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Co-translational mRNA decay in *Saccharomyces cerevisiae*

Wenqian Hu[1,‡], **Thomas J. Sweet**[1,‡], **Sangpen Chamnongpol**[2], **Kristian E. Baker**[1], and **Jeff Coller**[1,*]

[1]Center for RNA Molecular Biology, Case Western Reserve University, Cleveland, OH 44106 USA

[2]Affymetrix Inc., 26111 Miles Road, Cleveland, OH 44128 USA

Abstract

The rates of RNA decay and transcription determine the steady state levels of all mRNAs and both can be subject to regulation. While the details of transcriptional regulation are becoming increasingly understood, the mechanism(s) controlling mRNA decay remain unclear. In yeast, a major pathway of mRNA decay begins with deadenylation followed by decapping and 5'-3' exonuclease digestion. Importantly, it is hypothesized that ribosomes must be removed from mRNA before transcripts are destroyed. Contrary to this prediction, here we show that decay takes place while mRNAs are associated with actively translating ribosomes. The data indicate that dissociation of ribosomes from mRNA is not a prerequisite for decay and we suggest that the 5'-3' polarity of mRNA degradation has evolved to ensure that the last translocating ribosome can complete translation.

In eukaryotic cells, mRNA is predominately degraded by two alternative pathways that are both initiated by shortening of the 3' polyadenosine tail (deadenylation). Following deadenylation, either the 5' 7mGpppN cap is removed (decapping) and the message is digested exonucleolytically 5' to 3' or alternatively the transcript is destroyed 3' to 5' by the cytoplasmic exosome1. The two mechanisms of mRNA decay together determine basal mRNA levels thereby significantly contributing to overall gene expression.

Translation is postulated to be a key determinant in controlling mRNA decapping1. The translational initiation complex eIF-4F occupies the cap during translation implying that its binding must be antagonized and translational repression must ensue before decapping can occur1-4. This hypothesis is supported by several observations. First, translational initiation rate is inversely proportional to decapping rate3. Second, the decapping regulators Dhh1p and Pat1p are translational repressors and their role in promoting mRNA decapping is

*To whom correspondence should be addressed: jmc71@case.edu.
‡These authors contributed equally

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partially a function of this activity8,19. Third, mRNA decapping can occur at an unquantified level in ribosome-free cellular foci, termed P-bodies2. Collectively, a two-step model for mRNA decay has been proposed where ribosome dissociation is a necessary first step prior to mRNA decapping1-4.

Deadenylated mRNA remains on polyribosomes

The aforementioned model for mRNA decay predicts that following deadenylation but before decapping a ribosome-free state exists1-4. We reasoned that in a decapping defective cell (*dcp2*Δ), deadenylated RNA would accumulate in this ribosome-free state. We used sucrose-density gradients to survey mRNA ribosome association in wild-type (WT) and decapping defective cells (*dcp2*Δ). Greater than 90% of total cellular mRNA is analyzed by this method (data not shown) and ribosome-free ribonucleoprotein (RNP) structures can be clearly separated from polyribosomes (Fig. S2c). As predicted, inhibition of decapping did result in accumulation of deadenylated mRNA (Fig. S2a, b&f); however, the mRNAs continued to sediment deep into a sucrose gradient even when deadenylated (Fig. S2d, g&h). In fact, the sedimentation profiles of several mRNAs in *dcp2*Δ cells were indistinguishable from those in wild-type cells (Fig. S2d, g&h). The rapid sedimentation of these RNAs could occur either because they were sequestered in heavy particles (perhaps P-bodies)1,2 or because they were associated with ribosomes. The fact that sedimentation correlated with the length of the open reading frame (Fig. S2d, g&h) strongly suggested that the mRNAs were ribosome associated (see below).

