

# The *rpsO* mRNA of *Escherichia coli* is polyadenylated at multiple sites resulting from endonucleolytic processing and exonucleolytic degradation

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**The *rpsO* monocistronic messenger, encoding ribosomal protein S15, is destabilized upon polyadenylation occurring at the hairpin structure of the transcription terminator t1. We report that mRNA fragments differing from the monocistronic transcript by their 3' termini are also polyadenylated in the absence of polynucleotide phosphorylase and RNase II. Some of these 3' extremities result from endonucleolytic cleavages by RNase E and RNase III and from exonucleolytic degradation. Most of these mRNA fragments are destabilized upon polyadenylation with the exception of the RNA species generated by RNase III. RNase E appears to reduce the amount of poly(A) added at the transcription terminator t1.**

**Keywords:** 3' to 5' exonucleases/mRNA polyadenylation/mRNA stability/poly(A) polymerase I/RNase E

## Introduction

Post-transcriptional addition of poly(A) tails to the 3' ends of mRNAs, which was first identified in eukaryotic cells, now appears to be a characteristic of all living organisms (Brawerman, 1981; Littauer and Sorek, 1982; Cohen, 1995; Hajnsdorf *et al.*, 1995; O'Hara *et al.*, 1995). There is considerable evidence that the long poly(A) tails of eukaryotic mRNAs are structural determinants of their stabilities (Baker, 1993) while, in contrast, the shorter oligo(A) tails found at the 3' ends of some bacterial mRNAs (Sarkar *et al.*, 1978; Karnik *et al.*, 1987; Cao and Sarkar, 1992a, 1993) have been recently shown to promote their degradation (Hajnsdorf *et al.*, 1995; O'Hara *et al.*, 1995). The *pcnB* gene coding for poly(A) polymerase I (PAP I) of *Escherichia coli* has been cloned and sequenced (Liu and Parkinson, 1989; March *et al.*, 1989; Cao and Sarkar, 1992b), and a second poly(A) polymerase, PAP II, has been identified in a *pcnB* deletion strain (Kalapos *et al.*, 1994).

The *rpsO* mRNA, which encodes ribosomal protein S15 of *E. coli*, is one of the RNAs destabilized upon polyadenylation (Hajnsdorf *et al.*, 1995). The *rpsO* gene forms an operon with the downstream *pnp* gene, which encodes polynucleotide phosphorylase (PNPase) (Régnier and Portier, 1986; Régnier *et al.*, 1987), one of the exonucleases involved in mRNA degradation (Figure 1) (Ehretsmann *et al.*, 1992a). The most abundant *rpsO* transcript, hereafter referred to as P1-t1, is monocistronic

and extends from the promoter P1 to the Rho-independent transcriptional terminator t1 located just downstream of *rpsO* (Hajnsdorf *et al.*, 1994b) (Figure 1). The RNase E cleavage occurring in the dicistronic *rpsO-pnp* transcript at the M site just downstream of t1 (Figure 1), produces a mRNA species which cannot be distinguished from P1-t1 on a Northern blot (Régnier and Hajnsdorf, 1991; Hajnsdorf *et al.*, 1994b). Another endonucleolytic cleavage made by RNase III in the dicistronic transcript gives rise to a P1-RIII *rpsO* mRNA that is 82 nucleotide longer (Régnier and Portier, 1986; Hajnsdorf *et al.*, 1994b) (Figure 1). The initial and limiting step in the degradation of the *rpsO* message is the endonucleolytic cleavage made by RNase E at the M2 site, between the *rpsO* coding sequence and the hairpin of the transcriptional terminator, which protects the message from the exonucleolytic attack (Figure 1) (Régnier and Hajnsdorf, 1991; Hajnsdorf *et al.*, 1994b). Subsequently, the 3' to 5' exonucleases PNPase and presumably RNase II, carry out the rapid processive degradation of the P1-M2 RNase E processed molecule (Hajnsdorf *et al.*, 1994b; Braun *et al.*, 1996). Two intermediary products of this degradation pathway, fragments *b* and *c*, which differ from P1-M2 by their 3' termini (Figure 1) are detected when there is no PNPase in the cell (Braun *et al.*, 1996).

Simultaneous inactivation of the three ribonucleases implicated in the RNase E-dependent degradation pathway of the *rpsO* mRNA (RNase E, PNPase and RNase II) does not completely abolish the degradation of the *rpsO* transcript (Hajnsdorf *et al.*, 1994b, 1995). It has been demonstrated that a poly(A) tail added post-transcriptionally downstream of t1 renders the P1-t1 mRNA susceptible to the attack of unidentified RNases (Hajnsdorf *et al.*, 1995). Polyadenylation of P1-t1 requires the activity of PAP I (Hajnsdorf *et al.*, 1995). Moreover, characterization of a mRNA-poly(A) junction internal to the *rpsO* coding sequence suggested that polyadenylation could take place at multiple sites and contribute to the degradation of other *rpsO* mRNAs.

We show here that polyadenylation of *rpsO* occurs at many locations including the RNase E, RNase III and exonuclease cleavage sites. Our data also indicate that mRNAs processed by RNase E at M2 are polyadenylated more efficiently than the P1-t1 species harbouring the 3' terminal hairpin, and that polyadenylation contributes to the degradation of P1-M2 and other mRNA fragments.

## Results

### **The *rpsO* mRNA is polyadenylated at several sites in addition to t1**

The locations of polyadenylation sites at the 3' ends of the *rpsO* mRNAs were determined previously by amplification and cloning of cDNAs initiated with an

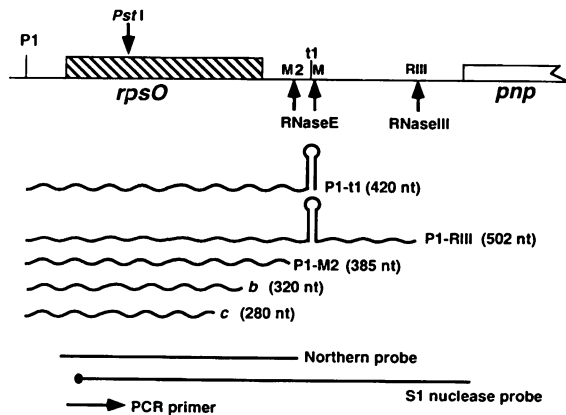
oligo(dT<sub>18</sub>) primer hybridized to poly(A) tails (Hajnsdorf *et al.*, 1995). Identification of one clone containing an mRNA–poly(A) junction at nucleotide 282, in the coding sequence of *rpsO*, in addition to 32 clones harbouring poly(A) tails at t1 (Hajnsdorf *et al.*, 1995) suggested that the *rpsO* mRNA is polyadenylated at sites other than t1. In order to test this possibility, we cloned, in bulk, the PCR fragments amplified from cDNA initiated with an oligo(dT<sub>18</sub>) primer. Our experimental procedure did not include gel purification of the major cDNA band, a step presumably favouring identification of the polyadenylation site at t1 (Hajnsdorf *et al.*, 1995). We assumed that the amounts of cDNAs obtained by reverse transcription and amplification and subsequently, the numbers of the cDNA

clones containing the different mRNA–poly(A) junctions would be proportional to the relative abundance of the *rpsO* mRNAs polyadenylated at the corresponding sites, if these mRNAs are at similar concentrations in the same RNA preparation.

