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Ribosomes inhibit an RNase E cleavage which induces the decay of the *rpsO* mRNA of *Escherichia coli*

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The hypothesis generally proposed to explain the stabilizing effect of translation on many bacterial mRNAs is that ribosomes mask endoribonuclease sites which control the mRNA decay rate. We present the first demonstration that ribosomes interfere with a particular RNase E processing event responsible for mRNA decay. These experiments used an *rpsO* mRNA deleted of the translational operator where ribosomal protein S15 autoregulates its synthesis. We demonstrate that ribosomes inhibit the RNase E cleavage, 10 nucleotides downstream of the *rpsO* coding sequence, responsible for triggering the exonucleolytic decay of the message mediated by polynucleotide phosphorylase. Early termination codons and insertions which increase the length of ribosome-free mRNA between the UAA termination codon and this RNase E site destabilize the translated mRNA and facilitate RNase E cleavage, suggesting that ribosomes sterically inhibit RNase E access to the processing site. Accordingly, a mutation which reduces the distance between these two sites stabilizes the mRNA. Moreover, an experiment showing that a 10 nucleotide insertion which destabilizes the untranslated mRNA does not affect mRNA stability when it is inserted in the coding sequence of a translated mRNA demonstrates that ribosomes can mask an RNA feature, 10–20 nucleotides upstream of the processing site, which contributes to the RNase E cleavage efficiency.
Keywords: mRNA stability/ribosomes/RNase E/translation

Introduction

The stability of mRNA is one of the parameters which determine the amount of proteins synthesized in the cell. Although recent investigations have significantly improved our knowledge of the ribonucleases involved in mRNA decay (Zilhao *et al*., 1993, 1996; Py *et al*., 1996; Cohen and McDowall, 1997) and of mRNA features which determine its stability (Bechhofer, 1993; Higgins *et al*., 1993; Xu *et al*., 1993; Hajnsdorf *et al*., 1995; O'Hara *et al*., 1995; Haugel-Nielsen *et al*., 1996), some aspects of mRNA turnover in bacteria, such as the role of ribosomes, still need to be clarified. Early observations showed that antibiotics such as puromycin, which causes premature termination of translation, and chloramphenicol, which slows down the rate of translation, have destabilizing and stabilizing effects respectively, on mRNA (Petersen, 1993). This suggests that ribosomes mask RNase targets on the mRNA. Accordingly, changes in translation efficiency and early translation termination modify the stability of some mRNAs (Nilsson *et al*., 1987; Baumeister *et al*., 1991; Yarchuk *et al*., 1991; Jain and Kleckner, 1993). Moreover, the recent demonstration that labile, ribosome-free, *lacZ* mRNA, resulting from uncoupling transcription and translation, can be stabilized by RNase E inactivation suggests that the untranslated mRNA is more sensitive to the attack of this endonuclease (Iost and Dreyfus, 1995; Marakova *et al*., 1995).

The *rpsO* mRNA, which encodes ribosomal protein S15, has been a fruitful model for studying both regulation of translation and degradation of mRNA. S15 autoregulates its expression by repressing the translation of its own message when it is synthesized in excess relative to the 16S rRNA (Philippe *et al*., 1993), and an endonucleolytic cleavage by RNase E initiates the degradation of the *rpsO* message (Régnier and Hajnsdorf, 1991). This cleavage, at the M2 site, removes the 3′ hairpin of the transcriptional terminator t1 which protects the monocistronic mRNA from the attack of 3′ exonucleases (Figure 1) (Hajnsdorf *et al*., 1994). The processed messenger is degraded rapidly by polynucleotide phosphorylase (PNPase) (Braun *et al*., 1996). The fact that only 10 nucleotides lie between the UAA termination codon and M2 suggests that RNA features recognized by RNase E might be within the limit of the region covered by the terminating ribosome. The bulk of ribosomes might, for example, hinder the access of RNase E to the cutting site or mask sequences which contribute to RNase E recognition (Naureckiene and Uhlin, 1996; Cohen and McDowall, 1997; Mackie *et al*., 1997). This structural organization prompted us to investigate whether ribosomes could inhibit the RNase E cleavage event which triggers the exonucleolytic degradation of the *rpsO* mRNA. We demonstrate, in this report, that the distance which separates the UAA termination codon and the M2 site affects the stability of the *rpsO* messenger and the efficiency of RNase E cleavage at M2. The mRNA is destabilized when nucleotides are inserted between the two sites and stabilized when the termination codon is brought closer to the processing site. These data support the idea that the stabilization of mRNA by translation results from the inhibition of the M2 RNase E cleavage by ribosomes.

Results

Construction of translated and untranslated rpsO genes

Prior to investigating whether ribosomes affect the efficiency of the RNase E cleavage at the M2 site, it was

