Degradation pathway of CopA, the antisense RNA that controls replication of plasmid R1

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RNA decay in bacteria is carried out by a number of enzymes that participate in the coordinated degradation of their substrates. Endo- and exonucleolytic cleavages as well as polyadenylation are generally involved in determining the half-life of RNAs. Small, untranslated antisense RNAs are suitable model systems to study decay. A study of the pathway of degradation of CopA, the copy number regulator RNA of plasmid R1, is reported here. Strains carrying mutations in the genes encoding RNase E, polynucleotide phosphorylase (PNPase), RNase II and poly(A) polymerase I (PcnB/PAP I) – alone or in combination – were used to investigate degradation patterns and relative half-lives of CopA. The results obtained suggest that RNase E initiates CopA decay. Both PNPase and RNase II can degrade the major 3'-cleavage product generated by RNase E. This exonucleolytic degradation is aided by PcnB, which may imply a requirement for A-tailing. RNase II can partially protect CopA's 3'-end from PNPase-dependent degradation. Other RNases are probably involved in decay, since in *rnb/pnp* double mutants, decay still occurs, albeit at a reduced rate. Experiments using purified RNase E identified cleavage sites in CopA in the vicinity of, but not identical to, those mapped *in vivo*, suggesting that the cleavage site specificity of this RNase is modulated by additional proteins in the cell. A model of CopA decay is presented and discussed.

Keywords: antisense RNA, RNA decay, RNase E, exoribonucleases, poly(A) polymerase

INTRODUCTION

RNA degradation in bacteria is a complex process. Structure and sequence elements in mRNAs are known to determine the stability of individual RNAs. Half-lives from <1 min to >50 min have been measured, but the motifs that govern these great differences in stability are only poorly understood. A great number of RNases have been identified (Deutscher, 1993). Most of them have roles in the processing of stable RNAs, but some are clearly involved in the degradation of mRNAs. Recent work indicated that RNA decay occurs by the concerted activity of a number of physically associated proteins, collectively referred to as the degradosome (Carpousis *et al.*, 1994; Py *et al.*, 1996; Miczak *et al.*, 1996). In

addition to the endoribonuclease RNase E and the 3'→5'-exonuclease polynucleotide phosphorylase (PNPase), additional proteins were shown to be part of this complex: the β -subunit of PNPase (enolase, a glycolytic enzyme), an RNA helicase possibly involved in disruption of RNA structure (RhlB) and the heat-shock protein and chaperonin DnaK. The chaperonin GroEL was found associated with this complex under some conditions (Miczak *et al.*, 1996; see also Sohlberg *et al.*, 1993). The significance of this latter finding is not clear, but recent work has demonstrated that purified GroEL can protect RNA from degradation *in vitro* (Georgellis *et al.*, 1995). Finally, a protein that may protect stem-loops from 3'-exonucleolytic degradation (EIF) also co-purifies with RNase E and PNPase (Py *et al.*, 1994). The recent establishment of an *in vivo*-like *in vitro* mRNA degradation system suggested that degradosomal components are polysomally located (Ingle & Kushner, 1996). Interestingly, an additional enzyme involved in RNA decay, the poly(A) polymerase PcnB/PAP I (Lopilato *et al.*, 1986; Cao & Sarkar, 1992), has

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Abbreviations: FL-CopA, full-length CopA; PNPase, polynucleotide phosphorylase; SL-E, RNase-E-generated 3'-stem-loop of CopA containing a 5'-tail.

previously been found associated with polysomes as well (Ingle & Kushner, 1996). Hence, although this enzyme has not yet been found stably associated with the degradosome, it may be located at/near the site where degradation occurs.

