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Accumulation of a mRNA decay intermediate by ribosomal pausing at a stop codon

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ABSTRACT

A RNA fragment which is protected from degradation by ribosome pausing at a stop codon has been identified in growing *Escherichia coli*. The fragment is 261 nt long and corresponds to the 3′-end of the mRNA expressed from a semi-synthetic model gene. The 5′-end of the RNA fragment, denoted rpRNA (ribosomal pause RNA), is located 13 bases upstream of the stop codon. *In vivo* decay of the complete mRNA and accumulation of rpRNA are dependent on the nature of the stop codon and its codon context. The data indicate that the rpRNA fragment arises from interrupted decay of the S3A′ mRNA in the 5′→3′ direction, in connection with a ribosomal pause at the stop codon. RF-2 decoding of UGA is less efficient than RF-1 decoding of UAG in identical codon contexts, as judged from rpRNA steady-state levels. The half-life of UGA-containing rpRNAs is at least 5 min, indicating that ribosomal pausing can be a major factor in stabilising downstream regions of messenger RNAs.

INTRODUCTION

The stop codons UAA (ochre), UAG (amber) and UGA (opal) are protein synthesis termination signals of mRNA reading frames (1,2). The UAA and UAG codons are recognised by release factor one (RF-1) and the UAA and UGA codons by release factor two (RF-2).

mRNA levels are highly dependent on translation, since stop codons in coding regions often induce transcription polarity (3). mRNA that escapes premature termination of transcription is degraded by endo- and/or exonucleolytic RNases (4). A protein complex containing both ribonuclease E (RNase E) and polynucleotide phosphorylase (PNPase) has been characterised in *Escherichia coli*. This complex can processively degrade mRNAs, after an initial RNase E endonucleolytic cleavage, which occurs frequently in the 5′-part of messages (5,6). Interestingly, the formation of such complexes and their activity seems to be dependent on both 5′-phosphorylation and 3′-polyadenylylation (7,8).

Stop codons that are placed early in coding regions have been shown to influence mRNA stability, in contrast to stop codons which are introduced further downstream (9–11). For instance, a study of *bla* transcripts in *E.coli* showed that UAA codons at positions 4 and 26 decrease mRNA stability, whereas stop codons introduced at positions 56 (UAG), 103 (UAG) and 192 (UAA) had no influence (10). However, recent reports show that there are exceptions to this rule, since UAA at codon position 15 in the mRNA for ribosomal protein S20 does not influence mRNA stability (12). Furthermore, UAA at codon position 10 in *lacZ* mRNA increases the stability of the entire transcript (13). This effect probably results from ribosomal pausing at the UAA stop codon, which protects the mRNA from RNase E cleavage at its 5′-end (14).

Ribosomal pausing has been shown to be important for expression of several genes coding for resistance to antibiotics that affect translation (15,16). The resistance of *Bascillus subtilis* to erythromycin is caused by elevation of *ermC* gene expression. The antibiotic induces a ribosomal pause in translation of the *ermC* leader peptide, which stabilises the downstream part of the mRNA, which encodes the *ermC* gene methylase (16).

In this article we describe an mRNA degradation intermediate (rpRNA; ribosomal pause RNA) of S3A′ mRNAs with an internal stop codon (17). rpRNA seems to be the result of interrupted mRNA degradation on the 5′-side of the ribosome, i.e. pausing at the stop codon.

MATERIALS AND METHODS

Chemicals and enzymes

[α-^{32}P]ATP was purchased from Amersham and restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase from Promega. The DNA Sequenase kit was from United States Biochemical Corporation. Deoxyoligonucleotides were made on a Gene Assembler Plus from Pharmacia.