Strain SK5704(pFB1) harbours the *pnp7* allele encoding inactive PNPase, and the *rmb500*<sup>ts</sup> and *ams1*<sup>ts</sup> alleles encoding thermosensitive RNase II and RNase E, respectively (Arraiano *et al.*, 1988). This strain, deficient for exonucleases (PNPase and RNase II) and RNase E at 44°C, is referred to as RNase E<sup>-</sup> exo<sup>-</sup>. It overproduces the *rpsO* mRNA due to the presence of the pFB1 plasmid harbouring *rpsO* and accumulates the polyadenylated form of P1-t1 at the non-permissive temperature (44°C) (Hajnsdorf *et al.*, 1995). Total RNA extracted 15 min after the temperature shift was used as template for reverse transcription initiated with the oligo(dT<sub>18</sub>) primer which contains a *Bam*HI cloning site upstream of 18 T residues. The oligo(dT<sub>18</sub>) primer and a second primer in the *rpsO* coding region (PCR primer, Figure 1), were used to amplify the *rpsO* cDNAs and the products were cleaved at the *Pst*I and *Bam*HI sites located in *rpsO* and the oligo(dT) primer, respectively and ligated into a cloning vector. Sequencing of the *rpsO*–poly(A) junctions of 104 clones allowed us to determine the relative abundance of mRNAs polyadenylated at 28 sites in *rpsO* (Table I). Because the transcription start site is the only 5' terminus identified upstream of the *Pst*I cloning site, we assume that each of these mRNAs has its 5' end at this location.

The majority of mRNAs (52%) are polyadenylated at the site previously characterized just downstream of t1 (Figure 2). Moreover, poly(A) tails fused to mRNA between nucleotides 275 and 291 (23% of the 104 clones) include the site previously characterized at position 282. They might result from polyadenylation of the RNA fragment *c* whose 3' ends map in this region (Braun *et al.*, 1996). In addition, 26 clones harbour poly(A) tails fused at 15 sites distributed throughout *rpsO* and the intercistronic region of the *rpsO*–*pnp* mRNA between positions 235 and 542 (Figure 2).

The poly(A) sequence downstream of position 243 identified in one clone (Figure 2) presumably resulted from annealing of the oligo(dT<sub>18</sub>) primer to the stretch of six As encoded by *rpsO*, and therefore probably does not correspond to a polyadenylation site (see below).



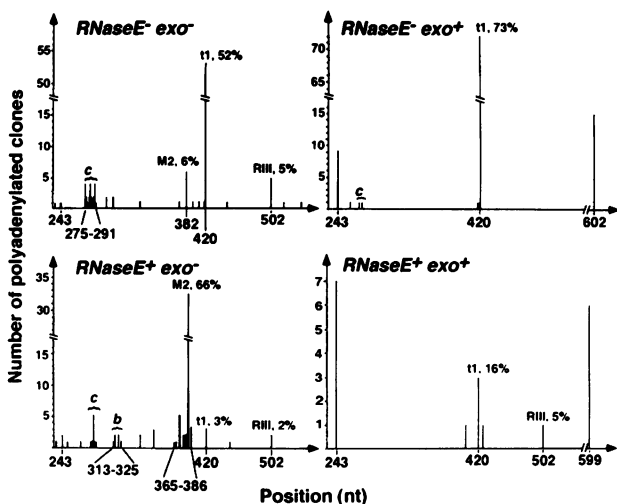
**Fig. 1.** Structure of *rpsO* transcripts. Transcription of the *rpsO*–*pnp* operon from the promoter P1 gives rise to the primary transcript P1-t1, as well as to the processed transcripts P1-RIII, P1-M2, *b* and *c* which differ from each other by their 3' termini (Régnier and Portier, 1986; Hajnsdorf *et al.*, 1994b; Braun *et al.*, 1996). These RNAs are shown by wavy lines beneath the genetic map and their lengths in nucleotides (nt) are indicated in parenthesis. Positions of the Rho-independent transcription terminator t1 (420), of the RNase E cleavage sites M2 (385) and M (423), of the proximal RNase III cleavage site RIII (502) and of the *Pst*I site (220) used for cloning of amplified cDNAs are indicated (Régnier and Portier, 1986; Régnier and Hajnsdorf, 1991). Numbering starts at the first nucleotide of the *rpsO* transcripts initiated at the upstream transcription start site (Régnier and Portier, 1986). The uniformly labelled *Dra*I–*Bgl*II RNA probe used for Northern blots and the 3'-labelled probe used for S1 nuclease mapping are shown beneath the transcripts. The dot indicates the 3' labelled *Hpa*II site of the S1 nuclease probe. The primer (PCR primer) used in combination with an oligo(dT<sub>18</sub>) primer for cDNA amplification is indicated by an arrow showing its polarity.

**Table I.** Localization of mRNA–poly(A) junctions in oligo(dT) primed *rpsO*–*pnp* cDNAs

Strain	Number of clones <sup>a</sup>	mRNA–poly(A) junctions <sup>b</sup>
RNase E <sup>-</sup> exo <sup>-</sup>	110 (6)	G235(1); C241(1); C243(1); T275(4); G276(1); C277(2); C279(1); T281(2); G282(4); G283(2); T285(1); C287(2); C289(4); G291(1); T302(2); G315(2); C343(1); C372(1); T373(1); C382(6); C389(1); G406(1); T419(1); C420(53); T463(1); G502(5); T519(1); T542(1)
RNase E <sup>+</sup> exo <sup>-</sup>	114 (20)	C236(1); C243(2); G250(1); G269(1); T281(1); G283(1); T284(5); T285(1); C289(1); C313(1); G315(2); T321(2); G325(1); C343(2); G352(3); T365(1); C367(1); G368(1); C372(5); T373(5); C378(2); T380(1); G381(2); C382(9); G383(32); T386(3); T407(1); C420(3); G466(1); G502(2)
RNase E <sup>-</sup> exo <sup>+</sup>	110 (10)	C243(9); C258(1); C274(1); C277(1); T417(1); T419(1); C420(71); G602(15)
RNase E <sup>+</sup> exo <sup>+</sup>	97 (78)	C243(7); G399(1); C420(3); G429(1); G502(1); T599(6)

<sup>a</sup>The total number of clones containing amplified cDNA sequenced to identify mRNA–poly(A) junctions is indicated. The numbers in parenthesis indicate those which do not contain *rpsO* sequences, or which contain several *rpsO* sequences because of multiple inserts.