Decapped mRNAs are found on polyribosomes

Because deadenylated mRNAs are the substrates for decapping3 we also assessed the sedimentation profiles of decapped RNAs. This was done in cells defective for the 5'-3' exonuclease (*xrn1*Δ). In these cells a stable decapped decay intermediate shortened by 2 nucleotides accumulates (indicated by - cap; Fig. 1a) and can be detected using quantitative primer extension analysis (Fig. S10)5-7. Interestingly, the decapped intermediate showed the same sedimentation profile as the deadenylated RNA (Fig. 1a); the vast majority (83-95%) of decapped mRNA being present in polyribosomes (Fig. 1a & d). To determine if the decay intermediate was associated with ribosomes, we took four approaches. First, introduction of a premature termination codon that shortened the ORF of *PGK1* by 393 codons resulted in a dramatic shift to significantly lighter fractions (Fig. 1b&c). Second, introduction of a stem-loop to limit translation8 caused a shift towards the top of the gradient for both capped and uncapped mRNAs (Fig. 1c). Third, treatment with EDTA (known to dissociate ribosomes) shifted the sedimentation to the top of the gradient (Fig. 1c). Finally, we showed that decapped mRNAs were associated with ribosomes by ribosome immunoprecipitation9 (Fig. S3).

To investigate ribosome-associated decapping further and to exclude the possibility that decapping had occurred prior to initiation of protein synthesis, we took a transcriptional-pulse chase approach using the *PGK1* mRNA reporter7. Using a circularization-based RT-PCR (cRT-PCR)10 analysis we noted that decapped RNA started to appear around 60 min following initiation of transcription (Fig. 2a-c). Separation of cell lysate into non-translating

and polyribosome-associated fractions indicated that when decapping is initiated at 60 min, the vast majority of decapped mRNA was polyribosome associated (Fig. 2d). To further exclude the possibility that association of uncapped mRNA with polyribosomes is a consequence of reloading ribosomes, we used a transcriptional shut-off approach3 with the *PGK1* reporter and monitored decapping using primer extension analysis. Transcription was arrested and further translation was blocked by addition of cycloheximide. Because cycloheximide inhibits ribosome elongation, newly initiated strands would be arrested at 80S11. Strikingly, mRNAs trapped on ribosomes continued to be decapped until greater than 50% was achieved after 120 min (Fig. 2e & f). In the absence of cycloheximide, the bolus of newly decapped mRNA sediments to the top of the gradient by 120 min (Fig. 2g), indicating that ribosomal run-off ensued. These results clearly show that decapping can occur when mRNAs are associated with actively translocating ribosomes.

Decapping occurs on polyribosomes when translation is slowed in *cis*

The foregoing studies were all conducted in *xrn1*Δ cells to allow for the enrichment of decay intermediates. To detect decay intermediates in wild-type cells, we designed a reporter with 10 consecutive rare codons (*PGK1*^{RC}; Fig. 3a). We reasoned that the presence of rare codons might slow ribosome transit12 and result in accumulation of decapped, ribosome-associated decay intermediates13. Importantly, the *PGK1*^{RC} reporter's decay is dependent on decapping and is not a major substrate for No-Go mRNA decay (Fig. S4)14. We analyzed the *PGK1*^{RC} reporter on sucrose gradients and detected decay intermediates using high resolution PAGE followed by Northern blot. Strikingly, using a 3' end-specific probe, decay intermediates of ~500 nt were detected in the region of the gradient associated with a single ribosome (i.e. 80S; Fig. 3b). In addition, mRNA intermediates of increasing length were also detected in polyribosome fractions and their size correlated well with possible ribosome occupancy (Fig. 3b). Addition of formaldehyde prior to cell lysis was used to ensure that the decay intermediates were generated *in vivo* (Fig. 3b), however, similar fragments were seen without formaldehyde treatment (Fig. S6a). A probe complementary to the 5' end of the mRNA failed to detect decay intermediates confirming that the truncated mRNA was trimmed from the 5' end (Fig. 3b & Fig. S5). Most importantly, polyribosome-associated decay intermediates were lost in *dcp2*Δ and *xrn1*Δ mutants (Fig. 3c & Fig. S6b), indicating their formation requires mRNA decapping and 5'-3' exonucleolytic digestion. Moreover, the *PGK1*^{RC} mRNA decay fragments were not a result of No-Go decay14 (Fig. S6c).