Growth medium and bacterial strains

LB and M9 based media were formulated according to Miller (18). The M9 medium was supplemented with glucose, thiamine and all amino acids at recommended concentrations (19). Ampicillin was used at 100 μg/ml in plates and 200 μg/ml in liquid medium. The *E.coli* strain MC1061 was used in cloning experiments (20). Translation assays were in XAc [wild-type, *trpT*$^{+}$(Su^{-}), with respect to tRNA genes] and CDJ64 [containing the *trpT*(Su9) suppressor gene] (21). The *E.coli* strain UY211, a rifampicin-sensitive derivative of XAc, was used for mRNA

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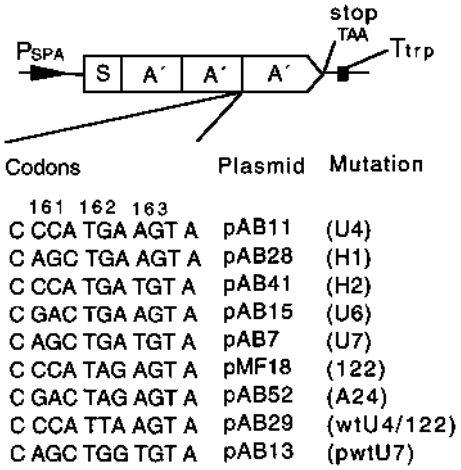

Figure 1. The semi-synthetic S3A′ reporter gene. Transcription is initiated from the P_{SPA} promoter and terminated at the T_{trp} transcription terminator. S and A′ denote the secretion signal peptide region and the synthetic antibody binding B domain region from protein A, respectively (24). The names of plasmids with their test codon context variants at positions 161–163 are given. The codon contexts are as defined elsewhere for *lacI–lacZ* gene fusions (17,35,36).

half-life determinations (22). *Escherichia coli* strains with the *prfB2* mutation are P1 transduction derivatives of XAc, containing the *zgc21*::Tn*10* marker with either the *prfB2* mutation (UY2687) or the wild-type RF-2 (*prfB*$^+$) allele (UY2688). *Escherichia coli* strain YN3231 was the source of the *prfB2* mutation and the *zgc21*::Tn*10* marker (23).

DNA manipulations

New codon context variants (pAB15, pMF18, pAB29 and pAB52) of the S3A′ gene were made as previously described (Fig. 1) (17). The A′ domain has earlier been referred to as Z or B′ (24–26).

RNA preparations and Northern blots

Escherichia coli cultures (20 ml) were inoculated using the M9-based medium described above. Cells were harvested in the mid log growth phase, in SS-34 centrifugation tubes containing crushed ice and chloramphenicol (200 µg/ml). Total RNA was prepared using the hot phenol method (27). Chloramphenicol was used, although later control experiments indicated that it gave neither improvement of the RNA preparation nor a change in the obtained results (data not shown). Rather, it is the rapid cooling on ice that gives good quality RNA. Samples containing 5µg total RNA were first fractionated on 5 or 6% Sanger gels in a minigel system at 15 mA/gel for 30 min (Midget system; Pharmacia) and then blotted overnight in a Midget minigel blotting unit at 20 V. The ^{32}P-labelled oligonucleotide probe ABS01 (5′-CGTTGTT-CTTCGTTTAAGTTAGG-3′), complementary to a sequence within each A′ domain of S3A′ mRNA (17,24), was used in hybridisation (20 pmol/filter).

Primer extension analysis

Primer extension analysis of S3A′ mRNA was made using ^{32}P-labelled primer ABP02 (5′-CTTACTTAAGCTTGGCTGC-AG-3′), which is complementary to a sequence at the 3′-end of S3A′ mRNA. The ABP02 primer was annealed to total *E.coli* RNA (2.5 µg) overnight at 30°C. Extensions were made using 50 U M-MuLV reverse transcriptase and reaction products were analysed on a 5% Sanger gel. Products from DNA sequencing reactions primed with the same primer were used as size markers.

RNA decay measurements

mRNA half-life was measured using a Northern blotting technique. A 250 ml culture of UY841 (UY211/pAB7) or UY931 (UY211/pAB11) was grown to the desired cell density (OD_{540} 0.5) and rifampicin was added (600 µg/ml) to stop transcription initiation. Samples were taken at regular intervals and RNA was prepared, blotted and probed as described above for Northern blots. The amount of probe bound to the filter was monitored with a PhosphorImager (Molecular Dynamics Inc). RNA half-lives were calculated by least squares analysis of a semi-logarithmic plot of mRNA concentration as a function of time.

