# Translatability of a plant-mRNA strongly influences its accumulation in transgenic plants

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## ABSTRACT

**Current knowledge of parameters affecting RNA stability is very restricted in plants. Here we investigated factors which might contribute to the stability of a particular plant messenger RNA. To this end, insertion and deletion mutants were made in two different exons and an intron of the transcribed region of a well characterised patatin gene (pgT5). Mutant genes were expressed under the control of a strong leaf-stem specific promoter (ST-LS1) and analysed *in vivo* in transgenic tobacco plants. Northern analysis revealed the importance of the translatability of the mature messenger RNA with respect to its accumulation in transgenic plants. Enlargement of the 3′ non-translated region by several hundred base-pairs reduced the steady state mRNA level slightly; the introduction of a stop codon leading to premature termination of translation of the RNA led to a dramatic decrease of the steady state mRNA level.**

## INTRODUCTION

The steady state level of a eucaryotic mRNA is determined by the frequency of transcriptional initiation and by the half life of the transcribed mRNA molecule.In addition differential splicing is known to influence RNA steady state levels e.g. in case of the transposition of the P elements. Studies of parameters affecting the stability *in vivo* of a messenger RNA molecule are very restricted in eucaryotic systems. General features of the eucaryotic messenger RNA, such as the 5′ cap structure and the 3′ poly A tail, determine the half life of the respective pre-mRNA in the nucleus and the mRNA in the cytoplasm in some particular cases analysed (1). The importance of both factors was shown in plant cells for the bacterial CAT gene using a transient expression system (2).

A relationship between ribosomal mRNA binding and mRNA decay has been reported. In several messengers, the absence of translating ribosomes beyond a certain position in the coding region, resulted in rapid mRNA decay. For the *E. coli bla* transcript for example, it was shown that premature termination of translation could destabilise the respective messenger RNA (3). Although this effect is most easily explained by increased accessibility of nucleases to the portion of the coding region not covered by ribosomes, no correlation was evident between length of the untranslated segment and the decrease of the half-life. In the human triosephosphate isomerase mRNA, the coding region of which consists of 249 codons, generation of translational termination signals close to the first ATG codon and as far as 189 codons downstream apparently led to the same extent of destabilisation (4).

In *Saccharomyces cerevisiae* randomly chosen mRNAs with different half-lives were analysed with respect to transcript stability. No obvious correlation between the stability of the different messengers and the number of ribosomes they carried *in vivo* was found (5). However, amber mutations in the URA3 gene and ochre mutations in the URA1 gene in yeast were shown to reduce the half-life of the encoded transcript; the closer the nonsense mutation was located to the start codon, the shorter the half-life (6, 7). This effect supports the idea that mRNA can be protected by the bound ribosomes in eucaryotic cells. Similar analyses carried out in *Dictyostelium discoideum* amoebae also supplied evidence for a correlation between mRNA decay and ribosome loading (8).

Patatin is a 40Kd protein mainly present in tubers, roots and flowers of potato (*Solanum tuberosum* L.) (9) and is encoded by a multigene family. One particular gene, pGT5 (10), was successfully overexpressed under the control of a strong leaf-stem specific promoter (ST-LS1) in leaves of tobacco plants (11). We took advantage of this *in vivo* system for analysing intrinsic parameters affecting the accumulation of this particular plant mRNA, encoding patatin. Parts of the transcribed region of the ST-LS1-patatin chimeric gene were mutagenised with the aim to test the importance of a) the presence of an open reading frame b) the structure and length of introns c) the structure and length of the 3′-untranslated region for the accumulation of the RNA. After Agrobacterium mediated transfer to tobacco plants, the steady-state mRNA levels in leaves of transgenic plants were quantitatively compared. Northern analysis revealed the importance of a continuous reading frame throughout the mature messenger RNA for its normal accumulation in tobacco leaves.

## MATERIALS AND METHODS

### Bacterial media and strains

All agrobacterial strains were grown in YEB medium (12). *E. coli* strains were grown in YT medium (13).

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### Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (13).