Antisense RNAs are suitable models for the study of RNA decay, since they are (i) small and untranslated, (ii) generally well-characterized with respect to structure and biological function and (iii) easily manipulated *in vitro*. We have previously characterized replication control of plasmid R1 and studied the structure and function of the replication control element, the antisense RNA CopA (Wagner & Nordström, 1986; for reviews see Wagner & Simons, 1994; Nordström & Wagner, 1994). CopA inhibits replication of plasmid R1 by binding to the target region, CopT, within the *repA* mRNA, to block the synthesis of the replication initiator protein RepA. In almost all cases, copy number regulator RNAs are constitutively synthesized and rapidly turned over (for an exception see Brantl & Wagner, 1996). Hence, rapid decay is a hallmark of CopA as well as of RNA I, the antisense RNA controlling the replication of plasmid ColE1 (Eguchi *et al.*, 1991). The decay of both RNAs is initiated by RNase E cleavage, which is a prerequisite for rapid subsequent degradation (Lin-Chao & Cohen, 1991; Söderbom *et al.*, 1997). Previous work also showed that the PcnB protein is needed for normal decay of these RNAs, and that RNase-E-generated cleavage products are stabilized in its absence (Xu *et al.*, 1993; He *et al.*, 1993; Söderbom *et al.*, 1997). Xu & Cohen (1995) presented evidence that the PcnB-dependent addition of adenosines to the 3'-end of RNA I creates a substrate for PNPase and is required for decay. The question of whether or not CopA also requires A-tails for decay is not yet settled (Söderbom *et al.*, 1997). The PcnB-dependence of RNA I and CopA degradation is different: in *pcnB* mutant strains, RNA I was stabilized more than tenfold (Xu *et al.*, 1993; He *et al.*, 1993), whereas only a twofold effect was seen for CopA (Söderbom *et al.*, 1997).

In an attempt to characterize the pathway of CopA decay in more detail, we used strains carrying mutant alleles of *rne* (encoding RNase E), *pnp* (PNPase), *rnb* (RNase II) or *pcnB* (PcnB/PAP I). Single and multiple mutant strains were constructed, and CopA accumulation and degradation patterns were analysed. The effect of the mutations present on the degradation of CopA was analysed by rifampicin run-out experiments. RNase E cleavage sites in CopA were mapped *in vitro* and compared to those previously identified *in vivo* (Söderbom *et al.*, 1997). Finally, we compared relative cleavage rates of RNase E on CopA and RNA I substrates *in vitro* and found that RNA I is more rapidly processed. Based on the results obtained, we discuss a tentative pathway of CopA decay.

METHODS

Bacterial strains and plasmids. The *Escherichia coli* strains used in this study are shown in Table 1. To introduce the *pcnB* deletion, we used P1 transduction as described by Söderbom *et al.*

(1997). For the experiments reported, cells were grown in L broth (Bertani, 1951) supplemented with 50 µg kanamycin ml⁻¹ for *ΔpcnB* strains. All strains containing the *rne-1/ams-1* mutation were grown in the presence of 50 µg thymine ml⁻¹. Growth of bacteria was at 30 °C unless otherwise indicated. Plasmid pKG339 (Jensen *et al.*, 1995) is a pSC101 replicon carrying a *copA* gene under the control of an IPTG-inducible promoter. When plasmid pKG339 was present, the medium contained 15 µg tetracycline ml⁻¹.

Analysis of CopA patterns *in vivo* by Northern analysis.

Strains containing plasmid pKG339 were grown at 30 °C, and at an OD₆₀₀ of about 0.2, half of the culture was transferred to 44 °C for 20 min. IPTG was added to 1 mM to all cultures, and samples were collected for RNA extraction after an additional 10 min. RNA preparation and Northern blot analyses were performed as described by Söderbom *et al.* (1997). The probe for CopA RNA was *in vitro* transcribed, uniformly [α -³²P]UTP-labelled CopT RNA (Hjalt & Wagner, 1995).

Decay of CopA in strains carrying mutations in genes encoding RNases and/or PcnB.

Cell growth was the same as above, except that rifampicin (Sigma) was added to 0.2 mg ml⁻¹ 10 min after IPTG induction of CopA transcription. Subsequently, aliquots were withdrawn at intervals for Northern blot analysis. Decay rates were calculated from semi-logarithmic plots based on PhosphorImager analysis of Northern blots. Corrections for loading were done by probing for 5S rRNA as described by Söderbom *et al.* (1997).

In vitro cleavages of CopA by RNase E.

Cleavages were performed either on 5'-end-labelled CopA (~0.4 pmol; generated by phosphorylation of *in vitro* transcribed CopA; Hjalt & Wagner, 1995) or of ³H-labelled CopA (~0.1 pmol), in a buffer containing 10 mM Tris/HCl, pH 7.9, 5 mM MgCl₂, 0.1 mM DTT and 100 mM NH₄Cl. Purified RNase E (Naureckiene & Uhlin, 1996) was a generous gift from Saule Naureckiene (Microbiology Dept, University of Umeå). Cleavages were performed in 40 µl reaction mixtures. The amount of enzyme added was tested in pilot experiments to obtain suitable time intervals. In the case of end-labelled CopA (experiment in Fig. 4), 4 µl aliquots were quenched in gel loading buffer containing 7 M urea. After heating for 3 min at 85 °C, the samples were loaded onto 8% sequencing gels. The dried gels were autoradiographed using Kodak X-Omat S or Amersham Hyperfilm MP.