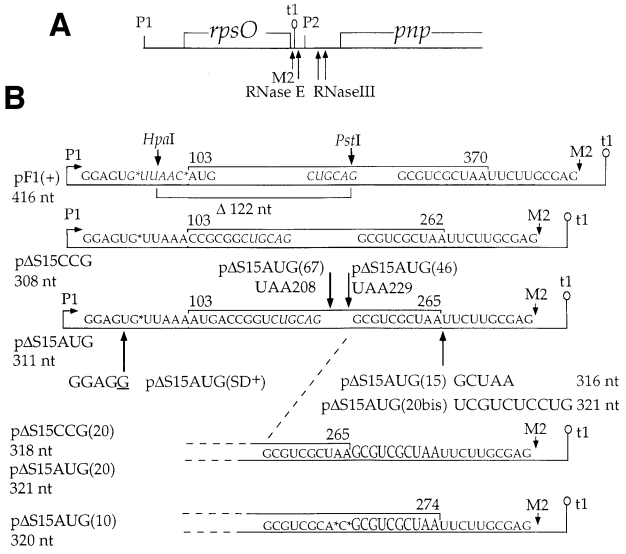

Fig. 1. Structures of the *rpsO–pnp* operon and of the P1-t1 *rpsO* mRNAs transcribed from mutant plasmids. (**A**) The promoters P1 and P2 of the operon, the internal Rho-independent terminator t1, the M2 RNase E site (upstream of t1), the RNase E site downstream of t1 and the RNase III sites are indicated on the genetic map showing *rpsO* and the beginning of *pnp* (Régnier and Portier, 1986; Régnier *et al*., 1987). (**B**) The P1-t1 transcript generated from the *rpsO* gene of plasmid pF1(+) is shown at the top. The coding sequence of S15 is overlined and the 122 nucleotide *Hpa*I–*Pst*I deletion is indicated beneath the mRNA. Nucleotides relevant for this study are shown on the diagrams. The *Hpa*I and *Pst*I recognition sites are shown in italics, and the T→G and A→C substitutions made to create the *Hpa*I site are marked with asterisks. The nucleotide sequences of adaptors 2 and 1 inserted between the *Hpa*I and *Pst*I sites are shown on the diagrams of the pΔS15CCG and pΔS15AUG mRNAs, respectively. The non-functional internally deleted coding sequence of pΔS15CCG begining with a CCG codon provided by adaptor 2 and the functional internally deleted coding sequence of pΔS15AUG begining with an AUG provided by adaptor 1 are overlined. The positions of the two mutations which introduce early UAA termination codons in pΔS15AUG are indicated above the map of this plasmid, together with the names of the resulting mutant plasmids. The arrow pointing upstream of the AUG codon shows the U→G modification of the Shine–Dalgarno sequence giving rise to the pΔS15AUG(SD$^+$) plasmid. The arrow pointing downstream of the coding sequence shows the nucleotides inserted immediately downstream of the UAA to produce plasmids pΔS15AUG(15) and pΔS15AUG(20bis). The structures of the ends of transcripts from plasmids pΔS15CCG(20), pΔS15AUG(20) and pΔS15AUG(10) are shown at the bottom of the figure. Inserted nucleotides giving rise to these plasmids are shown by larger characters. The UAA codon at the end of the coding sequence of pΔS15AUG(20) has been mutagenized into an AC sequence indicated by an asterisk on the map of pΔS15AUG(10). The ends of the coding sequences are overlined. The numbers in parentheses at the end of plasmid names correspond to the number of nucleotides between the UAA and the M2 processing site. The lengths of the transcripts in nucleotides (nt), the positions of the first and last nucleotides of the functional and non-functional reading frames and positions of base substitutions which create early termination codons are indicated. Numbering begins at the transcription start of *rpsO* (Régnier and Portier, 1986).

necessary to show that translation stabilizes the *rpsO* mRNA. For this purpose, we tried to construct a constitutively expressed, plasmid-borne *rpsO* gene, i.e. one whose translation efficiency does not depend on the concentration of free ribosomal protein S15, by mutagenizing nucleotides of the operator essential for repression by S15 (Portier *et al*., 1990). Since we failed to isolate clones of *Escherichia coli* expressing the full-length *rpsO* gene constitutively (maybe because overexpression of S15 is deleterious to the cell), we overcame this difficulty by deleting 122 nucleotides coding for the N-terminus of S15 (~30% of the structural gene) from the *rpsO* gene contained on pF1(+) to give plasmids pΔS15AUG and pΔS15CCG (Figure 1B). Seven of the 122 nucleotides deleted are involved in the formation of the translational operator (Philippe *et al*., 1993). pΔS15AUG harbours a functional AUG initiation codon at the beginning of the internally deleted *rpsO* gene, which supports the synthesis of active β-galactosidase when fused in-frame to *lacZ*. The failure to complement the cryosensitivity associated with the S15 deficiency of strain KY1447 (Yano and Yura, 1989) demonstrates that the truncated S15 protein, missing the 38 N-terminal amino acids, encoded by pΔS15AUG is not incorporated into active ribosomes. In addition, this polypeptide was not able to repress translation of the wild-type *rpsO* message, thus suggesting that it does not recognize the translational operator. The internally deleted *rpsO* gene of pΔS15CCG harbours a CCG triplet instead of the normal AUG initiation codon. Construction of plasmid-borne *lacZ* fusions to this construct demonstrates that the *rpsO* coding sequence of pΔS15CCG does not harbour cryptic translation initiation sites in any of the three reading frames. Similarly, the two out-of-frame Δ*rpsO′::lacZ′* fusions constructed with the AUG-containing *rpsO* gene of pΔS15AUG do not produce β-galactosidase. Thus, pΔS15AUG and pΔS15CCG are appropriate tools to study translational interference in RNase E processing at the M2 site and the resulting changes in mRNA stability.