<sup>b</sup>mRNA–poly(A) junctions indicate the position of the last nucleotide (G, T or C) identified upstream of the poly(A) sequences in clones of amplified cDNA. The number of clones harbouring stretches of As fused at the same position is indicated in parenthesis.



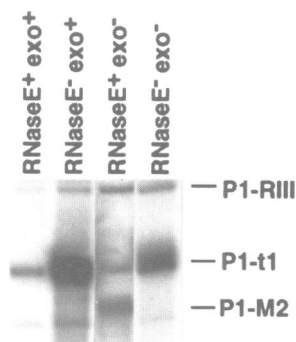
**Fig. 2.** Localization of polyadenylation sites in transcripts of the *rpsO-pnp* operon. The number of clones containing the different mRNA-poly(A) junctions listed in Table I have been plotted as a function of their positions in the *rpsO* transcript. The range of nucleotide positions on the abscissa extends from the *Pst*I cloning site at nucleotide 220 to the most 3' polyadenylation site identified at nucleotide 602. The regions indicated (275–291) and (313–325) correspond to the 3' extremities of the processed *c* and *b* *rpsO* fragments, respectively (Braun *et al.*, 1996). The M2 RNase E maturation site is downstream nucleotide 385. Position 420 corresponds to the Rho-independent transcription terminator t1 and position 502 to the proximal RNase III maturation site RIII (Régnier and Portier, 1986). Positions 243, 599 and 602 which presumably reflect initiation of oligo(dT) primed reverse transcription at encoded A-rich regions are also indicated (see Discussion). Locations of *c*, *b*, M2, t1 and RIII 3' extremities are indicated on the diagrams together with the percentage of *rpsO* cDNA clones harbouring stretches of As fused at t1, M2 and RIII.

Two new polyadenylation sites located at the proximal RNase III site (nucleotide 502), and three nucleotides upstream of the RNase E maturation site M2 (nucleotide 382) were identified in this study, and correspond to 5% and 6% of all *rpsO* clones, respectively (Figure 2). This raises the possibility that the 3' mRNA termini resulting from endonucleolytic cleavages by RNase III and RNase E can be polyadenylated.

#### The mRNA cleaved by RNase E at site M2 is polyadenylated

If polyadenylation detected in the vicinity of M2 in the RNase E<sup>-</sup> exo<sup>-</sup> strain takes place at the 3' end generated by RNase E, we should obtain more clones containing the M2-poly(A) junction by using as template RNA from the SK5726(pFB1) (*pnp7 mb500<sup>ts</sup>*) strain (Arraiano *et al.*, 1988) (referred to as RNase E<sup>+</sup> exo<sup>-</sup>), which accumulates the P1-M2 mRNA due to exonuclease deficiency (Hajnsdorf *et al.*, 1994b) (Figure 3).

Total RNA was isolated from the RNase E<sup>+</sup> exo<sup>-</sup> strain 15 min after the shift to 44°C, which inactivates RNase II and induces the accumulation of P1-M2. Then, polyadenylation sites were analysed as described above for the RNase E<sup>-</sup> exo<sup>-</sup> strain (Table I). It is striking that 66% of the 94 *rpsO* clones characterized contain stretches of As fused at M2 or a few nucleotides upstream, between positions 365 and 386 (Figure 2), demonstrating that the 3' end generated by RNase E is a substrate for PAP I and/or PAP II. These data also imply that the 3' terminus

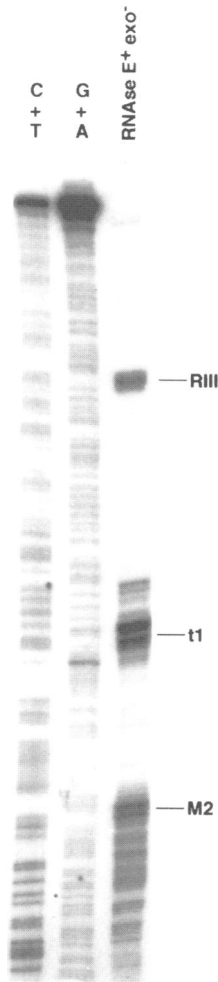


**Fig. 3.** Analysis of *rpsO* transcripts in strains of *E. coli* deficient for RNase E and/or exonucleases. Strains MG1693 (wt; RNase<sup>+</sup> exo<sup>+</sup>), SK5665 (*ans*; RNase<sup>-</sup> exo<sup>+</sup>), SK5726 (*pnp rmb*; RNase<sup>+</sup> exo<sup>-</sup>) and SK5704 (*ams pnp rmb*; RNase<sup>-</sup> exo<sup>-</sup>) transformed with pFB1 were grown at 30°C to OD<sub>650</sub> = 0.25 before being shifted to 44°C. Five micrograms of RNAs extracted from aliquots withdrawn 15 min after the shift were analysed on a Northern blot probed with the *Dra*I-*Bgl*II RNA probe. The positions of the three main *rpsO* mRNA species (P1-RIII, P1-t1 and P1-M2) are indicated beside the gels. The lane containing RNA from the RNase E<sup>+</sup> exo<sup>-</sup> strain was exposed for a longer time than the other lanes. Relative amounts of these three mRNAs in each strain are shown in Table II. The band which migrates slightly more rapidly than P1-M2 in the RNase E<sup>+</sup> exo<sup>+</sup>, RNase E<sup>-</sup> exo<sup>+</sup> and RNase E<sup>-</sup> exo<sup>-</sup> strains has not been considered in our analysis because the nature of the corresponding mRNA is not known.

resulting from the endonucleolytic cleavage might be slightly resected before being polyadenylated. To confirm this hypothesis we carried out S1 nuclease protection analysis which shows that processing at M2 gives rise to a set of 3' termini spread over at least 20 nucleotides upstream of M2 (Figure 4). These mRNAs are then presumably polyadenylated. S1 nuclease mapping does not detect the A residues added post-transcriptionally at the 3' ends of mRNAs. The large quantity of the mRNA 3' termini mapping at t1 presumably prevented detection of mRNA-poly(A) junctions corresponding to mRNA 3' ends mapping in the vicinity of this site (Figure 4).