In the case of unlabelled CopA, reactions were stopped by withdrawing aliquots at different times into phenol. After phenol extraction, the RNA was precipitated in the presence of 10 µg glycogen, redissolved in water, and subsequently reverse transcribed as in Söderbom *et al.* (1997). The cDNA products were electrophoresed on 8% sequencing gels. Sequencing ladders generated on *copA* DNA using the same primer were used as markers.

Relative cleavage activity of RNase E on CopA and RNA I substrates *in vitro*.

CopA and RNA I (0.2 pmol each, both 5'-phosphorylated by [γ -³²P]ATP and T4 polynucleotide kinase) were mixed in RNase E buffer (see above). RNase E was added, and aliquots were quenched in stop buffer as above, followed by gel analysis and autoradiography. Quantification of RNA I and CopA band intensities on the dried gel was performed using a Molecular Dynamics PhosphorImager 400S.

Oligodeoxyribonucleotides. The oligodeoxyribonucleotide used for primer extension analysis was FRED 53: 5'-AAA CCC CGA TAA TCT TCT TCA ACT TT-3'.

Table 1. Bacterial strains

Strain	Relevant genotype	Source/reference
SK 5665	<i>rne-1</i> (= <i>ams-1</i> , temperature sensitive) <i>thyA715 F</i> ⁻	Arraiano <i>et al.</i> (1988)
SK 5671	<i>rne-1 pnp-7 thyA715 F</i> ⁻	Arraiano <i>et al.</i> (1988)
SK 5715	<i>rne-1 rnb-500</i> (temperature sensitive) <i>thyA715 F</i> ⁻	Arraiano <i>et al.</i> (1988)
SK 5704Δ <i>pcnB</i>	<i>rne-1 pnp-7 rnb-500 ΔpcnB thyA715 F</i> ⁻	This work
SK 5715Δ <i>pcnB</i>	<i>rne-1 rnb-500 ΔpcnB thyA715 F</i> ⁻	This work
NDM 5003	<i>pnp-7 rnb-500 thr leu F</i> ⁻	K. Gerdes, Odense University, Denmark* (unpublished)
NDM 5003Δ <i>pcnB</i>	<i>pnp-7 rnb-500 ΔpcnB thr leu F</i> ⁻	This work
NDM 5004	<i>pnp-7 thr leu F</i> ⁻	K. Gerdes* (unpublished)
NDM 5004Δ <i>pcnB</i>	<i>pnp-7 ΔpcnB thr leu F</i> ⁻	This work
NDM 5005	<i>rnb-500</i> (temperature sensitive) <i>thr leu F</i> ⁻	K. Gerdes* (unpublished)
NDM 5005Δ <i>pcnB</i>	<i>rnb-500 ΔpcnB thr leu F</i> ⁻	This work
NDM 5006	<i>thr leu F</i> ⁻	K. Gerdes* (unpublished)
NDM 5006Δ <i>pcnB</i>	<i>ΔpcnB thr leu</i>	This work

* These strains correspond to the SK strain series (Donovan & Kushner, 1986) but were cured from a plasmid carrying an additional copy of the *rnb-500* allele.

Enzymes and chemicals. Chemicals and enzymes were purchased from Amersham or Sigma unless otherwise stated. Radioactive ribonucleotides were purchased from DuPont/New England Nuclear.

RESULTS

Involvement of RNase E, PNPase, RNase II and PcnB in the degradation of CopA *in vivo*

For this study, we constructed isogenic strains carrying mutations in either one of the genes encoding these four enzymes, alone and in combinations (Table 1; see Methods for details). The mutations in *rne* (*ams-1/rne-1*) and *rnb* (*rnb-500*) are alleles conferring temperature sensitivity (Donovan & Kushner, 1986). The point mutation in the *pnp* gene (*pnp-7*) almost completely abolishes activity (Donovan & Kushner, 1986). The

pcnB mutation used is a deletion of most of its gene (Masters *et al.*, 1993).