The stability of internally deleted rpsO mRNAs depends on translation efficiency

Stabilities of mRNAs originating from pΔS15AUG and pΔS15CCG were determined in the IBPC5321 wild-type bacteria (Plumbridge *et al*., 1985) by Northern blot analysis of *rpsO* mRNAs at various times after inhibition of transcription initiation by rifampicin. The major products of the chromosomal and the internally deleted plasmid-borne *rpsO* genes are the P1-t1 primary transcript (416 and ~310 nucleotides in length, respectively; Figure 1B) corresponding to the transcript beginning at the promoter, P1, and ending at the terminator, t1, and the less abundant P1-RIII mRNAs (Figure 1A) resulting from the RNase III processing of the read-through transcripts generated when transcription initiated at P1 does not stop at t1 (Figure 2). The rapid decay of the untranslated pΔS15CCG P1-t1 mRNA (half-life ~1.2 min) compared with the translated P1-t1 pΔS15AUG transcript (half-life ~3.5 min) suggests that this mRNA is stabilized by translation (Figure 2). This conclusion is also supported by the observation that the pΔS15AUG mRNA is three times more abundant than the untranslated pΔS15CCG mRNA (Figure 3) and by additional experiments showing that a mutation in the Shine–Dalgarno sequence which increases the frequency of translation (C.Portier, personal communication) stabilizes the *rpsO* mRNA. The half-life of the P1-t1 mRNA increases from ~3.5 to ~6 min when the GGAGU Shine–Dalgarno motif of pΔS15AUG is mutated to GGAGG [plasmid pΔS15AUG(SD+) in Figure 1] (Figure 2) and the GGAGG transcript is three times more abundant than the original GGAGU transcript (Figure 3).

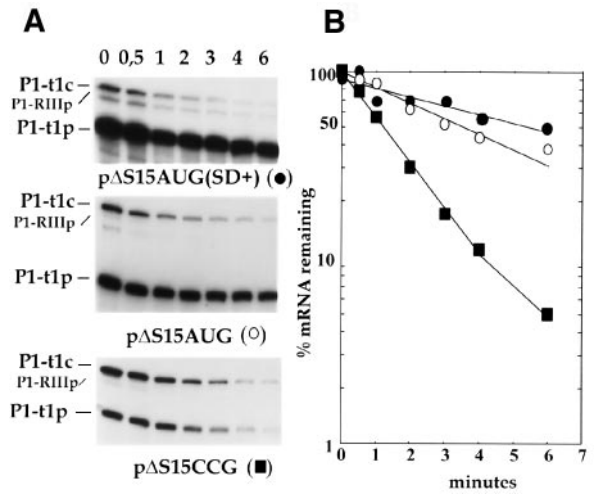

Fig. 2. Stability of the P1-t1 Δ*rpsO* mRNA depends on the efficiency of its translation. (**A**) The wild-type strain IBPC5321, transformed respectively with one of the pΔS15AUG(SD$^+$), pΔS15AUG or pΔS15CCG plasmids, was grown at 37°C until an $OD_{650\ nm}$ of 0.25. Rifampicin was added to a final concentration of 500 μg/ml at time 0. Five μg of total RNA, extracted from aliquots withdrawn at different times after rifampicin addition, indicated in minutes at the top of each lane, was analysed on Northern blots and probed for *rpsO* mRNA. The plasmids present in bacteria are indicated beneath each panel. Positions of the monocistronic *rpsO* mRNA (P1-t1) and of the RNase III-processed mRNA (P1-RIII) are shown on the left of the autoradiographs. Transcripts of chromosomal origin are indicated by 'c' while transcripts of plasmid origin are indicated by 'p'. (**B**) The relative amounts of the P1-t1p transcripts were expressed as a percentage of the highest value obtained and plotted as a function of time. Symbols used for each mutant plasmid are indicated in (A).

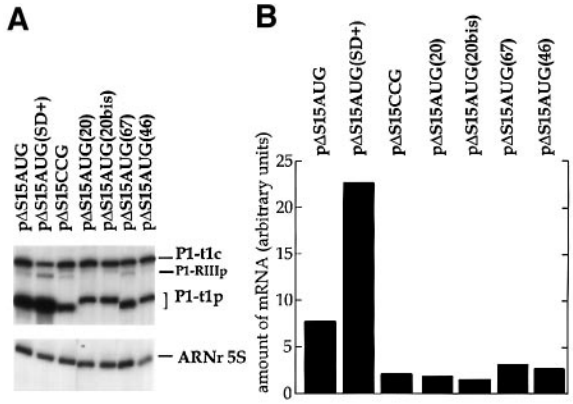

Fig. 3. The accumulation of the Δ*rpsO* P1-t1 transcripts of plasmid origin correlates with their stability. (**A**) Five μg of total RNA, extracted from strain IBPC5321 transformed with the plasmid indicated on the top of each lane, and grown at 37°C until an $OD_{650\ nm}$ of 0.25, was analysed by Northern blot as described in Figure 2. 5S rRNA was also quantified to correct for lane-to-lane variations in RNA loading. Positions of the 5S rRNA, of the monocistronic P1-t1c and P1-t1p *rpsO* transcripts and of the P1-RIIIp RNase III-processed mRNA are shown on the side of the autoradiograph. The *rpsO* mRNAs of pΔS15CCG and of pΔS15AUG(20) and pΔS15AUG(20bis) are three nucleotides shorter and 10 nucleotides longer, respectively, than those of pΔS15AUG, pΔS15AUG(SD$^+$), pΔS15AUG(46) and pΔS15AUG(67) (Figure 1B). (**B**) The relative amounts of P1-t1p, corrected for variations of loaded 5S rRNA, were plotted as a bar graph.