RESULTS

S3A′ mRNA

An artificial gene, S3A′, has been used previously for characterisation of ribosomal read-through of nonsense codons in *E.coli* strains during balanced growth (Fig. 1; 17,25). The S3A′ gene has three identical A′ domain sequences, which are based on the antibody binding B domain of the protein A gene from *Staphylococcus aureus* (17,24). In this report we analysed turnover of mRNA expressed from different S3A′ alleles. These alleles have an internal test codon at position 162, in the linker region between the second and third A′ coding domains. The test codon was either a stop codon (UGA or UAG) or a sense codon (UGG or UUA), with a variable codon context defined by the two flanking codons at positions 161 and 163 (17). The tight U7 (AGC UGA UGU) and the leaky U4 (CCA UGA AGU) codon contexts were characterised by 1 and 27% ribosomal read-through, respectively, in the tRNA suppressor-free *E.coli* strain XAc used here (17,21). We have reason to believe that the U4 context is exceptionally inefficient with respect to RF-2 interaction (28). This increased the time available for tRNA selection, which resulted in the observed high translation read-through of this UGA codon context, even in a strain lacking any opal tRNA suppressor.

mRNA was isolated from exponentially growing XAc cells harbouring plasmids with the different S3A′ alleles and analysed by Northern blotting using a probe (ABS01) complementary to a sequence within the three A′ coding domains. The results are shown in Figure 2. The S3A′ mRNA is 787 nt in length, as indicated. The amount of mRNA associated with the different alleles varied, even though equal amounts of total RNA were applied in each sample. The observed differences in steady-state mRNA levels were in direct correlation with the protein expression levels reported previously (17). This figure also shows the presence of an additional small RNA fragment which was expressed to a varying degree by strains with some of the different UGA context alleles of S3A′. This small RNA, denoted rpRNA for reasons which will be explained below, was not observed when the S3A′ gene had a sense codon as the test codon at position 162. Figure 2 also shows mRNA from S3A′ alleles with the UAG contexts 122 or A24. These codon contexts are identical

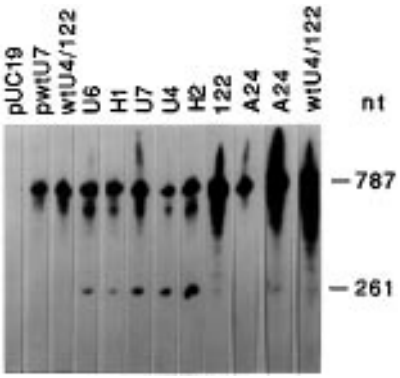

Figure 2. Northern blot of mRNA expressed from several S3A′ alleles. The *E.coli* strain XAc, with no suppressor tRNA [*trpT*$^{+}$(Su^{-})], was used. The name of each S3A′ allele is indicated (see Fig. 1 for codon context sequence). Hybridisation to the probe ABS01 shows full-length S3A′ mRNA (787 nt) and the 261 nt rpRNA fragment (see Fig. 3 for size determination). The first lane contains RNA from XAc with the pUC19 plasmid lacking the S3A′ gene. Lanes 2 and 3 show mRNA with contexts pwtU7 and wtU4/122, which both have a sense codon at position 162. Lanes 4–8 have mRNA with UGA contexts U6, H1, U7, U4 and H2, respectively. Lanes 9 and 10 show mRNA with UAG contexts I22 and A24. Lanes 11 and 12 show A24 and wtU4/122 mRNA after longer film exposure.

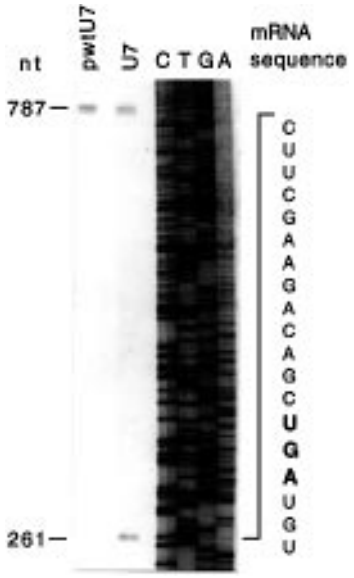

Figure 3. Primer extension analysis of mRNA with codon contexts pwtU7 and U7. Plasmids were expressed in the *trpT*$^{+}$(Su^{-}) *E.coli* strain XAc. The primer used (ABP02) is complementary to a sequence which is located 29 nt upstream of the 3′-end of each S3A′ mRNA. Both S3A′ alleles with the pwtU7 and the U7 contexts express the 787 nt S3A′ mRNA, however, only U7 yields an extra primer extension product, corresponding to a 261 nt rpRNA fragment. DNA sequencing (primer ABP02) of the U7 codon context plasmid was used to determine the size and 5′-end sequence of the 261 nt RNA template.

to two of the UGA codon contexts used (Figs 1 and 2). It can be seen that the amount of rpRNA was much lower in the case of UAG than was found for the corresponding UGA codon contexts.