Plasmid pKG339 (Jensen *et al.*, 1995) was chosen as the carrier of the *copA* gene for the following reasons. An IPTG-inducible promoter replaces the authentic *copA* promoter. Thus, CopA synthesis can be induced after temperature shifts in strains carrying the temperature-sensitive alleles mentioned above. The use of such a protocol implies that the newly synthesized CopA has never encountered these (active) enzymes. In addition, the absence of the *repA* promoter on pKG339 prevents transcription of CopT RNA. This is of importance, since CopT, if present, will bind to CopA, entailing RNase III cleavage (Blomberg *et al.*, 1990) and, possibly, the protection of CopA segments. Finally, the backbone of pKG339 is derived from plasmid pSC101, whose copy number is not controlled by an antisense RNA. This is

Fig. 1. CopA patterns in strains with combinations of *rnb*, *pnp* and *pcnB* mutant alleles. Strains with mutations in *rnb*, *pnp* and *pcnB* genes (Table 1) were grown at permissive temperature or shifted to non-permissive temperature. CopA synthesis was induced by IPTG from plasmid pKG339 and Northern blot analysis was performed as described in Methods. The figure shows an autoradiogram of such an analysis. The state of the different alleles is indicated by Δ, ts, mut or +.

Fig. 2. CopA patterns in strains with combinations of *rne*, *rnb*, *pnp* and *pcnB* mutant alleles. Strains with mutations in *rne*, *rnb*, *pnp* and *pcnB* genes (Table 1) were grown at permissive temperature or shifted to non-permissive temperature. The analysis and labelling of the lanes was as in Fig. 1.

particularly useful in strains carrying *pcnB* mutations that were shown to affect the copy numbers of R1 and ColE1 (see above).

Two sets of experiments were performed. Figs 1 and 2 represent analyses of CopA patterns on Northern blots in the presence and absence of active enzymes. In all cases and with all combinations of mutations, cells were grown at permissive temperature or shifted to non-permissive temperature before induction of CopA synthesis. The experiments shown in Fig. 3 are rifampicin run-outs. For easy comparison, the same time points were chosen in all experiments (Fig. 3a–j). Table 2 shows the half-life values obtained from the same experiment. Due to the absence of CopT the general pattern of CopA bands is simple: the upper band represents full-length CopA (FL-CopA), and the lower band the RNase-E-generated 3'-stem-loop of CopA containing a 5'-tail (SL-E; Söderbom *et al.*, 1997). An interpretation of the effects of the individual mutations and their contribution to the decay pathway is given below.

RNase E

In line with previous results, inactivation of RNase E results in stabilization of FL-CopA, irrespective of any of the other activities present (Figs 2 and 3b, f; Table 2; Söderbom *et al.*, 1997). This indicates that RNase E is the only enzyme responsible for this cleavage, and that the initial cut is required for subsequent degradation. At permissive temperature, RNase E cleaves CopA irrespective of the presence and absence of the three other activities. This suggests that RNase E is not dependent on these enzymes for cleavage site selection or activity. The absence of additional degradation intermediates that were detected previously (Söderbom *et al.*, 1997) is tentatively explained by the absence of CopT in the cell.

PcnB

Mutations in *pcnB* had previously been shown to increase the stability of SL-E (Söderbom *et al.*, 1997), whereas FL-CopA was not affected, indicating that its

activity is required only subsequent to RNase E cleavage. In all strains carrying a wild-type *pcnB* allele, FL-CopA was more abundant than SL-E (Figs 1–3; Table 2), and the *pcnB* mutation reversed the relative band intensities. Hence, the effect of PcnB is apparent both in the presence and absence of the other enzymic activities. We also note that the patterns observed do not indicate a significant extent of PcnB-dependent addition of adenosines, since the migration of both CopA bands appears unaffected by PcnB.

Exoribonucleases PNPase and RNase II

A mutation in the *pnp* gene showed remarkably small effects on the band patterns or the degradation rate of SL-E (Fig. 1, cf. lanes 1, 2 and 9, 10; Fig. 3d). PNPase is apparently unable to degrade FL-CopA, since in an *rne* mutant strain at non-permissive temperature, FL-CopA is stable (Fig. 2; Fig. 3b, 44 °C). So far, the similarity of band patterns and CopA half-lives in the presence or absence of PNPase suggests that RNase II (or possibly other enzymes) can functionally replace PNPase on an SL-E substrate or that PNPase normally is not involved (but see below).