### Constructions

All constructions were based upon a pUC derivative containing the wild type chimeric patatin gene (D8) (11). All insertion mutants were cloned as blunt ended fragments. The 470 fragment is a 456 bp *Pvu*II fragment derived from the T-DNA gene 2 (14) and linkered as indicated below: AGGCATGCCAGCTTGG-ATCCGG// CTGCTAG.....T-DNA gene 2.....CTCCAG// CC-GGATCCAAGCTGGCATGC. The linker sequence creates a 18bp inverted repeat. Final constructs were transferred as *EcoR*I/*Pst*I fragments to pMPK110 (15) and mobilised to *Agrobacterium* 3850Km (16).

### Northern analysis

Total RNA was isolated from tobacco leaves, separated by gel electrophoresis on 1.5% agarose in the presence of formaldehyde (17), blotted on Hybond N membranes and hybridised as described by Amasino (18). PolyA$^+$ RNA was isolated according to the suppliers' protocol (Pharmacia).

### In vitro translation and immunoprecipitation

Approximately 0.5−1.0 μg polyA$^+$ RNA were used for translation into radioactively ($^{35}$S-methionine) labelled proteins using a rabbit reticulocyte lysate (Promega), according to the suppliers' protocol. Immunoreactive polypeptides were precipitated overnight with patatin antiserum and separated by 12.5% SDS-PAGE. Labelled proteins were visualised by fluorography.

### Tobacco transformation

Tobacco leaves (*Nicotiana tabacum* var. W38) were used for *Agrobacterium*-mediated leaf disc transformation as described (19). Transformants were selected on 100mg/l kanamycin. Transgenic plants were analysed for the correct integration of chimeric gene by Southern analysis.

## RESULTS

### General strategy

As outlined in the Introduction, parameters affecting mRNA stability in plants are poorly understood. To tackle this problem a plant model system was devised. A typical plant gene, encoding patatin, was expressed in a plant background lacking patatin genes, namely *Nicotiana tabacum*. This was achieved by fusing two potato derived components: the coding region of a patatin gene and a strong leaf-stem specific promoter. Chimeric tobacco plants containing this construct (D8) express high amounts of patatin in leaves and stems (11).

As outlined in the Introduction, the knowledge about parameters influencing the accumulation of mRNA's in plant systems is very poor. We therefore decided initially to test the following parameters with respect to their importance for the stability of the patatin-mRNA ( cf. Figure 1):

a) Translatability of the mRNA. To this end, small deletions of either 25 or 21 nucleotides were introduced in the third exon of the patatin gene, which in case of the 25 nucleotides long deletion led to a premature stop of the open reading frame. Furthermore in order to test the possible influence of a more dramatic change of the primary structure of the mRNA, a 470 nucleotides long DNA-segment was inserted into the 3rd exon.

b) Primary structure and/or length of the mRNA. To this end the 3'-untranslated part of the mRNA contained in the seventh exon was modified by inserting a 470 bp long DNA segment.In order to distinguish the effect of an increase of the total length of the RNA from an effect on the structure of the RNA, this fragment was inserted in both orientations.

c) Primary structure and/or length of an intron. A 470 bp long DNA segment was inserted in the first intron thus extending the length of the intron from 379 to 849 bp. This mutation should not be maintained in the mature mRNA and hence affect only the pre-mRNA accumulation.

The model system (D8) consists of a transcriptional gene fusion: a leaf-stem specific promoter (ST-LS1) and the coding region of the patatin gene pgT5 (11). The 470 bp long DNA segment mentioned above is derived from the transcribed region of the T-DNA gene 2 of the Ti-plasmid pTi ACH5 (14). This fragment was chosen since this DNA fragment was shown not to influence the transcriptional activity when used in a transcriptional fusion (20). It consists of a 456 bp *Pvu*II fragment, flanked by 15 bp linker sequences, resulting in a 18 bp inverted repeat (Materials and Methods). All constructs were expressed and analysed in leaves of transgenic tobacco plants.

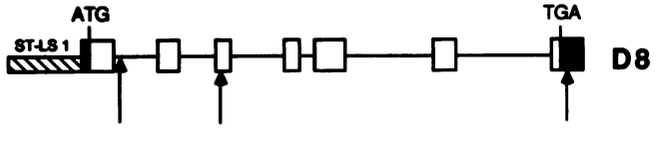


**Fig.1** Schematic drawing of the chimeric gene D8. Boxes represent the exons of the patatin gene pgT5, the introns are indicated by horizontal lines. The dotted parts represent the 3' and 5' untranslated regions. The promoter of the ST-LS 1 gene is shown in front of the transcribed region (dashed box). The patatin start (ATG) and stop (TGA) codon are indicated. The arrows locate the positions used for mutagenesis.

