

# Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA

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The polycistronic mRNA of the histidine operon is subject to a processing event that generates a rather stable transcript encompassing the five distal cistrons. The molecular mechanisms by which such a transcript is produced were investigated in *Escherichia coli* strains carrying mutations in several genes for exo- and endonucleases. The experimental approach made use of S1 nuclease protection assays on in vivo synthesized transcripts, site-directed mutagenesis and construction of chimeric plasmids, dissection of the processing reaction by RNA mobility retardation experiments, and in vitro RNA degradation assays with cellular extracts. We have found that processing requires (1) a functional endonuclease E; (2) target site(s) for this activity in the RNA region upstream of the 5' end of the processed transcript that can be substituted by another well-characterized *rne*-dependent cleavage site; (3) efficient translation initiation of the first cistron immediately downstream of the 5' end; and (4) a functional endonuclease P that seems to act on the processing products generated by ribonuclease E. This is the first evidence that ribonuclease P, an essential ribozyme required for the biosynthesis of tRNA, may also be involved in the segmental stabilization of a mRNA.

[Key Words: *Escherichia coli*; *Salmonella typhimurium*; *his* operon; mRNA processing; translation; RNase E; RNase P]

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mRNA decay in bacteria is mediated by the coordinated action of exonucleases and endonucleases (for review, see Petersen 1992). Polynucleotide phosphorylase (PNPase) and ribonuclease II (RNase II) are the two major 3' → 5' exonucleases involved in mRNA turnover (Donovan and Kushner 1986). Endoribonuclease III (RNase III) does not affect bulk mRNA stability (Babitzke et al. 1993) and is mostly involved in processing of the 30S rRNA precursor even though RNase III can initiate the functional decay of a few specific mRNAs (Court 1993). Endoribonuclease E (RNase E) was initially characterized as the enzyme that processes the precursor of 5S rRNA (Ghora and Apirion 1978). RNase E is the only endoribonuclease implicated thus far in general mRNA turnover (Arraiano et al. 1988; Mudd et al. 1990b; Babitzke and Kushner 1991). Many mRNAs were shown to contain sites whose cleavage was abolished in strains harboring temperature-sensitive *rne* mutations at the nonpermissive temperature (Kokoska et al. 1990; Mudd et al. 1990a; Gross 1991; Lin-Chao and Cohen 1991; Nilsson and Uhlin 1991; Régnier and Hajnsdorf

1991; Patel and Dunn 1992; Arraiano et al. 1993; Klug 1993; Mudd and Higgins 1993; Carpousis et al. 1994). *rne*-dependent cleavages might produce relatively stable processed transcripts, such as the T4 gene 32 (Mudd et al. 1988) and the *dicF* (Faubladier et al. 1990) and the *gltX* mRNAs (Brun et al. 1990). In different studies ribonuclease P (RNase P) and other nucleases responsible for stable RNA processing did not seem to be involved in mRNA degradation (Deutscher 1988).

We have previously identified in *Salmonella typhimurium* a rather stable 3900-nucleotide-long mRNA molecule derived by specific maturation of the *his* operon primary transcript. This species spans the five distal cistrons of the histidine gene cluster, and its formation requires a *cis*-acting element located 620 nucleotides upstream of the 5' end of the processed species and the presence of initiating ribosomes at the intercistronic barrier *hisC/hisB* located immediately downstream (Alifano et al. 1992).

We have now studied the molecular mechanisms of this specific processing in several exo- and endoribonuclease mutant strains of *Escherichia coli*. We have demonstrated that processing is reduced in RNase E mutants and that in the presence of nonfunctional exonucleolytic

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activities several *rne*-dependent cleavages are detected in the region spanning the *cis*-acting element. We have also shown that a well-characterized *rne*-dependent cleavage site can substitute for the element. The formation of the 5' end of the 3900-nucleotide species is impaired in RNase P mutants both in *in vivo* and *in vitro* assays. Additional *in vivo* evidence obtained confirmed the role of initiating ribosomes at the *hisB* start site in the processing reaction. These observations are consistent with a processing model in which cleavage by RNase P of *rne*-dependent endonucleolytic products stabilizes the distal part of a polycistronic transcript.

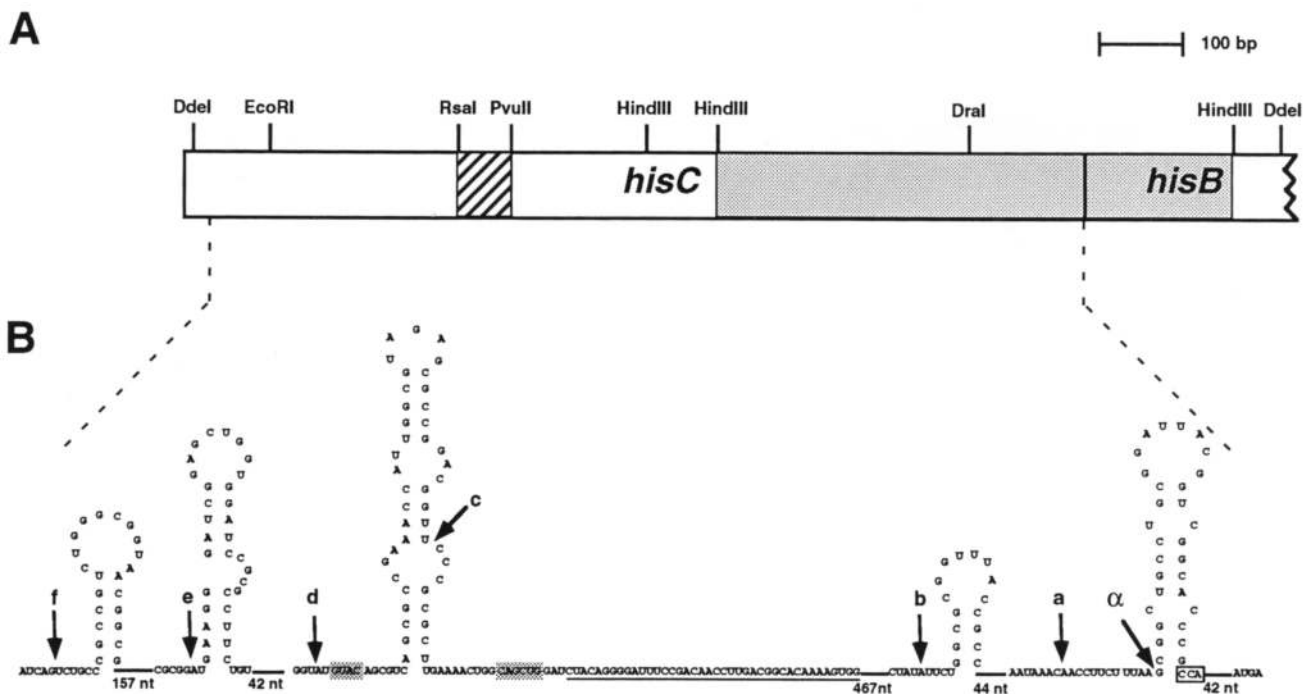
## Results

The processed 3900-nucleotide-long RNA derived from the primary transcript of the *his* operon of *S. typhimurium* is the most abundant species in the steady-state mRNA samples and has a half-life of 15 min compared with a half-life of ~2 min for the full-length transcript. The transcription pattern of the *his* operon in *E. coli* is indistinguishable from the one in *S. typhimurium*, including the processed species (Carlomagno et al. 1988; Alifano et al. 1992). We have therefore used as an experimental system the *S. typhimurium*-derived plasmid pUH1293, which contains all of the elements necessary