Upon inactivation of RNase II alone, a small but significant destabilization of SL-E is seen (e.g. Fig. 3c). This may suggest that active RNase II can protect SL-E from PNPase-dependent degradation, and that this protection is lost upon inactivation of RNase II (Fig. 3c, cf. 30 and 44 °C; Table 2). In the *rnb/pnp* double mutant strain at non-permissive temperature, SL-E is significantly stabilized (Fig. 3g) and persists for longer than 5 min. Thus, the relatively small effects of single *pnp/rnb* mutations on the decay rate of SL-E on the one hand, and the significant effect in the double mutant strain on the other, indicate that either of these enzymes can functionally substitute for the other. The lack of complete stabilization of SL-E in the *Exo⁻* strain at non-permissive temperature suggests that other RNases can replace RNase II and PNPase, although with reduced efficiency.

As indicated above, the most dramatic stabilization of SL-E is always associated with a *pcnB* lesion, i.e. SL-E is more stable in an *Exo⁺/ΔpcnB* strain than in an *Exo⁻/pcnB⁺* strain. The degree of stabilization in the absence of PcnB is only slightly affected by the activities of RNase II and PNPase. When the decay of SL-E was followed after rifampicin treatment in the presence or absence of PNPase, RNase II or both, only a minor additional stabilization of SL-E was seen (Fig. 3e, h, i, j; and F. Söderbom, unpublished). We conclude that both exonucleases require PcnB or polyadenylation for their proper activities.

RNase E cleaves CopA at two major sites *in vitro*

In a recent publication, we showed that RNase-E-dependent cleavage of CopA *in vivo* generates SL-E, and that the cleavage site was located within a single-

Fig. 3. Time courses of CopA decay in different mutant strain backgrounds. The protocol for this experiment is described in Methods. After addition of rifampicin, aliquots of the cultures were withdrawn for Northern blot analysis. Temperatures and time of sampling are indicated. The positions of FL-CopA and SL-E are indicated in (a). (a)–(j) represent experiments in the different strain backgrounds. The relevant genotypes are boxed, and the mutated alleles are indicated in bold. A quantification of this experiment is given in Table 2.

stranded region between the two CopA stem-loops (Söderbom *et al.*, 1997). The exact 5'-end of SL-E *in vivo* was mapped within the sequence GG ↓ UUUAA; the arrow indicates the position of cleavage. Since other proteins that could affect cleavage site selection are present *in vivo*, we tested for cleavage of 5'-end-labelled CopA using purified RNase E *in vitro*. Fig. 4 shows an autoradiogram of such an analysis. CopA was cleaved to generate two major 5'-fragments, here denoted E/1 and E/2. Their band intensities, determined by Phosphor-

Imager analysis, do not indicate a precursor–product relationship, i.e. cleavage by purified RNase E can occur at two sites with almost equal efficiency and rate *in vitro*. The significance of a third, minor band is unclear: its intensity is much less than that of the others (Fig. 4, '?'). The RNA species generated may stem from secondary cleavage since its appearance is delayed. Since 5'-end-labelled RNA was used in this experiment, the positions of the 3'-ends of the cleavage products could only be estimated from the size of the products.

Table 2. Decay of CopA in wild-type and mutant strain backgrounds

Relevant genotype*	Half-life at 30 °C (s)†		Half-life at 44 °C (s)†	
	FL-CopA	[SL-E]	FL-CopA	[SL-E]
<i>rne⁺ rnb⁺ pnp⁺ pcnB⁺</i>	50	[73]	58	[61]
<i>rne(ts) rnb⁺ pnp⁺ pcnB⁺</i>	53	[ND]	>300	[ND]
<i>rne⁺ rnb(ts) pnp⁺ pcnB⁺</i>	55	[80]	32	[40]
<i>rne⁺ rnb⁺ pnp-7 pcnB⁺</i>	56	[47]	73	[62]
<i>rne⁺ rnb⁺ pnp⁺ pcnBΔ</i>	59	[>300]	48	[>300]
<i>rne(ts) rnb(ts) pnp⁺ pcnB⁺</i>	75	[75]	>300	[ND]
<i>rne⁺ rnb(ts) pnp-7 pcnB⁺</i>	80	[ND]	115	[>300]
<i>rne⁺ rnb(ts) pnp⁺ pcnBΔ</i>	70	[>300]	56	[>300]
<i>rne⁺ rnb⁺ pnp-7 pcnBΔ</i>	74	[>300]	60	[>300]
<i>rne⁺ rnb(ts) pnp-7 pcnBΔ</i>	60	[>300]	61	[>300]

* Same denotation as in Fig. 3.

† Values derived from the experiment shown in Fig. 3. Relative band intensities were corrected using 5S rRNA loading controls. Values in brackets were intrinsically less reliable since decay of FL-CopA occurs via the SL-E intermediate.