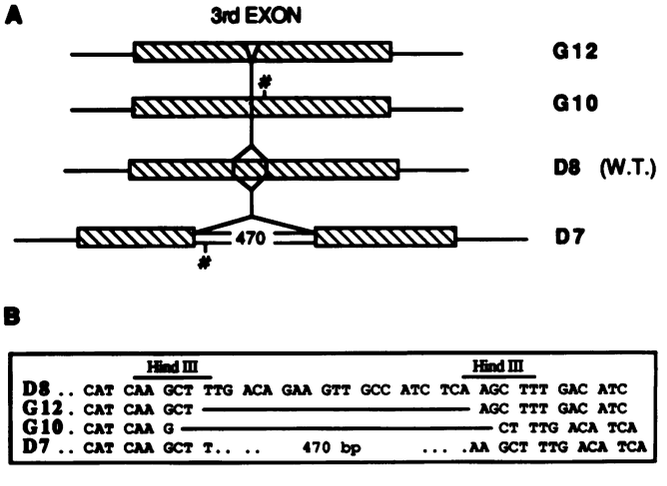


**Fig.2** A.Overview of the modifications around both *Hind* III sites in the 3rd exon of the pgT5 patatin gene. Boxes indicate the exon, stop codons are represented by '#'. D8 represents the wild type chimeric gene (w.t.).The insertion fragment derived from the T-DNA gene 2 is indicated by 470. B. Nucleotide sequences of the different modifications around the *Hind* III site(s) in the 3rd exon of the patatin gene. The 3bp clusters indicate the open reading frame of the different constructs. In case of D7 only the border sequences of the 470 bp insertion are shown.

### Mutagenesis of the 3rd exon

For the mutagenesis of the 3rd exon we took advantage of two closely linked *Hind*III restriction sites which are only by 25 bp apart. On the one hand this small *Hind*III fragment was deleted, resulting in the G10 construction. This deletion created a shift of the original reading frame which resulted in a premature stop codon (Fig. 2A, B). A second deletion (G12) only comprised 21 nt (Fig. 2A). In the latter case the reading frame is retained and hence this mutation should give rise to a deletion of 7 amino acids in the mature protein. In addition, the G10 deletion was combined with an insertion of the 470 bp fragment (D7). This modification was introduced in order to get an idea about the relative importance of creating a premature stop codon while leaving the primary structure of the RNA largely unaffected (as in the G10 construct) as compared to introducing a large modification in the primary structure of the RNA (i.e. by inserting the 470 bp fragment into the exon) in addition to creating a premature stop codon. All modifications and their respective reading frames are summarised in figure 2 in comparison to the wild type D8 chimeric gene. The mutant genes were transferred to tobacco plants via *Agrobacterium* T-DNA transformation (Materials and Methods). Leaves of transgenic plants were scored for the presence of patatin mRNA. It is a well-established fact that the expression level of transferred genes varies between independent transformants which is probably due to the influence of sequences neighbouring the integration site . In order to normalize for this variation, at least ten independent transformants per construct were analysed by northern blotting techniques. As a rule the maximal variation in the expression level for one construct did not exceed a factor of 2–3 ( data not shown).

Fig. 3 shows a summary of the Northern analysis, representing average patatin-producing plants. Since the D7 construction contains the 470 bp insert in one exon of the patatin gene, the D7 plants show a patatin transcript of about 2.0 Kb. A clear quantitative difference can be seen for the amount of transcript accumulating for the different constructs. Whereas G12 and D8 plants accumulate to about the same level, the G10 and D7 plants contain a much lower amount of the transcript. A semi-quantitative estimation of the differences as obtained by scanning autoradiograms reveals, that in case of D7 and G10, the amount of accumulating RNA is reduced by a factor 12 respectively 7 as compared to the D8 construct, whereas in case of the construct G12, the amount of RNA is reduced by a factor of 1.5 only.