We used four experiments to demonstrate that the sedimentation pattern of the *PGK1*^{RC} mRNA decay intermediates is a result of polyribosome association. First, we inhibited translation of the mRNA. Insertion of a stem-loop structure into the 5' UTR (SL-*PGK1*^{RC}; Fig. S7a) shifted the full-length mRNA to the top of the gradient, and no decay intermediates were detectable deep in the gradient (Fig. S7b). Second, we terminated ribosome elongation before rare-codon recognition by introduction of a stop codon upstream of the rare codon stretch (*PGK1*$^{PTC\text{-}RC}$; Fig. S7a). This experiment was performed in *upf1*Δ cells to prevent NMD15. Terminating ribosome translocation prior to the rare codons completely inhibited the formation of polyribosome-associated decay intermediates (Fig. S7c vs. S7d). Further demonstrating that ribosome recognition of the rare-codon stretch is

required, repositioning the rare codon stretch within the *PGK1* ORF resulted in a predictable size shift in polyribosome-associated decay fragments (Fig. S8). Finally, we performed affinity purification of polyribosomes9 and demonstrated that the decay fragments are ribosome bound (Fig. S9). In sum, these data strongly demonstrate that decapping can be detected on polyribosomes in wild-type cells if translational elongation is slowed in *cis*.

Endogenous mRNAs are decapped on polyribosomes in wild-type cells

The foregoing experiment utilzed a reporter harboring rare codons. To determine if endogenous mRNA in wild-type cells were also decapped when associated with ribosomes we developed a splinted ligation assay followed by RT-PCR (Fig. 3d). The RNA ligation mediated by the DNA splint is sequence specific16, thereby allowing us to directly detect the transient product generated by the decapping reaction (i.e. an RNA with 5' phosphate). Using this assay, decapped products from endogenous *PGK1* and *RPL41A* mRNA were detected in wild-type cells (Fig. 3e). A product was not detected in *dcp2*Δ cells (Fig. 3e), indicating formation requires decapping *in vivo*. Consistent with this, *in vitro* removal of the 5' cap by tobacco acid pyrophosphate (TAP) resulted in detection of RT-PCR products in both wild-type and *dcp2*Δ cells (Fig. 3e). Together, these data indicate that the splinted ligation/RT-PCR assay monitors 5' decapping. We performed this assay on RNA recovered from sucrose gradient fractions of wild-type cell lysate, and found that the decapped mRNAs from endogenous *PGK1* and *RPL41A* were predominately detected on polyribosomes (Fig. 3f). Notably the sedimentation pattern of the decapped mRNA correlates with the total mRNA detected by Northern blot (Fig. 3f) and mRNA ORF length (Fig. 3f). Consistent with our earlier findings (Fig. 2) the sedimentation of decapped mRNA on polyribosomes is unlikely a result of ribosome reloading since the decapped intermediate is exceptionally transient in a wild-type cell. Collectively, these data indicate that in wild-type cells, endogenous mRNA are decapped on polyribosomes.

Conclusions and perspective

In sum, we have shown that decapping and 5'-3' degradation of mRNA can occur when the transcripts are associated with actively translating ribosomes (Fig. S1). Co-translational degradation of mRNA has been previously hypothesized17,18. Here we experimentally demonstrate this hypothesis and show mRNA remains associated with active ribosomes during the process of mRNA decapping and exonucleolytic degradation. The data clearly indicate that sequestration into a ribosome-free state (e.g. P-bodies) is not a prerequisite for initiation of mRNA decay. These findings are consistent with the demonstration in yeast, *Drosophila*, and humans that mRNA metabolism can be uncoupled from P-body formation19-22. Moreover, they also help to explain why decay factors (e.g. hDCP2 and Xrn1p) have been found to co-sediment with polyribosomes18,23. Our findings raise several interesting mechanistic questions, for instance how mRNA half-lives are determined in the context of on-going translation. Moreover, it is unclear how the decapping machinery associates and functions on an actively translating mRNA. Interestingly, it has previously been proposed that decapping regulators promoted a ribosome-free state1,2,8,19; it now seems likely that they function in response to yet unknown cues to render the cap more accessible to the decapping enzyme during translation (Fig. S1).