for the processing event within *hisC* (Fig. 1A; Alifano et al. 1992). The different *E. coli* strains (Table 1) were transformed with this plasmid. Total RNA extracted from transformed strains was hybridized to a 5'-end-labeled 612-bp *S. typhimurium*-specific *Hind*III fragment spanning the 5' end of the 3900-nucleotide processed species (Fig. 1A, shaded area; Fig. 2, lane 1). This probe does not cross-hybridize to *his*-specific *E. coli* transcripts (Materials and methods). The hybrids were digested with S1 nuclease and analyzed on 5% polyacrylamide-urea denaturing gel. The same product corresponding to the 5' end of the processed transcript ( $\alpha$ ; Fig. 1B) was detected in RNA samples from *S. typhimurium* wild-type strain LT2 (Fig. 2, lane 2) or from *E. coli* strain SK5006 harboring the pUH1293 plasmid (Fig. 2, lane 3). This cleavage site is located 76 nucleotides upstream of the AUG initiation codon of the *hisB* cistron (Fig. 1B).

### *RNase E* mutants show reduced endonucleolytic-specific cleavages

The RNase-deficient strains SK5003 (*pnp7 rnb500*) and SK5721 (*pnp7 rnb500 rne1*) harboring pUH1293 were grown at 30°C to early logarithmic phase. One-half of the culture was then shifted to 44°C for 40 min before harvesting the cells for RNA isolation (see Materials and



**Figure 1.** (A) Physical and genetic map of the *hisCB* region of the *his* operon of *S. typhimurium*. The *DdeI* fragment cloned in plasmid pUH1293 and relevant restriction sites are shown. The shaded area indicates the 612-bp *Hind*III fragment used as a probe in S1 protection experiments. The hatched area indicates the fragment spanning the upstream *cis*-acting element required for processing. (B) Secondary structures and relevant sequences in the processed region. The arrows indicate the multiple *rne*-dependent cleavages (f–a) and the *rnp*-dependent cleavage ( $\alpha$ ) that corresponds to the 5' end of the processed species. The CCA consensus at the 3' end of the hairpin (Forster and Altman 1990) is boxed. The *RsaI* and *PvuII* restriction sites that delimit the *cis*-acting element are shaded. The region complementary to the primer used in the degradation assays (Fig. 3) is underlined. At the 3' end of the sequence is the *hisC*/*hisB* intercistronic barrier.

**Table 1.** Bacterial strains

Strain	Genotype	Reference
<i>S. typhimurium</i>		
LT2	wild type	Rechler et al. (1972)
<i>E. coli</i>		
SK5006	<i>thr leu</i> , pDK39 (Cm <sup>r</sup> <i>mb500</i> )	Arraiano et al. (1988)
SK5003	SK5006 <i>pnp7</i> <i>mb500</i>	Arraiano et al. (1988)
SK5721	SK5006 <i>rne1 pnp7</i> <i>mb500</i>	Arraiano et al. (1988)
SK5695	SK5006 <i>rne1</i>	Arraiano et al. (1988)
BF265	<i>arg gly try</i> <i>lacZ(Am)</i> Sm <sup>r</sup>	Kole et al. (1980)
A49	BF265 <i>rnpA49</i>	Kole et al. (1980)
4273	<i>lacZ(Am)</i> T6 <sup>r</sup> (Am) BF23 <sup>r</sup> (Am) <i>su</i> <sup>-</sup>	Kole et al. (1980)
ts709	4273 <i>rnpB709</i>	Kole et al. (1980)
HT115	<i>rnc14::ΔTn10</i>	Takiff et al. (1989)

methods). Strain SK5003 has an inactive *pnp* allele (Reiner 1969) and a *rnb*(Ts) mutation and produces only a partially temperature-sensitive RNase II enzyme (Donovan and Kushner 1986). Compared with SK5006, the exonucleases-deficient SK5003 strain accumulated intermediate breakdown products (a and b in Fig. 1B) and showed an increase of the processed species ( $\alpha$ ) at both temperatures (Fig. 2, lanes 4,5). In addition to *pnp* and *rnb*(ts) mutations, strain SK5721 harbors a temperature-sensitive *rne1* mutation (Arraiano et al. 1988). When grown at 30°C, this strain had a profile similar to SK5003 (Fig. 2, lane 7). However, when strain SK5721 was shifted to 44°C, the protection pattern was different. The amount of the  $\alpha$ , a, and b species was reduced, whereas the amount of full-length protected fragment was increased (Fig. 2, lane 6). These data suggest that the processing event leading to formation of the 3900-nucleotide species is *rne* dependent.

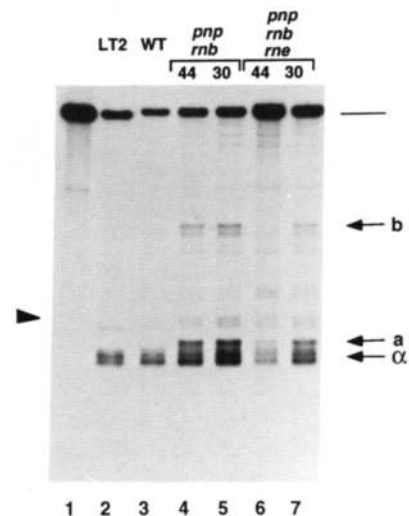
*The upstream cis-acting element contains RNase E target sites and can be replaced by a functionally equivalent element*

The genetic evidence that the processed species is generated by an *rne*-dependent mechanism (Fig. 2) led us to pursue more direct evidence using in vitro experiments. We have reported previously that processing requires an upstream *cis*-acting element within *hisC* (Fig. 1A, hatched region) that binds soluble factors in a mobility retardation assay (Alifano et al. 1992). This element was able to bind components present in an RNase E-enriched fraction and wild-type but not *rne1*-derived extracts (data not shown).