The rpRNA was also observed in Northern blots of mRNA expressed from the U7 context allele in the *E.coli* strain XL1-blue (data not shown); this strain contains a *recA* mutation (20). This observation suggests that rpRNA is not expressed from a truncated version of the S3A′ gene resulting from recombination between the first and last A′ segments. Furthermore, the size of such an mRNA, expressed from a hypothetical recombined SA′ gene, would be 385 nt, significantly longer than the 261 nt rpRNA analysed here (see below).

Primer extension analysis of S3A′ mRNA

S3A′ mRNAs with contexts U7 and pwtU7 were analysed by a primer extension experiment (Fig. 3). The U7 and pwtU7 contexts were used since the former, but not latter, is associated with a significant amount of rpRNA. The primer used (ABP02) was homologous to a sequence at the 3′-end of S3A′ mRNA. Only one primer extension product was observed in the case of pwtU7. This cDNA species corresponds to full-length S3A′ mRNA, as determined from a gel that was run for a longer time (data not shown). However, two cDNA products were observed in the primer extension reaction from mRNA with the U7 context. The long cDNA, which was also found in the pwtU7 mRNA-primed reaction, corresponds to full-length S3A′ mRNA. The small cDNA species was only present in the U7 mRNA-primed reaction, indicating that it corresponds to the rpRNA. The deduced 5′-end sequence of rpRNA is shown in Figure 3. It can be seen that the 5′-end of rpRNA is located 13 nt upstream of the internal stop codon. This finding indicates that the small RNA (rpRNA) is a mRNA decay intermediate which accumulates as a result of a pausing ribosome at the stop codon. The size of rpRNA, starting 13 nt upstream of UGA, is 261 nt, since its 3′-end should be identical to the 3′-end of full-length S3A′ mRNA.

The rpRNA is a degradation product of S3A′ mRNA

The fates of S3A′ mRNA and the rpRNA were monitored after addition of rifampicin to exponentially growing UY211 bacteria; this strain carries a plasmid with an S3A′ allele (UGA codon context U7 or U4). Samples were taken at regular time intervals and the RNA analysed by Northern blotting (Fig. 4). The antibiotic acts immediately, as can be seen in the expression levels of both species of S3A′ mRNAs, which showed exponential decay from the very beginning of the experiment. The initial steady-state level of the U7 S3A′ mRNA was three times higher than the level of U4 S3A′ mRNA. The amount of U7 S3A′ mRNA decreased during the course of the experiment, with a half-life of 1.5 min, whereas decay of the U4 S3A′ mRNA was faster, with a half-life of 0.6 min. The difference in steady-state levels of U7 and U4 mRNAs was therefore probably caused by different degradation rates.

The rpRNA associated with the U7 codon context accumulated until most of the full-length mRNA had been degraded, whereafter it decreased exponentially in amount. The rpRNA associated with the U4 codon context did not show this increase after addition of rifampicin.

Our results suggest that rpRNA is a decay intermediate of full-length S3A′ mRNA. However, it was not possible to unambiguously determine the half-life of rpRNA, because the proportion of full-length mRNA that gave rise to this fragment species was not known. Nevertheless, judging from the decay

