The EMBO Journal vol.13 no.14 pp.3368–3377, 1994

Roles of RNase E, RNase II and PNPase in the degradation of the *rpsO* transcripts of *Escherichia coli*: stabilizing function of RNase II and evidence for efficient degradation in an *ams pnp rnb* mutant

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Communicated by M.Grunberg-Manago

The *Escherichia coli rpsO* gene gives rise to different mRNA species resulting either from termination of transcription or from processing of primary transcripts by RNase E and RNase III. The main degradation pathway of these transcripts involves a rate-limiting RNase E cleavage downstream of the structural gene which removes the 3′ terminal stem–loop structure of the transcription terminator. This structure protects the message from the attack of 3′–5′ exonucleases and its removal results in very rapid degradation of the transcript by polynucleotide phosphorylase and RNase II. Polynucleotide phosphorylase is also able to degrade slowly the mRNA harboring the 3′ terminal hairpin of the terminator. In contrast, RNase II appears to protect the *rpsO* mRNA species which retains the 3′ hairpin structure. Rapid degradation of the *rpsO* mRNA is observed after inactivation of RNase II even in a strain deficient for RNase E and polynucleotide phosphorylase. The enzyme(s) involved in this degradation pathway is not known. We detected an unstable elongated *rpsO* mRNA presumably resulting from the addition of nucleotides at the 3′ end of the transcript.
Key words: 3′–5′ exonucleases/3′ hairpins/mRNA degradation/mRNA processing/RNase E

Introduction

The level of expression of a gene is determined by the efficiency of translation of its message and by the intracellular concentration of its mRNA which reflects the dynamic equilibrium between the frequency of transcription and the rate of decay. The 40-fold range of bacterial mRNA half-lives which vary from 0.5 to 20 min implies that the sensitivity of mRNA to degradation is one of the parameters that control gene expression (Pedersen *et al.*, 1978; Nilsson *et al.*, 1984). It has been suggested that the inherent metabolic instability of bacterial mRNA permits the rapid adaptation of the cell to changes in environmental conditions (Gros *et al.*, 1961; Jacob and Monod, 1961). Compared with the other principal gene-regulatory processes in bacteria (notably transcription and translation), the study of mRNA degradation has been neglected for a long time. However, investigations recently undertaken in this field are now beginning to yield information on cellular factors and RNA features involved in mRNA stability and degradation (Belasco, 1993).

It is generally believed that mRNA is degraded by coordinate activities of endonucleases and exonucleases (Apirion, 1973; Cannistraro *et al.*, 1986; Kennell, 1986). To date only five of the 20 RNases identified in *Escherichia coli* have been shown to contribute to the metabolism of mRNA (Deutscher, 1988). They include two exonucleases, namely polynucleotide phosphorylase (PNPase) and RNase II, and three endonucleases, i.e. RNase III, RNase E and RNase K (Belasco and Higgins, 1988; Ehretsmann *et al.*, 1992a). RNase II and PNPase both attack the 3′ extremity of RNAs which they degrade processively to mononucleotides (Grunberg-Manago, 1963; Spahr and Schlessinger, 1964). *In vivo*, the two enzymes can substitute for each other to degrade the same mRNAs (Donovan and Kushner, 1986). So far no exonuclease able to attack the 5′ end of RNA has been identified in bacteria (Deutscher, 1993). In addition to their function in rRNA maturation (King *et al.*, 1986) endonucleases RNase E and RNase III are involved in the processing of phage (Guarneros, 1988; Mudd *et al.*, 1988) and cellular mRNAs (Barry *et al.*, 1980; Régnier and Portier, 1986; Régnier and Grunberg-Manago, 1989; Downing *et al.*, 1990; Faubladier *et al.*, 1990; Régnier and Hajnsdorf, 1991) and have been shown to play a part in the control of gene expression (Saito and Richardson, 1981; Guarneros *et al.*, 1982; Altuvia *et al.*, 1987; Krinke and Wulff, 1987; Portier *et al.*, 1987; Bardwell *et al.*, 1989; Mudd *et al.*, 1990a). Inactivation of RNase E has a stabilizing effect on the bulk of mRNA (Ono and Kuwano, 1979; Mudd *et al.*, 1990b; Babitzke and Kushner, 1991) while RNase III only affects the stability of some individual messengers (Gitelman and Apirion, 1980; Takata *et al.*, 1987; Régnier and Grunberg-Manago, 1990). RNase E (Carpousis *et al.*, 1994) and RNase K make endonucleolytic cleavages in the 5′ leader of the *ompA* message. RNase K is implicated in the growth-rate dependent regulation of the expression of *ompA* (Lundberg *et al.*, 1990). RNase K and RNase E likely originate from the same gene (Melefors and von Gabain, 1991; Mudd and Higgins, 1993; Carpousis *et al.*, 1994).

Structural features at the 5′ and 3′ ends of mRNA determine the stability of many mRNAs. Some transcripts harboring a hairpin at their 5′ end are more resistant to the attack of RNases than the same transcripts harboring a single-stranded 5′ end (Bouvet and Belasco, 1992; Emory *et al.*, 1992). Also, primary transcripts which exhibit at their 5′ ends the stem–loop structure of an RNase III cutting site are more stable than the corresponding RNase III processed messages lacking this stem–loop (Régnier and Grunberg-Manago, 1990). At the 3′ end of mRNA, the hairpin structures of Rho-independent terminators and REP sequences stabilize the mRNA pre-

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sumably because they have the capability to impede 3′ exonucleases catalyzing the 3′–5′ processive exonucleolytic degradation of mRNA (Schmeissner *et al.*, 1984; Newbury *et al.*, 1987; Belasco and Higgins, 1988; Chen *et al.*, 1988; Gross and Hollatz, 1988; Plamann and Stauffer, 1990; Régnier and Hajnsdorf, 1991; Higgins *et al.*, 1993). Evidence that the degradation of most messengers is initiated by endonucleolytic cleavages implies that 3′ stem–loop structures can provide an effective block to 3′–5′ exonucleases *in vivo*. The fact that 3′ hairpins protect RNA against PNPase and RNase II more efficiently *in vivo* than *in vitro* led to the idea that protein factors may help to impede the exonucleases at the stem–loop structures (McLaren *et al.*, 1991).

One model, derived from studies on transcripts of a few operons, postulates that messages are irreversibly inactivated by endonucleolytic cleavages occurring at the 5′ end of the transcript (Cannistraro *et al.*, 1986; Kennell, 1986). Then, free mRNA progressively emerging after passage of the translating ribosomes would be cut by endonucleases into short RNA fragments which are degraded to mononucleotides by 3′–5′ exonucleases (Cannistraro *et al.*, 1986). Alternatively the messenger could be cleaved at successive sites by an endonuclease migrating along the RNA (Belasco and Higgins, 1988; Emory and Belasco, 1990). This model accounts for the global 5′–3′ degradation of several mRNAs. In a second model, derived from the study of the *int* mRNA from phage λ and the *rpsO* transcript from *E.coli*, which code respectively for integrase and ribosomal protein S15, the initial cleavages by RNase III or RNase E which occur in the 3′ non-coding sequence of the mRNA initiate the rapid degradation of the message by RNase II and PNPase (Régnier and Hajnsdorf, 1991; Schmeissner *et al.*, 1984). In that model RNAs are degraded in the global 3′–5′ direction.