Finally, we note that co-translational mRNA degradation makes sense from an evolutionary point of view. Specifically, the three steps of decay each serve to systematically limit translational events without interfering with them. Deadenylation may reduce translational efficiency perhaps through loss of the poly(A) binding protein, Pab1p15 or association of decapping regulators8. mRNA decapping inhibits further translation initiation events. Finally, degradation from the 5' end while the mRNA is ribosome associated ensures decay does not impede residual ribosomes undergoing translocation. In this way, the final polypeptide expressed prior to the mRNA being destroyed is full length and functional.

Methods Summary

All experiments were performed using early log phase cells grown at 24 °C in synthetic medium containing appropriate sugars. RNA and polysome analyses were performed as described previously8. The cRT-PCR assay was performed as described previously10 with oJC620 for reverse transcription and oJC620/oJC635 for PCR amplification. The *PGK1*^{RC} reporter was generated from fragments amplified from a previously described *PGK1* reporter7 using oJC558/oJC556 and oJC557/oJC559; fragments were combined to produce a template for amplification of full length *PGK1*^{RC} using oJC558/oJC559, followed by cloning onto the *PGK1* reporter backbone at the BamHI and HindIII sites. Affinity purification of polyribosomes was performed as described previously9. Detection of endogenous decapped mRNA was achieved by ligating an RNA adaptor (oJC706) to the 5' end of decapped mRNA via splinted ligation, removal of the DNA splint by DNase I, cDNA synthesis by Superscript II reverse transcriptase using a gene specific primer, and DNA amplified by PCR using a primer complementary to the RNA adaptor and a gene specific primer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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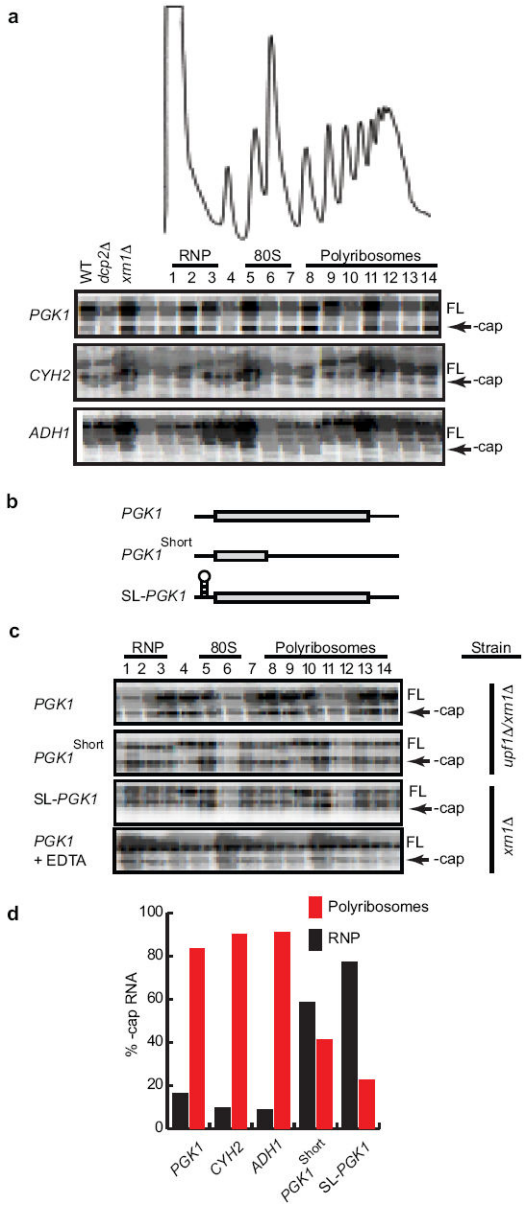