Early termination of translation destabilizes the translated ΔrpsO mRNA

In order to investigate whether the stabilizing effect of translation is due to the inhibition of RNase E cleavage at the M2 site by ribosomes, we introduced premature UAA stop codons at positions 208 and 229 in the coding sequence of the internally deleted *rpsO* gene of pΔS15AUG. The two resulting plasmids, named pΔS15AUG(46) and pΔS15AUG(67), generate mRNAs which harbour 46 and 67 nucleotides, respectively, between the UAA codon and the M2 site (Figure 1B). Figure 4 shows that the pΔS15AUG(46) P1-t1 transcript is significantly destabilized (half-life ~1.2 min) compared with the original pΔS15AUG mRNA harbouring the UAA codon 10 nucleotides upstream of M2 (half-life ~3.5 min). This destabilization is confirmed by the 3-fold reduction in its intracellular concentration (Figure 3). Similarly, the pΔS15AUG(67) transcript is ~2.5 times less abundant than the original pΔS15AUG mRNA (Figure 3). It can be concluded from these data that ribosomes mask a destabilizing motif located within the stretch of 46 nucleotides located just upstream of M2. It could be the M2 cutting site or an upstream sequence required for RNase E recognition.

Insertion of nucleotides between the UAA termination codon and the M2 site destabilizes the mRNA

The other approach used to confirm that the length of the untranslated sequence in the vicinity of M2 affects the efficiency of RNase E processing was to increase the distance between the UAA termination codon and the M2 site of pΔS15AUG by inserting arbitrarily chosen nucleotides (Figure 1B). Duplication of the last 10 nucleotides of the coding sequence of *rpsO*, just downstream of the UAA termination codon, gives rise to the pΔS15AUG(20) plasmid which harbours the same stretch of 20 nucleotides upstream of M2 as pΔS15AUG (Figure 1B). However, pΔS15AUG(20) harbours 20 nucleotides between the functional UAA codon and the M2 site instead of 10 as in pΔS15AUG. As observed above for early termination, the pΔS15AUG(20) mRNA harbouring an extended untranslated region upstream of M2 is significantly destabilized (half-life ~1.65 min) (Figure 4) and four times less abundant (Figure 3) compared with the original pΔS15AUG mRNA (half-life ~3.5 min). The fact that insertion of a different 10 nucleotide sequence at the same position [plasmid pΔS15AUG(20bis) of Figure 1] has a similar destabilizing effect on translated mRNA (half-life ~1.4 min, data not shown) and reduces the amount of mRNA 4-fold (Figure 3) indicates that distance, but not nucleotide content, between the UAA codon and the M2 site is responsible for destabilization of the *rpsO* mRNA.

Elongation of the coding sequence stabilizes the mRNA

This conclusion has been reinforced strongly by the experiment below showing that the pΔS15AUG(20) mRNA harbouring 20 nucleotides between the UAA and M2 is stabilized when a mutation in the stop codon allows translation to terminate only 10 nucleotides upstream of M2. This has been achieved by changing the UAA termination codon of pΔS15AUG(20) into an AC sequence, giving rise to the pΔS15AUG(10) plasmid in which the elongated reading frame of *rpsO* terminates

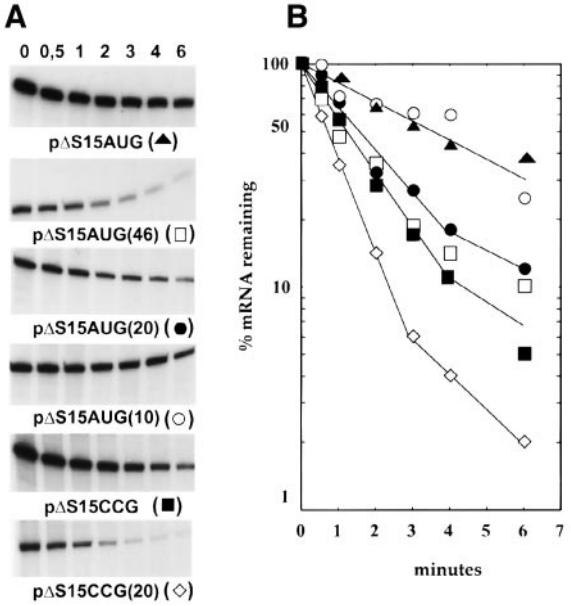

Fig. 4. Modifications of the length of ribosome-free mRNA and of mRNA structure in the vicinity of the M2 RNase E site affect the stability of Δ*rpsO* mRNA. (**A**) IBPC5321, transformed with pΔS15AUG, pΔS15AUG(46), pΔS15AUG(20), pΔS15AUG(10), pΔS15CCG or pΔS15CCG(20) (Figure 1B), was grown at 37°C until an $OD_{650\ nm}$ of 0.25, and total RNA extracted from aliquots withdrawn at different times after rifampicin addition (0), indicated in minutes at the top of the picture, was analysed on Northern blots as described in Figure 2. Plasmids present in the bacteria are indicated under the autoradiographs showing decay of the P1-t1 transcript of plasmid origin. (**B**) The relative amounts of the P1-t1p mRNAs transcribed from each plasmid were plotted as described in Figure 2. Symbols used for each strain are indicated in (A).

10 nucleotides upstream of M2 (Figure 1B). It is striking that the pΔS15AUG(10) mRNA is significantly more stable (half-life ~3.2 min) than the pΔS15AUG(20) mRNA (half-life ~1.65 min) (Figure 4). We verified that the stability of the pΔS15AUG(10) mRNA is not due to the fact that it harbours a nine nucleotide insertion instead of 10 as in pΔS15AUG(20). In fact, insertion of five nucleotides downstream of the UAA termination codon in plasmid pΔS15AUG(15) (Figure 1B) is sufficient to reduce the mRNA half-life from ~3.5 to ~2.8 min (data not shown).