Fig. 4. RNase E cleavage of 5'-end-labelled CopA *in vitro*. Conditions for cleavage and gel analysis are given in Methods. The figure shows an autoradiogram of the analysis. The times of sample withdrawal are indicated. 'CopA' shows the position of the uncleaved RNA, 'E/1' and 'E/2' represent the two major, RNase-E-cleaved 5'-fragments generated, and '?' is a minor cleavage product (see Results). Lane C shows CopA incubated in the absence of RNase E for 190 min, and the marker lane (M) contains radioactively labelled *MspI*-generated DNA fragments of plasmid pBR322.

To analyse the 3'-cleavage products, we performed a similar cleavage test using unlabelled CopA. Aliquots withdrawn from the time course experiment were analysed by reverse transcription to determine the 5'-ends of the cleaved 3'-CopA fragments (see Methods for details). Fig. 5 shows such an analysis. Due to the very stable structure of the 3'-half of CopA, numerous reverse transcription stops are present. One strong such site is indicated by an asterisk. In contrast to this band, whose intensity is unchanged over the time course, two bands increase in intensity and, hence, are due to RNase E cleavage. The exact 5'-ends corresponding to these two RNA fragments can be read off the sequence ladder and are shown in Fig. 6. These 5'-end points are in agreement with the deduced 3'-end points of E/1 and E/2 in Fig. 4. Therefore, the same denotation is used in Fig. 5. Interestingly, the two cleavage sites are different from the one mapped *in vivo* (Söderbom *et al.*, 1997; see also Blomberg *et al.*, 1990), indicating that other proteins present in the cell may influence RNase E cleavage site selection *in vivo*. In summary, the combined results of the experiments shown in Figs 4 and 5 suggest that two major endonucleolytic, RNase-E-dependent, cleavages occur in CopA *in vitro*. The 5'-fragment represented by the minor band (Fig. 4, '?') may be derived from either E/1 or E/2 by exonucleolytic activity present as a minor contaminant in the RNase E preparation.

RNA I is cleaved more rapidly than CopA by purified RNase E *in vitro*

Both RNA I and CopA are substrates for RNase E *in vivo* and *in vitro*. The overall *in vivo* decay rate of CopA is slightly higher than that of RNA I (He *et al.*, 1993; Xu *et al.*, 1993; Söderbom *et al.*, 1997). RNase E is responsible for the initial cleavage step in the decay of

Fig. 5. Determination of 5'-ends of *in vitro* RNase E-cleaved CopA. Conditions for RNase E cleavage of CopA as well as reverse transcription analysis are given in Methods. The times of sample withdrawal are indicated. Lane C shows reverse transcription products generated on CopA incubated for 175 min in the absence of RNase E. The cDNA products corresponding to the 5'-end of CopA and to the two major cleaved species, 'E/1' and 'E/2', are indicated. The asterisk indicates the position of a strong, structure-dependent and RNase E-independent, reverse transcription stop. The sequencing ladders (A, C, G, T) were generated using the same primer as the one used for reverse transcription.

Fig. 6. Localization of *in vitro* and *in vivo* RNase E cleavage sites on CopA. The secondary structure of CopA is shown (Wagner & Nordström, 1986; C. Malmgren and others, unpublished). The *in vivo* cleavage site mapped previously (Söderbom *et al.*, 1997) and the two major sites identified *in vitro* (Fig. 5) are indicated. The location of the reverse transcription primer relative to the CopA sequence is shown.

both RNAs. We therefore asked whether RNase E acts more rapidly on a CopA substrate than on RNA I. End-labelled CopA and RNA I were mixed, and RNase E was added to initiate cleavage. Cleavages were followed over a time course of 60 min. Fig. 7(a) shows an autoradiogram of such an experiment and Fig. 7(b) shows a quantification of the ratio of the RNA I/CopA band intensities as a function of time. Clearly, in the competition between these two substrates for RNase E, RNA I is preferred (or more rapidly cleaved). It is worth emphasizing that this experiment was done without addition of other components of the degradosomal machinery, and that the folding of the two RNAs under *in vitro* conditions may be somewhat different from their structures *in vivo*.

