Amplification of ribonuclease II (*rnb*) activity in *Escherichia coli* K-12

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Received 7 October 1982; Revised and Accepted 1 December 1982

ABSTRACT

A 7.1 kb *Hind*III-*Xho*I fragment of *E. coli* DNA which contains the structural gene for ribonuclease II (*rnb*) has been cloned in the recombinant plasmid pDK24. At least two constitutively expressed genes are encoded on the fragment as shown by maxicell analysis. On denaturing polyacrylamide gels RNase II appears as a single 72,000 dalton species. The approximate site of transcription initiation of the *rnb* gene has been mapped. Although derivatives of *E. coli* harboring pDK24 contained 10-fold more RNase II activity than wild type strains without the plasmid, the degradation rate of mRNA was similar in all strains tested. Strains deficient in both RNase II and polynucleotide phosphorylase appear inviable.

INTRODUCTION

Ribonuclease II (RNase II) is one of the most active RNA exonucleases in cell lysates of *E. coli* (1). The enzyme hydrolyzes single-stranded RNA processively in the 3' to 5' direction producing 5'-nucleotide monophosphates. DNA and polynucleotides with secondary structure are resistant to hydrolysis by RNase II (2). Previous results have indicated that RNase II consisted of a single subunit of approximately 75,000 daltons (2-4). However, Leineweber and Philipps (5) suggested that RNase II was composed of three dissimilar subunits of approximately 40,000, 33,000, and 26,000 daltons.

The enzyme has been implicated in the turnover of both stable RNA and mRNA, since the degradation rates of both stable RNA and mRNA were decreased in RNase II deficient strains of *E. coli* compared to RNase II⁺ controls (6-8). Additionally, a second RNase mutant that had increased levels of RNase II-like activity also had increased rates of degradation of both stable RNA and mRNA compared to wild type controls (9-11). However, these two mutations were mapped at different locations on the *E. coli* genome (28 min and 74 min, respectively) (12-13).

The results reported in this paper show that the structural gene for

RNase II is located approximately 3 kb from the pyrF locus at 28 minutes. In contrast to previous results no change in the degradation rate of mRNA was observed either in the absence of RNase II activity or in the presence of a ten-fold increase over wild type levels.

MATERIALS AND METHODS

Bacterial Strains

The isogenic *E. coli* strains SK4803 (rnb-296 pyrF⁺ kan^S) and SK4804 (rnb⁺ pyrF⁺ kan^S) were constructed by P1 cotransduction of the pyrF and rnb genes. The RNase II deficient strain S296 (rnb-296 pyrF⁺ kan^S) (14) was used as donor and SK3921 (rnb⁺ pyrF::Tn5 kan^R) as recipient. Pyr⁺ transductants which grew on minimal media in the absence of uridine were assayed for RNase II activity as described below.

RNase II Assay

Cells were grown at 37° in M9 minimal medium (plus ampicillin if strains harbored plasmid) to a Klett reading of 50 (No. 42 green filter), centrifuged, resuspended in 1 ml of RNase II buffer (20mm Tris pH 8.0, 0.1 M KCl and 1 mM MgCl₂) (13,15) and sonicated in an ice-water bath. After pelleting cell debris, 20 µl of supernatant (diluted 50-fold in RNase II buffer) was added to 180 µl of RNase II buffer containing 20 nmoles (200 nCi) of (³H)-polyadenosine, mixed and incubated at 37°. At various time intervals 50 µl samples were withdrawn and added to 100 µl of ice cold 10% trichloroacetic acid. Samples were left on ice for at least 30 min, subsequently centrifuged and 100 µl of supernatant were counted in 5 ml of a Triton-X100 based scintillation fluid. One unit of RNase II activity is defined as 1 µMole of AMP solubilized per hour. Protein concentrations were determined by the method of Lowry *et al.* (16).

mRNA Half-Life

To measure the half-life of pulse labeled RNA, strains were grown at 37° in M9 minimal medium (plus ampicillin if strains harbored plasmid) to a Klett reading of 50, 1.0 µCi/ml (³H)-uridine was added, followed 1.0 min later by rifampicin and nalidixic acid (400 mg/ml and 30 mg/ml, respectively). At 1 min intervals, 0.5 ml samples were withdrawn and precipitated in 0.5 ml 10% trichloroacetic acid containing 0.2 mg/ml of carrier tRNA. After 2 hr on ice, samples were filtered through Whatman GF/C filters and the acid precipitable counts determined. The half-life was determined by linear regression analysis of the data points.