As in the RNase E<sup>-</sup> exo<sup>-</sup> strain, poly(A) sequences were also fused in the region extending from nucleotides 275 to 291 (10% of the clones), at the RNase III site (2% of the clones) and at the upstream nucleotide 243 (2% of the clones) (Table I and Figure 2). Moreover, 16 clones contained stretches of As fused at 11 sites which were mostly (10 out of 11) different from those identified in the RNase E<sup>-</sup> exo<sup>-</sup> strain (Table I). Some of them between nucleotides 313 and 325 might result from polyadenylation of the *b* decay intermediate (Braun *et al.*, 1996) (Figure 1).

Strikingly, mRNA polyadenylated at t1 represented only 3% of the total polyadenylated *rpsO* mRNA in the RNase E<sup>+</sup> exo<sup>-</sup> strain, in contrast to 52% in the RNase E<sup>-</sup> exo<sup>-</sup> strain (Figure 2). We wished to verify that the relative numbers of clones harbouring poly(A) sequences at t1 and M2 reflected the amounts of cDNAs initiated at the poly(A) tails of P1-t1 and P1-M2 in the RNase E<sup>+</sup> exo<sup>-</sup> strain. For this purpose, oligo(dT)<sub>18</sub> primed cDNAs were amplified using the 5' labelled internal primer as above, and the radioactive PCR products were analysed in a polyacrylamide urea gel (Figure 5). Quantification of this gel using a PhosphorImager showed that P1-M2 gives rise to ~16 times more amplified cDNA than P1-t1. This is in agreement with the data in Table I showing that cloning of amplified cDNAs yielded ~20 times more clones

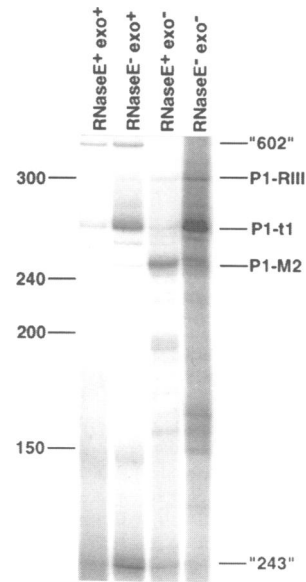


**Fig. 4.** Location of mRNA termini generated upon RNase E cleavages. A culture of strain SK5003 (*pnp rnb*; RNase E<sup>+</sup> exo<sup>-</sup>) was grown at 30°C to OD<sub>650</sub> = 0.25 and shifted to 44°C. Twenty micrograms RNA extracted from an aliquot withdrawn 30 min after the temperature shift were mixed with the 3'-labelled S1 probe shown in Figure 1, denatured, hybridized, digested with S1 nuclease and analysed in a 8% polyacrylamide-urea gel (lane RNase E<sup>+</sup> exo<sup>-</sup>). Sequence ladders generated by chemical cleavages of the 3'-labelled probe at G+A and C+T are shown. Positions of the RIII, t1 and M2 3' extremities are indicated.

harbouring poly(A) at M2 (between positions 365 and 386) than at t1 (positions 419 and 420). This result might reflect either the large difference of the relative amounts of the P1-t1 mRNA species in the two strains (Figure 3), or preferential polyadenylation at M2 in the RNase E<sup>+</sup> exo<sup>-</sup> strain.

**The M2 3' termini are more efficiently polyadenylated than t1 in the RNase E<sup>+</sup> exo<sup>-</sup> strain**

To evaluate the efficiencies of polyadenylation at t1, M2 and RIII in the RNase E<sup>-</sup> exo<sup>-</sup> and the RNase E<sup>+</sup> exo<sup>-</sup> strains, we compared the numbers of clones originating from mRNAs polyadenylated at these sites to the amounts of the P1-t1, P1-M2 and P1-RIII mRNA species detected on a Northern blot (Table II). Because the Northern blot shown in Figure 3 allows quantification of only these three major mRNA species, we only took into account those clones harbouring poly(A) tails at t1, M2 or RIII in the calculations shown in Table II. The large amount of



**Fig. 5.** Amplification of oligo(dT) primed *rpsO* cDNA. Aliquots of RNAs from strains MG1693 (wt; RNase<sup>+</sup> exo<sup>+</sup>), SK5665 (*ams*; RNase E<sup>-</sup> exo<sup>+</sup>), SK5726 (*pnp rnb*; RNase E<sup>+</sup> exo<sup>-</sup>) and SK5704 (*ams pnp rnb*; RNase E<sup>-</sup> exo<sup>-</sup>) analysed in the Northern blot of Figure 3 were reverse transcribed with the BH<sub>2</sub>T<sub>18</sub> oligo(dT) primer. Then, this primer was used in combination with the 5' end labelled internal PCR primer indicated in Figure 1 for amplification of the resulting cDNAs. The radioactive products were analysed in an 8% polyacrylamide-urea gel together with radioactive DNA fragments whose lengths are indicated on the left of the autoradiograph. DNA fragments presumably resulting from the amplification of cDNAs initiated at the polyadenylated 3' end of P1-t1, P1-M2 and P1-RIII, or at encoded A-rich regions located downstream of nucleotides '243' and '602', are indicated on the right. Amounts of amplified cDNA fragments corresponding to P1-M2 and P1-t1 in the RNase E<sup>+</sup> exo<sup>-</sup> strain were quantified with a PhosphorImager.

P1-t1 mRNA in the RNase E<sup>-</sup> exo<sup>-</sup> strain (88% of the three major mRNA species) suggests that the prevalence of mRNA polyadenylated at t1 in this strain [83% of poly(A) fused at t1, M2 and RIII] simply reflects the relative abundance of this mRNA species (Table II). Accordingly, mRNA of this strain mostly gives rise to an amplified cDNA fragment corresponding to P1-t1 (Figure 5).

In contrast, the strong discrepancy observed in the RNase E<sup>+</sup> exo<sup>-</sup> strain between the relative amounts of mRNAs polyadenylated at M2 and t1 (93% and 4% of the clones, respectively) and the relative abundances of the P1-M2 and P1-t1 mRNAs (58% and 28% respectively, Table II), suggest that P1-M2 transcripts contain a much higher proportion (at least 10 times) of polyadenylated molecules than the P1-t1 mRNAs (Table II).