DISCUSSION

In this communication, we investigated the involvement of four proteins in the concerted degradation of CopA, the copy number regulator of plasmid R1. In a previous publication, we showed that CopA decay is initiated by RNase E cleavage, resulting in an extremely unstable 5'-fragment and a 3'-fragment, SL-E. The latter degradation intermediate is further degraded in a PcnB-dependent manner. Here, we extend this analysis by

using host strains carrying lesions in the genes encoding RNase II and PNPase. These two exoribonucleases are considered to be the major enzymes with a 3'→5' specificity in *E. coli*. In addition, they have been implicated in mRNA decay (Nierlich & Murakawa, 1996) as well as in the decay of regulatory RNAs (e.g. RNA-OUT, antisense RNA of IS10; Pepe *et al.*, 1994). The use of multiply mutated strains at both permissive and non-permissive temperature (the *rnb* and *rne* alleles used encode heat-labile proteins) enabled us to compare the CopA degradation patterns (Figs 1 and 2) and the decay of FL-CopA and SL-E after rifampicin addition (Fig. 3; Table 2).

The conclusions from this analysis re-emphasize the key role of RNase E in mediating the initial cleavage. Irrespective of the presence or absence of any of the other activities, inactivation of RNase E resulted in almost complete stabilization of full-length CopA. The major RNase E-generated CopA fragment, SL-E, was unstable in the presence of PcnB, but stabilized in its absence. Whether this effect depends on 3'-adenylation cannot be decided based on the experiments reported here but appears likely since a similar but more pronounced PcnB-dependent effect on the decay of RNA I has been shown to be due to polyadenylation (Xu & Cohen, 1995). A recent publication from the Gerdes group also shows that the rapid degradation of Sok, the

Fig. 7. Relative cleavage rates of RNA I and CopA by RNase E *in vitro*. Mixtures of 5'-end-labelled RNA I and CopA were subjected to RNase E cleavage. (a) Autoradiogram of the analysis. The gel was run so that the resulting small cleavage products are not visible. The times of sample withdrawal are indicated. The marker lane (M) represents *Msp*I-cleaved, 32 P-labelled pBR322 DNA. Lanes 'RNA I', 'CopA' and 'RNA I + CopA' are incubations of these RNAs in the absence of RNase E. (b) PhosphorImager quantification of the RNA I and CopA band intensities in (a), represented as relative ratios of RNA I/CopA. The ratio of the intensities of the input RNAs (lane RNA I + CopA) was set to unity.

negative regulator RNA of the post-segregational killing system (*hok/sok*), is dependent on A-tailing by PcnB (Mikkelsen & Gerdes, 1997). It is worth noting that the degree of SL-E stabilization obtained in the series of isogenic strains used in this study exceeds that seen in a different strain background (Söderbom *et al.*, 1997). The activities responsible for this difference are not known.

Both exoribonucleases were able to degrade SL-E, indicated by stabilization in the absence of both functional enzymes (Fig. 3g). However, since some residual degradation occurred under these conditions, other enzymic activities must be able to partially replace the two major exoribonucleases. The relatively minor effects on apparent half-life of SL-E in the single mutant cases suggest that RNase II and PNPase can functionally replace each other (Fig. 3c, d). Both enzymes appear to require the presence of PcnB to promote SL-E decay. Inactivation of RNase II also revealed a different effect. SL-E was more stable at permissive than at non-permissive temperature (Fig. 3c). The degree of stabilization was approximately threefold in three separate experiments and suggests that, when RNase II is present, the rate of PNPase-dependent SL-E degradation is decreased, i.e. since RNase II processivity is impeded by stable stem-loops (Guarneros & Portier, 1991; McLaren *et al.*, 1991), binding (or stalling) of this enzyme at the 3'-stem-loop could protect SL-E from PNPase activity. Alternatively, RNase II could act by removing short A-tails, converting the RNA into a less suitable substrate for PNPase. A similar RNase-II-dependent protection of RNA-OUT (Pepe *et al.*, 1994) and *rpsO* mRNA (Hajnsdorf *et al.*, 1994) was previously reported. The two effects of RNase II are indicated schematically in Fig. 8.

In conclusion, CopA decay most likely involves at least the four genes/gene products studied here, and the possible involvement of additional exonucleases is suggested by the pattern obtained in the *Exo⁻* strain. The steps in the degradation pathway of CopA are summarized in Fig. 8. Decay is initiated by RNase E, followed by PcnB action on SL-E (most likely via addition of short A-tails), which in turn is a prerequisite for rapid 3'-exonucleolytic degradation by PNPase and/or RNase II. Our data do not permit us to assess the relative contributions of these two latter enzymes.

