased translation of the secondary *CAN1/LUC* mRNA was due to overall decreased translational capacity of the nuclease-treated *upf1Δ* extracts, we also analyzed the distribution of the *can1-100* mRNA (Fig. 8B, right panel; Fig. 8C, white boxes) used to prime the reactions. We observed a similar distribution pattern for the primary *can1-100* mRNA in extracts containing or lacking Upf1, further indicating that decreased *CAN1/LUC* translation in *upf1Δ* extracts is most likely a consequence of translational termination at a PTC in the absence of Upf1.

Addition of WT ribosomal subunits enhances translation in *upf1Δ* extracts

Although Upf1 has been shown to cosediment with polyribosomes (Peltz et al. 1993b; Atkin et al. 1995; Yamashita et al. 2009), relatively little is known about the nature of this association. Since the results of Figures 7 and 8 suggested that Upf1 might enhance the ability of ribosomes to recycle to another round of translation, we evaluated the effect of adding purified WT ribosomal subunits to *upf1Δ* extracts. We reasoned that the absence of Upf1 in these extracts might lead to the accumulation of defective 40S and/or 60S subunits and that the possible amelioration of the translation defects upon addition of the WT subunits would shed light on which subunit was affected in the absence of Upf1. Figure 9A (right panel) shows that addition of purified 40S or 60S subunits to non-nucleated *upf1Δ* extracts was sufficient to enhance translation of the *CAN1/LUC* mRNA. WT extracts (Fig. 9A, left panel), on the other hand, did not show any increases in translation upon addition of either ribosomal subunit. The stimulatory activity of 40S and 60S subunits in *upf1Δ* extracts suggested that either both subunits were affected by the absence of Upf1 or that only one subunit was affected but that the other contained bound Upf1. Indeed, Figure 9B shows that Upf1 preferentially copurified with 40S subunits, whereas it was markedly re-

duced in the 60S subunit fraction. Since the subunit purification protocol (Algire et al. 2002) involves high salt washes, this result indicates that Upf1 can form a stable association with 40S subunits, and probably accounts in part for the increase in luciferase activity observed upon their addition to *upf1Δ* extracts. In turn, this suggests that the stimulatory activity of the purified 60S subunits may reflect a relative inactivity of the endogenous 60S subunits in *upf1Δ* extracts.

DISCUSSION

In addition to well-documented roles targeting nonsense-containing mRNAs for rapid decay (Jacobson and Izaurralde

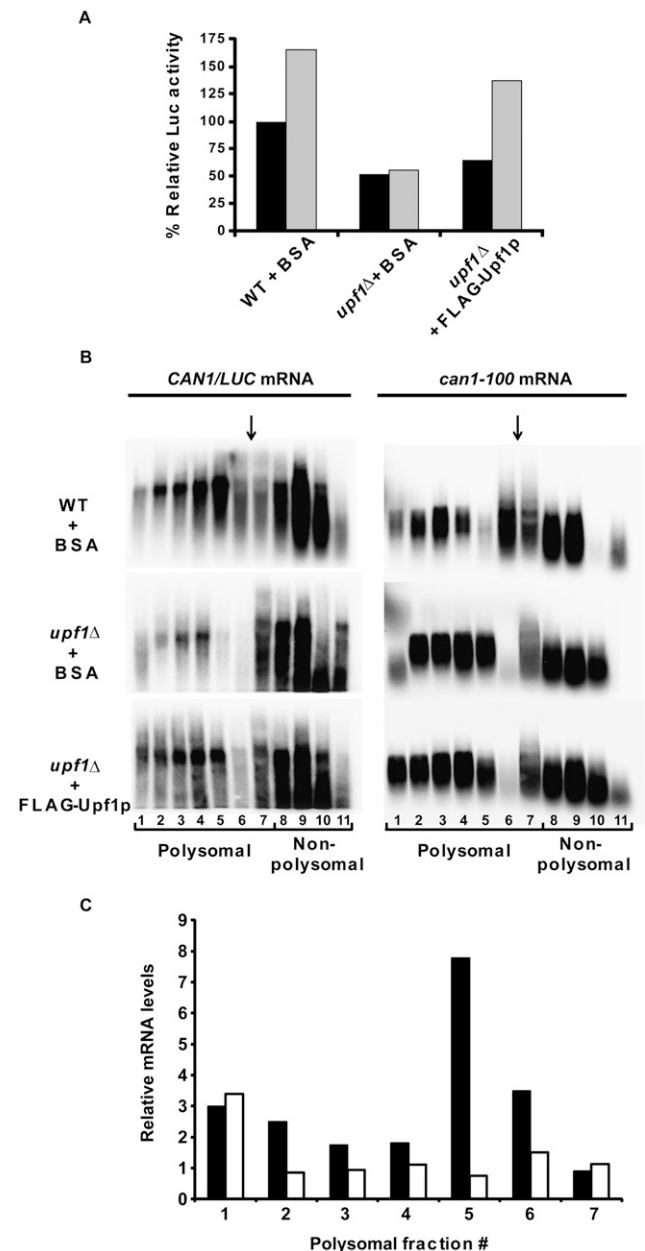


FIGURE 8. (Legend on next page)