The isolation of clones polyadenylated at M2 in the RNase E<sup>-</sup> exo<sup>-</sup> strain (Figure 2), in which P1-M2 cannot be detected (Figure 3), might result from the high yield of PCR amplification of molecules present at very low concentration in the RNA preparation. Alternatively, this might also indicate that M2 is efficiently polyadenylated.

**The P1-M2 transcript is stabilized in a strain deficient for PAP I**

Because polyadenylation was reported to reduce the stability of several transcripts including the *rpsO* P1-t1 mRNA (Xu *et al.*, 1993; Hajnsdorf *et al.*, 1995; O'Hara

**Table II.** Polyadenylation of P1-t1, P1-RIII and P1-M2 mRNA species

Strain	RNA species	mRNA (%) <sup>a</sup>	Poly(A) mRNA (%) <sup>b</sup>
RNase E <sup>-</sup> exo <sup>-</sup>	P1-RIII	12	8
	P1-t1	88	83
	P1-M2	0	9
RNase E <sup>+</sup> exo <sup>-</sup>	P1-RIII	14	3
	P1-t1	28	4
	P1-M2	5	93

<sup>a</sup>[mRNA(%)] is the ratio of the amount of one of the three mRNA species (P1-t1, P1-M2 or P1-RIII) to the total amount of *rpsO* mRNA present in the bands of P1-t1+P1-M2+P1-RIII mRNAs quantified with a PhosphorImager from the Northern blot in Figure 3.

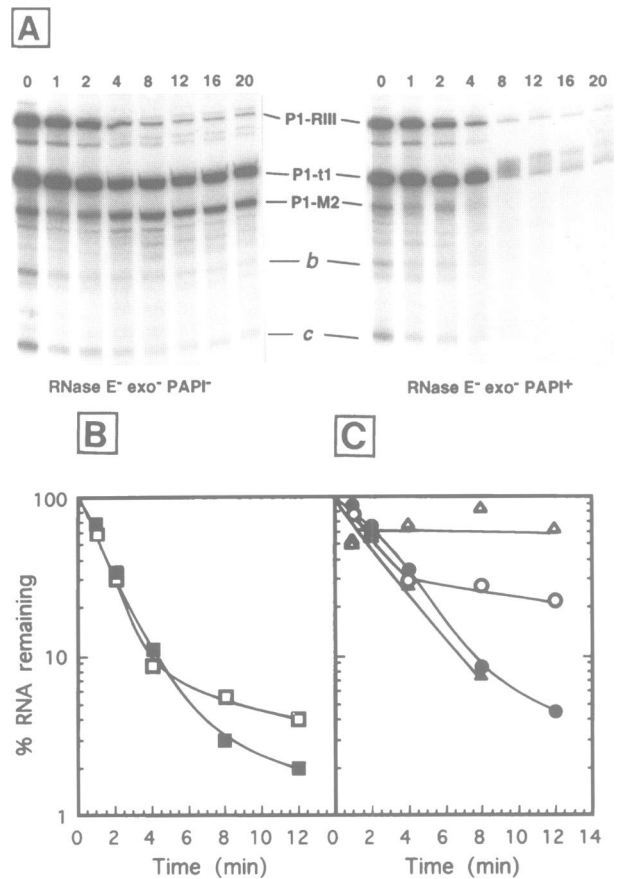
<sup>b</sup>[poly(A) mRNA(%)] represents the proportion of P1-t1, P1-M2 or P1-RIII in the population of polyadenylated *rpsO* mRNAs. It is the ratio of the number of clones containing stretches of As fused at t1 (downstream positions 419 and 420), M2 (between positions 365 and 386) or RIII (downstream position 502) to the total number of clones corresponding to mRNAs polyadenylated at t1, M2 and RIII (Table I).

*et al.*, 1995), we investigated whether the P1-M2 mRNA detected in both IBPC674 (RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>+</sup>) and IBPC673 (RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>-</sup>) (Hajnsdorf *et al.*, 1995) at permissive temperature is more stable in the PAP<sup>-</sup> strain than in the isogenic PAP<sup>+</sup> bacteria. These two strains harbour the *rnb500*<sup>ts</sup> and *ams1*<sup>ts</sup> mutations. The Northern blot shown in Figure 6 demonstrates that P1-M2 is detectable for at least 20 min after inhibition of transcription by rifampicin in the RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>-</sup> cells shifted to 44°C at the time of antibiotic addition, to inactivate RNase E and RNase II. In contrast this RNA species is no longer detectable 8 min after addition of the antibiotic to the RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>+</sup> cells. These data suggest that P1-M2 is destabilized upon polyadenylation by PAP I. The observation that the RNA fragment *c* and, to a smaller extent, the fragment *b* also appear to be stabilized in the absence of PAP I, is consistent with the finding that they are both polyadenylated (Figure 2). Interestingly, this experiment also shows that the decay rate of P1-RIII is not altered in a PAP I deficient strain (Figure 6), indicating that polyadenylation at its 3' end generated by RNase III does not modify its stability.

**Most poly(A) tails of *rpsO* mRNAs are detected at t1 when cells contain exonucleases**

All of the mRNA-poly(A) junctions described above were determined using RNA from exonuclease deficient strains. These strains contain a higher fraction of polyadenylated mRNAs than strains containing exonucleases, presumably because RNase II and PNPase compete against the elongation of poly(A) tails (Cao and Sarkar, 1992a; Hajnsdorf *et al.*, 1995). We therefore analysed the mRNA poly(A) junctions in strains containing PNPase and RNase II, in order to investigate whether exonucleases have an effect on the abundance of different poly(A) mRNA species. For this purpose, strains MG1693(pFB1) (wt) and SK5665(pFB1) (*ams1*<sup>ts</sup>) (Arraiano *et al.*, 1988) (referred to as RNase E<sup>+</sup> exo<sup>+</sup> and RNase E<sup>-</sup> exo<sup>+</sup>, respectively) were grown for 15 min at 44°C before RNA preparation, as described above.

Nearly all the clones of amplified cDNAs corresponding to polyadenylated *rpsO* mRNA isolated from the RNase



**Fig. 6.** PAP I destabilizes the processed P1-M2 *rpsO* transcript. (A) Strains IBPC674 (SK5704 *pcnB*<sup>+</sup>; RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>+</sup>) and IBPC673 (SK5704 *pcnB*<sup>-</sup>; RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>-</sup>) were grown at 30°C until the OD<sub>650</sub> reached 0.25. Then, rifampicin was added to a final concentration of 500 µg/ml and the culture was shifted to 44°C. RNAs extracted at the times indicated in min above each lane were analysed on a Northern blot probed with the *DraI*-*Bgl*I RNA probe. The positions of the P1-RIII, P1-t1, P1-M2, *b* and *c* transcripts are shown beside the autoradiograph. The P1-M2 band detected in this experiment is not visible in the RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>+</sup> strain 15 and 30 min after the temperature shift (Figure 3, right lane). (B and C) Northern blots of (A) were quantified with a PhosphorImager and the relative amounts of P1-RIII (B) (□, ■), P1-t1 (C) (○, ●) and P1-M2 (C) (△, ▲) remaining in the *pcnB*<sup>+</sup> (filled symbols) and *pcnB*<sup>-</sup> (open symbols) strains were plotted as a function of time. Radioactivity in bands *b* and *c* was too low to be quantified.