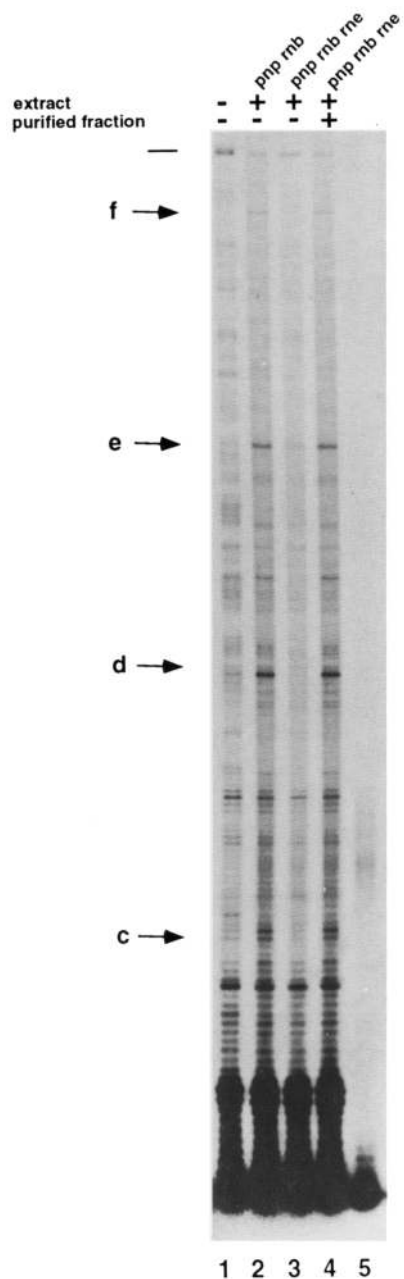
A single processed species ( $\alpha$  in Fig. 2) was present in the parental strain, whereas other intermediates could only be detected in exonucleases-defective hosts (a and b in Fig. 2). The relative position of the 5' ends of the a and b *rne*-dependent intermediates was deduced from sequence ladders run in parallel (data not shown). They

map upstream but in close proximity to the 5' end of the processed species ( $\alpha$ ) and quite 3'-distal to the *cis*-acting element (Fig. 1B). This suggests that other unstable exonucleases-sensitive breakdown products could be present but not detectable with the S1 protection assay.

To detect additional endonucleolytic cleavages, we extended our analysis to the sequences surrounding the *cis*-acting element by an in vitro degradation assay. A cold RNA was obtained in vitro by runoff transcription of the recombinant linearized plasmid pGEM1293 (Materials and methods). This cold transcript was incubated at 44°C for 5 min with S30 extracts obtained from strains SK5003 (*pnp7 rnb500*) and SK5721 (*pnp7 rnb500 rne1*) shifted to 44°C for 40 min. A 5'-end-labeled 40-nucleotide-long DNA primer (Fig. 3, lane 5), complementary to the region immediately downstream from the *cis*-acting element (Fig. 1B, underlined), was annealed to the breakdown products and elongated with reverse transcriptase. The complementary DNA obtained from the untreated runoff RNA is a ladder of nonspecific degradation products and/or premature transcription stops, including some full-length molecules (Fig. 3, lane 1). After incubation of the RNA with the extract derived from strain SK5003, several bands were detected above background, four of which were most prominent (c-f in Fig. 3, lane 2). These intermediate products were reduced when



**Figure 2.** Formation of the 3900-nucleotide species is *rne* dependent. The probe (Fig. 1A) labeled at its 5' end (lane 1) was hybridized to 20  $\mu$ g of total RNA extracted from wild-type *S. typhimurium* strain LT2 (lane 2), from *E. coli* strain SK5006 harboring the pUH1293 plasmid (WT) (lane 3), from strains SK5003 (*pnp rnb*) and SK5721 (*pnp rnb rne*) carrying the same plasmid and grown at 30°C (lanes 5 and 7, respectively) or shifted to 44°C (lanes 4 and 6, respectively). Hybrids were treated with S1 nuclease, and the DNA protected fragments were analyzed on a 5% polyacrylamide-urea denaturing gel. The bar indicates the full-length protected hybrids, and the arrows the 5' ends of the hybrids (b, a, and  $\alpha$ ) sensitive to inactivation of the *rne* locus. The arrowhead at left indicates the hybrid formed by transcripts initiating at the internal P2 promoter (Alifano et al. 1992).



**Figure 3.** Several *rne*-dependent cleavages are present in the upstream region. Cold RNA obtained *in vitro* from plasmid pGEM1293 [Materials and methods] was incubated with S30 extracts derived from strains SK5003 (*pnp rnb*) and SK5721 (*pnp rnb rne*). The cleavage products were analyzed by primer extension with the oligonucleotide (lane 5) shown in Fig. 1B. Untreated runoff transcript was incubated at 44°C for 5 min before the analysis (lane 1). The runoff transcript was incubated with SK5003 extract (lane 2), SK5721 extract (lane 3), RNase E-enriched fraction was added to SK5721 extract before incubation (lane 4). The bar indicates the full-length extended product. Arrows indicate the more prominent *rne*-dependent cleavage sites (c,d,e, and f).

the extract was derived from the triple mutant SK5721 (Fig. 3, lane 3) and were increased when the RNase E-en-

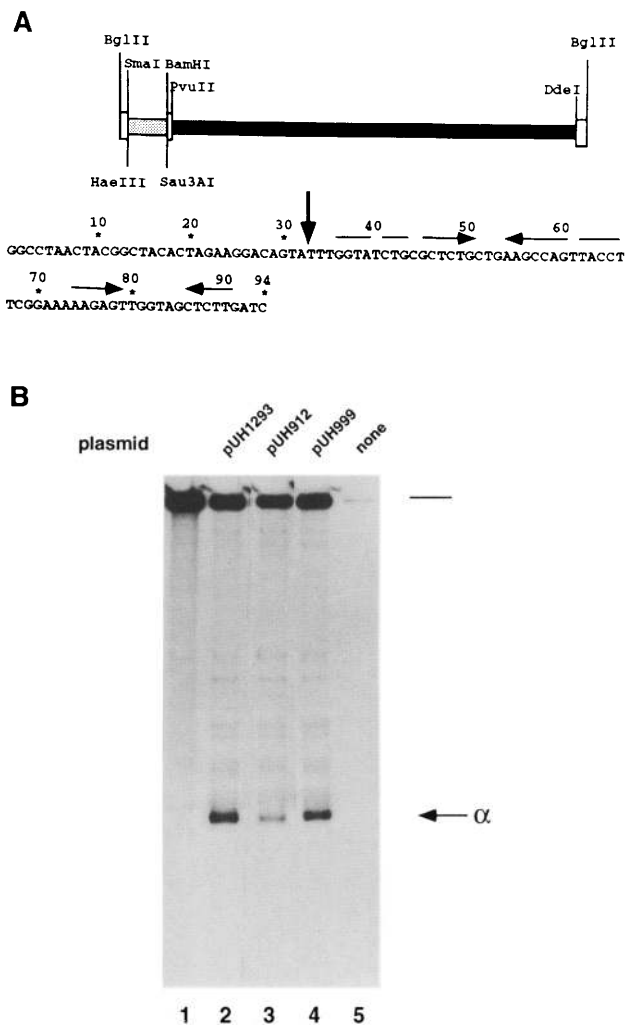
riched fraction was added to the SK5721 extract before RNA treatment (Fig. 3, lane 4). The relative position of the 5' ends of the cleavage products was deduced from sequence ladders run in parallel (data not shown) and is presented in Figure 1B. The d band corresponded to a cleavage site located in a single-stranded region immediately upstream from the *cis*-acting element (Fig. 1B) and was also detectable *in vivo* as a faint band in an S1 mapping assay (data not shown). This experiment proved the presence of multiple *rne*-dependent cleavage sites within and upstream of the *cis*-acting element.