The method of Kepes (17) was used to measure the functional half-life

of β -galactosidase mRNA. Strains were grown at 37° in M9 minimal medium to a Klett reading of 30, cAMP was added to 1.5 mM followed 3 min later by isopropyl- β -D-thiogalactoside (IPTG) (0.5 mM final concentration). One min after IPTG addition, rifampicin was added to 400 mg/ml. At 1 min intervals 0.5 ml samples were withdrawn and added to 0.5 ml of an ice cold aqueous solution of 2 mM NaAzide plus 100 mg/ml chloramphenicol. β -galactosidase activity was determined for each sample as described by Miller (18). Use of chloroform plus 0.01% SDS, in place of toluene, to solubilize cell membranes gave more consistent results.

Construction of Plasmid Subclones

Five μ g of plasmid DNA was digested with 5 units of the appropriate restriction enzyme for 2 hr at 37° in a total volume of 10 μ l containing 6 mM Tris pH 8.0, 6 mM MgCl₂, 60 mM NaCl and 6 mM dithiothreitol. After digestion, samples were successively incubated at 65° for 15 min, 37° for 10 min, and 0° for 10 min. Plasmid DNA was ligated by adding ATP (to 1 mM), 10 U of T4 DNA ligase and incubating overnight at 15°C. *E. coli* strains were transformed by the method of Kushner (19).

Assay for polynucleotide phosphorylase (PNPase)

PNPase was qualitatively assayed in cell lysates by the ³²Pi-ADP exchange reaction described by Grunberg-Manago (20) and modified by Reiner (21).

RESULTS

Analysis of Recombinant Plasmid DNA

The recombinant plasmid pDK07 contains the *E. coli pyrF* gene (orotidine decarboxylase) as part of a 15 kb BamHI fragment cloned in the BamHI site of pBR325 (W.P. Donovan and S.R. Kushner, manuscript in preparation). Since mutations which affect RNase II activity in *E. coli* are 80% linked by P1 cotransduction to pyrF (22), it seemed possible that pDK07 contained the structural gene for RNase II (rnb). To test this hypothesis a RNase II deficient strain (SK4803 rnb-296) was transformed with pDK07. Of 10 ampicillin resistant transformants tested, all contained RNase II activity (data not shown).

Restriction Enzyme Analysis of the rnb Region

The 15 kb BamHI fragment of pDK07 was shown to contain 5 EcoRI sites, 3 SalI sites, 3 SphI sites and 1 each XhoI and HindIII sites. The relative locations of the restriction sites are shown in Fig. 1. Plasmid subclones were constructed by digesting the 15 kb BamHI fragment with appropriate

restriction enzymes followed by ligation into digested plasmid vehicles as described in Materials and Methods and Fig. 1. SK4803 (RNase II⁻) was transformed with plasmid subclones and Ap^R transformants were assayed for RNase II activity. SK4803 remained RNase II deficient when harboring either plasmid pDK10, pDK11 or pDK20 but became RNase II⁺ in the presence of either plasmid pDK24 or pDK09 (Figure 1). Removal of either the 1 kb SalI fragment from pDK24 (pDK29) or the 2.5 kb and 1.2 kb SphI fragments from pDK24 (pDK28) inactivated RNase II activity (Figure 1).

Analysis of RNase II Activity

Extracts of SK4803 (rnb-296), SK4804 (rnb⁺), SK4860 (rnb-296/pDK24 rnb⁺) and SK4765 (rnb⁺/pDK24 rnb⁺) were assayed for RNase II activity. There was a dramatic increase in RNase II activity in a rnb-296 mutant harboring pDK24 compared to a rnb-296 strain without the plasmid (Table I). Additionally, a 10-fold increase in RNase II activity was seen in a rnb⁺ strain harboring pDK24 compared to a rnb⁺ strain without the plasmid (Table