Figure 1. Decapped mRNA is associated with polyribosomes

(**a**) Primer extension analysis on endogenous *PGK1*, *CYH2*, and *ADH1* mRNA was performed on RNA isolated from sucrose gradient fractions of an *xrn1*Δ cell lysate. RNP, 80S, and polyribosomes are indicated above fraction numbers. FL, full length mRNA; - cap, decapped mRNA. Primer extension analyses on total RNA (15 μg) from WT, *dcp2*Δ, and *xrn1*Δ cells are shown on left side of each panel to indicate −cap mRNA is observed only in *xrn1*Δ cells. (**b**) Representation of *PGK1* reporter, *PGK1* reporter with a PTC (*PGK1*^{short}), and *PGK1* reporter with a stem-loop in its 5' UTR (SL-*PGK1*). (**c**) Primer extension on RNA from sucrose gradient fractions from lysates of *upf1*Δ*/xrn1*Δ cells expressing *PGK1* reporter or *PGK1*^{short} reporter, and from *xrn1*Δ cells expressing SL-*PGK1* or *PGK1* reporter. In the bottom panel, lysates from *xrn1*Δ cells expressing the *PGK1* reporter were incubated in presence of 50 mM EDTA prior to loading on sucrose gradients. (**d**)

Quantification of – cap mRNAs as a percentage of total reverse transcription product in RNP and polyribosome fractions.