2007; Franks and Lykke-Andersen 2008), the Upf factors of the NMD pathway also appear to have translational regulatory functions. Results from both yeast and mammalian cells have implicated possible roles for Upf1, Upf2/Nmd2, and Upf3 in the regulation of translational termination, repression, and reinitiation (Czapinski et al. 1998; Muhrad and Parker 1999; Maderazo et al. 2000; Wang et al. 2001; Isken et al. 2008; Ivanov et al. 2008). To consider the possible general translational involvement of these factors in yeast, we sought in this study to first examine whether *in vitro* results supporting a Upf role in translational reinitiation (Amrani et al. 2004) could be recapitulated *in vivo*. Using a series of *PGK1/LUC* reporter constructs, we defined a cell-based assay in which ~2.5% of the ribosomes that prematurely terminate the *PGK1* ORF subsequently reinitiate and translate the *LUC* ORF. Relative to wild-type cells, strains that lacked the function of any one of the three *UPF* genes manifested markedly reduced reinitiation activity in this assay (Fig. 1D), but reinitiation per se played no role in activating NMD (Fig. 3). These observations not only served to confirm and elaborate the earlier results we had obtained in cell-free extracts (Amrani et al. 2004), but also led us to investigate in greater detail the mechanism by which the Upf proteins influence translational activity.

Prior studies indicated that Upf1 regulation of translation might not be limited to nonsense-containing mRNAs. For example, yeast cells expressing WT-like *CUP1* reporters showed reduced Cup1 levels when *UPF1* was deleted (Muhrad and Parker 1999). Furthermore, *in vitro* translation of the WT synthetic *CAN1/LUC* mRNA in yeast extracts was found to require Upf1 (Amrani et al. 2004), and mammalian cell-free extracts containing hyperphosphorylated Upf1 showed reduced translation of synthetic *Renilla LUC* mRNA (Isken et al. 2008). Consistent with these observations, we not only confirmed that optimal translation of the *CAN1/LUC* mRNA is Upf1-dependent,

FIGURE 8. Purified FLAG-Upf1 restores efficient translational activity to *upf1Δ* extracts. Cell-free translation extracts were prepared from WT or *upf1Δ* cells and treated with MN for 5 min at 25°C. Extracts were incubated with 270 ng of BSA or FLAG-Upf1 for 10 min on ice and added to translation mixes containing 500 ng of *CAN1* (A) or *can1-100* (A–C) mRNA and incubated for 7 min at 18°C. *CAN1/LUC* mRNA (300 ng) was then added and reactions were further incubated for 25 min at 18°C. (A) Relative luciferase activities in the reactions detailed on the x-axis primed with the (black boxes) *CAN1* or (gray boxes) *can1-100* mRNAs. (B) *can1-100*-primed translation reactions were set up as in A, terminated by the addition of CHX (2 mM), and subsequently fractionated on 7%–47% sucrose gradients. The fractions were then analyzed by Northern blotting using a DNA probe specific for the *CAN1/LUC* (left panel) or the *can1-100* (right panel) mRNA. Polysomal and non-polysomal fractions are indicated, as is the position of the 80S peak (vertical arrow) in each blot. (C) Relative mRNA levels in polysome fractions. The amount of *CAN1/LUC* (black boxes) or *can1-100* mRNA (white boxes) in each polysomal fraction of B was determined by phosphorimaging. The y-axis depicts the ratios of those amounts in *upf1Δ* extracts supplemented with FLAG-Upf1 versus BSA.