E<sup>+</sup> exo<sup>+</sup> and RNase E<sup>-</sup> exo<sup>+</sup> strains had poly(A) tails fused at t1 (Table I and Figure 2). Only one clone polyadenylated at the proximal RIII site, and two clones corresponding to polyadenylation at new sites located in the hairpin of the transcription terminator and ~10 nucleotides downstream t1, were also found in the wild-type strain (Figure 2). Moreover, three clones isolated from the RNase E<sup>-</sup> strain harboured poly(A) tails in the vicinity of the 3' end of the *c* fragment.

A large part of the clones obtained in these experiments with mRNAs of the RNase E<sup>-</sup> exo<sup>+</sup> and RNase E<sup>+</sup> exo<sup>+</sup> cells (24% and 68%, respectively) contain poly(A) tails fused downstream of nucleotides 243, 599 or 602 (Figure 2), presumably due to the presence in the message of an encoded A-rich sequence able to direct annealing of the oligo(dT<sub>18</sub>) primer (Figure 7). In fact, it was possible to amplify an oligo(dT<sub>18</sub>) primed cDNA fragment beginning at the stretch of six As located downstream nucleotide

"243" rpsO GAGCAC←AAAAAGATCA  
 "599" rpsO CTGTCT←AAGAAAGAGAAAGGATA  
 "602" rpsO TCTAAG←AAAGAGAAAGGATA  
 GroE AAGTTG←AAATGAAAGAGAAAAAGCA

**Fig. 7.** Oligo(dT) primed reverse transcription may be initiated at encoded A-rich sequences. The encoded A residues of *rpsO* and *groEL* which presumably hybridize with the oligo(dT<sub>18</sub>) primer to initiate reverse transcription are underlined. The arrows starting from the junctions between the stretches of As and the sequences of *rpsO* and *groEL* (upstream) identified in several cDNA clones indicate the putative initiation sites of cDNA synthesis primed by the oligo(dT<sub>18</sub>) primers. The numbers in quotation marks indicate the positions of the last encoded *rpsO* nucleotide identified upstream of the poly(A) sequence. Nineteen clones containing the *groEL*-poly(A) sequences were obtained by reverse transcription and amplification of RNase E<sup>+</sup> exo<sup>+</sup> RNA. Preferential cloning of this DNA fragment is presumably due to the presence upstream of a *Pst*I site, of a sequence able to anneal 13 of the 20 nucleotides of the internal PCR primer at 36 and 45°C. These temperatures were found to optimize amplification of *rpsO* DNA fragments. The high yield of PCR fragments obtained under these conditions probably results from a better annealing of the oligo(dT<sub>18</sub>) primer to poly(A) tails. However, the internal primer whose melting temperature is 62°C probably forms non-specific hybrids at these temperatures.

243 using an *in vitro* synthesized *rpsO* transcript as a template (data not shown). In addition, it was interesting to point out that we obtained many more of these clones resulting from reverse transcription primed with oligo(dT<sub>18</sub>) at nucleotides 243, 599 or 602 with RNA from exonuclease-replete strains which contain a low fraction of polyadenylated mRNAs, than from RNA of the exonuclease deficient strains which exhibit long and/or abundant poly(A) tails. These data, which imply that these clones do not reflect polyadenylation of mRNA, confirm that they result from reverse transcription priming at internal A-rich sequences. This conclusion was re-inforced by the observation that numerous clones lacking *rpsO* sequences, obtained from RNase E<sup>+</sup> exo<sup>+</sup> RNAs (Table I), contained fragments of the *groEL* gene, which were also reverse transcribed from an encoded A-rich region (Figure 7).

A striking discrepancy between the RNase E<sup>-</sup> exo<sup>+</sup> and RNase E<sup>+</sup> exo<sup>+</sup> strains is the proportion of clones which contains *rpsO* cDNA (Table II). The large difference in P1-t1 *rpsO* mRNA concentrations in the two strains (Figure 3) presumably explains why we obtained a much higher proportion of *rpsO* clones from the RNase E<sup>-</sup> exo<sup>+</sup> strain (100 out of 110 clones analysed) than from the RNase E<sup>+</sup> exo<sup>+</sup> strain (19 out of 97 clones analysed) (Table I). However, our data indicate that this discrepancy might also result from a reduction of the proportion of molecules polyadenylated at t1 in the RNase E<sup>+</sup> strain. This proportion can be estimated in each strain by comparing the number of clones containing a t1 poly(A) junction, which reflects the amount of P1-t1 mRNA harbouring a poly(A) tail at t1, with the number of clones containing a mRNA-poly(A) junction at nucleotide 243, which reflects the total amount of the P1-t1 mRNA able to anneal the primer at the internal stretch of six As. Table III shows that the ratio of clones corresponding to polyadenylated P1-t1 to the number of clones corresponding to total P1-t1 is much higher in the RNase E<sup>-</sup> strain than in the RNase E<sup>+</sup> strain, suggesting that the t1 termini harbour

**Table III.** Internal versus terminal initiation of reverse transcription of the P1-t1 *rpsO* mRNA in the RNase E<sup>+</sup> and RNase E<sup>-</sup> strains<sup>a</sup>

Strain	t1	243(6A)
RNase E <sup>+</sup> exo <sup>+</sup>	3	7
RNase E <sup>-</sup> exo <sup>+</sup>	72	9

<sup>a</sup>The number of clones harbouring stretches of As at the terminator t1 and downstream nucleotide 243 in each strain were taken from Table I.

less poly(A) tails in the presence of active RNase E. This negative effect of RNase E might also in part explain why P1-t1 contains a lower proportion of polyadenylated molecules than P1-M2 in the RNase E<sup>+</sup> exo<sup>-</sup> strain (Table II).