The untranslated mRNA is also destabilized by a 10 nucleotide insertion

Since modifications of RNA structure can alter efficiency of RNase E cleavages (Bouvet and Belasco, 1992; Mackie and Genereaux, 1993; McDowall *et al*., 1995; Naureckiene and Uhlin, 1996; Nilsson *et al*., 1996), we have investigated whether the nucleotides inserted in pΔS15AUG(20), 10 nucleotides upstream of M2, modify the stability of the untranslated mRNA. Figure 4 shows that the pΔS15CCG(20) untranslated mRNA which harbours the same insertion as pΔS15AUG(20) is also destabilized (half-life ~0.75 min) compared with the original pΔS15CCG mRNA which has a half-life of ~1.2 min. These data suggest that a duplication of 10 nucleotides induces a modification in mRNA structure which affects the efficiency of the RNase E cleavage occurring 10 nucleotides downstream.

Destabilization of transcripts is correlated with an increased processing efficiency at M2

If the destabilization of the mutant mRNAs, described above, is due to the increased cleavage efficiency at M2, these mRNAs should give rise to P1-M2 mRNA species, which can be detected in a strain deficient for 3′ exonucleases: PNPase and RNase II (Hajnsdorf *et al*., 1994). As expected, inactivation of the thermosensitive RNase II at 44°C in the PNPase-deficient SK5726 strain (Arraiano *et al*., 1988) transformed with pΔS15AUG, pΔS15-AUG(67) or pΔS15AUG(20) causes the accumulation of the 382 nucleotide P1-M2 mRNA, originating from the chromosomal copy of *rpsO*, and of mRNAs corresponding to the sizes of the transcripts of plasmid origin processed at M2 (Figure 5A). These latter mRNAs are ~277 nucleotides in length in cells transformed with pΔS15AUG or with pΔS15AUG(67) which has the premature UAA stop codon. The slightly longer mRNA (~287 nucleotides) appearing in cells transformed with pΔS15AUG(20) (Figure 5A) shows that this P1-t1 mRNA is also processed at the M2 site located downstream of the 10 nucleotide insertion (Figure 1B). These data demonstrate that the extension of the untranslated region by inserting nucleotides downstream of the coding sequence or by creating an early termination codon does not generate additional processing sites which might account for the destabilization of the mRNAs. Strikingly, after the shift to 44°C, P1-M2 mRNA originating from pΔS15AUG is far less abundant than the equivalent molecule generated from the mutated plasmids pΔS15AUG(20) and pΔS15AUG(67). This observation suggests that the translated mRNA of pΔS15AUG, which has 10 nucleotides between the UAA stop codon and M2, is processed inefficiently at M2 and agrees with our hypothesis that the unstable mRNAs of pΔS15AUG(20) and pΔS15AUG(67) are processed more efficiently at this site than the pΔS15AUG transcript. These data also imply that mutations which facilitate access to the M2 site increase the fraction of molecules which follow the RNase E-dependent pathway of decay initiated at M2 and that most pΔS15AUG mRNAs are degraded independently of this cleavage. It is worth pointing out that this pΔS15AUG mRNA, lacking the translational operator, appears also to be processed less efficiently at M2 than the full-length transcript of chromosomal origin which gives rise to a larger amount of P1-M2. This probably reflects better accessibility of the M2 site resulting from translational repression of the chromosomal transcript.

Destabilization of mutated mRNAs depends on RNase E

If destabilization of the mutant P1-t1 mRNAs results from increased accessibility to the M2 site, these mRNAs would be expected to be more stable in a strain deficient for RNase E. In fact, the decay of the P1-t1 transcripts of chromosomal (not shown) and plasmid origin is much slower in an IBPC681 (*rne-1*^{ts}) strain than in the isogenic IBPC694 wild-type strain (Hajnsdorf *et al*., 1995), after inactivation of RNase E at 44°C (Figure 5B and C). The half-lives of the pΔS15AUG(20) and pΔS15AUG(67) P1-t1 transcripts are ~1.2 min in the wild-type strain and ~3.5 and ~4.5 min, respectively, in the RNase E-deficient strain at 44°C. Moreover, consistent with the conclusion