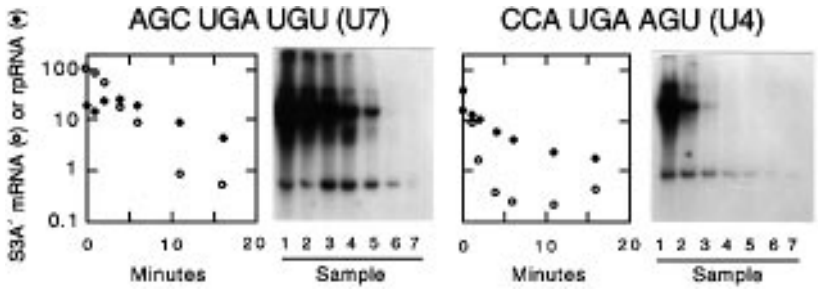

Figure 4. Decay of mRNAs analysed by Northern blot of U7 context mRNA (left) and U4 context mRNA (right). Total cellular RNA from the rifampicin-sensitive and suppressor-negative *trpT*(Su⁻) *E.coli* strain UY211 was isolated at indicated time intervals after rifampicin addition. The amount of radioactivity resulting from binding of the ABS01 probe was also measured by phosphorimager densitometry. A semi-logarithmic plot of the results is shown against time to the left of the respective blots. All values are plotted as percentages of the initial concentration of the full-length U7 mRNA. Furthermore, corrections were made, taking into account that each molecule of rpRNA has only one probe binding site, while S3A′ mRNA has three.

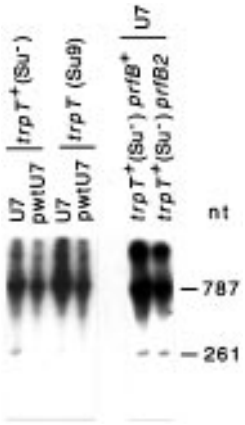

Figure 5. Northern blot analysis of mRNAs with the U7 or pwtU7 codon contexts. Lanes 1–4: expression of S3A′ alleles in the suppressor-negative *trpT*$^{+}$(Su^{-}) *E.coli* strain XAc (lanes 1 and 3) and the suppressor *trpT*(Su9) strain CDJ64 (lanes 2 and 4). Lanes 5 and 6: expression of the U7 codon context allele in the suppressor-negative *trpT*$^{+}$(Su^{-}), *prfB*$^{+}$ and *prfB2 E.coli* strains UY2687 and UY2688, respectively.

curves at time points when S3A′ mRNA levels were substantially decreased and replenishment of the RNA fragment pool was negligible, it can be estimated that the rpRNAs associated with the U7 and U4 contexts both had half-lives of ~5 min.

Increased read-through of UGA decreases the amount of the S3A′ mRNA decay intermediate

Figure 5 shows a Northern blot analysis of S3A′ mRNAs expressed in some *E.coli* strains. The UGA codon in the U7 context was decoded by a wild-type tRNA^{Trp} at 1% efficiency in the suppressor-free XAc strain and at 6% efficiency in CDJ64 by a *trpT*(Su9) suppressor form of the same tRNA (17). It can be seen that the relative steady-state level of rpRNA was lower in the *trpT*(Su9) strain compared with strain XAc. Thus increased read-through of the stop codon by a suppressor tRNA caused less protection from degradation of the fragment. On the other hand, the relative amount of rpRNA was only slightly increased in a *prfB2* (RF-2) mutant strain as compared with its *prfB*$^{+}$ counterpart (Fig. 5).

DISCUSSION

The small RNA species described here is a degradation intermediate of the mRNA expressed from the semi-synthetic model gene S3A′ (Fig. 4; 17) and appears only when S3A′ mRNA has an internal stop codon (Fig. 2). The small RNA carries the UGA codon close to its 5′-end (Fig. 3) and the relative level of this RNA species, compared with full-length mRNA, is decreased in a strain with the *trpT*(Su9) UGA suppressor tRNA (Fig. 5). These findings suggest that the small RNA is formed in connection with ribosomal pausing at UGA, because of slow binding of RF-2; this small RNA is therefore referred to as rpRNA (ribosomal pause RNA). The UAG codon contexts give lower steady-state levels of rpRNA than the UGA contexts. This suggests less efficient protection against RNA decay by ribosome pausing at UAG than at UGA. Therefore, UAG decoding by RF-1 appears to be faster than UGA decoding by RF-2.

The 5′-end of rpRNA is located 13 bases upstream of the stop codon (Fig. 3). In another study, using mRNA coding for bovine preprolactin in an *in vitro* translation system, it was deduced from nuclease treatment of the translation reactions that ribosomes tend to pause at a UAA codon and cover 12–13 bases upstream of this stop codon (29). This finding using extracts from eukaryotic cells is in line with our results in growing bacteria and supports the idea that rpRNA is a decay intermediate of full-length S3A′ mRNA, caused by ribosomal pausing at a stop codon, which delays further 5′→3′ degradation of the mRNA.