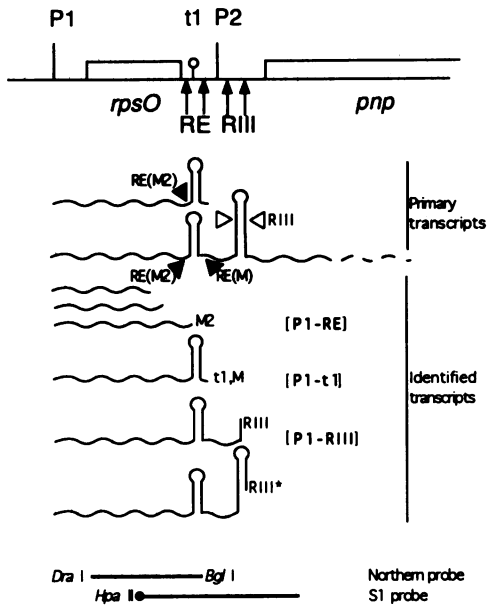

Fig. 1. Structure of the *rpsO* transcripts. The structures of the primary transcripts and of the post-transcriptionally processed messengers identified by Northern blot and S1 mapping are shown by wavy lines under the map of the *E.coli* chromosome showing *rpsO* and the beginning of *pnp*. The promoter P1, the RNase E (RE) maturation sites (M2 and M) and the RNase III (RIII) maturation sites are located on the map and on the primary transcripts. The structures of the anti-RNA probe used in Northern blot experiments and of the 3′ end-labelled DNA used for S1 nuclease mapping are shown at the bottom of the figure. The labelled extremity of the latter probe is indicated by a dot. The identities of 3′ ends of each identified transcript (M2, t1, M, RIII, RIII*) are indicated. RIII and RIII* refer respectively to the upstream and downstream cleavage sites occurring on both sides of the potential hairpin located upstream of *pnp* (Régnier and Portier, 1986). The names of the transcripts used in the article (P1-RE, P1-t1 and P1-RIII) are indicated between brackets.

The *E.coli rpsO* gene is transcribed either as a P1-t1 monocistronic message terminated at the Rho-independent transcription terminator t1 located downstream of *rpsO* or as a bicistronic *rpsO pnp* transcript coding for ribosomal protein S15 and PNPase (Figure 1) (Portier and Régnier, 1984; Régnier and Portier, 1986; Régnier *et al.*, 1987). RNase III and RNase E cleave these transcripts at several sites referred to as RIII, M and M2 between *rpsO* and *pnp* (Figure 1) (Régnier and Hajnsdorf, 1991; Régnier and Portier, 1986). The dramatic stabilization of P1-t1 observed in an *rne*3071 strain deficient for RNase E demonstrates that its degradation is triggered by RNase E (Régnier and Hajnsdorf, 1991).

We demonstrate here that the main degradation pathway of the *rpsO* mRNA involves an RNase E cleavage which removes the 3′ stabilizing hairpin and gives rise to an unstable species that is rapidly degraded by RNase II and/or PNPase. Our data also demonstrate that active RNase II is involved in the stabilization of the P1-t1 *rpsO* transcripts which retain the 3′ hairpin and that this species is rapidly degraded in a strain deficient for PNPase, RNase II and RNase E. Moreover we detect an elongated mRNA presumably resulting from the addition of nucleotides at the 3′ end which appears in a strain deficient for the 3′–5′ exonucleases.

Results

The rpsO mRNA resulting from the processing of P1-t1 by RNase E accumulates in a strain deficient for exonucleases

In order to investigate whether the messenger resulting from the processing by RNase E at the M2 site, upstream of the 3′ terminal hairpin of the P1-t1 *rpsO* transcript (Figure 1), is degraded by 3′–5′ exonucleases we have compared on Northern blots the *rpsO* mRNAs present in the SK5003 *pnp rnb* double mutant and the isogenic wild

Table I. Abbreviations for RNase E- and exonuclease-deficient strains

Abbreviation	Strain[a]	Ribonuclease genes[d]
Wild type	SK5006[b]	*ams*$^+$ *pnp*$^+$ *rnb*$^+$
pnp	SK5004[b]	*ams*$^+$ *pnp*7 *rnb*$^+$
rnb	SK5005[b]	*ams*$^+$ *pnp*$^+$ *rnb*500(ts)
pnp rnb	SK5003[b]	*ams*$^+$ *pnp*7 *rnb*500(ts)
ams	SK5665[c]	*ams*1(ts) *pnp*$^+$ *rnb*$^+$
ams pnp	SK5671[c]	*ams*1(ts) *pnp*7 *rnb*$^+$
ams rnb	SK5715[c]	*ams*1(ts) *pnp*$^+$ *rnb*500(ts)
ams pnp rnb	SK5704[c]	*ams*1(ts) *pnp*7 *rnb*500(ts)

[a]Complete genotypes of strains are listed in Table III.
[b,c]The superscripts b and c refer to two sets of isogenic strains. Strains marked b contain a multicopy plasmid carrying *rnb*500(ts).
[d]Alleles of RNase genes are specified.

type strain SK5006 (Table I) after a temperature shift to 44°C. This double mutant, which harbors the *pnp7* and *rnb500* alleles, is deficient for PNPase at 30°C and nearly completely deficient for 3′–5′ exonucleases PNPase and RNase II at 44°C (Table I). A strain harboring the *pnp7* allele exhibits only ~6% of the phosphorolytic activity of wild type *E.coli* (Reiner, 1969). The *pnp7* gene appears to be a nonsense mutant (Arraiano *et al.*, 1988) and the *rnb500* mutation produces a thermosensitive RNase II inactive at 44°C (Donovan and Kushner, 1986). The *pnp rnb* double mutant is not viable at 44°C. At 30°C the *pnp rnb* mutant (Figure 2) and the isogenic wild type strain (data not shown) both contain only two major mRNAs of 415 and 500 nucleotides. The most abundant species (415 nucleotides) has the size of the P1-t1 *rpsO* transcript terminated at t1 (Figure 2). This band probably also contains the *rpsO pnp* cotranscript processed by RNase E at the M site located 2–5 nucleotides downstream of the transcription termination site (Figure 1) (Régnier and Portier, 1986; Régnier and Hajnsdorf, 1991). However, because we do not discriminate between these two molecules in this report they are referred to as P1-t1 (Figure 1). The less abundant species of 500 nucleotides is referred to as P1-RIII (Figure 1) because it results from the RNase III processing of the *rpsO pnp* bicistronic transcript (see below). At the non-permissive temperature an additional mRNA species which migrates in the gel as a molecule of ~375 nucleotides is present in the exonuclease-deficient strain but not in the wild type strain (Figure 2). The size of this RNA suggests that it is the P1-RE *rpsO* transcript processed by RNase E at M2 which is 385 nucleotides in length (Figure 1). This mRNA species is not detected at 44°C in the IBPC661 RNase E-deficient strain, isogenic to the SK5003 *pnp rnb* strain (Figure 2), which harbors the *ams1* mutant gene coding for a thermosensitive RNase E inactivated at 44°C (Table III) (Kuwano *et al.*, 1977; Cormack *et al.*, 1993).

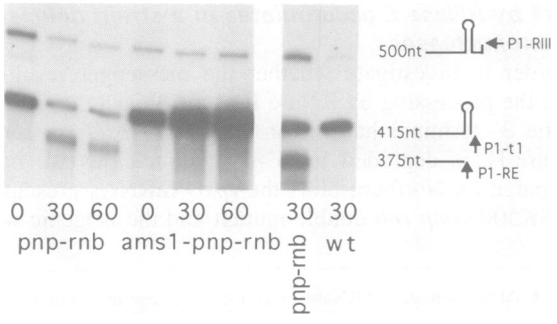

Fig. 2. Accumulation of the RNase E processed *rpsO* transcript in an exonuclease-deficient strain. Isogenic strains SK5003 (*pnp rnb*), SK5006 (wild type) and IBPC661 (*ams1 pnp rnb*) were grown at 30°C to an OD_{650nm} of 0.25 (mid log phase) then shifted to 44°C to inactivate RNase II and RNase E. IBPC661 has a genetic background different from strain SK5704 which is referred to as *ams pnp rnb* (Table I). RNAs were prepared from aliquots of the cultures withdrawn at the time of the shift (0) and after 30 min and 60 min of growth at 44°C. Five micrograms of total RNA were analyzed on Northern blots and probed with *rpsO* anti-RNA. The genotypes of the strains and the time of growth at 44°C are indicated beneath the picture. The structure and the size of the different mRNA species visible on the blots: P1-RE, P1-t1 and P1-RIII are shown on the right of the picture. Sizes were deduced from the migration of radioactive DNA fragments: pBR322 cleaved by *Msp*I (New England Biolabs) and a 123 nt ladder (Gibco BRL) transferred to the membrane.