These differences in the mRNA steady-state levels could either be due to a modification of the structure of the mRNAs in the G10 and D7 mutants compared to the D8 and G12 constructs or due to the fact that D8 and G12 contain a continuous open reading frame in contrast to D7 and G10. Therefore the translatability of the D8 and G12 derived patatin mRNA was examined. To this end polyA$^+$ RNA derived from D8 and G12 plants was *in vitro* translated in a reticulocyte system; patatin proteins were immunoprecipitated and separated by SDS-PAGE. Lane 'D8' in figure 4 reveals two major polypeptides: the larger one corresponds in size to the expected patatin pre-protein (with leader peptide), whereas the smaller one is most likely due to translation initiation from an internal ATG. A second ATG is present in the original reading frame 27 codons downstream of the first one and is surrounded by a Kozak consensus sequence. Lane 'G12' in figure 4 shows two polypeptides similar to those in the lane 'D8'. This illustrates that the G12 mRNA is indeed translatable. As expected the polypeptides in the G12 lane are slightly shifted to a lower molecular weight compared to the D8 lane, which is due to the deletion of 7 amino acids. Western analysis of G12 plants did not reveal patatin protein in leaves, indicating the instability of this truncated protein *in vivo* (data not shown).

### Mutagenesis of the 3′ untranslated region

The constructions previously described interfere directly with the reading frame of the patatin gene. A drastic effect on the mRNA

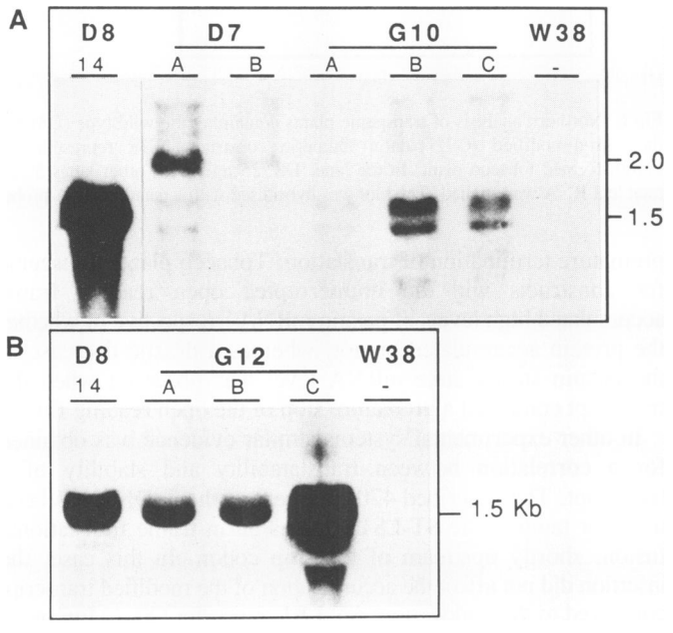


**Fig.3.** Northern analysis of transgenic plants containing the G10 and D7 (upper panel, A) or G12 (lower panel, B) construct. The RNA of independent transformants, indicated by letters above each lane, were separated by agarose gel electrophoresis and blotted on Hybond N. The blots were hybridised to the patatin cDNA clone Pat 58. All lanes contain approximately 50 $\mu$g of total leaf RNA. D8–14 represents a 'wild type' control, whereas W38 represents an untransformed tobacco plant. The autoradiogram in panel A was overexposed in order to visualise very weak signals. The double band in the G10 lanes is due to a blotting artefact and was not consistantly observed. The molecular weight marker is indicated in Kb.

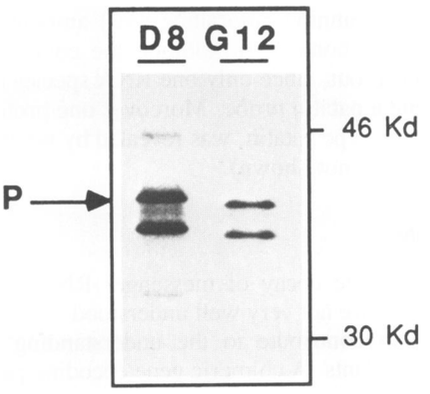


**Fig.4.** Translatabilty of D8 and G12 derived mRNA. PolyA$^+$ RNA was isolated from transgenic D8 and G12 plants, translated *in vitro* in a rabbit reticulocyte system and immunoprecipitated with patatin antiserum. The polypeptides obtained were separated by SDS-PAGE and visualised by fluorography. The molecular weight of the expected patatin preprotein is indicated by 'P'.

stability was observed when large parts of the mRNA were not translatable.