Figure 1. Restriction map and subclones of the 15 kb BamHI fragment of *E. coli* DNA. pDK24 was obtained by a HindIII-XhoI simultaneous digestion of the 15 kb BamHI fragment followed by ligation into HindIII- SalI digested pBR322 plasmid DNA. pDK09 was derived by a partial EcoRI digestion of the 15 kb fragment followed by ligation into pBR325. pDK28 was obtained by removal of both the 0.9 kb and the 2.8 kb. SphI fragments from pDK24 and religation. pDK29 was obtained by removal of the 1.0 kb SalI fragment from pDK24 and religation. Plasmid vehicles are not shown. Subclones were transformed into SK4803 (RNase II⁻) and ampicillin resistant transformants were assayed for RNase II activity. The presence or absence of functional RNase II activity on each plasmid is indicated. Gene assignments were determined by subclone complementation tests and maxicell analysis. The arrow indicates direction of transcription of rnb. Gene X encodes a 40,000 dalton protein whose function is unknown. Capital letters refer to enzymes as follows: B = BamHI; H = HindIII; Pv = PvuII; R = EcoRI; S = SalI; Sp = SphI; X = XhoI. The SphI site in parenthesis in pDK24 was not present in pDK07

I). When K^+ , which is required for RNase II activity (1), was omitted from the reaction mixture, RNase II activity was reduced by over 95% (Table I).

Analysis of Maxicells Containing Recombinant Plasmids

The number and molecular weight of proteins genetically specified by pDK24 were determined by transforming pDK24 and various subclones into the maxicell strain CSR603. Maxicells were irradiated, labeled with (^{35} S)-methionine, and proteins subsequently analyzed by polyacrylamide gel electrophoresis and autoradiography as described by Sancar *et al.* (23). Two large polypeptides, 72,000 and 40,000 daltons, were synthesized from pDK24 in addition to the β -lactamase protein (Figure 2, lane A-2). If the 1.0 kb *Sal*I fragment was deleted from pDK24 (pDK29, Fig. 1) only a 67,000 dalton

Figure 2. Polyacrylamide gel electrophoresis of polypeptides synthesized in maxicells. Maxicells were prepared as described by Sancar *et al.* (23), labeled with (^{35} S)-methionine, subjected to electrophoresis in a 12.5% acrylamide gel, and autoradiographed. The plasmids present in the maxicell preparations were (A-1) pDK29; (A-2) pDK24; (A-3) pDK28; (B-1) pDK11; (B-2) pBR325. Numbers in margins represent kilodaltons. Arrows in the margins specify the ampicillin, tetracycline and chloramphenicol resistance determinants.

protein was observed (Fig. 2, lane A-1). If the 2.5 kb and 1.2 kb SphI fragments were deleted from pDK24 (pDK28, Fig. 1) only the 40,000 dalton protein was obtained (Fig. 2, lane A-3). Maxicells harboring pDK11 (Fig. 1) synthesized a single major protein of 43,000 daltons in addition to the β -lactamase and Tc^R proteins (Fig. 2, Lane B-1). Neither plasmid pDK28, pDK29 nor pDK11 complemented rnb-296 mutants.

Half-Life of mRNA in Strains Harboring pDK24

In order to determine the effect of RNase II on the degradation rate of mRNA, half-life determinations were carried out in strains which contained various levels of RNase II activity. The chemical half-life of pulse labeled RNA was found to be similar in SK4803 (rnb⁺), SK4804 (rnb-296), and SK4765 (rnb⁺/pDK24 rnb⁺), although the levels of RNase II activity differed significantly in these strains (Table I and Fig. 3). Likewise the functional half-life of β -galactosidase mRNA was found to be identical within experimental error in SK4803, SK4804, and SK4765 (Table I and Fig. 4).

Apparent Inviability of Strains Deficient in Both RNase II and PNPase

The pyrF and rnb genes are greater than 80% linked by P1 cotransduction (13). Using SK4802 (rnb-296 pyrF::Tn5 kan^r pnp⁺) as donor and SK3915 (rnb⁺ pyrF⁺ kan^s pnp⁺) as recipient 7/8 (88%) Kn^r Pyr⁻ transductants became RNase II deficient. In contrast, with an isogenic PNPase deficient recipient SK3917 (rnb⁺ pyrF⁺ kan^s pnp-7) 0/39 Kn^r Pyr⁻ transductants were RNase II deficient. When SK3917 was transformed with the recombinant DNA plasmid

Table I. mRNA half-life and RNase II activity in strains harboring pDK24.