The hypothesis that P1-RE accumulates in the *pnp rnb* exonuclease-deficient strain has been verified by comparing the 3′ ends of *rpsO* mRNAs of this mutant with that of the isogenic wild type strain at non-permissive temperature for RNase II. RNAs extracted from both strains protect from S1 nuclease digestion a subfragment of the 3′ labelled probe resulting from its annealing with the P1-t1 *rpsO* mRNA (Figure 3). As expected, a shorter fragment of the probe is protected by a *rpsO* mRNA present in the *pnp rnb* double mutant but not in the isogenic wild type strain at the non-permissive temperature (Figure 3). Its size locates the 3′ end of this mRNA ~40 nucleotides upstream of the 3′ end of P1-t1 in the vicinity of the M2 RNase E processing site (Figure 1). More precise localization, by comparison of the electrophoretic migration of protected fragments with DNA sequence ladders, shows that some of the 3′ ends of this mRNA map at the position of the M2 RNase E site (Régnier and Hajnsdorf, 1991) (data not shown). These data are consistent with previous models proposing that endonucleases and particularly RNase E initiate the degradation of mRNA (Belasco and Higgins, 1988; Régnier and Hajnsdorf, 1991; Higgins *et al.*, 1993).

A series of *rpsO* mRNA 3′ extremities mapping just upstream of the M2 RNase E site is visible on the autoradiograph of the S1 nuclease protection experiment performed with total RNA from the *pnp rnb* strain (Figure 3). This heterogeneity probably reflects the persistence, in the exonuclease-deficient strain, of a 3′–5′ exonu-

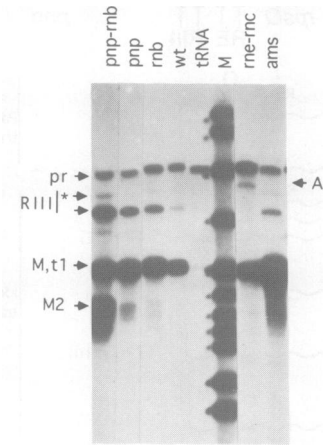

Fig. 3. Identification of the *rpsO* transcript processed by RNase E at M2. Total RNAs were prepared from aliquots of strains SK5003 (*pnp rnb*), SK5004 (*pnp*), SK5005 (*rnb*), SK5006 (wild type), IBPC637 (*rne rnc*) and IBPC642 (*ams*) grown at 30°C to an OD_{650nm} of 0.25 before being shifted to 44°C. Aliquots were withdrawn 30 min later. Twenty micrograms of RNAs were then mixed with the 3′ labelled probe shown in Figure 1, denatured, hybridized, digested by S1 nuclease and analyzed on an 8% polyacrylamide/50% urea gel. The 'tRNA' lane is a control experiment in which 20 µg tRNA were added instead of mRNA. Lane M shows the migration of the 5′ end-labelled fragments of pBR322 cleaved by *Msp*I which were used as size markers. Genotypes of the strains are indicated at the top of each lane. The 3′ ends responsible for the protection of the probe: M2, M, t1, RIII and RIII* (see Figure 1) are indicated on the left of the picture. 'pr' shows the location of the full length probe and A refers to a fragment ending at the bottom of the potential hairpin which is recognized by RNase III (Régnier and Portier, 1986). This RNA species probably results from the impeding of the 3′–5′ exonucleolytic degradation by the hairpin in the RNase III-deficient cells.

cleolytic enzyme(s) which nibbles the 3′ end of P1-RE. This exonucleolytic degradation probably also accounts for the smear detected on Northern blots of *pnp rnb* mRNA (Figure 2 and data not shown).

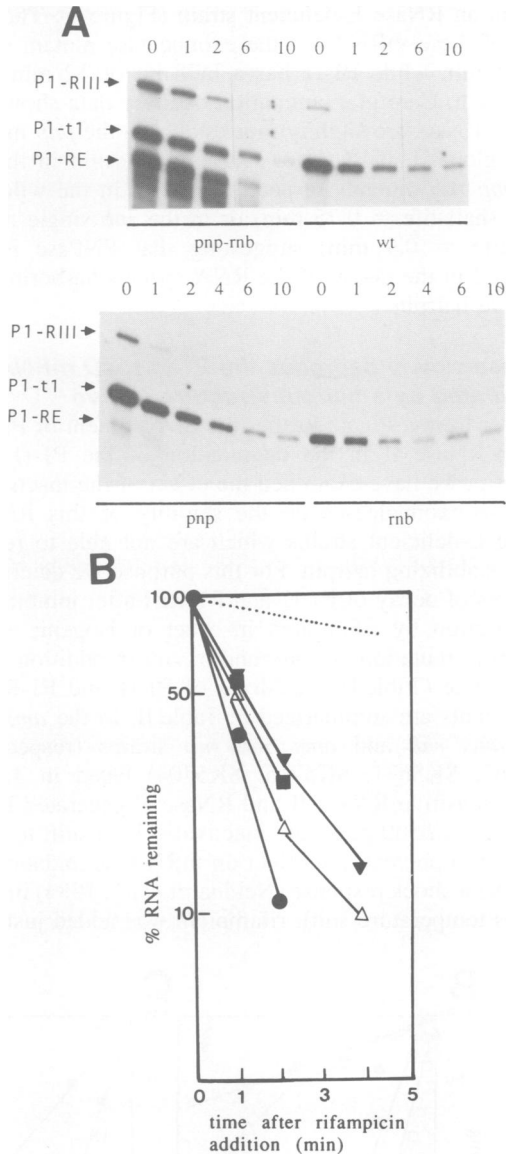

Fig. 4. The RNase E cleavage at M2 is a limiting step of the decay of the *rpsO* mRNA. Strains SK5003 (*pnp rnb*), SK5004 (*pnp*), SK5005 (*rnb*) and SK5006 (wild type) were grown at 30°C to an OD_{650nm} of 0.25 before being shifted to 44°C. Rifampicin was added at a final concentration of 500 μg/ml 30 min after the shift and total RNA was prepared from aliquots withdrawn immediately (0) and at successive times after the addition of the antibiotic which are indicated in minutes at the top of each lane. (**A**) Equal amounts of total RNA (5 μg) were separated on polyacrylamide urea gels, electrotransferred to a nylon membrane, probed for *rpsO* mRNA and autoradiographed. Genotypes are indicated beneath panels showing autoradiographs of the Northern blots. P1-RE, P1-t1 and P1-RIII show the locations of the different *rpsO* mRNA species. (**B**) After densitometric quantification of the different *rpsO* mRNA species the relative amounts of P1-RIII+P1-t1 *rpsO* mRNAs in each lane were expressed as percent of the value at the time of rifampicin addition in the same strain and plotted as a function of time: *pnp* (▼), *pnp rnb* (■), *rnb* (△) and wild type (●). The points of the biphasic kinetics which were not used to estimate the initial decay rate of the *rpsO* mRNA are not included in (B). The kinetic of decay of the P1-t1 *rpsO* mRNA in the RNase E-deficient strain SK5665 taken from Figure 6B is shown by a dotted line.