Our RNA decay study shows that the U4 mRNA decays two to three times faster than the U7 context mRNA (Fig. 4). This finding is correlated with a 3-fold lower steady-state level of the U4 mRNA species. The observed difference in stability between U7 and U4 mRNAs is remarkable, in view of the fact that the only differences between these two mRNAs are the codon on the 5′-side and the first base on the 3′-side of the internal UGA codon. We also find that the kinetics of rpRNA production is less complex for U4 rpRNA than for U7 rpRNA. The level of U4 rpRNA decreases exponentially during the sampling period. In contrast, the U7 rpRNA level seems to decrease during the first minute, then increase until most S3A′ mRNA has been broken down, and finally decrease exponentially.

An initial lag period is observed in the functional decay of many mRNA species in *E.coli* when rifampicin is used to stop transcription initiation (30–32). Addition of rifampicin stops the production of mRNA, causing increased levels of free ribosomes

and translation factors as mRNA decays. This leads to increased translation initiation at mRNAs with under-saturated ribosome binding sites, since protein synthesis in *E.coli* is normally limited by the number of free ribosomes (23). It is possible that the difference in accumulation observed for rpRNAs with the U7 or U4 codon contexts originates from such secondary effects after rifampicin addition.

The half-life of UGA codon-containing rpRNAs is at least 5 min. This time corresponds to 0.003 decoding events/s, which is many orders of magnitude slower than the normal rate of translation *in vivo*, ~4–22 events/s (33). Even if most natural stop codons are decoded faster by the release factors than found for the UGA contexts analysed here, a considerable portion of ribosomes should be trapped at stop codons in growing bacteria. Such temporary inactivation of ribosomes by slow termination is unlikely, since protein synthesis in *E.coli* seems to be limited by the number of free ribosomes (34). The efficiency of translation termination would in such a case have a strong influence on gene expression and be a bottleneck for cellular growth. However, we see no specific effects on cellular growth, induced by UGA codon context versions of the S3A′ gene used here (data not shown). Thus even if ribosomal pausing at the stop codon is significant and could be of importance for gene expression, it is expected to be considerably shorter than the estimated 5 min half-life of rpRNA.

A ribosomal pause at the stop codon would allow other ribosomes to initiate and stack behind the first pausing ribosome. The result of this would be a ladder-like pattern of several fragments on the gels used for RNA separation, arising from protection against degradation by two or more ribosomes. This is not seen, and it is therefore an intriguing observation that rpRNA seems to result from protection by pausing of a single ribosome at the stop codon. The apparent precursor–product relationship between S3A′ mRNA and rpRNA (Fig. 4) indicates that rpRNA is produced by 5′-end-initiated decay of S3A′ mRNA. A protein complex containing RNase E and PNPase may, after an initial 5′-end cleavage, give a 5′→3′ directed decay of many mRNAs (5,6). Cleavage by this, or by some other RNase complex, could trim the mRNA on the 5′-side of the last translating ribosome. This RNase activity might track the mRNA until it reaches a pausing ribosome, whereby further decay will be delayed. Alternatively, ribosome pausing at a stop codon could be a signal to some RNase to bind and cleave the unprotected mRNA 13 nt upstream of the stop codon. Both of the indicated models predict that rpRNA production is directly correlated with the duration of ribosome pausing at the stop codon. Thus fast termination at UAG should give a lower level of rpRNA compared with slow termination at UGA, as indeed is observed. Release of the presumed RNase and a successful termination event would give a free, relatively stable rpRNA, with a half-life that is independent of stop codon efficiency, as is observed for U7 and U4 rpRNAs. These rpRNAs both have a half-life of ~5 min.

The relative level of rpRNA should be higher in a *prfB2* than in a *prfB*$^+$ strain (Fig. 5), because of much slower termination and a longer pause in the former case. We observe only a minor increase in the *prfB2* strain. It appears that extended ribosomal pausing is ineffective in producing more rpRNA, suggesting that the relevant RNA decay pathway is already saturated at some step. Altogether, we favour a S3A′ mRNA decay model with 5′→3′ processivity, where decay can be temporarily halted by ribosome pausing at stop codons. Complete degradation of the fragment containing the stop codon (rpRNA) will then be achieved by other RNases after release of the ribosome from the stop codon in connection with termination of translation.

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