Characterization of the *rnc-97* mutation of RNAaseIII: a glycine to glutamate substitution increases the requirement for magnesium ions

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The *rnc-97* mutation of the *Escherichia coli* double-stranded-RNA-specific ribonuclease III (RNAaseIII) was previously isolated by virtue of the lethal expression of RNAaseIII in *Saccharomyces cerevisiae*. Here we show that *rnc-97* is a single point mutation causing the substitution of glycine 97 by glutamic acid. The mutation eliminates the lethal phenotype of RNAaseIII expression in yeast and reduces fourfold the effect of RNAaseIII expression on bacteriophage gy1 propagation in *E. coli*. Mutant RNAaseIII-G97E and wild-type RNAaseIII were purified according to published procedures. The apparent molecular masses of the two enzymes on SDS polyacrylamide gels are the same but they differ in pI (6.85 for RNAaseIII-G97E and 7.3 for RNAaseIII). Whereas the two enzymes (under standard assay conditions) do not show a great difference in activity towards double-stranded RNA and defined single-stranded RNAaseIII substrates, they differ dramatically (20-fold or more) under conditions of Mg²⁺ limitation. The hypothesis that limitation of Mg²⁺ ions *in vivo* is responsible for the phenotypes of the *rnc-97* mutation in *S. cerevisiae* and *E. coli* is discussed.

Introduction

RNAaseIII is a double-stranded RNA (dsRNA)-specific ribonuclease from *Escherichia coli*. The enzyme recognizes certain dsRNA structures; it is involved in processing the transcript of the rRNA gene cluster precursor and in maturation of certain bacteriophage and bacterial mRNAs (Dunn & Studier, 1973; King *et al.*, 1986; Portier *et al.*, 1987; Studier & Dunn, 1983). *In vitro* RNAaseIII can also nonspecifically cleave RNA–RNA duplexes more than 20 base pairs long, producing fragments with an average size of 15 base pairs (Robertson & Dunn, 1975). The *rnc* gene, which encodes RNAaseIII, is located at 55 min on the *E. coli* chromosome (Studier, 1975); the gene has been cloned (Watson & Apirion, 1985) and sequenced (March *et al.*, 1985; Nashimoto & Uchida, 1985). The *rnc* operon contains three genes, with *era* and *recO* immediately downstream of the *rnc* gene (Takiff *et al.*, 1989). RNAaseIII is a dimer composed of two identical subunits, each of molecular mass approximately 25 kDa (Dunn, 1976; March & Gonzalez, 1990). There are no

reports on the biochemical characterization of mutant RNAaseIII enzymes. Very few mutations in *rnc* have been isolated; the most important of these is *rnc-105*, which renders the enzyme inactive (Dunn & Studier, 1973; Studier & Dunn, 1983). The mutation, isolated more than 15 years ago, is a missense mutation changing the 44th residue, glycine, to aspartic acid (Kindler *et al.*, 1973; Nashimoto & Uchida, 1985).

RNAaseIII of *E. coli* has been previously cloned into an expression vector in *Saccharomyces cerevisiae* and has been shown to cause abnormal cell morphology and cell death upon induction (Pines *et al.*, 1988). Mutants selected for resistance to RNAaseIII induction were subsequently isolated. The genetic alteration leading to this resistance was localized within the RNAaseIII-coding region, but not in the yeast chromosome, suggesting that *S. cerevisiae* contains some essential RNA which is susceptible to *E. coli* RNAaseIII. In this study we purified and characterized one of these RNAaseIII mutant proteins in an attempt to gain an understanding of the molecular basis of its phenotypes in *S. cerevisiae* and in *E. coli*.

Methods

Strains and plasmids. The *E. coli* and *S. cerevisiae* strains, bacteriophages and plasmids used in this study are shown in Table 1. The general yeast, bacterial and phage techniques used have been

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Abbreviation: dsRNA, double stranded RNA.

Table 1. *Bacterial and yeast strains, bacteriophages and plasmids used*

Description		Reference/source
<i>E. coli</i>		
BL15	<i>rnc</i> ⁺ (F ⁻ <i>rna-19 rel-1</i>)	Dunn & Studier (1975)
BL107	<i>rnc</i> ⁻ (F ⁻ <i>his bio rna-19 rnc-105 rel-1</i>)	Dunn & Studier (1975)
A6008	<i>rnc-14::ΔTn10</i> (ΔTn10 inserted into the <i>rnc</i> gene)	Altuvia <i>et al.</i> (1991)
<i>S. cerevisiae</i>		
DMMI-15A	<i>leu2 ura3 ade2 his5</i>	Pines <i>et al.</i> (1988)
Bacteriophages		
T7	<i>E. coli</i> phage	Studier & Dunn (1983)
gyl	<i>E. coli</i> phage	Davidov <i>et al.</i> (1992)
Plasmids		
pIN-III	<i>E. coli</i> expression vector	Masui <i>et al.</i> (1983)
pIN-pPY	<i>rnc</i> gene under the control of the <i>lpp-lac</i> promoter of pIN-III	This study
pIN-SP97	<i>rnc-97</i> gene under the control of the <i>lpp-lac</i> promoter of pIN-III	This study
pPY2	<i>rnc</i> gene under the control of the <i>S. cerevisiae</i> <i>GAL10</i> promoter	Pines <i>et al.</i> (1988)
pSP97	<i>rnc-97</i> gene under the control of the <i>S. cerevisiae</i> <i>GAL10</i> promoter (previously SP1)	Pines <i>et al.</i> (1988)
pAR2656	Encodes the <i>E. coli</i> <i>rnc</i> RNAaseIII cleavage sites	Rosenberg <i>et al.</i> (1987)
pET4	Encodes the T7 R1·1 RNAaseIII cleavage site	Bardwell <i>et al.</i> (1989)
pAR3903*	Encodes the T7 R0·3 RNAaseIII cleavage site	W. Studier
pAR2652*	Encodes the T7 R18·5 RNAaseIII cleavage site	W. Studier