Deletion of the *cis*-acting element causes a severe reduction of the 3900-nucleotide processed species (Alifano et al. 1992). The evidence provided above suggested a prominent role for this element in binding and subsequent cleavage mediated by RNase E. We tested whether a canonical RNase E target region could substitute for the native element. The region harboring the RNase E target site of RNAI of plasmid ColE1 (Tomcsanyi and Apirion 1985; Bouvet and Belasco 1992) was religated in the correct position within pUH912, a plasmid without the *cis*-acting element. The resulting plasmid, pUH999 (Fig. 4A), was introduced into parental strain SK5006, and the RNA produced was analyzed in an S1 mapping experiment using the *Hind*III probe (Fig. 1A) labeled at its 5' end (Fig. 4B, lane 1). The comparison between the protection pattern of RNAs derived from strain SK5006 harboring the complete plasmid pUH1293 (Fig. 4B, lane 2) or the deleted plasmid pUH912 (Fig. 4B, lane 3), with that obtained from RNA derived from the same strain harboring the hybrid plasmid pUH999 (Fig. 4B, lane 4), showed that the processed species ( $\alpha$ ) was produced in an amount comparable to that obtained in the presence of the natural element. Therefore, it seems that the *cis*-acting element is necessary for the formation of the 3900-nucleotide species simply because it is an RNase E target site and it can be replaced by a functionally equivalent RNA region.

#### *RNase P* participates in the formation of the 3900-nucleotide processed species

The 5' end of the processed species must be produced by an endonucleolytic cleavage. Because formation of the  $\alpha$  species is not entirely *rne* dependent (Fig. 2), we examined its production in other endoribonuclease mutants. Formation of the 3900-nucleotide species was not affected in the RNase III<sup>-</sup> host HT115 (data not shown), indicating that RNase III activity does not participate in the processing event.

Next, we tested the influence of RNase P on the formation of the stable species  $\alpha$ . We used strains harboring mutations affecting either the C5 protein (A49) or the M1 RNA subunit (ts709) (Schedl and Primakoff 1973; Kole et al. 1980; Guerrier-Takada et al. 1983). Cells containing the *mnpA49* mutation do not grow at temperatures above 42°C. However, even under permissive growth conditions, the activity of RNase P is considerably reduced in cells bearing this mutation (Schedl and Primakoff 1973). We therefore transformed the parental



**Figure 4.** The region containing the ColE1 *me*-dependent site can substitute for the upstream *cis*-acting element. (A) The boxes indicate the different fragments used to construct the pUH999 hybrid plasmid [(open) polylinker of the pUH912 plasmid, (shaded), fragment spanning the *me*-dependent site of ColE1 (Bouvet and Belasco 1992); (solid), *PvuII*-*DdeI* insert carried by pUH912 (Alifano et al. 1992)]. Relevant restriction sites used for the construction are indicated at the top. (Bottom) The sequence of the fragment derived from ColE1 with its potential secondary structures (convergent arrows). The vertical arrow indicates the *me*-dependent cleavage site. (B) Total RNA (20  $\mu$ g) extracted from strain SK5006 (lane 5) harboring the recombinant plasmids pUH1293 (lane 2), pUH912 (lane 3) and pUH999 (lane 4) was hybridized to the probe (Fig. 1A), labeled at its 5' end (lane 1), and analyzed as in Fig. 2 in an S1 protection assay. The bar indicates the full-length extended product, and the arrow the 5' end of the 3900-nucleotide processed species ( $\alpha$ ).

strain BF265 and its congenic derivative A49 (*rnpA49*) with the plasmid pUH1293 and grew them at 37°C. The parental strain 4273 and its congenic derivative ts709 [*rnpB709*(Ts)] were transformed with the same plasmid and grown at 30°C to logarithmic phase, after which one-half of each culture was shifted to 44°C for 40 min. RNAs derived from the transformed strains were ana-

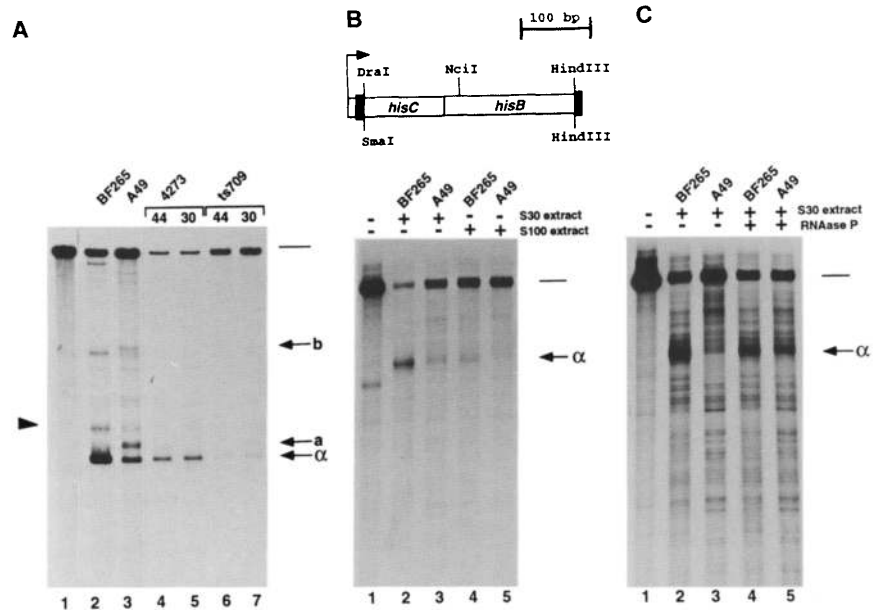
lyzed by S1 mapping by use of the *HindIII* fragment (Fig. 1A) labeled at its 5' end as a probe (Fig. 5A, lane 1). Compared with BF265 (Fig. 5A, lane 2) in mutant strain A49 a decreased amount of the  $\alpha$ -processed species and a concurrent increase in the amount of full-length protected hybrids were detected. Accumulation of the intermediate *me*-dependent a and b species was also observed (Fig. 5A, lane 3). Similar results were obtained when strain A49 was grown at 30°C and then shifted to 44°C (data not shown). The  $\alpha$  band was present in strain 4273 grown at 30°C or shifted to 44°C (Fig. 5A, lanes 4,5). In mutant strain ts709 there was an increase of the full-length protected fragment. The  $\alpha$  species almost disappeared at the nonpermissive temperature (Fig. 5A, lane 6) and was very faint even at the permissive temperature (Fig. 5A, lane 7). The different amount of *his*-specific transcripts could be attributable to the different genetic background of the parental and derivative strains.