Strain	Genotype	Plasmid	RNase II (U/mg protein)		Chemical half-life of pulse labeled RNA (min)	Functional half-life of β -galactosi- dase mRNA (min)
			+K ⁺	-K ⁺		
SK4803	<u>rnb-296</u>	none	< 0.1	< 0.1	2.7	1.3
SK4804	<u>rnb</u> ⁺	none	13.0	0.5	2.8	1.3
SK4860	<u>rnb-296</u>	pDK24	88.4	3.3	---	---
SK4765	<u>rnb</u> ⁺	pDK24	127.0	5.0	2.8	1.2

SK4860 and SK4765 are pDK24 transformed derivatives of SK4803 and SK4804, respectively. RNA half-life and RNase II activity were determined as described in Materials and Methods.

pKAK7 ($\text{amp}^r \text{pnp}^+$, K. Armstrong and S.R. Kushner, manuscript in preparation) and used as a recipient, RNase II⁻ transductants were obtained at the expected frequency (data not shown). One of the RNase II⁺ transductants was designated SK4752 [$\text{rnb}^+ \text{pnp-7/pKAK7} (\text{amp}^r \text{pnp}^+)$] while a RNase II⁻ transductant was designated SK4753 [$\text{rnb-296 pnp-7/pKAK7} (\text{amp}^r \text{pnp}^+)$].

Additional experiments tested the linkage of the *argG* and *pnp* loci in RNase II deficient recipients. Thus while *pnp* is normally >75% cotransduced with *argG* (24), when SK4818 ($\text{rnb-296 argG6 pnp}^+$) was used as a recipient 0/10 Arg⁺ transductants inherited the *pnp-7* allele. In control experiments with SK4819 ($\text{rnb}^+ \text{argG6 pnp}^+$) as a recipient, 15/19 (78.9%) of the Arg⁺ transductants became PNPase deficient. A pDK24 ($\text{amp}^r \text{rnb}^+$) transformant of SK4818 (SK4867) inherited the *pnp-7* allele at the expected frequency (9/10). One of the PNPase deficient transductants was designated SK4869 [$\text{rnb-296 pnp-7/pDK24} (\text{amp}^r \text{rnb}^+)$] and a *pnp*⁺ control strain SK4868 [$\text{rnb-296 pnp}^+/\text{pDK24} (\text{amp}^r \text{rnb}^+)$].

Figure 3. Chemical half-life of pulse labeled RNA in strains having various levels of RNase II activity. Strains were pulse labeled with (³H)-uridine and 1 min later rifampicin plus nalidixic acid were added as described in Materials and Methods. At 1 min intervals 0.5 ml aliquots were withdrawn and precipitated in 10% trichloroacetic acid. ○-○ SK4803 (*rnb-296*); □-□ SK4804 (*rnb*⁻); △-△ SK4765 (*rnb*⁻/pDK24 *rnb*⁺).

Figure 4. Chemical half-life of pulse labeled RNA in strains having various levels of RNase II activity. Strains were pulse labeled with (^3H)-uridine and 1 min later rifampicin plus nalidixic acid were added as described in Materials and Methods. At 1 min intervals 0.5 ml aliquots were withdrawn and precipitated in 10% trichloroacetic acid. o-o SK4803 (rnb-296); □-□ SK4804 (rnb⁺); Δ-Δ SK4765 (rnb⁺/pDK24 rnb⁺).

In order to confirm the transduction results, attempts were made to cure either pKAK7 (amp^r pnp⁺) or pDK24 (amp^r rnb⁺) from rnb-296 pnp-7 double mutants. As shown in Table 2 it was not possible to cure either plasmid from the double mutant strain. In contrast, the plasmids were

Table II. Plasmid curing from PNPase RNase II deficient strains

Strain No.	Genotype	Plasmid	Number of Colonies	
			Ap ^R	Ap ^S
SK4867	<u>pn</u> p ⁺ <u>arg</u> G6 <u>rnb-296</u>	pDK24 (<u>amp</u> ⁺ <u>rnb</u> ⁺)	60	30
SK4868	<u>pn</u> p ⁺ <u>arg</u> G ⁺ <u>rnb-296</u>	pDK24	32	58
SK4869	<u>pn</u> p-7 <u>arg</u> G ⁺ <u>rnb-296</u>	pDK24	210	0
SK4751	<u>rnb</u> ⁺ <u>pn</u> p-7 <u>pyr</u> F::Tn5	pKAK7 (<u>amp</u> ⁺ <u>pn</u> p ⁺)	170	40
SK4752	<u>rnb</u> ⁺ <u>pn</u> p-7 <u>pyr</u> F ⁺	pKAK7	54	46
SK4753	<u>rnb-296</u> <u>pn</u> p-7 <u>pyr</u> F ⁺	pKAK7	100	0

Analysis of plasmid curing was carried out by growing the strains for approximately 100 generations in Luria broth not containing ampicillin. The cultures were subsequently streaked onto Luria agar plates and individual colonies tested by replica plating for ampicillin resistance.

readily lost from either pnp-7 or rnb-296 single mutants.