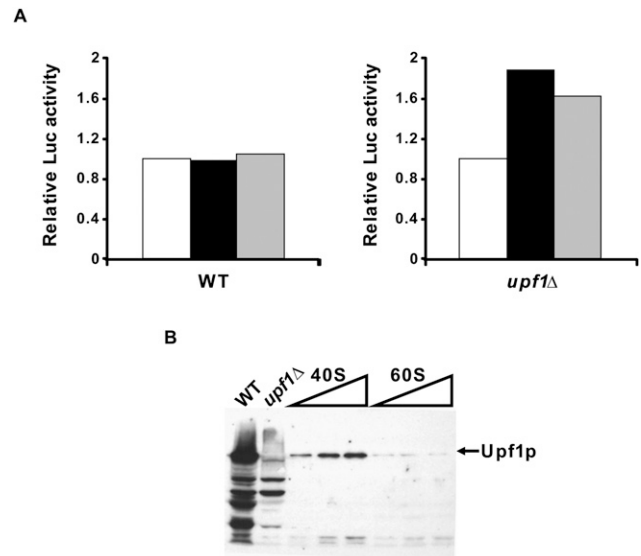


FIGURE 9. 40S and 60S ribosomal subunits enhance translation in *upf1Δ* extracts. Cell-free translation extracts were prepared from WT or *upf1Δ* cells. (A) Extracts were supplemented with 100 ng of *CAN1/LUC* mRNA and then incubated with BSA (white boxes), purified 40S subunits (black boxes), or purified 60S subunits (gray boxes) for 60 min at 18°C. Luciferase activity is depicted relative to the value obtained with the BSA control for each extract. The graph depicts the average of two experiments. (B) Western blot of increasing amounts of purified 40S (5, 10, and 15 μg) and 60S (5, 10, and 15 μg) subunit preparations probed with polyclonal anti-Upf1 antibody. WT and *upf1Δ* extracts (5 μg each) were run as controls.

but that Upf2/Nmd2 and Upf3 are also required (Fig. 4; data not shown). Moreover, using a novel assay for ribosome reutilization, we found that elimination of any one of the Upf proteins led to reduced availability of ribosomes for translation initiation on *CAN1/LUC* mRNA (Fig. 4D; data not shown). The significance of these results was underscored by the observation that the translational defect(s) of *upf1Δ* extracts could be overcome by the addition of purified recombinant Upf1 (Figs. 5, 8). Collectively, these experiments suggested that Upf1 plays a direct role in regulating the initiation efficiency of ribosomes that enter a new cycle of translation.

A further understanding of the apparent initiation defect was obtained by attempting to identify the stage of initiation that was impaired. Toeprinting analyses of the *CAN1/LUC* initiator AUG codon indicated that although *upf1Δ* extracts had fewer 80S ribosomes at the AUG than did WT extracts, the two extracts exhibited similar 40S recruitment, suggesting that a loss of Upf1 affects subunit joining (Fig. 6). Indeed, the addition of purified WT 40S or 60S ribosomal subunits to *upf1Δ* extracts stimulated translation in these extracts, but not in WT extracts (Fig. 9A). Further analyses showed that Upf1 was preferentially associated with 40S subunits, suggesting that enhanced translation observed upon 40S addition could, in part, be explained by the ribosome-bound Upf1 (Fig. 9B). In combination with the

toeprinting results, these data implied that (1) the increase in luciferase activity detected upon addition of 40S subunits to *upf1Δ* extracts was most likely Upf1-mediated and (2) the stimulation of translation occurring in *upf1Δ* extracts upon addition of 60S subunits was probably attributable to the accumulation of inactive 60S subunits in those extracts.

It is becoming increasingly apparent that factors involved in regulating translation may have multiple functions and that this multitasking may link the initiation pathway to those for termination and/or NMD (Amrani et al. 2008; Saini et al. 2009). Moreover, it has been shown that (1) eIF3, eIF1, and eIF1A, as well as the eIF3j subunit, not only play a role in initiation, but also stimulate the in vitro dissociation of post-termination 80S complexes (Pisarev et al. 2007); (2) at elevated temperatures, yeast strains harboring the temperature-sensitive *prt1-1* mutation (in eIF3) lose their abilities to both initiate translation and promote NMD (Welch and Jacobson 1999); (3) yeast cells with mutations in both *SUI1* (encoding eIF1) and *UPF1* manifest synthetic lethality (Harger and Dinman 2004); and (4) phosphorylated mammalian Upf1 can interact with eIF3, and this interaction appears to inhibit cap-dependent in vitro translation (Isken et al. 2008). Consistent with these observations, we find that yeast *upf1Δ* extracts are unable to recycle ribosomes efficiently from a nonsense-containing mRNA (Figs. 7, 8), a result suggesting that events at termination can influence the subsequent activity of ribosomal subunits (Khoshnevis et al. 2010; Pisarev et al. 2010). Since our in vitro translation system does not support NMD (e.g., see Fig. 8B; Amrani et al. 2004), we hypothesize that Upf1's role in promoting ribosome dissociation and/or recycling from nonsense-containing mRNAs represents an indepen-

dent function that occurs concurrently or prior to its role in promoting decapping. How these events may be linked remains to be elucidated. Since premature termination in yeast is considerably less efficient than normal termination (Amrani et al. 2004), one possibility is that the post-peptide hydrolysis recruitment of eIF3, and perhaps other initiation factors, to a prematurely terminating ribosome is Upf1-dependent. Inclusion of Upf1 would allow termination complex remodeling and efficient 80S dissociation, yielding a 40S subunit capable of reinitiating translation and a 60S subunit capable of being primed for subsequent translation cycles (Amrani et al. 2006).