Further proof of the involvement of RNase P in the processing event was obtained by an in vitro degradation assay. A 183-nucleotide-long labeled RNA (Fig. 5B, bottom, lane 1), obtained by in vitro transcription of the recombinant linearized plasmid pGEM322 (Fig. 5B, top; Materials and methods), was incubated at 44°C for 20 min with S30 and S100 extracts obtained from strains BF265 and A49. Formation of the processed species ( $\alpha$ ) was drastically reduced after incubation with mutant (Fig. 5B, bottom, lane 3) S30 extracts compared with the parental (Fig. 5B, bottom, lane 2) ones. The activity responsible for the processing of the substrate was only recovered in the S30 fraction, because formation of the  $\alpha$  species was severely affected when the riboprobe was incubated with parental (Fig. 5B, bottom, lane 4), and more so with mutant (Fig. 5B, bottom, lane 5), S100 extracts. Similar results were obtained when cold RNA from plasmid pGEM322 was primer extended with a 5'-end-labeled oligonucleotide after incubation with the different extracts (data not shown). Next, we performed a reconstitution experiment by adding a purified preparation of RNase P (kindly provided by Dr. S. Altman, Yale University, New Haven, CT) to S30 extracts of parental and mutant strains (Fig. 5C). Addition of the pure enzyme to the S30 extract prepared from mutant strain A49 restored processing of the labeled substrate (Fig. 5C, lane 5) as evidenced by the presence of the  $\alpha$  species in amounts comparable with those detected with S30 extracts derived from parental strain BF265 in the absence (Fig. 5C, lane 2) or presence (Fig. 5C, lane 4) of the purified RNase P preparation. However, when the purified *E. coli* RNase P was directly challenged in vitro under the appropriate conditions with the same 183-nucleotide substrate, in the absence of any other component of the cellular extract, cleavage did not occur at all (Cecilia Guerrier-Takada, pers. comm.).

#### *Ribosomes initiating at the intercistronic barrier hisC/hisB are necessary for the formation of the processed transcript*

By use of drugs inhibiting the translation initiation step,

**Figure 5.** Efficient formation of the 3900-nucleotide species requires active RNase P and components of the S30 fraction. (A) Total RNA (20  $\mu$ g) derived from strains harboring the plasmid pUH1293 was hybridized to the probe (Fig. 1A), labeled at the 5' end (lane 1), and analyzed as in Fig. 2. Strain BF265 (lane 2) and its A49 (*rnpA49*) derivative (lane 3) were grown at 37°C; strain 4273 and its ts709 derivative (*rnpB709*) were grown at 30°C (lanes 5,7) or shifted to 44°C (lanes 4,6). The bar indicates the full-length protected probe, and the arrows the 5' ends of the processed species (b,a, and  $\alpha$ ). The arrowhead at left indicates the hybrid formed by transcripts initiating at the internal P2 promoter (Alifano et al. 1992). (B) (Top) The boxes indicate the different fragments used to construct the pGEM322 hybrid plasmid [(solid) polylinker of the pGEM3Z vector plasmid; (shaded, far left) region of the T7 promoter; (arrow) transcription initiation site; (open) *DraI*–*HindIII* 322-bp insert derived from the *hisC*/*hisB* region]. Relevant restriction sites used for the construction, and the internal *NciI* site used to linearize the plasmid for in vitro transcription are indicated. (Bottom) Labeled RNA (lane 1) obtained in vitro from plasmid pGEM322 (Materials and methods) was incubated with S30 extracts derived from strains BF265 (lane 2) and A49 (lane 3), and with S100 extracts derived from strains BF265 (lane 4) and A49 (lane 5). (C) Labeled RNA (lane 1) obtained in vitro from plasmid pGEM322 (Materials and methods) was incubated with S30 extracts derived from strains BF265 and A49 in the absence (lanes 2,3) and in the presence (lanes 4,5) of purified RNase P. The cleavage products were analyzed on a 6% polyacrylamide–urea denaturing gel. The bar indicates the full-length RNA, and the arrow the 5' end of the processed product ( $\alpha$ ).