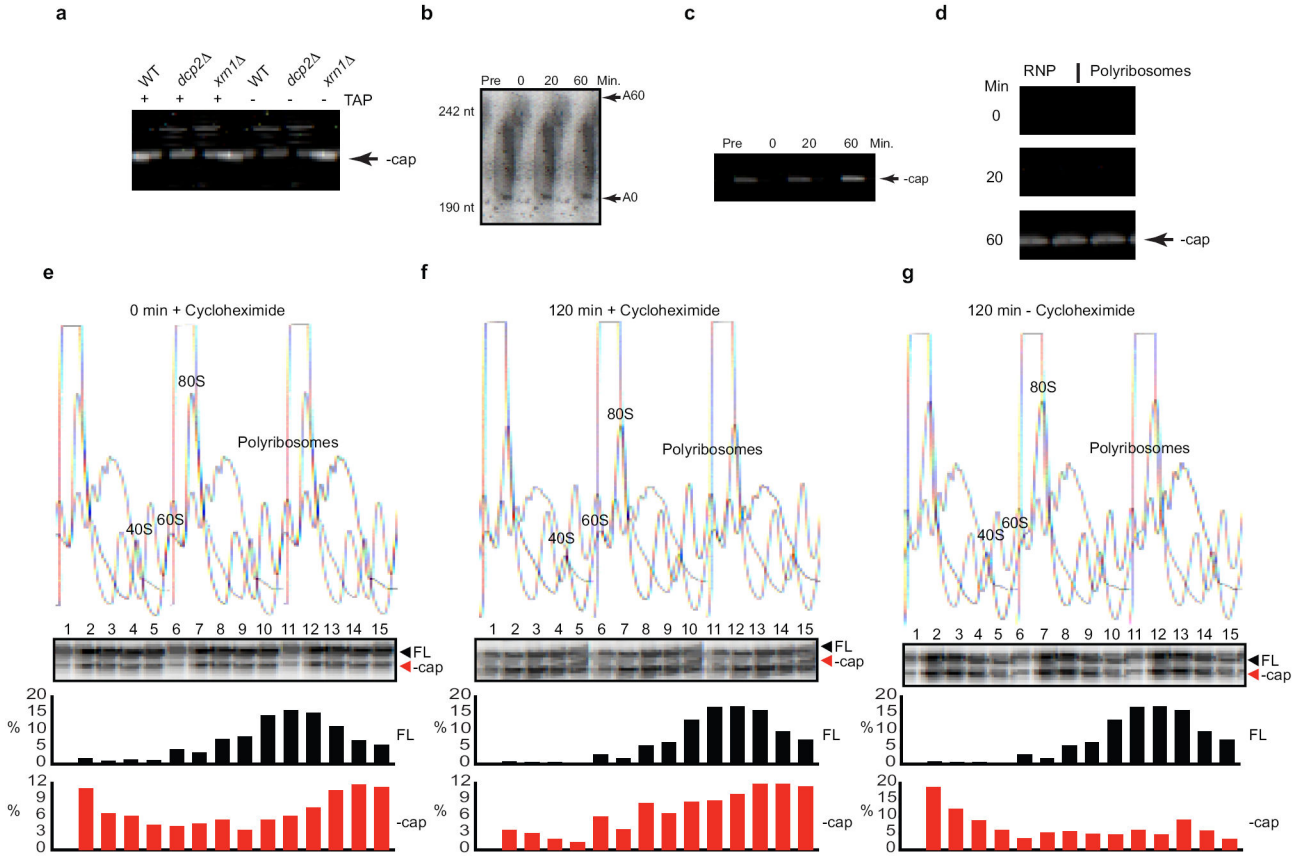

Figure 2. mRNA decapping is initiated on polyribosomes

All experiments in Fig. 2 were performed using cells expressing *PGK1* reporter under control of the *GAL1* promoter. (**a**) Total RNA from WT, *dcp2*Δ, and *xrn1*Δ cells was treated with (+) or without (-) tobacco acid pyrophosphatase (TAP) and circularization RT-PCR (cRT-PCR) was performed to detect decapped *PGK1* reporter. (**b-d**) Transcriptional pulse-chase of *PGK1* performed in *xrn1*Δ cells. (**b**) Poly(A) tail status of *PGK1* was analyzed by oligonucleotide-directed RNase H cleavage, PAGE and Northern analysis. (**c**) Decapping of *PGK1* mRNA monitored by cRT-PCR. (**d**) Cell lysates from the pulse-chase were separated on sucrose gradients. RNA from gradient fractions was pooled into non-translating (RNP) and polysome pools and decapped *PGK1* was detected by cRT-PCR. (**e-g**) Transcriptional shut-off of *PGK1* was performed in *xrn1*Δ cells. Lysates from cells at 0 min after shut-off (**e**), 120 min after shut-off in the presence of 25 μg/mL cycloheximide (**f**), and 120 min after shut-off without cycloheximide (**g**) were separated ny sucrose gradients. RNA from gradient fractions was analyzed by primer extension for *PGK1* reporter. The quantifications of full length (FL) and decapped (-cap) mRNA as a percentage of total extension product are shown for each time point.