The pathway of CopA decay is similar to that of RNA I and Sok, but shows some differences. RNase E cleavage is, in all three cases, required as the initial step (Lin-Chao & Cohen, 1991; Söderbom *et al.*, 1997; Mikkelsen & Gerdes, 1997), and PcnB activity (in RNA I and Sok: polyadenylation; Xu & Cohen, 1995; Mikkelsen & Gerdes, 1997) is needed to provide exoribonucleases with proper substrates. The degree of stabilization of the RNase-E-generated 3'-fragments SL-E (of CopA; twofold in Δ *pcnB*; Söderbom *et al.*, 1997) and RNA I₅ (of RNA I; >10-fold; He *et al.*, 1993; Xu *et al.*, 1993; Xu & Cohen, 1995) is however different. If we speculate that the effect in both cases is mediated by A-tails, this may indicate that longer tails or a higher stoichiometric fraction of tails must be added to RNA I than to CopA to permit exonucleolytic degradation. Since previous attempts to identify polyadenylated CopA species *in vivo* have failed (Söderbom *et al.*, 1997; and unpublished), and the band patterns in Figs 1–3 do not indicate a significant degree of tailing, the question of whether PcnB affects CopA degradation directly or via its polyadenylation activity is still unresolved.

Upon polyadenylation of RNA I, the degradation rate by PNPase is drastically increased both *in vitro* and *in vivo* (Xu & Cohen, 1995), suggesting that this enzyme is the most important one in PcnB-dependent decay of RNA I₅. Similarly, *in vivo* data suggest that PNPase is the most important exonucleolytic activity on Sok substrates (Mikkelsen & Gerdes, 1997). In the case of CopA, a dominant role of PNPase is not supported, since the absence of this enzyme alone has only minor effects on CopA decay. Interestingly, results by Xu & Cohen (1995) suggested that PNPase is also required for full RNase E activity on RNA I. RNase E activity was stimulated approximately sevenfold. Again, CopA decay is different in that no quantitatively comparable effect is supported (cf. Fig. 3a, d).

is conceivable that RNA structure is important: RNA I may fold into a clover-leaf structure in which the 5'- and 3'-ends are in close proximity. This might facilitate direct interaction between enzymes acting at either end, such as RNase E and PNPase, to mutually stimulate each other's activities. This may not be the case for CopA. Similarly, the length of the protruding 3'-tail seems to be different: CopA has a longer template-encoded tail than RNA I and Sok (~ six and two to three nucleotides, respectively). RNase II and PNPase require single-stranded extensions of sufficient length 3' of stable RNA structures to initiate degradation, and polyadenylation is known to aid RNase II in degradation of otherwise resistant structures (Coburn & Mackie, 1996a, b). Hence, CopA's longer tail may render polyadenylation quantitatively less important than in the case of RNA I (and possibly Sok). If so, this might also explain the difficulty of identifying A-tails on CopA (see above).

A clear indication that RNase E activity is modulated by other factors present *in vivo* can be found in the identification of RNase E cleavage sites on CopA *in vitro* (Figs 4–6). Two major cleavage sites were mapped. Both cleavages appear to occur independently, since bands consistent with these sites were found both when 5'- and 3'-fragments were analysed. Neither one of these sites coincides precisely with the one mapped *in vivo* (Söderbom *et al.*, 1997), which is located between the two scissions generated *in vitro*. Site E/1 conforms to the known preference of RNase E for A/U-rich, single-stranded RNA segments (McDowall *et al.*, 1994), whereas E/2 does not, although it is used with equal efficiency. Similar discrepancies between *in vitro* and *in vivo* cleavages by RNase E have been reported previously (Braun *et al.*, 1996; Kaberdin *et al.*, 1996; Naureckiene & Uhlin, 1996), emphasizing the role of additional factors assisting RNase E in cleavage site selection in the cell. Hence, this endonuclease is still a 'wonderfully mysterious enzyme' (Cohen & McDowall, 1997) and other factors that determine its cleavage site specificity remain elusive.

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somal complex, are probably involved in fine-tuning of degradation activity. From our experience, *in vivo* analyses of decay in mutant host strains can yield useful information, but the uncertainties created by, for example, unwanted and unanticipated side effects – such as altered regulation of one enzyme due to the absence of another (e.g. Zilhão *et al.*, 1996) – make interpretations difficult. Reconstitution of complete degradosomal activities to obtain *in vivo*-like RNA decay *in vitro* (e.g. Py *et al.*, 1996) should eventually result in a better understanding of RNA turnover.

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