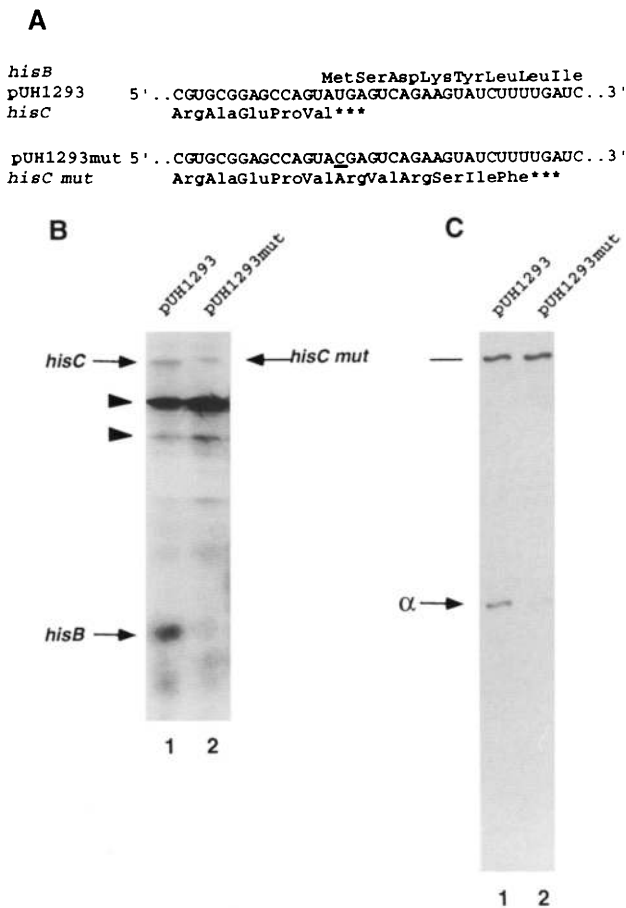
we have shown previously that formation of the initiation complex at the *hisB* cistron plays a major role in the processing event (Alifano et al. 1992, 1994). Further proof was obtained with a mutation that directly affected translation initiation. A mutation that changed the AUG initiation codon of the *hisB* cistron to ACG was constructed by in vitro site-directed mutagenesis of plasmid pUH1293 (Materials and methods) to produce plasmid pUH1293mut. At the same time, the mutation changed the UGA stop codon of the *hisC* cistron to CGA, generating a *hisC* peptide with a 6-amino-acid carboxy-terminal extension (Fig. 6A,B). The truncated *hisB*-encoded peptide produced by pUH1293 (Fig. 6B, lane 1) was not detected when pUH1293mut was transcribed and translated with an S30 extract (Fig. 6B, lane 2). Total RNAs derived from strain SK5006 transformed with plasmids pUH1293 and pUH1293mut were hybridized to the *HindIII* 5'-end-labeled probe (Fig. 1A) and analyzed by S1 mapping. The protection pattern showed comparable amounts of protection of the entire probe with both RNAs, whereas the band corresponding to the processed species  $\alpha$ , present in discrete amount in the strain harboring pUH1293 (Fig. 6C, lane 1), was considerably reduced in the strain carrying pUH1293mut (Fig. 6C, lane 2). From these data we obtained a further and more direct demonstration that the presence of ribosomes initiating translation at the *hisB* cistron is important for the formation of the processed species.

## Discussion

*rne*-dependent endonucleolytic cleavages are necessary for the maturation of the 3900-nucleotide species

The data presented in this paper demonstrate that formation of the 3900-nucleotide processed species initiates with *rne*-dependent endonucleolytic cleavages at multiple internal sites within *hisC*. The amount of the processed transcript and of minor breakdown products was significantly reduced in the *pnp rmb rne* triple mutant at the nonpermissive temperature compared with the congenic *pnp rmb* double mutant in vivo (Fig. 2). Compared with the parental strain, the strain harboring *pnp* and *rmb* mutations accumulated a larger amount of the processed species, and additional processing intermediates (a and b in Fig. 2) were detected. This finding is consistent with previous reports on the stabilization of products in strains with defective 3' exonucleases (Mackie 1989; Plamann and Stauffer 1990; Jain and Kleckner 1993). It is believed that in a wild-type host, a significant fraction of transcripts is cleaved endonucleolytically at more than one site, and the resulting internal fragments are rapidly degraded from their 3' ends (Jain and Kleckner 1993).

We have shown previously by an RNA binding–shift assay that the *cis*-acting element needed for generation of the stable 3900-nucleotide species consists of an RNA



**Figure 6.** The uncoupling of translation at the intercistronic boundary *hisC/hisB* affects the maturation of the 3900-nucleotide species. (A) Effects of the mutation introduced in pUH1293mut on the translation product of *hisC* and *hisB* cistrons. For pUH1293 the sequence is indicated in the middle, the amino acid sequence of the *hisB* product is indicated at the top, and that of the *hisC* product at the bottom. For pUH1293mut, the nucleotide sequence is indicated on the top and the amino acid sequence of the fused *hisC/hisB* peptide at the bottom. The mutated C nucleotide in the intercistronic barrier is underlined. (B) Analysis on a 12% SDS-polyacrylamide gel of the peptides produced in a transcription-translation coupled system by the recombinant plasmids pUH1293 (lane 1) and pUH1293mut (lane 2). Arrowheads indicate the position of the  $\beta$ -lactamase peptides; arrows indicate the positions of the *his*-specific gene products. (C) Total RNA (20  $\mu$ g) derived from strain SK5006 transformed with pUH1293 and pUH1293mut (lanes 1,2) was analyzed as in Fig. 2. The bar indicates the full-length protected probe, and the arrow the 5' end of the mature species ( $\alpha$ ).

region that is able to bind soluble factor(s) in S30 extracts (Alifano et al. 1992). We have now observed that the binding activity was absent in extracts derived from the *rne1* mutant strain and was present in a wild-type RNase E-enriched fraction. In this study we demonstrate that a different *rne*-dependent site, harboring only one RNase E target (Bouvet and Belasco 1992), can substitute for the region spanning the multiple RNase E sites (Fig. 4).

These evidences confirm that the formation of the mature species is an *rne*-dependent process, requiring at least one target site upstream of the 5' end. The cleavage site (d) identified in the in vitro degradation assay (Fig. 3) is located immediately upstream of the putative stem-loop structure corresponding to the *cis*-acting element and cleavage occurs at a degenerate consensus sequence GU/AUG for RNase E (Mackie 1991). Detection of the processing intermediate produced by this cleavage in vivo is difficult, probably because of its instability even in a *pnp rnb* background (see Results). The additional cleavage sites identified in vitro in the region of the element (c, e, and f) do not appear to play a major role in vivo. Deletion of these cleavage signals does not affect significantly the production of the 3900-nucleotide processed species (Alifano et al. 1992). It is possible that a hierarchy of target sites exists in vivo, depending on their consensus. Alternatively, these multiple target sites might be less accessible to RNase E in vivo because of interference by translating ribosomes.

#### *RNase P* participates in the maturation of the 3900-nucleotide species

Alternative mechanisms can be proposed for the production of the 3900-nucleotide processed transcript by *rne*-dependent cleavage of an upstream region. (1) The upstream *cis*-acting element serves as an entry site for a processive endoribonuclease uniquely responsible for the maturation event. It has been proposed that a 5'-binding endonuclease exists in *E. coli* that is capable of binding the 5' end of mRNA molecules and migrating or looping downstream until a recognition site for endonucleolytic cleavage is encountered (Bouvet and Belasco 1992; Di Mari and Bechhofer 1993). Given its involvement in the decay of many mRNAs, RNase E has been suspected to be this enzyme. In our system we cannot conclude that RNase E is the only activity involved and that it works exclusively with this intrinsic 5'  $\rightarrow$  3' processivity. We observed that deletion of the cleavage sites c-f did not impede cleavage at the downstream a and b *rne*-dependent sites (data not shown) and reduced but did not abolish production of the processed species ( $\alpha$ ) (Fig. 4; Alifano et al. 1992). (2) The initial cleavage at the upstream element might trigger downstream decay by a 5' exonuclease that is temporarily arrested at the stem-loop structure at the 5' end of the mature 3900-nucleotide species. However, no 5' exonucleolytic activities have been isolated from *E. coli* up until now (Petersen 1992). (3) Another endonuclease acting on substrates originated by *rne*-dependent cleavages might be involved in generation of the mature 3900-nucleotide species. RNase E-inactivating cleavages have been reported in translated segments of many mRNAs (McCarthy et al. 1991; Klug 1993). The initial cleavages by RNase E functionally inactivate the mRNA molecules, leaving them unprotected by ribosomes as naked RNA and capable of forming structures recognized by other endonucleases. RNase III predominantly cuts untranslated regions of mRNAs, because of the higher order structure of the rec-

ognition site that is impeded by translating ribosomes (Court 1993). In our system, the absence of RNase III did not cause a detectable effect on formation of the 3900-nucleotide species.