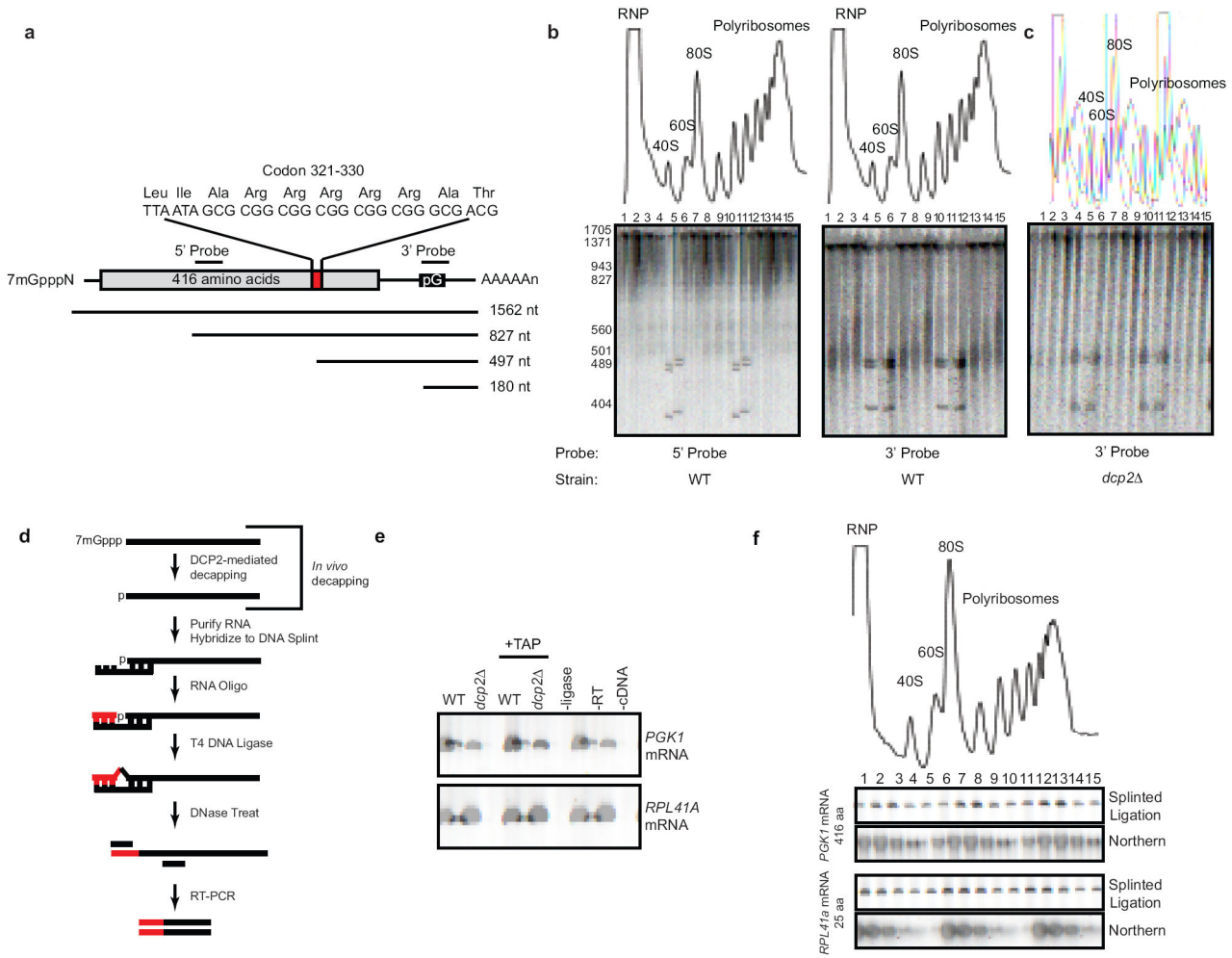

Figure 3. mRNA decapping occurs on polyribosomes in wild-type cells

The *PGK1*^{RC} reporter is depicted (**a**). (**b**) Northern blot analysis of *PGK1*^{RC} mRNA after sucrose gradient fractionation. RNA detected using a 5' or 3' probe as depicted in (**a**). The same analysis as in (**b**) performed in *dcp2*Δ cells (**c**). (**d**) Splinted-ligation RT-PCR assay to detect endogenous decapped mRNA in wild-type cells. An RNA adaptor is ligated specifically to decapped mRNA via a DNA splint by T4 DNA ligase. The DNA splint is removed by DNase I treatment and ligation product is detected by RT-PCR using a gene and adaptor specific primers. The PCR product is indicative of decapped mRNA. (**e**) Splinted-ligation RT-PCR analysis for endogenous *PGK1* and *RPL41A* mRNAs on total RNA from WT and *dcp2*Δ cells. + TAP: total RNA treated with tobacco acid pyrophosphatase to remove the 5' cap *in vitro*; −ligase: no T4 ligase; -RT: no reverse transcriptase; -cDNA: no cDNA template added to PCR. (**f**) RNA recovered from sucrose gradient fractions of wild-type cell lysate was analyzed by splinted-ligation RT-PCR or Northern blot using gene specific probe.