## Discussion

This paper presents the first evidence that polyadenylation can occur at processing sites in *E. coli* mRNA. We demonstrated that polyadenylation occurs at the M2 RNase E cleavage site upstream of the hairpin transcription terminator of *rpsO* if this message is processed by RNase E *in vivo*, while nearly no poly(A) tails are detected at this site in an RNase E deficient strain. This suggests that poly(A) tracts fused upstream of transcription termination sites of *lpp* mRNA (Cao and Sarkar, 1992a), *colE1* RNA I of *E. coli* (Xu *et al.*, 1993) and *hag* mRNA of *Bacillus subtilis* (Cao and Sarkar, 1993), reflect polyadenylation at processing sites. However, our data, showing that a substantial number of mRNA-poly(A) clones can result from reverse transcription initiated at encoded A-rich regions, indicate that these *lpp* and *hag* polyadenylated mRNAs might also result from hybridization of the oligo(dT) primer to the stretches of As encoded in these genes just upstream of their respective transcription terminators. Poly(A) tails were also detected at the position of the RNase III cleavage downstream of *rpsO*, in agreement with previous data suggesting that post-transcriptional addition of A residues occurs at an RNase III processing site of the phage T7 early transcript (Rosenberg *et al.*, 1974). The 3' ends of the decay intermediates (fragments *b* and *c*), generated exonucleolytically in the absence of PNPase (Braun *et al.*, 1996), also appear to be polyadenylated. The scattering of polyadenylation sites over 17 nucleotides in the vicinity of the *c* fragment 3' end (between nucleotides 275 and 291) is consistent with the heterogeneity of the 3' ends of this exonucleolytically generated fragment (Braun *et al.*, 1996) and with the persistence of residual exonucleolytic activity of RNase II and other RNases in the exonuclease deficient strains at 44°C (Donovan and Kushner, 1986; Hajnsdorf *et al.*, 1994b; O'Hara *et al.*, 1995). Probably, this residual activity also accounts for the heterogeneity of the *rne*-dependent mRNA-poly(A) junctions mapping between two and 20 nucleotides upstream of M2.

As observed previously for the P1-t1 mRNA (Hajnsdorf *et al.*, 1995), we show here that P1-M2 is more stable in a PAP I deficient strain also devoid of PNPase, RNase II and RNase E. These data indicate that PAP I participates in the polyadenylation of P1-M2, and that the polyadenylated species can be degraded by unidentified exo and/or endo-

ribonucleases. Polyadenylation of P1-M2 presumably gives rise to a smear of heterogeneous extended molecules not detectable in Northern blots of RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>+</sup> RNA (Figure 6). Polyadenylation appears also to be involved in the degradation of the *b* and *c* small decay intermediates. It is interesting to point out that lack of polyadenylation in the RNase E<sup>-</sup> exo<sup>-</sup> PAP I<sup>-</sup> (Figure 6) and RNase E<sup>-</sup> exo<sup>+</sup> PAP I<sup>-</sup> strains (data not shown) has no effect on the stability of P1-RIII. This might reflect the fact that P1-RIII is polyadenylated by another enzyme (e.g. PAP II), or that P1-RIII is degraded independently of its poly(A) tail even in the absence of PNPase, RNase II and RNase E. This also suggests that sequences just upstream of the polyadenylation site can modulate the destabilizing effect of poly(A) tails.

We deduced from the proportions of amplified cDNAs harbouring mRNA-poly(A) junctions at t1 and M2 that P1-t1 contains a significantly lower proportion of polyadenylated molecules than P1-M2 in RNase E<sup>+</sup> exo<sup>-</sup> strains, and that P1-t1 seems to be less efficiently polyadenylated in the presence of RNase E. A possible explanation is that the binding of PAP I to P1-t1 mRNA is impeded by RNase E bound at the M2 site (Hajnsdorf *et al.*, 1994b), or associated in a complex with the Exonuclease Impeding Factor bound at the t1 hairpin (Causton *et al.*, 1994). Alternatively, the lower proportion of polyadenylated P1-t1 might reflect the greater instability of these molecules in the RNase E<sup>+</sup> strain. Moreover, it is also possible that the preferential amplification of M2 poly(A) junctions compared with those at t1 in the RNase E<sup>+</sup> strain is due to the presence of longer poly(A) tails which exhibit a higher affinity for the primer. Similarly, the small number of *rpsO* clones polyadenylated at t1 obtained with the RNA of the RNase E<sup>+</sup> exo<sup>+</sup> strain might reflect the fact that poly(A) tails are shorter in the presence of RNase E.

Finally, we wish to stress the limits of the technique employed and the significance of the data obtained. Comparisons between Northern blots and polyadenylation patterns detect a reliable difference of polyadenylation frequency between P1-t1 and P1-M2 in the exonuclease deficient strains (Table II), which mainly yields clones containing *rpsO* cDNA. Probably, one has to be cautious in assigning polyadenylation frequencies from the few *rpsO* clones obtained with RNA of RNase E<sup>+</sup> exo<sup>+</sup> strains, which yields numerous artefacts. Attention must also be drawn to the fact that our method allows us to compare proportions of polyadenylated molecules for each mRNA species, but does not give any indication as to the abundance of polyadenylated molecules. In fact, Northern blots fail to distinguish between tail-less and (A)tail-containing mRNAs, and patterns of mRNA-poly(A) junctions do not relate to their concentrations.

In spite of the amino acid sequence homology with terminal transferase (Masters *et al.*, 1990) suggesting that PAP I might have a preferential affinity for stably folded 3' termini such as transcription termination hairpins, polyadenylation at M2 and RIII demonstrates that PAP I also recognizes unstructured termini and therefore, like mammalian poly(A) polymerases, presumably does not exhibit specificity for particular mRNA motifs (Keller, 1995). The latter conclusion is very strongly supported by the observation that polyadenylation can occur at 45

of the 185 nucleotides of *rpsO* mRNA extending between positions 235 and 420 (t1) (Table I).

The fact that many polyadenylation sites are spread throughout *rpsO* in cells deficient for exonucleases and RNase E indicates that the *rpsO* mRNA is cleaved at many sites despite the inactivation of PNPase, RNase II and RNase E.