Surprisingly, the amount of the 3900-nucleotide RNA was severely reduced in two hosts defective of the RNase P function (Fig. 5A). RNase P is an evolutionary conserved essential ribozyme and is required for the endonucleolytic cleavage of precursor tRNAs to generate the 5' termini of tRNA molecules (Altman 1990; Altman et al. 1993). In the attempt to define a minimal consensus sequence or RNA conformation recognized by this enzyme, it has been reported that any RNA could be targeted by a custom-designed external guide sequence (EGS) for specific cleavage by RNase P in vivo or in vitro. It has been hypothesized that any RNA molecule that may fold into a short stem-loop structure followed by a 3' distal NCCA consensus sequence might constitute a potential substrate for RNase P (Forster and Altman 1990). The 34-nucleotide sequence at the 5' end of the processed 3900-nucleotide species seems to satisfy this requirement (Fig. 1B). A substrate comprising this sequence was also cleaved in vitro in the presence of wild-type but not of RNase P mutant S30 cellular extracts (Fig. 5B). According to previous findings indicating that RNase P cosediments with the ribosomes under the conditions used to prepare the extracts (Vioque et al. 1988), the activity responsible for cleavage of the substrate was predominantly recovered in the S30 and not in the S100 fraction. We can exclude the possibility that RNase P affects the processing event acting on RNase E function. In the absence of a functional RNase P, *rne*-dependent cleavages (a and b) located upstream of the final processing site ( $\alpha$ ) accumulated in vivo (Fig. 5A), similar to what was observed in the *pnp rnb* background. The reconstitution experiment with purified RNase P added to the mutant-derived S30 extract (Fig. 5C) excludes that lack of cleavage of the substrate is attributable to indirect effects of the *rnp* mutation on the composition and activity of the extract. These observations suggest that RNase P itself cleaves the substrate and support the hypothesis that RNase P acts on processing products generated by RNase E.

It has been reported that the 5' end of a transcript plays a major role in determining the rate of its decay. Considerable stability is conferred by stem-loop structures sequestering the 5' end of RNA molecules (Bouvet and Belasco 1992). RNase E, which controls the decay of many RNAs, cuts in single-stranded regions preceding stem-loop structures and leaves protruding 5' ends, thus destabilizing the targeted molecules (Bouvet and Belasco 1992; Ehretsmann et al. 1992). On the contrary, cleavages of precursor molecules by RNase P produce 5' ends that are embedded in secondary structures (Altman 1990). We suggest that this may account for the relevant stability of the RNase P-dependent processed species.

We have shown previously that processing of the 3900-nucleotide species requires binding of the ribosomes at the ribosomal binding site of *hisB* cistron in an in vitro system (Alifano et al. 1992). One might suspect that

RNase P affects the processing event by interfering with translation of *hisB*. However, at least in the *rnpA49* genetic background, translation rate is not significantly modified (Schedl and Primakoff 1973). In this study we showed that efficiency of the processing event producing the 3900-nucleotide species was severely reduced by a mutation disrupting both the termination codon of *hisC* and the initiation codon of *hisB* (Fig. 6). According to current models (Kozak 1983; Alifano et al. 1988), both ribosomal binding and initiation of translation of the *hisB* cistron are impeded in this mutant and this may alter the conformation of the intermediates produced by *rne*-dependent cleavages. This explanation supposes that the substrate for *rnp*-dependent cleavage must fold into the appropriate structure. Ribosomes initiating translation of the *hisB* cistron might favor the formation of the *rnp*-targeted structure, allowing RNase P to cleave the mRNA efficiently. The requirement of ribosomes for the processing reaction may account for the failure of the purified RNase P to cleave the substrate in vitro in the absence of other components of the cellular extract (C. Guerrier-Takada, pers. comm.; see Results) and for the ability of purified RNase P added to mutant-derived S30 extract to restore processing (Fig. 5C).

The model for the proposed for the processing event of the *his* operon primary transcript leading to the production of the 3900-nucleotide species is shown in Figure 7. It may be articulated in the following steps: (1) RNase E (or an *rne*-controlled endonucleolytic activity) cleaves the full-length mRNA at multiple internal sites located within the *hisC* cistron. (2) Ribosomes translating the *hisC* cistron proceed until they reach the *hisC/hisB* intercistronic barrier, whereas the intermediate products are degraded by 3' exonucleases. (3) The 5' region of the distal *his* RNA segment, produced by endonucleolytic cleavage, being uncovered by ribosomes, may assume an appropriate structure recognized by RNase P. Formation of this RNA structure is, in turn, dependent on ribosomes bound at the translation initiation site of the *hisB* cistron. (4) RNase P cleaves the targeted RNA at the bottom of the stem-loop structure located at the 5' end of the 3900-nucleotide species. (5) The stem-loop structure that sequesters the 5' end of the 3900-nucleotide species (Bouvet and Belasco 1992) and translation of the *hisB* cistron may be responsible for the considerable stability of this processed transcript.

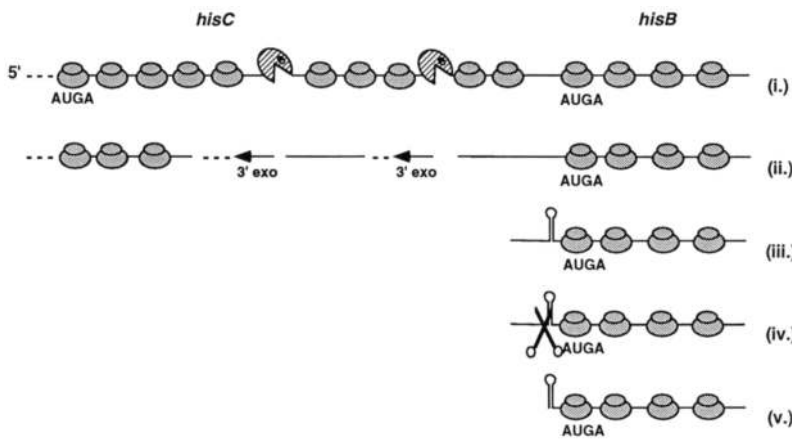
## Materials and methods

### Bacterial strains and plasmids

The strains used in this study are listed in Table 1. The strains were grown in minimal medium M9CA (Maniatis et al. 1982) supplemented with 0.5% glucose and 50  $\mu$ g/ml of ampicillin as required.