It remains to be seen whether the association between Upf1 and 40S subunits reflects a cause or consequence of ribosome dissociation, whether other NMD factors show a similar association with ribosomal subunits, and what other factors may be present in a Upf-dependent manner. Upf1 has intrinsic ATPase and helicase activities (Czaplinski et al. 1995), and it will be of interest to test whether these activities are principally required for ribosome remodeling at a premature termination codon or for the stimulation of mRNA decay.

MATERIALS AND METHODS

Yeast strains, vectors, and transformations

The yeast strains used in this study, and their genotypes, are listed in Table 1. Strain YRG467 was constructed by transforming HFY467 with plasmid pRS315-NMD2(C-Sal) (He et al. 1996). For in vivo studies, strains were grown in selective media (Amberg et al. 2005) at 30°C, or at 24°C for temperature-sensitive

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference
HFY114	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100</i>	He et al. (2003)
HFY115	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, nmd2::HIS3</i>	He et al. (2003)
HFY467	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, nmd2::HIS3, upf1::URA3</i>	He and Jacobson (1995)
HFY861	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, upf3::HIS3</i>	He et al. (1997)
HFY872	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, upf1::URA3, upf3::HIS3</i>	He et al. (1997)
HFY874	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, upf3::HIS3, nmd2::URA3</i>	He et al. (1997)
YRG467	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, nmd2::HIS3, upf1::URA3 + [pRS315-NMD2(C-Sal)]</i>	This study
RY262	<i>MATa, his4-519, ura3-52, rpb1-1</i>	Herrick et al. (1990)
SWP154(-)	<i>MATa, upf1-Δ1::URA3, ura3-52, rpb1-1, leu2-1, his4-38</i>	Peltz et al. (1993a)
MBS	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, [rho+], L-o, M-o</i>	Iizuka and Sarnow (1997)
NA101	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, [rho+], L-o, M-o, upf1::HIS3</i>	Amrani et al. (2004)
NA102	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, [rho+], L-o, M-o, nmd2::HIS3</i>	Amrani et al. (2004)
NA103	<i>MATa, ade2-1, his3-11,15, leu2-3,112, trp1-1, ura3-1, can1-100, [rho+], L-o, M-o, upf3::HIS3</i>	This study

strains. Yeast transformations were performed by the rapid protocol (Soni et al. 1993), and the yeast vectors utilized included pRIP1 (Parker and Jacobson 1990), pRS314 (Sikorski and Hieter 1989), YEplac22 (Gietz and Sugino 1988), and pRS316 (Sikorski and Hieter 1989). For in vitro translation experiments, MBS and strains derived from it (NA101, NA102, and NA103) were grown in YEPD at 25°C for extract preparation (Amrani et al. 2004).

Plasmid-borne expression constructs

The constructs used for in vivo expression analyses are described in Table 2. The oligonucleotides used in this study are listed in Table 3. Construct 1, a chimeric *PGK1/LUC* gene (Fig. 1A), consists of the *PGK1* 5'-untranslated region (UTR); the first 5% of the *PGK1* open reading frame (ORF) followed by a UAG stop codon (contextually a premature termination codon); an 82-nt *PGK1* sequence element (derived from a 106-nt segment previously designated as a DSE) (see Table 2; Peltz et al. 1993a) that contains two AUG codons (out-of-frame with the upstream *PGK1* ORF) fused in-frame to the *LUC* ORF lacking its normal start codon;

and the 3'-UTR of *PGK1*. All constructs for in vivo luciferase expression experiments contain a 5'-BamHI site and a 3'-HindIII site and were subcloned into vectors by excising with BamHI/HindIII. PCR fragments 1 and 2 were generated using the *mini-PGK1* allele as a template. This allele contains a 58-nt tag in its 3'-UTR to allow later detection of its mRNA on a Northern blot (Peltz et al. 1993a). Fragment 1 was generated using the M13/pUC Reverse Amplification primer (BRL) and primer 3-106-82 and digested with BamHI and PstI restriction enzymes. Fragment 2 was generated using primer 5-PPGKH and the M13/pUC Forward Amplification primer (BRL) and digested with BamHI and HindIII. Fragment 3 was generated by PCR using pXP1 as a template (a kind gift from Paul Dobner, University of Massachusetts Medical School) and primers 5-PSLUCP and 3-PLUCSP, and digested with PstI. Fragments 1 and 2 were ligated into pGEM-3Z(f)+ (Promega) and cut with BamHI/HindIII. The resulting construct was cut with PstI, incubated with calf intestinal alkaline phosphatase (CIAP), and ligated with fragment 3 to generate construct 3. Construct 1 was generated by digesting construct 3 with Sall to remove a 10-base-pair (bp) fragment and religating to