In order to test the effect of enlarging the 3′ untranslated region of the patatin mRNA, the 470 fragment was inserted in a convenient *Ava* I restriction site located in the 3′ untranslated region 12 bp downstream of the patatin stop codon and in front of potential polyadenylation signals. In order to avoid orientation-dependent effects the fragment was inserted in both sense and antisense orientation, resulting in the G5 and G6 constructs respectively. These were transferred via Agrobacterium to tobacco plants (Materials and Methods). Transgenic plants were scored for the presence of patatin mRNA and protein by northern and western blotting techniques (Fig. 5). An RNA species migrating at a molecular weight of approximately 500bp larger than the D8 control is observed in RNA extracted from G5 and G6 plants. Similar amounts of patatin mRNA were found for both constructs, indicating that the orientation of the inserted fragment does not influence the steady state mRNA level. However G5 and G6 plants accumulate about 3 fold less patatin mRNA (estimated by scanning autoradiograms) when compared to the D8 control. In order to compare RNA amounts, a control hybridisation was performed with the cDNA of the ST-LS1 gene which showed that the same amount of total RNA was applied into each lane. The level of patatin protein, determined by western analysis, correlates well with the mRNA level (Fig.5).

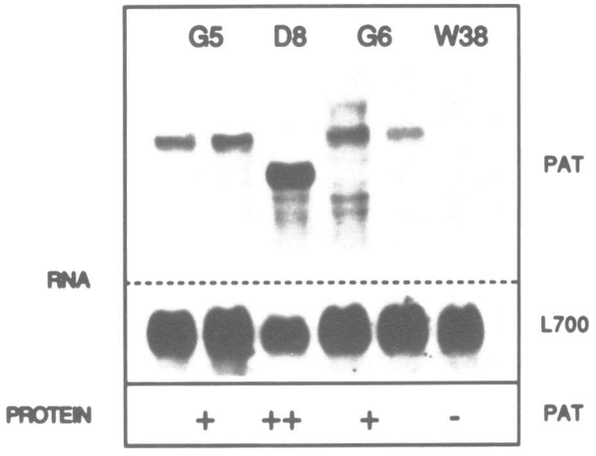


**Fig.5.** Northern analysis of transgenic plants containing either the wild type chimeric patatin gene (D8), or the G5 and G6 derivatives. W38 stands for an untransformed tobacco plant. The upper part shows the results of a northern analysis ( 50$\mu$g total RNA per lane) using a labelled patatin cDNA probe (PAT). The middle part shows a control hybridisation with a ST-LS1 cDNA probe (L700). The lower part summarizes a western analysis using a polyclonal patatin antiserum.

### Mutagenesis of the 1st intron

So far modifications have been described which affect different exons of the transcribed region. Those mutations were hence maintained on the mature mRNA. The eucaryotic gene in addition provides a refined system, which allows experiments modifying the transcribed region without changing the mature mRNA, namely mutations in an intron. In order to test whether or not these mutations are of any importance when they are not present in the mature mRNA, the 470 bp fragment was inserted into an intron, thus allowing to monitor effects of the inserted fragment on the pre-mRNA production.

The 470 bp fragment was cloned into the 1st intron of the D8 chimeric patatin gene in the *EcoR*V restriction site, which is located about 100 bp downstream of the 5′ splice site (G7). This insertion enlarges the 1st intron from 379 to 849 bp. The construction was transferred to tobacco plants which were scored for the presence of patatin mRNA (Fig. 6). Leaves of transgenic plants produce similar or even slightly higher ( about two-fold as estimated by scanning autoradiograms) amounts of patatin mRNA as the D8 control. In addition the enlarged intron is efficiently spliced out, since only one RNA species of 1.5Kb is detectable using a patatin probe. Moreover one protein, similar in size as the wild type patatin, was revealed by western analysis of G7 plants (data not shown).