Plasmids pUH1293, pUH912, and pGEM136 have been described previously (Alifano et al. 1992). Plasmid pGEM1293 was obtained by cloning in the *Sma*I site of the polylinker of vector pGEM3Z (Promega) the insert carried by plasmid pUH1293 (Fig. 1A) in the correct orientation. Plasmid pGEM322 was constructed by insertion of a 322-bp *Dra*I-*Hind*III fragment (Fig. 5B)





**Figure 7.** Model for the processing of the polycistronic *his* mRNA. The *top* drawing shows the active mRNA segment spanning the *hisC* and *hisB* cistrons with translating ribosomes. Two internal cleavages by ribonuclease E (hatched forms) are also shown. The subsequent sequential steps are detailed in the Discussion. Arrows indicate the 3' → 5' degradation of naked mRNA segments by 3' exonucleases. The structure that forms at the 5' end of the processed species in the presence of ribosomes bound at the *hisC/hisB* intercistronic region (AUGA) is represented by a hairpin, which is then cleaved by ribonuclease P (scissors) at the root of the stem-loop.

into the polylinker of pGEM3Z digested with *Sma*I and *Hind*III. Plasmid pUH999 was constructed by ligation of a 94-bp *Hae*III-*Sau*3AI fragment, spanning the RNase E target site of plasmid ColE1 (Bouvet and Belasco 1992), into the polylinker of plasmid pUH912 digested with *Sma*I-*Bam*HI. Plasmid pUH1293mut was obtained by site-directed mutagenesis (Kunkel et al. 1987) with a kit (Boehringer Mannheim) and the purified oligonucleotide 5'-GATACTTCTGACTCGTACTGGCTCCGCACG-3'. The construction was verified by DNA sequence analysis.

*In vitro* expression of plasmids pUH1293 and pUH1293mut was obtained in S30 extracts from *E. coli*, making use of a translation kit (Amersham) and following the instructions of the producer. We have indicated previously that the *hisC* cistron in plasmid pUH1293 was not translated, as it lacks the natural AUG start codon (Alifano et al. 1992). We have subsequently found that the cistron is translated at low efficiency starting from a GUG initiation codon (data not shown; Fig. 6B).

#### DNA procedures

Plasmid DNA was purified according to standard procedures (Sambrook et al. 1989). DNA fragments were isolated through acrylamide slab gels and recovered by electroelution (Sambrook et al. 1989). 5' End-labeling with polynucleotide kinase and nick translation were performed as described (Sambrook et al. 1989).

DNA sequencing was performed by the dideoxy chain termination procedure (Sanger et al. 1977). For the high-resolution S1 nuclease mapping experiments the ladder channels were obtained by the same procedure (Sanger et al. 1977).

#### RNA preparation and hybridization procedures

Total bacterial RNA was extracted from logarithmically growing cells by the guanidine hydrochloride procedure described previously (Grisolia et al. 1982).

RNA-DNA hybridization, S1 nuclease digestion, and analysis of the hybrids on polyacrylamide denaturing gels were performed with the same conditions described previously (Carlo-magno et al. 1985). Because the strains that we used were *his*<sup>+</sup>, we ensured that *his E. coli* mRNA did not cross-hybridize to the *S. typhimurium* probe (Fig. 4B, lane 5).

#### RNA-binding assays

Crude (S30) extracts and RNase E-enriched ammonium sulfate-fractionated extracts were prepared according to the procedure

described by Mackie (1991), except that M9CA medium was employed in the bacterial growth.

The gel mobility retardation assays using a uniformly labeled RNA substrate derived from plasmid pGEM136 were performed as described previously (Alifano et al. 1992).

#### *In vitro* degradation assays

Plasmid pGEM1293 was linearized at the *Pst*I site of the polylinker and transcribed *in vitro* in a reaction mixture containing 500 μM each NTPs, 10 mM dithiothreitol, 40 mM Tris-HCl (pH 7.5), 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 μM NaCl, and 5 units of T7 RNA polymerase (Boehringer) for 60 min at 38°C. Template DNA was digested with 1 U/μg of DNA from RQ1 RNase-free DNase (Promega) and the RNA purified by phenol extraction and ethanol precipitation. One-tenth of the *in vitro*-made RNA was incubated with S30 extracts for 5 min at 44°C in a buffer containing 25 mM Tris-HCl (pH 8.5), 5 mM MgCl<sub>2</sub>, 60 mM KCl, 100 mM NH<sub>4</sub>Cl, 0.1 mM dithiothreitol, 5% (wt/vol) glycerol, and 50 μg/ml of yeast RNA. The protein concentration was 1 μg/μl. The samples were extracted twice with a 1:1 mixture (vol/vol) of 0.1% SDS-saturated phenol and chloroform/isoamyl alcohol to eliminate contaminating proteins bound to RNA and then precipitated with 0.3 M Na-acetate (pH 5) and 2.5 volumes of ethanol. The degradation products were then analyzed by primer extension as described by Fisher and Wray (1989) by use of a 40-nucleotide synthetic oligonucleotide complementary to the region underlined in Figure 1B.

Plasmid pGEM322 was linearized at the internal *Nci*I site (Fig. 7B) and transcribed *in vitro*, and the RNA was purified as described above except that the reaction mixture contained 100 μM ATP, CTP, and GTP, 10 μM UTP, and 30 μCi of [α-<sup>32</sup>P]UTP (400 Ci/mmol). S30 extracts were prepared according to Zubay (1973), and S100 fractions were obtained by ultracentrifugation of the S30 extracts for 45 min at 4°C. One-twentieth of the *in vitro* made RNA was incubated with S30 or S100 extracts for 20 min at 44°C in the buffer system described by Zubay (1973). The protein concentration was 1 μg/μl. For the reconstitution experiment the RNase P holoenzyme was reassembled *in vitro* as described previously (Vioque et al. 1988). The degradation products were analyzed on 6% polyacrylamide denaturing gels.

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## Ribonuclease E provides substrates for ribonuclease P-dependent processing of a polycistronic mRNA.

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