As expected from previous reports showing that the *ams*1 and *rne*3071 mutations are allelic and deficient for RNase E at 44°C (Mudd *et al.*, 1990b; Babitzke and Kushner, 1991; Melefors and von Gabain, 1991; Tarasevic-iene *et al.*, 1991) we found that the accumulation of *rpsO* mRNA observed in the *rne* strain at non-permissive temperature (Régnier and Hajnsdorf, 1991) also occurs in the SK5665 *ams* strain at 44°C (see Figure 8 below).

PNPase and RNase II are both involved in the degradation of the RNase E processed P1-RE rpsO mRNA

The roles of PNPase and RNase II in the degradation of P1-RE have been investigated by comparing the accumulation of this RNA species at 44°C in the SK5004 *pnp* and SK5005 *rnb* single mutants, and in the *pnp rnb* double mutant and wild type isogenic strains above (Table I). The single mutants have the same alleles (*pnp*7 or *rnb*500) which are present in combination in the double mutant. Northern blot analysis shows that a small amount of P1-RE is detected in the *pnp* mutant but not in the *rnb* strain (Figure 4) even when the autoradiograph is exposed for a long time (data not shown). In confirmation of this result the S1 nuclease analysis detects the 3′ ends of P1-RE in the *pnp* but not in the *rnb* strain (Figure 3). However, in both Northern blots and S1 nuclease analysis the intensity of the signal corresponding to P1-RE in the *pnp* mutant is much lower than in the *pnp rnb* double mutant. These data clearly demonstrate that the RNase II activity in a PNPase-deficient strain and PNPase in the RNase II-deficient cells are sufficient for the 3′ exonucleolytic degradation of P1-RE *in vivo*. However, they indicate that PNPase is more efficient than RNase II.

The P1-RIII RNase III processed transcript is also degraded by RNase II and PNPase

Both Northern blot and 3′ S1 nuclease mapping analysis of *rpsO* transcripts show signals in wild type and exonuclease-deficient strains corresponding to the RNase III processed molecules (RIII and RIII* in Figure 3 and P1-RIII in Figure 5) which are not detected in the *rne rnc* (Figure 3) and *rnc* (Figure 5) RNase III-deficient strains. P1-RIII refers to the molecule processed at the RNase III site proximal to t1 (Figure 1). The stabilization of P1-RIII observed at 44°C after inhibition of transcription initiation by rifampicin in the *pnp rnb* double mutant compared with the isogenic wild type strain and the *pnp* and *rnb*

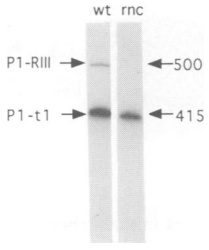

Fig. 5. Identification of the RNase III processed *rpsO* mRNA. Five micrograms of RNA from strains N3433 (wild type) and IBPC633 (*rnc*) were analyzed on a Northern blot and probed for *rpsO*. P1-t1 and P1-RIII locate the different *rpsO* mRNA species. Genotypes of the strains (wild type and *rnc*) are indicated at the top of the lanes and lengths of mRNA (in nucleotides) on the right of the picture.

single mutants demonstrates that this mRNA species can be degraded by both PNPase and RNase II (Figure 4A). The higher amount of the P1-RIII transcript obtained in the *pnp* mutant strain relative to the *rnb* mutant suggests that this species is degraded more efficiently by PNPase than by RNase II (Figure 4A). It appears that both endonucleolytically processed *rpsO* mRNAs, harboring a 3′ extremity unable to fold into a stable hairpin (P1-RE and P1-RIII), are degraded by PNPase and RNase II.

The RNase E cleavage is the limiting step in the decay of the rpsO transcript

The accumulation of P1-t1 in RNase E-deficient strains harboring the *rne*3071 or *ams*1 mutations and the failure to detect the P1-RE species in a wild type strain raise the possibility that the RNase E cleavage which removes the 3′ terminal hairpin structure is the limiting step in the decay of P1-t1. If this is true the disappearance of the *rpsO* mRNAs which contain this RNase E site (P1-t1 + P1-RIII) should be independent of the exonucleolytic activity of the cell. The degradation of P1-RIII by exonucleases probably does not affect these kinetics since it presumably yields an RNA species similar in structure to P1-t1. As predicted, the decay rates of P1-t1 and P1-RIII measured at 44°C after addition of rifampicin in wild type and isogenic *pnp, rnb* and *pnp rnb* mutants are much faster than in an RNase E-deficient strain (Figure 4). The half-lives of these mRNAs in the exonuclease mutant strains are ~1 min while P1-t1 has a half-life of 12 min in an *ams* strain. Careful examination of these data shows that these mRNAs are slightly more stable in the *pnp* mutants (their global half-lives are 1.25 and 1.1 min in the *pnp* and *pnp rnb* mutants respectively) than in the wild type strain (half-life = 0.75 min) or in the *rnb* single mutant (half-life = 0.9 min) suggesting that PNPase is also involved in the decay of the RNA species harboring a 3′ terminal hairpin.

Table II. Half-lives of *rpsO* mRNAs in RNase mutants

Strains	Half-life (minutes)	
	P1-t1	P1-RIII[a]
ams	12	0.6
ams pnp	15	0.6
ams rnb	1.8	0.6
ams pnp rnb	2.5	1.9

These half-lives are deduced from the kinetics of Figure 6. The half-life of P1-t1 in a wild type strain at the same temperature (44°C) is ~0.75 min.

[a]These estimations of half-lives of P1-RIII are given to facilitate the comparison of its stability in different strains.

PNPase slowly degrades the P1-t1 rpsO mRNA terminated by a hairpin structure in vivo

In order to investigate further the involvement of PNPase and/or RNase II in the degradation of the P1-t1 *rpsO* transcript we have examined the effect of the inactivation of these exonucleases on the stability of this RNA in RNase E-deficient strains which are not able to remove the 3′ stabilizing hairpin. For this purpose we determined the rates of decay of P1-t1 and P1-RIII after inhibition of transcription by rifampicin in a set of isogenic strains harboring mutations in *pnp* and/or *rnb* in addition to the *ams*1 allele (Table I). Half-lives of P1-t1 and P1-RIII in the mutants are summarized in Table II. In the *ams, ams pnp, ams rnb* and *ams pnp rnb* strains (respectively SK5665, SK5671, SK5715, SK5704) listed in Table I, thermosensitive RNase II and RNase E generated by the *ams*1 and *rnb*500 genes are inactivated by a shift to 44°C. In order to prevent any effect on mRNA degradation due to the heat shock response (Neidhardt *et al.*, 1984) induced by this temperature shift, rifampicin was added just prior