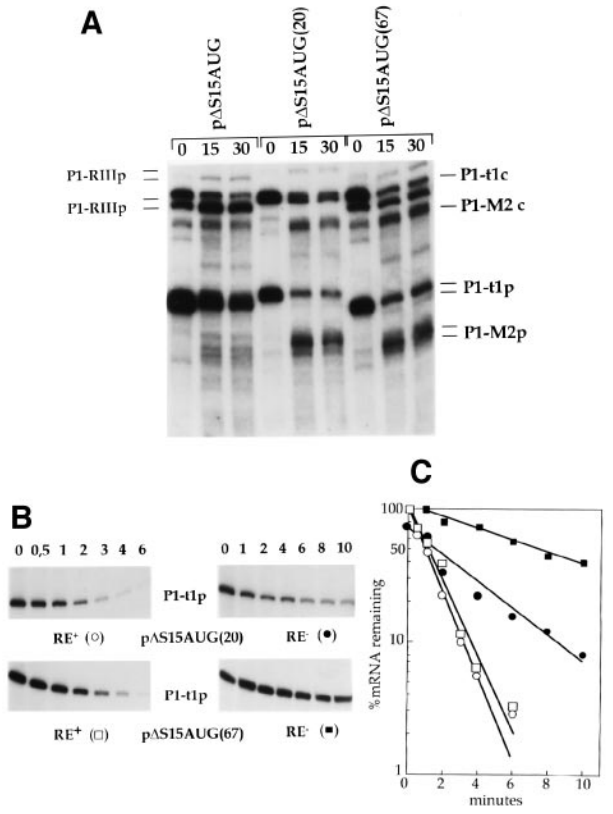

Fig. 5. Destabilization of Δ*rpsO* mRNA is due to a better efficiency of the RNase E cleavage at the M2 site. (**A**) Strain SK5726 (PNPase$^-$, RNase II^{ts}), transformed with pΔS15AUG, pΔS15AUG(20) or pΔS15AUG(67) and grown at 30°C, was shifted to 44°C at time 0. Five μg of total RNA, prepared from aliquots withdrawn at the time of the shift or 15 and 30 min later (time is indicated at the top of each lane), was analysed on Northern blots as described in Figure 2. Positions of the different transcripts and plasmids present in bacteria are indicated as in Figure 2. P1-M2 refers to mRNAs processed by RNase E at M2. The P1-t1p, P1-M2p and P1-RIIIp transcripts synthesized from pΔS15AUG(20) migrate more slowly than the equivalent mRNAs from pΔS15AUG or pΔS15AUG(67) which are 10 nucleotides shorter. (**B**) Stabilization of the Δ*rpsO* mRNA in an RNase E-deficient strain. Strains IBPC681(RNase E^{ts}, RE$^-$) and IBPC694 (RNase E$^+$, RE$^+$) transformed with pΔS15AUG(20) or pΔS15AUG(67) were grown at 30°C until an $OD_{650\ nm}$ of 0.25. Rifampicin was added to a final concentration of 500 μg/ml and the cultures shifted immediately to 44°C (time 0). Five μg of total RNA extracted from aliquots withdrawn at the different times indicated at the top of each lane was analysed as described in Figure 2. The plasmids present in the RE$^+$ and RE$^-$ strains are indicated, beneath the autoradiographs, between the phenotype of the strains. (**C**) The relative amounts of P1-t1p transcripts were quantified and plotted as in Figure 2. Symbols used for each strain are indicated in (B).

that translation protects the pΔS15AUG mRNA from cleavage by RNase E, we found that this mRNA is only slightly stabilized in an *rne*$^-$ strain. Its half-life is ~2 min in an *rne*$^+$ strain and ~3.5 min in an *rne*$^-$ strain (data not shown).

Discussion

We demonstrate in this report that cleavage by RNase E at the M2 site, which initiates the exonucleolytic decay of the *rpsO* mRNA, is sensitive to the presence of ribosomes, and we propose that this accounts for the stabilization of the message by translation. Even though the cleavage occurs outside the coding sequence, ribosomes can modulate its efficiency. It is striking that all mutations which increase the length of ribosome-free mRNA upstream of the processing site cause the destabilization of the mRNA and/or the reduction of its intracellular concentration and that, in contrast, a mutation which brings the termination codon closer to the processing site stabilizes the mRNA. This suggests that the M2 site is more accessible to RNase E if the surrounding mRNA is not occupied by ribosomes. Accordingly, early termination and insertion mutant mRNAs are cleaved more efficiently than the original molecules. RNase protection and toeprint experiments which demonstrated that a ribosome covers ~15 nucleotides downstream of the first nucleotide of the codon in its P site (Steitz, 1969; Hartz *et al*., 1991) suggest that the steric hindrance of ribosomes standing at the UAA stop codon presumably prevents the access of the catalytic domain of RNase E to the processing site located 10 nucleotides downstream of the UAA. Therefore, transcripts harbouring early termination codons and insertions might be destabilized because ribosomes are no longer an obstacle to RNase E access if translation terminates 20 nucleotides or more upstream of M2. However, our data suggest that ribosomes can also mask sequences, in the vicinity of M2, which belong to the RNase E recognition/cleavage site. Indeed, the destabilization of the untranslated pΔS15CCG(20) mRNA resulting from a 10 nucleotide insertion indicates that RNase E interacts with the sequence upstream of M2. The fact that the same insertion has no effect on mRNA stability when it is part of the coding sequence of a translated mRNA [the half-lives of the pΔS15AUG(10) and pΔS15AUG mRNAs are ~3.2 and ~3.5 min respectively] indicates that its destabilizing effect is impaired by ribosomes. One possible explanation is that the addition of 10 bases increases the number of unpaired nucleotides lying between the potential stem–loop at the end of the *rpsO* coding sequence and the transcriptional terminator t1 (Figure 6) and thus facilitates processing of the untranslated mRNA by RNase E (Mackie and Genereaux, 1993; McDowall *et al*., 1995; Naureckiene and Uhlin, 1996). In contrast, ribosomes presumably prevent access of RNase E to this region when the 10 nucleotide insertion is a part of the coding sequence of the translated pΔS15AUG(10) mRNA. These data indicate that ribosomes can sterically hinder access of RNase E to the processing site and to upstream structural motifs which contribute to the recognition/cleavage of the mRNA. This set of data provides strong experimental support for the hypothesis that mRNAs, reported to be stabilized by translation, harbour rate-limiting endonucleolytic cleavage site(s), located in (or close enough to) the coding sequence which are masked by ribosomes upon translation (Nilsson *et al*., 1987; Baumeister *et al*., 1991; Yarchuk *et al*., 1991; Jain and Kleckner, 1993; Rapaport and Mackie, 1994; Iost and Dreyfus, 1995).