TABLE 2. Pertinent features of constructs used in this study

Construct	Description
Mini- <i>PGK1</i>	Contains the 5'-UTR and first 5% of the <i>PGK1</i> ORF followed by a premature termination codon, a 106-nt <i>PGK1</i> sequence element required for NMD sensitivity that contains two AUG codons, and the 3'-UTR of <i>PGK1</i> (Peltz et al. 1993a). There is an NheI restriction site at the UAG stop codon that terminates the <i>PGK1</i> ORF prematurely. This <i>PGK1</i> construct and all other related constructs were subcloned into various vectors with a 5' BamHI site and a 3' HindIII site.
Construct 1	Similar to the mini- <i>PGK1</i> allele (above), except that the 106-nt sequence element was truncated to 82 nt (retaining the two AUG codons) and the <i>LUC</i> coding region (minus its endogenous AUG start codon) was inserted immediately 3' to it. The <i>LUC</i> coding region is in-frame with the two AUG codons of the 82-nt sequence element, generating a 1716-bp ORF in the +1 reading frame relative to the <i>PGK1</i> ORF. A Sall site joins the 82-nt sequence element and the 5' end of the <i>LUC</i> coding region; a PstI site joins the 3' end of the <i>LUC</i> coding region and the <i>PGK1</i> 3'-UTR. As in mini- <i>PGK1</i> , an NheI site lies at the UAG codon.
Construct 2	Identical to construct 1, except that each of the two AUGs of the 82-nt <i>PGK1</i> sequence element were deleted. The first ORF following the <i>PGK1</i> premature termination codon begins at an AUG codon 82 nt into the <i>LUC</i> coding region.
Construct 3	Similar to construct 1, except that the <i>LUC</i> coding region is in the +2 reading frame, relative to the <i>PGK1</i> ORF, and out of frame with the two AUGs of the 82-nt <i>PGK1</i> sequence element. There is a 10-bp linker between the 82-nt <i>PGK1</i> sequence element and the <i>LUC</i> coding region containing the sites 5'-Sall-PstI-Sall-3'. Digestion with Sall and religation to close yielded construct 1.
Construct 4	Identical to construct 1, except that the premature UAG codon between the <i>PGK1</i> ORF and the 82-nt <i>PGK1</i> sequence element was deleted.
Construct 5	Identical to construct 1, except that the premature UAG codon of <i>PGK1</i> plus the first nucleotide of the 82-nt <i>PGK1</i> sequence element were deleted, generating an ORF that begins at the <i>PGK1</i> AUG initiation codon and ends at the <i>LUC</i> termination codon.
Construct 6	The <i>PGK1</i> ORF and the 82-nt <i>PGK1</i> sequence element of construct 1 were deleted between the <i>PGK1</i> AUG initiation codon and the beginning of the <i>LUC</i> coding region. A Sall site lies at the junction of the <i>PGK1</i> 5'-UTR and the <i>LUC</i> coding region.
Construct 7	A stem-loop cassette consisting of a strong stem-loop preceded by a 30-nt leader was inserted into construct 1 between the premature UAG codon of <i>PGK1</i> and the 82-nt <i>PGK1</i> sequence element. NheI sites are present at the UAG codon and between the stem-loop and the 82-nt <i>PGK1</i> sequence element. A Sall site lies between the 82-nt <i>PGK1</i> sequence element and the <i>LUC</i> coding region. A PstI site is at the <i>LUC</i> UAA termination codon.
Construct 8	Identical to construct 8, except that the UAG termination codon of <i>PGK1</i> was deleted, generating an ORF starting at the <i>PGK1</i> initiation codon and ending at the <i>LUC</i> termination codon.