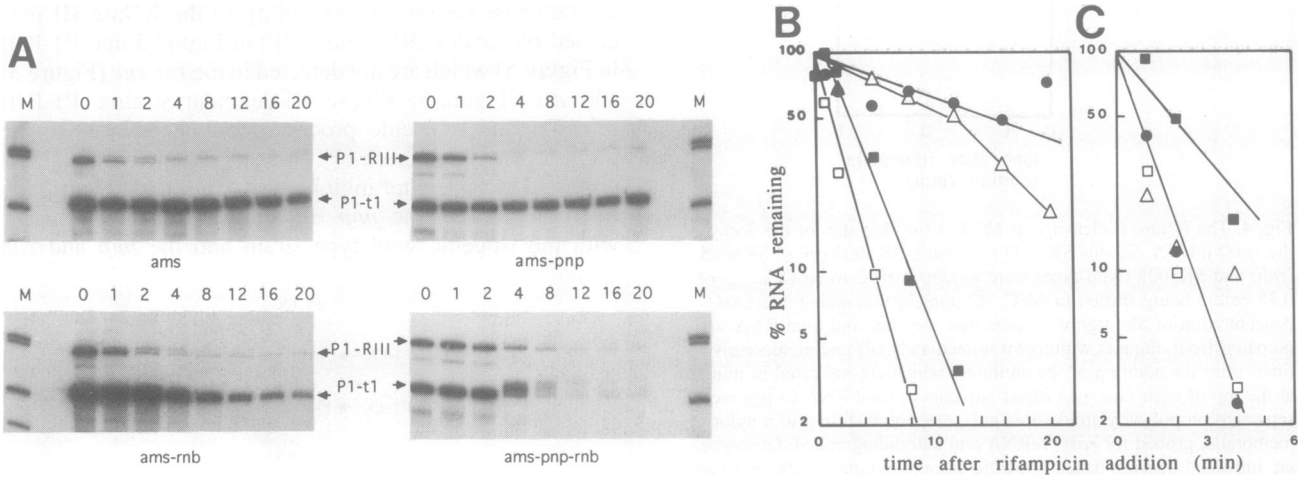

Fig. 6. Role of exonucleases in the stability of the *rpsO* mRNA. (**A**) Strains SK5665 (*ams*), SK5671 (*ams pnp*), SK5715 (*ams rnb*) and SK5704 (*ams pnp rnb*) were grown at 30°C until the OD_{650nm} had reached 0.25. Rifampicin was added to a final concentration of 500 μg/ml and the culture shifted immediately to 44°C. Five micrograms of total RNA extracted from aliquots withdrawn at the different times following antibiotic addition indicated at the top of each lane were then analyzed on Northern blots and probed for *rpsO* mRNA. The genotypes of strains are indicated beneath each panel. (**B** and **C**) The relative amounts of both P1-t1 (panel B) and P1-RIII (panel C) mRNAs in the *ams* (△), *ams pnp* (●), *ams rnb* (□) and *ams pnp rnb* (■) strains were quantified with a PhosphorImager, expressed as a percentage of the highest value obtained and plotted as a function of time. The faint band migrating just above the P1-RIII mRNA species which is destabilized in the *ams rnb* and the *ams pnp rnb* strains probably corresponds to a mRNA harboring a 3′ terminal hairpin (see text). However, since the structure of this molecule is not known it has not been taken into account in the quantifications.

to the temperature shift. The slow degradation of P1-t1 observed in the *ams* strain (half-life = 12 min) compared to the SK5006 wild type strain (half-life = 0.75 min) demonstrates that thermosensitive RNase E is very rapidly inactivated after a shift to 44°C (Figure 6). The slightly longer half-life measured in the *ams pnp* double mutant (15 min) is compatible with the hypothesis that PNPase participates in the degradation of the P1-t1 *rpsO* mRNA which is largely but not completely protected from exonucleases by a 3′ terminal hairpin (Figure 6). In agreement with this notion we found that the BL321 *rnc* strain deficient for RNase III which contains 10 times more PNPase activity than the isogenic BL322 *rnc*$^+$ strain (Portier *et al.*, 1987) degrades the *rpsO* mRNA about twice as fast as the wild type strain (Figure 7). Half-lives are 2 min in the *rnc* strain and 5 min in the wild type at 37°C. The detection by S1 nuclease mapping of a series of 3′ extremities in the IBPC642 *ams* strain, mapping just upstream of the site of transcription termination reinforces the conclusion that the 3′ end of P1-t1 is processed exonucleolytically (Figure 3).

The P1-t1 rpsO mRNA which is terminated by a hairpin structure is destabilized in the ams pnp rnb strain

Surprisingly, the inactivation of thermosensitive RNase II at the non-permissive temperature in the *ams rnb* strain has a destabilizing effect on the P1-t1 *rpsO* mRNA harboring the 3′ hairpin of the terminator (Figure 6A). Figure 6B shows that its half-life drops from 12 min in the *ams* strain to <2 min in the *ams rnb* double mutant. These data suggest that *in vivo* RNase II may protect P1-t1 from RNases.

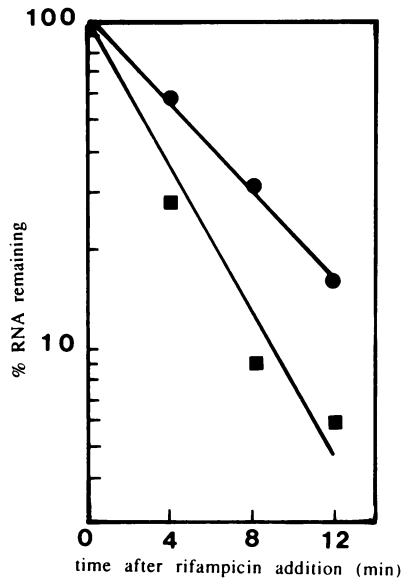

Fig. 7. *rpsO* mRNA decays more rapidly in a strain overproducing PNPase. Rifampicin was added to a final concentration of 500 µg/ml in cultures of strains BL321 (*rnc*) and BL322 (wild type) growing exponentially at 37°C. RNA prepared from aliquots withdrawn at successive times after rifampicin addition were analyzed on Northern blots and probed for *rpsO*. The relative amounts of *rpsO* mRNA were quantified with a PhosphorImager, expressed as a percentage of the value at the time of rifampicin addition (time 0) and plotted as a function of time; wild type (●), *rnc* (■).

Because PNPase was found to be able to degrade slowly the P1-t1 *rpsO* mRNA with a 3′ hairpin the destabilization of this species following the inactivation of RNase II could be due to an increased sensitivity of this mRNA to PNPase. However, this RNA species remains unstable in an *ams pnp rnb* strain (half-life = 2.5 min). Thus, the transcript's degradation after RNase II inactivation occurs in cells deficient for PNPase (Figure 6A and B). However, the slight stabilization observed in the *ams pnp rnb* strain relative to the *ams rnb* strain (Figure 6 and Table II) might indicate that the modest contribution by PNPase to the degradation of P1-t1 can be inhibited by active RNase II.

To exclude the possibility that the destabilization of P1-t1 in the *ams rnb* and *ams pnp rnb* strains is an artefact caused by the use of rifampicin, we verified that, in the

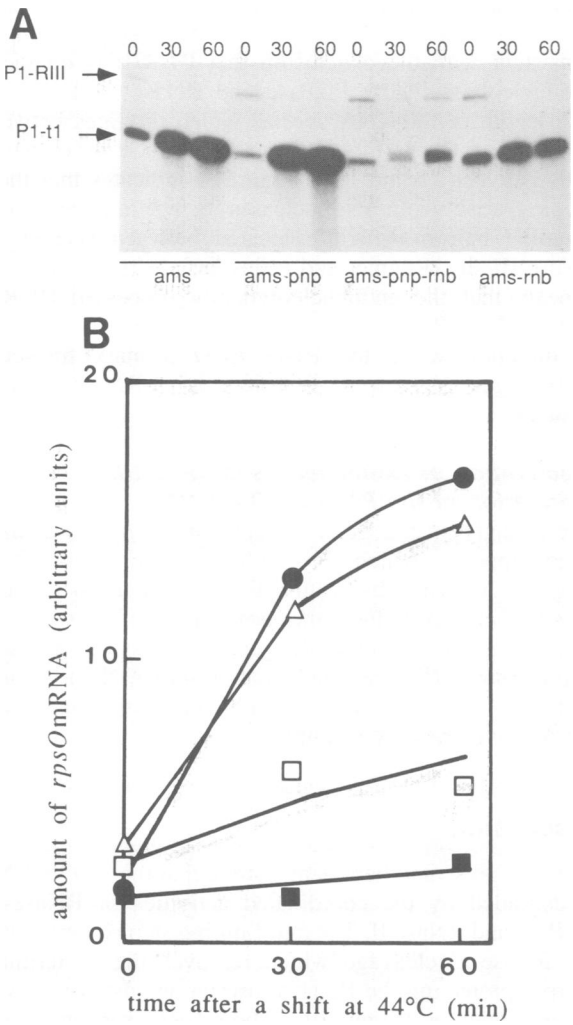

Fig. 8. Accumulation of *rpsO* mRNA in RNase mutants at non-permissive temperature. (**A**) Strains SK5665 (*ams*), SK5671 (*ams pnp*), SK5715 (*ams rnb*) and SK5704 (*ams pnp rnb*) were grown at 30°C up to an OD_{650nm} of 0.25 before shifting to 44°C. Total RNA extracted from aliquots withdrawn at different times after the shift of temperature, indicated in minutes at the top of each lane, was analyzed on a Northern blot (5 µg) and probed for *rpsO* mRNA. The genotypes of the strains are indicated beneath the autoradiograph. (**B**) The relative amounts of P1-t1 in the *ams* (△), *ams pnp* (●), *ams rnb* (□) and *ams pnp rnb* (■) strains were quantified with a Hoefer densitometer and plotted as a function of time. Shorter exposure shows that the fuzzy bands of P1-t1 visible in the *ams pnp rnb* strain 30 and 60 min after the shift are not detectable in the other strains.