The observation that mutations which increase the access of RNase E to the M2 site destabilize the mRNA is consistent with our prior finding that the RNase E cleavage at M2 is rate-limiting in *rpsO* mRNA decay (Régnier and Hajnsdorf, 1991; Hajnsdorf *et al*., 1994). On the other hand, the P1-t1 mRNAs transcribed from pΔS15AUG and pΔS15AUG(SD$^+$), where cleavage at M2 is presumably very inefficient [as estimated from the low

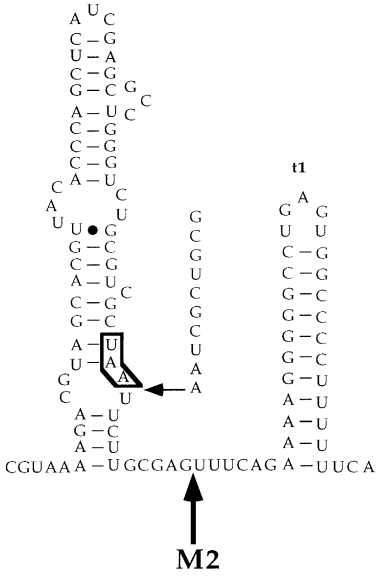

Fig. 6. Potential secondary structure at the end of the *rpsO* transcript. The 10 nucleotide insertion which gives rise to pΔS15AUG(20) and pΔS15CCG(20) takes place at the bottom of the potential secondary structure formed by the end of the coding sequence of *rpsO* (Braun *et al*., 1996). The UAA termination codon is boxed. The stem and loop of the transcriptional terminator t1 and the M2 RNase E site are also shown (Régnier and Portier, 1986).

amount of P1-M2 produced from pΔS15AUG (Figure 5) and pΔS15AUG(SD$^+$) (data not shown)], are still relatively unstable (half-lives of 3.5 and 6 min, respectively). This is in agreement with our previous conclusion that the *rpsO* mRNA can also be degraded independently of RNase E (Hajnsdorf *et al*., 1994). This transcript could, for example, be cleaved at the site identified in the 5′-untranslated region (Hajnsdorf *et al*., 1996) and by unidentified RNases which specifically attack polyadenylated molecules (Hajnsdorf *et al*., 1995). The influence of ribosomes on the processing at M2 does not exclude the possibility that this cleavage is made by RNase E interacting with the 5′ end of the message, as proposed for other RNAs (Bouvet and Belasco, 1992; Emory *et al*., 1992), with the poly(A) tail (Huang *et al*., 1998) or with the degradosome (Carpousis *et al*., 1994). The simultaneous contribution of several mechanisms to the degradation of the *rpsO* mRNA [e.g. the RNase E-dependent pathway initiated at M2, and the poly(A)-dependent pathway] (Hajnsdorf *et al*., 1996) probably explains why mutations which improve the accessibility of the M2 RNase E site only increase the decay rate by a factor of 2 or 3. The preferential degradation of these mutated mRNAs by the RNase E-dependent pathway probably also accounts for the enhanced production of the P1-M2 mRNA species.

The stabilizing effect of translation on the *rpsO* mRNA suggests that this transcript is degraded rapidly in the cell when its translation is repressed by an excess of free ribosomal protein S15. This might explain why the full-length mRNA which contains the binding site for the translational repressor appears to be processed more efficiently at M2 (Figure 5A) and less stable (see above) than the constitutively translated transcript generated from pΔS15AUG. A similar control of mRNA decay has already been proposed for transcripts of the α (Singer and Nomura, 1985) and L11 (Cole and Nomura, 1986) operons, which are also destabilized under conditions of repression. The coupling between translation and decay may serve to adjust the amounts of mRNAs to the yield of ribosomal proteins and to minimize the pools of ribosomal proteins necessary to repress the translation of mRNAs. If the *rpsO* mRNA is not degraded rapidly under conditions where S15 is in excess, one could imagine a situation where accumulation of mRNA would titrate out the repressor and lead to restoration of gene expression. In this context, it is a paradox that the messenger of ribosomal protein S20 is stabilized under similar conditions (Mackie, 1987).

In conclusion, our data demonstrate that the *rpsO* mRNA degradation can be mediated by an endonucleolytic cleavage event that depends on translation. The protection of translated mRNAs implies that the 3′ to 5′ exonucleolytic process of decay triggered by this cleavage concerns mainly untranslated (or poorly translated) mRNAs and, therefore, probably generates principally translationally inactive mRNA fragments. If it applies to other transcripts, this mechanism should minimize the synthesis (from mRNA fragments lacking a translation stop codon) of tagged truncated polypeptides which are degraded specifically in the cell (Keiler *et al*., 1996).

Materials and methods

Strains and growth conditions

Bacteria were grown in Luria–Bertani (LB) medium supplemented with 50 μg/ml thymine for *thy*$^-$ cells and with 50 μg/ml spectinomycin for transformed strains. mRNA processing and decay were analysed in IBPC5321 (wild-type) (Plumbridge *et al*., 1985) and in the isogenic strains SK5726 [*ThyA715, rnb500, pnp7*/pDK39(Cm^R *rnb500*)] (Arraiano *et al*., 1988), IBPC681 (*ThyA715, rne-1, zad*::Tn*10* Tet^R) and IBPC694 (*ThyA715, zad*::Tn*10* Tet^R) (Hajnsdorf *et al*., 1995). IBPC681 was obtained by curing the pRS415 plasmid from IBPC670 (Hajnsdorf *et al*., 1995). Reversion of the cryosensitivity of KY1447 (*rpoH11 suhD3*) was used to test the synthesis of functional S15 from plasmids (Yano and Yura, 1989).