TABLE 3. Oligonucleotide primers used in this study

Oligonucleotide	Sequence
3-106-82	5'-AAAAGTGCAGTCGACGCCAGCCAGCTGGAATACCTT-3'
5-PPGKH	5'-AAAAGTGCAGGATCTCCCATGTCTACTGGT-3'
5-PSLUCP	5'-AAAAGTGCAGTCGACGCCAAAAACATAAGAAAG-3'
3-PLUCSP	5'-AAAAGTGCAGTTACAATTTGGACTTCCGCC-3'
PGKDATG	5'-AGTCCTAGCTAGCTAGGACTTCATCATTGCTGCTTCTCTGCTGCC AACACCAAGACTGTCACTGACAA-3'
pGEM-SEL	5'-CTATAGGGCGATATCGAGCTCG-3'
PGKΔTAG	5'-AGAGTTCTAGCTAGCGACTTCATCATTGCT-3'
FUSION	5'-ATTCCAGCTGGCTGGGTGACCTGCAGGCA-3'
PGKDELINIT	5'-ACAAATATAAAAACATCTTTATCTTCAAAG-3'
106DEL83	5'-TTCCAGCTGGCTGGCTCGACGCCAAAAACA-3'
106DEL1	5'-GTTCTAGCTAGCTAGACTTCATCATTGCTG-3'
PGKUAGSAL2	5'-GTCGACCCTAGCTAGCTAGAAGCTCTGAT-3'
NHELINKERA	5'-CTAGCTAGAGCGTGATATCAGGCCCTAG-3'
NHELINKERB	5'-CTAGCTAGAGGCCCTGATATCAGCGCTAG-3'
SALLINKERA	5'-TCGACAGCGCTGATATCAGGCCCTG-3'
SALLINKERB	5'-TCGACAGGCCCTGATATCAGCGCTG-3'
HPR1A	5'-TGGGCAGAAGCCTTGACAGCGGCTTC-3'
HPR2A	5'-GAAGCCGCTGCAAGGCTTCTGCCAAT-3'
HPL1A	5'-GTCGGTCCAGAAGTTGAAGCCGCTGTCAAGGCTTCT GCCAAT-3'
HPL2A	5'-TGGGCAGAAGCCTTGACAGCGGCTTCAACTTCTGGA CCGAC-3'
HAIRPINL1	5'-GTCGGTCCAGAAGTTGAAGCCGCTGTCAAGGCTTCT GCTTAAT-3'
HAIRPINL2	5'-TAAGCAGAAGCCTTGACAGCGGCTTCAACTTCTGGA CCGAC-3'
HAIRPINR1	5'-TAAGCAGAAGCCTTGACAGCGGCTTC-3'
HAIRPINR2	5'-GAAGCCGCTGTCAAGGCTTCTGCTTAAT-3'
DSTOP1	5'-CATCAGAGTTCTAGCTAGCAGCGCTGATATCAGGGT CG-3'
DSTOP2	5'-CGACCCTGATATCAGCGCTGCTAGCTAGAAGCTCTGA TG-3'
SAL-PST	5'-TCGATGCA-3'

close. BamHI/HindIII fragments containing construct 3 and construct 1 were then subcloned into the centromeric yeast vectors pRIP1 (cut with BamHI/HindIII) and pRS314 (cut with SmaI), and the high-copy plasmid, YEplac112 (cut with BamHI/HindIII). The resulting constructs were transformed into strains HFY114, YRG467, HFY115, HFY861, HFY467, HFY872, and HFY874.

Construct 2 was generated with the Chameleon Site-Directed Mutagenesis Kit (Stratagene) using the following strategy: plasmid pRIP1 containing construct 1 was digested with BamHI/SalI to yield an 800-bp fragment containing the 5'-end of construct 1 from the *PGK1* start to the junction of the 82-nt *PGK1* sequence element and *LUC* and an 8-kb fragment containing the remainder of the construct and pRIP1. The smaller fragment was subcloned into pGEM-3Z(f)+ to yield pGEM-3Z(f)+/PGKBS. The resulting plasmid was mutagenized with primers PGKDATG and pGEM-SEL. A BamHI/SalI fragment containing the mutagenized construct was then ligated with the 8-kb fragment described above to yield construct 2. Construct 5 was generated similarly, with primers PGKΔTAG and pGEM-SEL, followed by mutagenic primer FUSION. These constructs were also placed into pRS314 and YCplac22. Construct 4 was generated using primers PGKDELINIT and PGKΔTAG. Construct 6 was generated as follows: An ~560-bp fragment was made by PCR using pGEM-3Z(f)+/PGKBS as a template and primers M13 reverse and PGKUAGSAL2, and cut with BamHI/SalI. This fragment was ligated into the 8-kb fragment described above to yield construct 6. All of the above constructs were transformed into HFY114, YRG467, HFY115, HFY861, HFY467, HFY872, and HFY874.

To generate construct 7, construct 1 was subcloned into pGEM-3Z(f)+ and cut with NheI. Linkers NHELINKERA and NHELINKERB were annealed together and ligated into the NheI site. These constructs were sequenced for orientation such that NHELINKERA reads 5' → 3'. The resulting construct [pGEM-(Sal)*lucNHE*] was digested with StuI to cut within the NHE linker and treated with CIAP. Oligonucleotide primers HPR1A and HPR2A were annealed together, and HPL1A and HPL2A were annealed together. The resulting short double-stranded fragments (designated HairpinR and HairpinL, respectively) were isolated on a 15% acrylamide gel and purified by the crush-and-soak method (Maxam and Gilbert 1977). Ligations were performed such that HairpinR and HairpinL were ligated together for 0 and 5 min before addition of the StuI-cut pGEM-(Sal)*lucNHE*. Colonies from the above ligations were screened by colony PCR for 162-bp inserts containing HairpinR and HairpinL (as opposed to double HairpinR or double HairpinL inserts) and sequenced for orientation such that HairpinL was 5' of HairpinR. The resulting BamHI/HindIII fragment-containing construct 7 was subcloned into YCplac22. This construct was transformed into HFY114, YRG467, HFY115, HFY861, HFY467, HFY872, and HFY874. Construct 8 was made by deleting the UAG stop codon

of construct 7 using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). The mutagenic primers were DSTOP1 and DSTOP2. This construct was transformed into yeast strains HFY114, YRG467, HFY115, HFY861, HFY467, HFY872, and HFY874.