absence of the antibiotic, the accumulation of the stable P1-t1 *rpsO* mRNA resulting from the inactivation of RNase E in the *ams* and *ams pnp* strains is dramatically reduced in the *ams rnb* and *ams pnp rnb* strains deficient for RNase II (Figure 8). Assuming that transcription is not significantly affected by the exonuclease mutations these data suggest that P1-t1 is destabilized by the *rnb* mutation and that this instability cannot be caused only by PNPase.

Unlike the P1-t1 mRNA terminated by the 3′ hairpin, the P1-RIII mRNA has a single-stranded sequence extending 80 nucleotides downstream of t1 (Figure 1). The stability of this species is not affected by RNase II inactivation in the *ams rnb* and *ams pnp rnb* strains. In fact this RNA species is more stable and more abundant relative to P1-t1, in the *ams pnp rnb* mutant deficient for exonucleases than in the isogenic *ams, ams pnp* and *ams rnb* strains (Figure 6A and C and Table II). This is consistent with the conclusion that P1-RIII is degraded exonucleolytically by PNPase and RNase II (Figure 4). The length of the RNA species which migrates between P1-RIII and P1-t1 (Figure 6A) and the location of the 3′ ends between t1 and RIII (Figure 3) indicates that these species arise from exonucleases being impeded by a potential hairpin structure located between t1 and the RNase III site (Régnier and Hajnsdorf, 1991). It therefore appears that the endonucleolytically processed P1-RIII and P1-RE mRNAs are degraded by RNase II in the 3′–5′ direction while the P1-t1 *rpsO* primary transcript harboring a 3′ hairpin is protected by RNase II from RNases.

Inactivation of exonucleases induces the elongation of the P1-t1 rpsO mRNA

Interestingly, in the triple mutant (*ams pnp rnb*) a fuzzy band appears which is slightly longer than P1-t1 (Figure 6A). The observation that a shift at 44°C alone is sufficient to induce the production of a smear of heterogeneous molecules longer than P1-t1 in the *ams pnp rnb* strain (Figure 8 and data not shown) demonstrates that these molecules do not result from a modification of RNA polymerase by rifampicin.

Discussion

The data presented here demonstrate that the *rpsO* mRNA is degraded by the coordinated activities of RNase E, PNPase and RNase II. The rate-limiting step is presumably the RNase E cleavage which removes the 3′ terminal hairpin protecting the P1-t1 transcript against the activity of exonucleases (Figure 9) . The P1-RE truncated *rpsO* mRNA lacking this motif is degraded by the 3′–5′ exonucleases PNPase and RNase II. This latter step is so rapid that the P1-RE mRNA cannot be detected in a wild type strain. Although no other cutting sites dependent on the wild type allele of the *rne/ams* gene were found upstream of the terminator hairpin *in vivo* (Régnier and Hajnsdorf, 1991) it cannot be completely excluded that the *rpsO* message is cleaved at other sites by RNase E. Similarly the *glyA* mRNAs devoid of their 3′ protective hairpins are also sensitive to both 3′–5′ exonucleases (Plamann and Stauffer, 1990). Detection of P1-RE in the *pnp* but not in an *rnb* strain suggests that the RNase E generated 3′ extremity is degraded more rapidly by PNPase than by RNase II. This could reflect a difference in affinity of the two enzymes for the 3′ extremity and/or the existence of pause sites (maybe secondary structures) which specifically slow down the progression of RNase II. In agreement with this hypothesis, RNase II has been described to be more impeded by secondary structures than PNPase (Thang *et al.*, 1967; Gupta *et al.*, 1977; McLaren *et al.*, 1991). The *sib* hairpin at the 3′ end of the *int* message was also found to be more resistant to RNase II than to PNPase (Guarneros and Portier, 1990).

Our data also suggest that PNPase is able to degrade the P1-t1 *rpsO* mRNA harboring the 3′ terminal hairpin (Figure 9). This is in agreement with previous conclusions that PNPase can degrade highly structured RNAs completely (Thang *et al.*, 1967). However, the weak stabilization observed in an *ams pnp* strain relative to the *ams* strain or in the *pnp* strain relative to the wild type strain demonstrates that in wild type cells, the degradation of the *rpsO* mRNA is mostly mediated by the RNase E cleavage.

Interestingly, we found that the inactivation of RNase II has opposite effects on the stability of the *rpsO* mRNA species differing by the structure of their 3′ extremities. It is striking that the stable P1-t1 mRNA species protected from exonuclease attack by a 3′ terminal hairpin is destabilized upon RNase II inactivation while the P1-RE and P1-RIII unstable molecules harboring unstructured 3′ ends sensitive to PNPase and RNase II are stabilized. These data suggest that RNase II protects the RNA species terminated by a hairpin from the degradation machinery. The existence of such factors able to improve the stabilizing effect of 3′ terminal hairpins was postulated on the basis of the different stabilities conferred upon a molecule by such structures *in vivo* and *in vitro* (McLaren *et al.*, 1991). Based on the properties of RNase II it can be proposed that this protection results from the stalling of this nuclease just downstream of the hairpin which stops

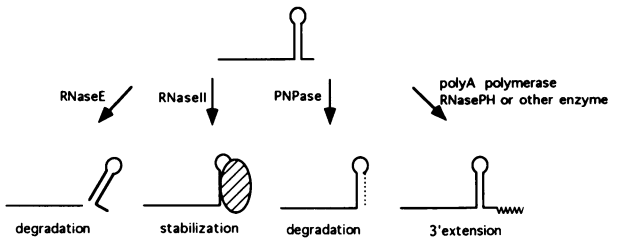

Fig. 9. Interactions of the 3′ terminal hairpin of the *rpsO* mRNA with RNases and polymerases. The recognition site of RNase E was proposed to include an AU-rich, single-stranded cutting site and a hairpin structure located downstream (Ehretsmann *et al.*, 1992b). The hairpin structure of the transcription terminator is likely to be the site of interaction between the *rpsO* mRNA and RNase E cutting this RNA at M2 (Régnier and Hajnsdorf, 1991). The fact that elongated *rpsO* mRNAs are observed in the *ams pnp rnb* triple mutant but not in the *pnp rnb* mutant suggests that active RNase E could prevent 3′ extension of the molecule maybe by binding the terminal hairpin. RNase II, impeded by the terminator stem–loop structure, presumably protects the RNA from the attack of PNPase and other unidentified RNases. PNPase slowly degrades the base paired regions of the hairpin before degrading the upstream message. The 3′ stem–loop might also be a preferential site of interaction for enzymes able to add ribonucleotides to the 3′ end of mRNA (Cao and Sarkar, 1992).