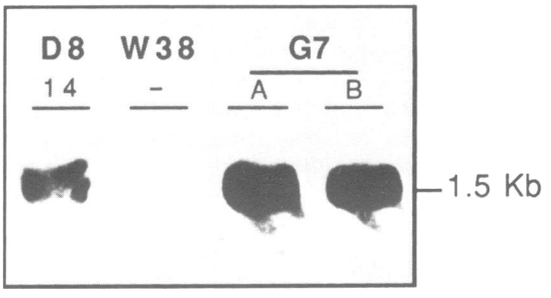


**Fig.6.** Northern analysis of transgenic plants containing the wild type (D8) and the intron-modified (G7) patatin chimeric construct. W38 represents an untransformed tobacco plant. In the lane 'D8' 25 $\mu$g, in all other lanes 50 $\mu$g total leaf RNA was applied. The blot was hybridised with a patatin cDNA probe.

## DISCUSSION

Factors affecting the decay of messenger RNA molecules in higher organisms are not very well understood. A model system was designed to contribute to the understanding of mRNA accumulation in plants. A chimeric gene encoding patatin under the control of the ST-LS1 promoter, was overexpressed in a heterologous plant system, which normally does not express this gene. Modifications were introduced in the transcribed region of this chimeric plant gene. These insertion and deletion mutants either retained the original reading frame, or resulted in a premature termination of translation. Tobacco plants transgenic for constructs with an uninterrupted open reading frame accumulated high levels of patatin mRNA irrespective of whether the protein accumulated or not, whereas a drastic decrease of the patatin steady state mRNA level was observed when the transcript contained a premature stop of the open reading frame.

In other experimental systems similar evidence was obtained for a correlation between translatability and stability of a transcript. The described 470 fragment of the T-DNA has been used for tagging the ST-LS1 gene as an in-frame translational fusion, shortly upstream of the stop codon. In this case, the insertion did not affect the accumulation of the modified transcript compared to the endogenous ST-LS1 transcript (20). Moreover, with respect to the patatin gene, the insertion of 84 or 147 bp in the 4th exon allowing continuous translation in the original reading frame did not influence the steady-state mRNA level (21). Indirect evidence supporting the idea that enhanced translatability of a mRNA can stabilise the encoded transcripts comes from experiments dealing with a mutant form of the Kunitz trypsin inhibitor gene from soybean. A mutant allele containing a frame-shift mutation resulted in 100 fold reduction of the steady state mRNA compared to the wild type gene (22).

Another parameter which has been postulated to be involved in RNA stability is the size of the transcript. The enlargement by 470 bp of the 3′ untranslated region (G5 and G6) resulted in a patatin mRNA of about 2 kb which is still translated into protein. However the abundance of this transcript is significantly lower compared to the wild type (D8). As indicated before, the stability of the patatin mRNA might be partially correlated with the proportion of the transcript which is translated. The insertions into the 3′ end (G5 and G6) enlarge the untranslated region by 470 bp and hence a larger part of the mRNA might be exposed to ribonucleases in the cytoplasm.

The 470 fragment was introduced into the 1st intron of the chimeric D8 gene (G7), in order to analyse whether or not the extension of the pre-mRNA could affect its nuclear decay rate. No quantitative difference in expression was observed compared to the wild type D8 or to the endogenous ST-LS1 gene. This indicates that the effects of the introduced fragment are exerted in the mature RNA molecule and not at the level of transcription of the gene or processing of the pre-mRNA. Another example of pre-mRNA extension has been reported for the β-glucuronidase gene. Insertion of a 189bp intron did not affect the overall expression of the marker gene (23).

In conclusion all transgenic plants tested containing mutant patatin genes with an enlarged untranslatable part of the mature mRNA accumulate significantly less patatin mRNA compared to constructions where longer portions can be translated. The explanation for these data remains an open question. One possibility is that the modifications of the patatin mRNA might alter and/ or destabilise the secondary structure of the transcript. However theoretical predictions of the secondary structure of the mRNAs of the D8, G10 and G12 constructs, according to the method of Zuker (24) indicated no significant influence of both deletions on the wild type D8 mRNA conformation (data not shown).

The effects of premature termination cannot be easily explained in terms of physical masking of the mRNA by ribosomes, although protection of a unique nuclease-sensitive site is a possibility. These effects could also be attributed to ribosome-induced cleavages during the termination process, when this process takes place at some distance from the normal termination site (25). Whether or not the translating ribosomes directly protect the messenger RNA for specific or unspecific ribonucleases needs to be further elucidated.

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