The *CAN1/LUC* ("Fusion") construct used as a template for the preparation of synthetic mRNA for in vitro translation experiments has been described elsewhere (Amrani et al. 2004). pRS316pA-*CAN1* and pRS316pA-*can1-100* templates were derived by ligating an EcoRI/HindIII fragment of plasmid CFE86 (containing the complete *CAN1* gene; a kind gift from Chunfang Li) or plasmid CFE87 (which contains the complete *can1-100* gene; a kind gift from Chunfang Li) into pRS316pA linearized by cleavage with EcoRI and HindIII. To generate pRS316pA, oligonucleotide primers Xho-pA-Asp (5'-[phos]TCGAG(A)₇₅G-3') and Asp-pT-Xho (5'-[phos]GTACC(T)₇₅C-3') were annealed, alkaline phosphatase-treated (Roche), and ligated into XhoI/Asp718-linearized pRS316.

Measurement of luciferase activity in cells, Bradford assays, and Northern analyses

Yeast cultures (30 mL) were grown in selective medium overnight at 24°C to an OD₆₀₀ of 0.7–1.2. Four milliliters of each culture was removed, and the cells were pelleted and frozen at –70°C for later RNA extraction. An additional 20 mL of each culture was pelleted and washed twice with 1× PBS (0.01 M NaH₂PO₄, 0.15 M NaCl). The cells were resuspended in 2 mL of 1× PBS and split between two microcentrifuge tubes. These were then pelleted and stored

at -70°C to await glass bead extraction. The frozen cell pellets were resuspended in 200 μL of luciferase extraction buffer (LEB) (0.1 M KPO_4 at pH 7.8, 1 mM DTT, 1 mM PMSF). Acid-washed glass beads (200 mg; Sigma 425–600 μm) were added, and the tubes were then vortexed for 30 sec and placed on ice for 30 sec, alternately, for 5 min. The tubes were then spun in a microcentrifuge at 14,000 rpm for 5 min at 4°C , and the supernatant (extract) was placed into a fresh tube on ice. The following components were then added to a luminometer cuvette: 100 μL of LEB, 20 μL of extract, and 350 μL of luciferase assay buffer (LAB) at pH 7.8 (25 mM glycylglycine at pH 7.8, 15 mM KPO_4 at pH 7.8, 15 mM MgSO_4 , 4 mM EGTA, 2 mM ATP, 1 mM DTT). Assays were performed in a Monolight 2010 luminometer by injecting 100 μL of 200 μM D-luciferin and potassium salt (Analytical Luminescence Labs) in 25 mM glycylglycine (pH 7.8) and measuring light output for 10 sec. Extracts were stored at -70°C following luciferase assays until Bradford assays were performed. Bradford assays were performed on a Beckman DU7400 Spectrophotometer with the Bio-Rad Protein Assay Kit according to the microassay protocol using 10 μL of extract per assay. RNA was prepared from frozen cell pellets as described previously (Herrick et al. 1990) and quantitated on a Beckman DU7400 Spectrophotometer. Aliquots (10 μg) of total RNA were loaded onto a 1% agarose/formaldehyde/MOPS gel and electrophoresed, blotted, and hybridized as described previously (Herrick et al. 1990). Transcripts derived from the different constructs were detected with an oligonucleotide (Peltz et al. 1993a) that hybridizes to the 3'-UTR of the respective construct mRNAs but does not hybridize to the endogenous *PGK1* mRNA. mRNA signals were quantitated using a Betascope (Betagen) blot analyzer. mRNA levels were normalized for loading by stripping the blots and rehybridizing with a random-primed probe targeted to the *CYH2* mRNA. Final luciferase activity was expressed as relative light units (RLUs) of 20 μL of extract, per microgram of protein in 20 μL of extract, per unit of construct mRNA in 10 μg of total RNA, normalized for loading differences with the *CYH2* mRNA signal. Each set of assays performed on a given day included three cultures of construct 1 in strain HFY114 on a YCp plasmid, and each Northern blot from each set of assays contained the RNAs from these three cultures. The average luciferase activity (expressed in RLU/microgram of protein per unit of mRNA) of these three cultures was assigned a value of 100% to which all other luciferase activities in a set of assays were compared.