its processive degradation of the mRNA (Figure 9) (Mott *et al.*, 1985). Messengers which do not have stable hairpin structures able to stop RNase II degradation are stabilized upon RNase II inactivation in the absence of PNPase. How can the stalled RNase II stabilize an mRNA? Assuming that RNase II and PNPase compete for binding at the 3′ end of mRNA, it could be that stalled RNase II inhibits PNPase exonucleolytic degradation by sequestering RNA 3′ ends as suggested for the IS10 antisense RNA (Pepe *et al.*, 1994). However, the rapid decay of the *rpsO* mRNA in the *ams pnp rnb* strain implies that RNase II also protects against degradation enzymes distinct from RNase E and PNPase which have not yet been identified. We believe that measurements of mRNA decay which are performed in RNase-deficient strains under lethal conditions reflect the normal physiological activity of RNases involved in mRNA degradation for two reasons. First, the destabilization of P1-t1 observed in the *ams pnp rnb* triple mutant at 44°C is coincident with a stabilization of the P1-RIII mRNA. These observations exclude that a non-specific extracellular or periplasmic nuclease e.g. RNase I is imported in the cytoplasm and degrades RNA. Second, since rifampicin is added just before the shift at 44°C, the elongation and the destabilization of P1-t1 occurring after this shift are obviously catalyzed by enzymes preexisting in the growing cells. It is possible that broad specificity nucleases identified in the cells e.g. RNase I* (Cannistraro and Kennell, 1991) RNase M (Cannistraro and Kennell, 1989) and RNase R (Deutscher, 1988; Srivastava *et al.*, 1992) are involved in this degradation process. More likely, other identified or unidentified 3′–5′ exonucleases (Kasai *et al.*, 1977; Deutscher, 1993) together with residual phosphorolytic activity of PNPase (Reiner, 1969), which could account for the degradation of the RNase E processed *rpsO* mRNA observed in a *pnp rnb* strain (Figures 3 and 4), could degrade the P1-t1 *rpsO* mRNA in the absence of RNase II. Residual activity of RNase E might also participate in this degradation (Mudd *et al.*, 1988; Melefors *et al.*, 1993; Hajnsdorf *et al.*, 1994). It is also possible that other factors such as RNA helicases (Schmid and Linder, 1992) can melt the hairpin which is no longer protected by RNase II and facilitate the action of residual 3′ exonucleases present in the *ams pnp rnb* mutant. However, in the absence of any direct evidence that RNase II protects the *rpsO* mRNA from the RNA degradation machinery it cannot be excluded that inactivation of RNase II indirectly stabilizes the *rpsO* mRNA. For example RNase II could be associated in a complex with other RNases and act as a negative regulator of their activity. In this case inactivation of RNase II would induce an enhancement of ribonucleolytic activity.

Very interestingly, the complete inactivation of the 3′–5′ exonucleolytic activity in the *ams pnp rnb* strain leads to the appearance of a fuzzy band of *rpsO* mRNAs, slightly longer than the *rpsO* primary transcript, ranging from 415 to 440 nucleotides. These molecules, appearing 4 min after the inhibition of transcription initiation by rifampicin, cannot result from the activation of an upstream promoter. They might originate either from the elongation of the shorter P1-t1 terminated mRNA or from the exonucleolytic degradation of the slightly longer P1-RIII species (Figure 6A). The appearance of longer and longer molecules as a function of time argues in favor of a progressive elongation of P1-t1. Moreover, the fact that the fuzzy band only appears in the *ams pnp rnb* strain deficient for PNPase and RNase II and not in the *ams pnp* or *ams rnb* strains, which still contain 3′ exonucleolytic activity, suggests that the activity which elongates the RNA is in competition with 3′ exoribonucleases and therefore that it results from the addition of nucleotides to the 3′ end of mRNA.

Identification of several messengers harboring short poly(A) tails and of polyadenylated RNAI of ColE1 plasmids suggests that adenine nucleotide residues could be polymerized at the 3′ end of the *rpsO* mRNA (Karnik *et al.*, 1987; Cao and Sarkar, 1992a; He *et al.*, 1993; Xu *et al.*, 1993). In agreement with this hypothesis increased polyadenylation of mRNA and elongation of the *rpsO* mRNA are both observed in strains deficient for exonucleases (Cao and Sarkar, 1992). The demonstration that polyadenylation of RNAI is dependent on poly(A) polymerase encoded by the *pcnB* gene suggests that this enzyme might be responsible for the elongation of the *rpsO* mRNA (He *et al.*, 1993; Xu *et al.*, 1993). However, in the absence of direct evidence that the *rpsO* mRNA is polyadenylated at its 3′ end by poly(A) polymerase, it cannot be excluded that RNase PH, which was shown to add short polynucleotide tails to the 3′ end of RNA *in vitro*, can also catalyze this reaction *in vivo* (Ost and Deutscher, 1990). Elongation of the *rpsO* mRNA in the *ams pnp rnb* strain implies that this reaction is not catalyzed by PNPase. We are now characterizing the 3′ tail of the *rpsO* mRNA and the enzyme involved in its synthesis.

The recent finding that the inactivation of poly(A) polymerase is correlated with the stabilization of RNAI supports the idea that, in bacteria, polyadenylated transcripts are destabilized (He *et al.*, 1993; Xu *et al.*, 1993). Accordingly, it is striking that in the *ams pnp rnb* strain the 3′ extension and the destabilization of the *rpsO* mRNA occur simultaneously. However, the destabilization, in the *ams rnb* strain at 44°C, of P1-t1 which is apparently not elongated suggests that the two processes may not be correlated. We are now investigating if, as in eukaryotes (Brawerman, 1987), the 3′ tail of bacterial mRNA is a structural determinant of their stability.

Hairpin structures were already known to stabilize mRNA by impeding the processive degradation catalyzed by RNase II and PNPase and to be a recognition site for endonucleases (Guarneros, 1988; Régnier and Hajnsdorf, 1991). The data presented in this paper indicate that, in addition to impeding the exonucleases, the formation of a complex between a hairpin and RNase II protects the upstream message from the attack of other nucleases (Figure 9). The 3′–5′ processive activity of RNase II might help the formation of the RNase II hairpin stabilizing complex by guiding RNase II to its target. The intriguing question now arising is the nature of the RNase(s) implicated in the degradation of the *rpsO* mRNA in the cells deficient for RNase E, RNase II and PNPase and how they interact with the hypothetical RNase II–3′ hairpin complex. Moreover our data lead us to consider the possibility that 3′ hairpins are recognition sites for the enzyme(s) able to polymerize nucleotides at the 3′ end of

RNA and that these 3′ extensions of bacterial mRNAs might be one of the determinants of their stability.

Materials and methods

Strains and growth conditions

The bacterial strains used in this report are listed in Table III. JM109 was used for preparation of plasmids. IBPC642 results from the P1 transduction of the *ams* allele of HAK117*zcf*229::Tn*10* into IBPC5321 and IBPC661 from the P1 transduction of the *ams* allele of strain CH1828 into SK5003. IBPC661 was selected as Tet^R ts colonies unable to grow at 42°C while the recipient SK5003 *rnb*(ts) grows at 42°C but not at 44°C. Strains IBPC633, IBPC637, N3433, BL322, BL321 and IBPC642 were grown in MOPS–Tricine medium supplemented with 1 μg/ml thiamine, 0.4% (w/v) glucose, 2 mM potassium phosphate and 0.5% (w/v) casamino acids. Strains SK5003, SK5004, SK5005, SK5006 and IBPC661 were grown in LB medium supplemented with 20 μg/ml chloramphenicol and strains SK5665, SK5671, SK5704 and SK5715 in LB medium supplemented with 50 μg/ml thymine. LB medium was used to allow a better expression of the exonuclease deficiency at 44°C (Donovan and Kushner, 1986).

General methods

Restriction digests, DNA purification, ligation, transformation and gel electrophoresis were performed essentially as described in Sambrook *et al.* (1989).