It is striking that, with the exception of t1, most polyadenylated mRNA species, including M2, could be identified in the exonuclease deficient strains but not in strains containing PNPase and RNase II. An initial explanation might be that these termini do not become accessible to PAP I due to the processivity of 3' to 5' exonucleases and because PNPase is associated with RNase E (Carpousis *et al.*, 1994; Py *et al.*, 1994) and might therefore be delivered to the M2 3' end immediately after the processing step. A second possibility is that once polyadenylated these mRNAs are degraded so rapidly by exonucleases that they cannot be detected by the method we have used. Accordingly, poly(A) tails were proposed to improve the affinity of PNPase for RNA I of ColE1 plasmids, and to facilitate processive degradation through secondary structures (Xu and Cohen, 1995). Moreover, the pre-eminence of RNase II which represents 90% of the poly(A) degrading activity in crude extracts (Deutscher and Reuven, 1991), the higher velocity of poly(A) degradation by this nuclease compared with other homopolymers (Singer and Tolbert, 1965) and the absence of detectable elongated (polyadenylated) *rpsO* mRNAs in a PNPase deficient strain containing RNase II (Hajnsdorf *et al.*, 1994b) strongly suggest that RNase II could contribute to the degradation of poly(A) tails and carry out the complete degradation of the polyadenylated mRNA fragments in the absence of impeding secondary structures (Gupta *et al.*, 1977; McLaren *et al.*, 1991).

In contrast, detection of mRNAs polyadenylated at t1 in strains containing exonucleases suggests that the stable hairpin of the terminator offers a barrier to nucleases even when the mRNA is polyadenylated. It has been proposed that RNase II stalls at t1 (Hajnsdorf *et al.*, 1994b; Pepe *et al.*, 1994). It can also be hypothesized that RNase II removes oligo(A) tails and dissociates when it reaches t1 (Coburn and Mackie, 1996). The mRNA molecule could undergo several rounds of polyadenylation and de-adenylation by PAP I and RNase II before being completely degraded by PNPase or other ribonucleases. Such a de-adenylating function of RNase II might also explain why mRNAs terminated by a hairpin are destabilized upon RNase II inactivation (Hajnsdorf *et al.*, 1994b; Pepe *et al.*, 1994). This dynamic view of the structure of mRNA 3' end is reminiscent of the exchanges of the 3' terminal adenosine nucleotide of tRNAs catalysed by ribonuclease T and nucleotidyl transferase (Deutscher, 1990).

We have demonstrated here that bacterial mRNAs are polyadenylated at 3' termini generated by RNase E, RNase III and exonuclease cleavages. In this respect, bacterial poly(A) polymerase(s) are similar to eukaryotic enzymes which polyadenylate mRNA at processing sites (Keller, 1995). A question arising is whether *E.coli* PAPs have affinity for processing complexes as in mammalian cells (Keller, 1995), or for other features of mRNA sequence or structure. On the other hand, there are now many indications that mRNA fragments such as the P1-M2, P1-

RIII, *b* and *c* fragments of the *rpsO* mRNA and the RNase E processed RNA I of ColE1 plasmids (Xu *et al.*, 1993) are polyadenylated. The destabilization resulting from this polyadenylation indicates that poly(A) tails play a major role in the rapid elimination of intermediary products of mRNA decay in addition to its role in the degradation of the primary transcript.

## Materials and methods

### Strains and growth medium

Isogenic ribonuclease deficient strains SK5704 (*ams1<sup>ts</sup> pnp7 rnb500<sup>ts</sup>*), SK5726 [*pnp7 rnb500<sup>ts</sup> pDK39(Cm<sup>R</sup> rnb500<sup>ts</sup>)*] and SK5665 (*ams1<sup>ts</sup>*) (Arraiano *et al.*, 1988) were transformed with plasmid pFB1 (Hajnsdorf *et al.*, 1995) and grown in LB medium supplemented with thymine (40 µg/ml) and ampicillin (100 µg/ml). The isogenic wild-type strain MG1693 transformed with pFB1 was grown in the same medium, and strain SK5003 [*pnp7 rnb500<sup>ts</sup> pDK39(Cm<sup>R</sup> rnb500<sup>ts</sup>)*] (Donovan and Kushner, 1986) was grown in LB medium supplemented with 20 µg/ml chloramphenicol. IBPC673 (SK5704 *pcnB<sup>-</sup> pRS415*) and IBPC 674 (SK5704 *pcnB<sup>+</sup> pRS415*) (Hajnsdorf *et al.*, 1995) were grown in LB medium supplemented with thymine (40 µg/ml) and ampicillin (50 µg/ml).

### RNA preparation and analysis

Total RNA was prepared as described previously (Hajnsdorf *et al.*, 1994b) from 10 ml aliquots of cultures grown as mentioned in the figure legends. RNA preparations were analysed by Northern blotting as described (Hajnsdorf *et al.*, 1994b) and probed with antisense *rpsO* RNA synthesized by *in vitro* transcription from the pEHa4 plasmid containing the *rpsO* *DraI*-*Bgl*II fragment, extending from the translation initiation codon to the transcription terminator t1 of *rpsO* (Hajnsdorf *et al.*, 1994a) (Figure 1). Northern blots were quantified with a PhosphorImager (Molecular Dynamics).

S1 nuclease mapping was performed as previously described (Hajnsdorf *et al.*, 1994b) with the 3'-labelled probe shown in Figure 1.

### cDNA synthesis and cloning of amplified mRNA-poly(A) junctions

cDNAs were synthesized from the BH<sub>2</sub>T<sub>18</sub> primer as previously described (Hajnsdorf *et al.*, 1995) except that reverse transcription was carried out in 75 mM Tris-HCl pH 8.3, 15 mM MgCl<sub>2</sub>, 4 mM DTT, 112 mM KCl and 1 mM of each of the four dNTPs.

25% of the synthesized cDNA (5 µl) was amplified with the BH<sub>2</sub>T<sub>18</sub> primer and the internal PCR primer (Figure 1) as described previously (Hajnsdorf *et al.*, 1995). The PCR fragments were cleaved with *Pst*I and *Bam*HI and cloned, in bulk, into the pT3T718U vector. Single-stranded DNAs produced in JM101TR were sequenced.

PI-t1 RNA was transcribed *in vitro* by SP6 RNA Polymerase under conditions described by the manufacturer (Promega) from a PCR generated template extending from nucleotides 1 to 420 of *rpsO* amplified from the pB15.6 plasmid. Primers used for the amplification were 5'-ATT-AAGGTGACACTATAGCCGCTTAACGTCGCGTAAATTG-3' and 5'-GAAAAAAGGGGCCACTCAGG-3', the underlined sequence corresponding to the sequence of the SP6 promoter.

## Acknowledgements

We thank M.Grunberg-Manago and M.Springer for encouragement and discussions, G.Mackie for communication of results prior to publication, and D.Stern and M.Dreyfus for critical reading of the manuscript. This work was funded by CNRS (URA1139), University Paris 7 and the European Union (Human Capital and Mobility ERB CHRX CT 93-0263). J. H.-N. is supported by a grant from Forskerakademiet (Denmark).

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Received on January 30, 1996; revised on March 21, 1996