General methods

Restriction digests, DNA purifications, PCR amplifications, ligations, transformations and gel electrophoresis were performed as described (Sambrook *et al*., 1989). Oligonucleotides were synthesized by Oligo Express (Paris, France).

Construction of pΔS15AUG and pΔS15CCG

The 796 bp *Hpa*I–*Hin*dIII fragment carrying the *rpsO* gene from pFB1 (Hajnsdorf *et al*., 1995) was first inserted into the polylinker of pCL1921 (Lerner and Inouye, 1990) digested by *Sma*I and *Hin*dIII to give pCL1. The *Eco*RI fragment carrying the origin of replication of bacteriophage f1 (f1 ori) from pD4 (Dotto *et al*., 1981) was inserted into pCL1 digested by *Eco*RI to create pCF1(+), a plasmid whose f1 ori directs synthesis of single-stranded DNA with the polarity of the *rpsO* messenger. The *Eco*RI site of pCF1(+), located at position 3990, was removed by filling the extremities of the plasmid partially digested by *Eco*RI with Klenow enzyme. An *Hpa*I site was then created and the 122 bp *Hpa*I–*Pst*I fragment of pF1(+) (Figure1B) was deleted and substituted by synthetic adaptors 1 and 2 to produce plasmids pΔS15AUG and pΔS15CCG, respectively (Figure 1B). Adaptor 1 is obtained by annealing oligonucleotides 5′-AAAATGACCGGTCTGCA-3′ and 5′-GACCGGTCATTTT-3′ and adaptor 2 by oligonucleotides 5′-AAACCGCGGCTGCA-3′ and 5′-GCCGCGGTTT-3′.

Construction of translational fusions between the ΔrpsO genes of pΔS15AUG and pΔS15CCG and the lacZ reporter gene

Gene fusions were constructed by inserting PCR-amplified fragments of the Δ*rpsO* genes of pΔS15AUG or pΔS15CCG into the *lacZ* gene of the pRS414 plasmid (Simons *et al*., 1987). The upstream primer

used for the amplification (5′-GGATGTTG*GAATTC*GAGCTCGG-3′) hybridizes between positions –68 and –47 relative to the transcription start site of *rpsO* and carries an *Eco*RI site (in italics). Three downstream primers (5′-CG*GGATCC*AGGCGCTCGATGAGCTGGG-3′, 5′-CG*GGATCC*CCAGGCGCTCGATGAGCTGGG-3′ and 5′-CG*GGATCC*CCAGGCGCTCGATGAGCTGGG-3′), which hybridize between nucleotides +233 and +253, at the end of the coding sequence of *rpsO*, and harbour 5′ extensions carrying *Bam*HI sites (in italics) staggered by one nucleotide, were used so that the ligation of amplified fragments to the *Bam*HI site of *lacZ* creates *rpsO′*::*lacZ′* fusions in the three reading frames of the Δ*rpsO* genes. PCR fragments digested by *Eco*RI and *Bam*HI were cloned into the identical sites of pRS414. The level of β-galactosidase activity in the Δ*lac* strain, AB5311 (Springer *et al*., 1985), transformed with the six recombinant plasmids, was estimated as described (Miller, 1972).

Mutagenesis

The pCF1(+) and pΔS15AUG uracil-containing single-stranded DNAs were used for site-directed mutagenesis (Kunkel *et al*., 1987). Plasmids pF1(+), pΔS15AUG(SD$^+$), pΔS15AUG(67), pΔS15AUG(46), pΔS15AUG(15), pΔS15AUG(20) and pΔS15CCG(20), pΔS15AUG(20bis) and pΔS15AUG(10) were created with oligonucleotides 5′-CTTAGAGACATGTTAACACTCCAAAG-3′, 5′-CCGGTCATTTTAACCCTCCAAAG-3′, 5′-GCTACGTCTTTACGTTACAGGTAGTCGAGC-3′, 5′-GATGAGCTGGGTTTAGTTAACTACGTCTTTACGTTTCAGG-3′, 5′-CTGAAACTCGCAAGAATTAGCTTAGCGACGCAG-3′, 5′-CTGAAACTCGCAAGAATTAGCGACGCTTAGCGACGCAGAC-3′, 5′-CTGAAACTCGCAAGAACAGGAGACGATTAGCGACGCAGAC-3′ and 5′-CTCGCAAGAATTAGCGACGCGTGCGACGCAGACCCAGGCGC-3′, respectively. The mutated or inserted nucleotides relevent to this study are underlined. All mutants were verified by sequence analysis.

RNA analysis

RNA preparations and Northern blot analysis on polyacrylamide gels hybridized with the 3′ *rpsO* probe were performed as described previously (Braun *et al*., 1996). 5S rRNA was probed with a 5′-end-labelled oligonucleotide, 5′-ACTACCATCGGCGCTACGGC-3′, which anneals to the 5S rRNA between nucleotides 61 and 80. After hybridization with the 5S rRNA and the 3′ *rpsO* probes, the blots were washed three times for 10 min at room temperature with 2× SSPE and 0.1% SDS; 1× SSPE is 180 mM NaCl, 10 mM sodium phosphate pH 7.5 and 1 mM EDTA. RNAs on blots were quantified by PhosphorImager. Half-lives were deduced from the slope of the semi-logarithmic plot of amounts of mRNA versus time after rifampicin addition. Standard deviation of the half-lives of P1-t1c mRNA estimated in 17 independent experiments at 37°C allows the error of half-life measurements to be calculated as ± 25%.

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