Probes

The anti-RNA used to probe the *rpsO* messengers was prepared by *in vitro* transcription from the pEHa4 plasmid. This plasmid carries the *DraI–BglI* fragment extending from the translation initiation codon to the transcription terminator of *rpsO*, excised from plasmid pB15-6 (Figure I) (Plumbridge and Springer, 1983). After treatment with the Klenow fragment of DNA polymerase in order to make blunt its two extremities it was ligated to the pGEM5Z(f^-) plasmid (Promega) linearized at *Eco*RV. Anti-RNA complementary to the *rpsO* transcript was transcribed *in vitro* by T7 RNA polymerase from pEHa4 linearized at the *SalI* site of the polylinker in the presence of [α-^{32}P]UTP (3000 Ci/mmol) according to the supplier's instructions.

The 3′ labelled probe used to locate the 3′ extremities of the *rpsO* mRNA by S1 nuclease protection was prepared from a PCR-amplified DNA fragment of 435 nucleotides extending from the 5′ part of the *rpsO* coding sequence to the intercistronic region downstream of the RNase III processing sites (Figure 1). Oligonucleotides were synthesized with a Gene Assembler Plus (Pharmacia LKB, Sweden). The sequences of the upstream and downstream primers were respectively 5′-GCAAA-CGACACCGGTTCTAC-3′ and 5′-GTACCTTACGGCACTGGTGT-3′. Plasmid pB15.6 carrying the *rpsO pnp* region of the *E.coli* chromosome was used as a template. After phenol extraction and ethanol precipitation the amplified DNA fragment was cut at the *Hpa*II site located in *rpsO* to give a DNA fragment of 424 bp harboring a 5′ protruding extremity. After purification on a polyacrylamide gel and elution this fragment was labelled at the *Hpa*II site by the Klenow fragment of DNA polymerase I and [α-^{32}P]dCTP 3000 Ci/mmol as previously described (Régnier *et al.*, 1987).

RNA preparations

RNAs from cells grown in MOPS–Tricine medium were prepared as previously described (Bardwell *et al.*, 1989). When bacteria were grown in LB medium, 10 ml aliquots were rapidly mixed with an equal volume of ethanol preequilibrated at –70°C and cells were pelleted 10 min at 6000 r.p.m. in the JA20 rotor of a Beckman centrifuge. Then they were resuspended in 1.5 ml of cold 10 mM Tris–HCl pH 7.3, 10 mM potassium chloride, 5 mM magnesium chloride buffer before mixing with 1.5 ml of 20 mM Tris–HCl pH 7.9, 200 mM NaCl, 40 mM EDTA, 1% SDS lysis buffer. Cells were lysed for 2 min at 95°C and the tubes were chilled on ice. RNAs were extracted twice at 65°C with 3 ml of phenol saturated with water and once with chloroform at room temperature. RNAs were then precipitated, washed with ethanol and dissolved in diethylpyrocarbonate-treated water.

S1 nuclease mapping

S1 nuclease mapping was performed as previously described (Régnier and Portier, 1986) except that RNAs and the radioactive DNA probe, both dissolved in water, were lyophilized prior to solubilization in the hybridization buffer. Radioactive DNA fragments protected from S1 nuclease digestion were analyzed on an 8% polyacrylamide–50% urea gel.

Northern blots

Five micrograms of RNA preparations were separated on 6% polyacrylamide/50% urea sequencing gels and electrotransferred to Hybond N^+ (Amersham) in an IDEA Scientific (Minneapolis, MN) cell filled with 10 mM Tris–acetate pH 7.8, 0.5 mM EDTA, 5 mM sodium acetate buffer. RNA was fixed to the membrane in 50 mM NaOH for 5 min. Then the membrane was neutralized for 5 min in 2 × SSPE before being prehybridized and hybridized to the ^{32}P-labelled RNA probe essentially as previously described except that hybridization was carried out at 55°C and washes at 68°C (Régnier and Hajnsdorf, 1991). The amounts of probe hybridized to the *rpsO* mRNA were quantified with a

Table III. Strains used

Strain	Genotype and relevant markers	Source/origin
BL322	*thi*-1, *argH*1, *gal-6*, *galY*1, *mtl-2*, *xyl-7*, *malA*1, *ara-13*, *str-9*, *tonA*2, SupE44	W.Studier
BL321	BL322 *rnc*105	W.Studier
CH1828	MC1061, *ams*1(ts), *zce*726: Tn*10*	Mudd *et al.* (1990b)
HAK117	F^-, *thr*, *leu*, *proA*, *argE*, *rpsL*, *gal*, *ams*1(ts)	Ono and Kuwano (1979)
N3433	HfrH, *lacZ*43, λ^-, *relA*1, *spoT*1, *thi*-1	D.Apirion
IBPC5321	F^-, *thi-1*, *argG*6, *argeE*3, *his-4*, *mtl-1*, *xyl-5*, *tsx-29*, *rpsL*, Δ*lacX74*	Plumbridge *et al.* (1985)
IBPC633	N3433, *rnc*105, *nadB*51::Tn*10*	Régnier and Hajnsdorf (1991)
IBPC637	N3433, *rne*3071(ts), *rnc*105, *nadB*51::Tn*10*	Régnier and Hajnsdorf (1991)
IBPC642	F^-, *thi-1*, *arg*G6, *arg*E3, *his-4*, *mtl-1*, *xyl-5*, *tsx-29*, *rps*L, Δ*lac*X74, *ams1*(ts), *zcf*229::Tn*10*	This work
IBPC661	F^-, *thr*, *leu*, *rnb*500(ts) pDK39[Cm^R*rnb*500(ts)], *pnp7*, *ams1*(ts), *zce*726::Tn*10*	This work
SK5003	F^-, *thr*, *leu*, *rnb*500(ts), *pnp7*, pDK39[Cm^R, *rnb*500(ts)]	Donovan and Kushner (1986)
SK5004	F^-, *thr*, *leu*, *pnp7*, pDK39[Cm^R*rnb*500(ts)]	Donovan and Kushner (1986)
SK5005	F^-, *thr*, *leu*, *rnb*500(ts)pDK39[Cm^R*rnb*500(ts)]	Donovan and Kushner (1986)
SK5006	F^-, *thr*, *leu*, pDK39[Cm^R*rnb*500(ts)]	Donovan and Kushner (1986)
SK5665	F^-, *thy*A715, *ams*1(ts)	Arraiano *et al.* (1988)
SK5671	F^-, *thy*A715, *ams*1(ts), *pnp*7	Arraiano *et al.* (1988)
SK5704	F^-, *thy*A715, *ams*1(ts), *pnp*7, *rnb*500(ts)	Arraiano *et al.* (1988)
SK5715	F^-, *thy*A715, *ams*1(ts), *rnb*500(ts)	Arraiano *et al.* (1988)
JM109	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_k^-, m_k^+), *relA*1, *SupE*44, λ^-, Δ(*lac-proAB*), F′, (*traD*36, *proAB*, $lacI^q$, *lacZ*ΔM15)	

PhosphorImager (Molecular Dynamics, Sunnyvale, CA) or by densitometric analysis of autoradiographs (Hoefer Scientific Instruments).

Acknowledgements

We are grateful to M.Grunberg-Manago and M.Springer for providing supports and facilities. We are indebted to H.Krisch, A.Carpousis and J.Plumbridge for critical reading of the manuscript and to C.Arraiano for fruitful discussions. S.Kushner and C.Arraino are acknowledged for the gift of strains and R.Simons for communication of results prior to publication. This work was supported by grants from the Université Paris VII to P.R. (Contrat DRED), from CNRS (URA 1139), from ARC and from Ministère de la Recherche et de l'Espace (contract no. 92 C 0315).

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Received on October 25, 1993; revised on April 7, 1994