DISCUSSION

A 7.1 kb HindIII-XhoI DNA fragment carrying the *E. coli* rnb gene (RNase II) has been cloned in the plasmid pBR322 (composite plasmid pDK24). Strains transformed with pDK24 contained ten-fold higher levels of RNase II activity than rnb⁺ controls (Table I).

Maxicell results indicated the presence of at least two genes on the 7.1 kb fragment, encoding proteins of 72,000 and 40,000 daltons. The 72,000 Mr species observed in Fig. 2, lane A-2 most likely represents RNase II, a result in close agreement with the 75,000 figure reported by previous workers (2,3). The 67,000 Mr species encoded by pDK29 (Fig. 1; Fig. 2, lane A-1) represents a truncated RNase II protein, as does the 43,000 Mr species encoded by pDK11 (Fig. 1; Fig. 2, lane B-1). These results permitted the determination of the direction of transcription for RNase II as indicated by the arrow in Figure 1. Since the 40,000 dalton polypeptide was still observed with plasmid pDK28 (Fig. 2, lane A-3) the gene encoding this protein must be located to the left of rnb and is most likely not cotranscribed with rnb (Fig. 1). The function of the 40,000 dalton protein is unknown.

An unexpected finding was that strains which had significantly different levels of RNase II activity degraded mRNA at similar rates. No significant difference was found in the half-life of pulse labeled RNA among RNase II deficient strains, wild type strains or strains having a 10-fold increase in RNase II activity (Table I). Likewise, no difference was found among these strains in the functional half-life of β -galactosidase mRNA. These results suggest that RNase II does not have a primary role in degrading mRNA, in contrast to previous suggestions (6-11). There are several possible explanations for these conflicting observations: 1. Although the *E. coli* strains used by Lennette *et al.* (10) and Simon and Apirion (11) more rapidly degraded mRNA and had increased levels of ribonuclease activity compared to wild type controls, it was not demonstrated conclusively that the increased ribonuclease activity was in fact RNase II. In addition, the mutation in their strain was not cotransducible with pyrF (12-13); 2. Several studies have shown that the initial step in mRNA degradation is an endonucleolytic cleavage of the mRNA producing oligonucleotides which are subsequently degraded exonucleolytically (25-28). If endonucleolytic cleavage is the rate limiting step then an excess of RNase

II would not significantly increase the rate of degradation of mRNA; 3. RNase II may not have an *in vivo* role in degrading mRNA; 4. Kivity-Vogel and Elson (6) found an increase in the half-life of mRNA in RNase II⁻ mutants compared to wild type controls. However, these mutants were also deficient in polynucleotide phosphorylase (PNPase) (21). All the strains used in the experiments reported here had wild type levels of polynucleotide phosphophorylase. It is possible that the presence of PNPase masks the effect of RNase II in degradation of mRNA.

In order to examine this latter possibility, attempts were made to construct a strain deficient in both PNPase and RNase II. As described in the text and in Table II, rnb-296 pnp-7 double mutants were only obtained if a recombinant plasmid carrying either pnp⁺ or rnb⁺ was present in the same cell. No plasmid curing was observed from such strains even though pDK24 and pKAK7 were readily lost from appropriate control derivatives (Table II). These results strongly suggest that the double mutant is inviable. It should be noted that Kinscherf and Apirion have previously reported the construction of a pnp-13 rnb-464 double mutant (29). However, strains carrying either pnp-13 or rnb-464 have been shown to contain high levels of residual PNPase or RNase II activity (13,24,30).

The above described results are the first indication of a possible absolute requirement for both RNase II and PNPase in cell viability. In addition, they strengthen the hypothesis that PNPase and RNase II can substitute for each other *in vivo*. Accordingly, changes in the pattern of mRNA degradation may only be observed in the absence of both enzymes. Isolation of temperature sensitive RNase II mutants by *in vitro* mutagenesis of pDK24 (rnb⁺) plasmid DNA should help confirm this model.

ACKNOWLEDGEMENTS

The work was supported in part by Grants from the National Institutes of Health to S.R.K. (GM28760 and GM27994). W.P.D. is supported by a N.I.H. Predoctoral Training Grant.

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