Extract preparation, synthetic mRNA, in vitro translation, and toeprinting assays

Saccharomyces cerevisiae strains MBS, NA101, NA102, and NA103 were used to make extracts for in vitro translations as described (Iizuka and Sarnow 1997; Wu et al. 2007; He et al. 2008). Capped and polyadenylated *CAN1/LUC* mRNA was synthesized with the SP6 mMessage mMachine Kit (Ambion) as per the manufacturer's protocol, from HindIII-linearized plasmids. Capped and polyadenylated *CAN1* and *can1-100* mRNAs were synthesized in vitro from KpnI-linearized pRS316pA-*CAN1* and pRS316pA-*can1-100* plasmids using the T7 mMessage mMachine Kit (Ambion) as per the manufacturer's protocol. Translation assays were as described (Amrani et al. 2004; Wu et al. 2007), except that each assay was performed at 18°C without prior treatment of the extract with micrococcal nuclease (MN), unless otherwise specified, and used

100 μg of extract and 100 ng of the *CAN1/LUC* mRNA unless specified otherwise. When used, MN pre-treatment of extracts was performed for 5 min at 25°C (Amrani et al. 2004). Protein concentrations of translation extracts were measured using the BCA Protein Assay Kit (Pierce). Translation reactions were incubated as specified in the figure legends and frozen in a dry ice/ethanol bath until luciferase assays were to be performed. The latter were done with a TD20/20 luminometer (Promega), as described previously (Amrani et al. 2004). For translation reactions involving FLAG-Upf1 purified from yeast (a kind gift from Stephanie Kervestin), 270–360 ng of FLAG-Upf1 or BSA was preincubated with extracts on ice for 10 min prior to adding the rest of the components of the translation reactions. For add-back of purified 40S and 60S ribosomal subunits, 5 μg of purified 40S subunits, 10 μg of purified 60S subunits, or BSA was added to the reaction without prior incubation with extracts. For toeprinting reactions, translations were terminated by incubation for 3 min with 2 mM cycloheximide or GMP-PNP (Sigma), aliquotted, frozen in a dry ice/ethanol bath, and stored at -70°C until reverse transcription reactions were to be performed (Amrani et al. 2008) with oligomer #105 (5'-CGTCTGTGGTCTGTTTGTGAAGCC-3'). All in vitro translation experiments, except add-back experiments with ribosomal subunits, were performed at least three times with independently prepared extracts. Add-back experiments with ribosomal subunits were done at least twice with independently prepared extracts.

Sucrose gradient analyses

For in vitro mRNA:ribosome association assays, the equivalent of six translation reactions were incubated for 30 min at 18°C , stopped by the addition of 2 mM CHX, and fractionated on an 11 mL of a 7%–47% sucrose gradient. The gradients were scanned at A_{254} , and the resulting absorbance profiles were used to determine the position of the polysomal and non-polysomal fractions (Mangus and Jacobson 1999; Amrani et al. 2008). RNA was extracted from each fraction and analyzed by Northern blotting as described previously (Mangus and Jacobson 1999), using random-primed probes directed against the *LUC* ORF or the 3' half of the *can1-100* ORF (Amrani et al. 2008). All experiments were done at least twice with independently prepared extracts. Analyses of polysome distributions in vivo were performed as described previously (Mangus and Jacobson 1999).

Ribosome subunit preparation and Western blot analysis

We used a procedure for purification of 40S and 60S ribosomal subunits that had been described previously (Algire et al. 2002), except that we used 9L of strain MBS grown in YPD at 25°C to an OD_{600} of 1.0. Fractions enriched for 40S and 60S subunits were concentrated in Amicon Ultra-15 100K NMWL filter devices (Millipore). Protein concentrations were measured using the BCA Protein Assay Kit (Pierce). The 40S fraction contained 18S rRNA but no detectable 25S rRNA, and the 60S fraction contained 25S rRNA but no detectable 18S rRNA, as visualized on a 1% formaldehyde-agarose gel stained with ethidium bromide. Upf1 association with 40S and 60S ribosomal subunits was assessed by Western blot analysis using the methods and the anti-Upf1 antibody described previously (Mangus and Jacobson 1999).

ACKNOWLEDGMENTS

We thank Meng-Jiao Shi, Paul Dobner, Marcus Johansson, and Feng He for technical advice and reagents; members of the Jacobson laboratory for comments on the manuscript; and Stephanie Kervestin for a gift of FLAG-Upf1 protein. This work was supported by grants to A.J. from the National Institutes of Health and the Human Frontier Science Program.

Received November 4, 2009; accepted June 28, 2010.

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