* Unpublished plasmids analogous in structure to pAR2656 and pET4, but encoding the respective RNAaseIII cleavage sites. These plasmids were kindly provided by William Studier (Brookhaven National Laboratory, USA).

described elsewhere (Davidov *et al.*, 1992; Maniatis *et al.*, 1982; Pines *et al.*, 1988). LB for phage experiments was supplemented with 0·1% (w/v) MgSO₄.

dsRNAase activity assay. Total *S. cerevisiae* RNA was incubated with samples to be assayed, and then subjected to electrophoresis on a 0·7% agarose gel as previously described (Pines *et al.*, 1988). RNAaseIII activity was detected by specific degradation of yeast killer L dsRNA and observed on ethidium-bromide-stained gels.

Cleavage of RNAaseIII specific sites. The RNA was prepared *in vitro* from T7 expression vectors encoding RNAaseIII cleavage sites. These plasmids were linearized with *EcoRV* (pET-4, pAR2656, pAR2652) or *EcoRI* (pAR3903) and runoff transcripts were produced using T7 RNA polymerase, precisely as detailed by the Stratagene transcription kit. Approximately 5 µg of transcript was obtained from 1 µg of template. These transcripts are referred to in the text as single-stranded RNAaseIII specific substrates. The standard RNAaseIII assay contained 100 ng RNAaseIII and 400 ng RNA in a total volume of 15 µl. The assay buffer contained 0·02 M-Tris, pH 8·0; 0·1 M-KCl; 0·01 M-MgCl₂; 0·1 mM-dithiothreitol; 0·1 mM-EDTA. Incubations were carried out at 37 °C for 30 min. Reactions were stopped by addition of loading buffer (22%, v/v, glycerol, 100 mM-EDTA, 0·04%, w/v, bromophenol blue) containing 90% (v/v) formamide. The reaction products were separated on 8% (w/v) polyacrylamide gels in the presence of 8 M-urea and visualized by staining with ethidium bromide.

Polyacrylamide gel isoelectric focusing. Analytical isoelectric focusing was performed in 125 x 125 mm slabs of a fabric-reinforced polyacrylamide gel (Serva) of 5% T, 3% C and 150 µm thickness, containing 5% (v/v) Servalyt carrier ampholytes covering the pH range 3–10. Samples (10 µl) were applied in slots of 7 x 1 mm at a distance of 2·7 cm from the cathode, using the Serva applicator strip.

The run was carried out at 8–10 °C for 2·5 h, at 3 W constant power. A mixture of 25 mM-L-aspartic acid and 25 mM-L-glutamic acid was used as anolyte, and a mixture of 23 mM-L-arginine, 27 mM-L-lysine and 1·79 M-ethylenediamine as catholyte. Serva's protein test mixture no. 9 or Pharmacia's high-pI markers (pI 5–10·5) were used to calibrate the pH gradient. The gels were stained with Coomassie Brilliant Blue R-250.

Immunoblotting. Samples were subjected to polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) or polyacrylamide gel isoelectric focussing. Western blotting was performed using Hoefer's TE 70 Semiphor semi-dry transfer unit, with nitrocellulose 0·2 µm. Towbin's transfer buffer, pH 8·3, was used containing 192 mM-glycine, 25 mM-Tris, 1·3 mM-SDS and 15% (v/v) methanol. Transfer was performed for 45 min under 100 mA constant current. The presence of RNAaseIII was detected with anti-RNAaseIII polyclonal antibodies (Pines *et al.*, 1988) and iodinated protein A essentially according to Towbin *et al.* (1979) and as we have previously described (Shonberger *et al.*, 1991).

DNA manipulations and sequencing. To map the *rnc-97* mutation, a *SalI*-*BstXI* 570 bp fragment was reciprocally exchanged between pSP97 and pPY2 (appropriate DNA restrictions and ligations were performed). Each of the resulting hybrid plasmids was transformed into *S. cerevisiae* DMMI-15A and examined for lethal expression exactly as described by us previously (Pines *et al.*, 1988).

To express the wild-type and the mutant RNAaseIII in *E. coli*, 850 bp *SalI*-*BamHI* fragments from pPY2 or from pSP97 were inserted into the *XbaI* and *BamHI* sites of the pIN-III (Masui *et al.*, 1983) expression vector (*SalI* and *XbaI* sites were changed to blunt ends with the large fragment of DNA polymerase I). The resulting plasmids are designated pIN-pPY and pIN-SP97 respectively. The cloned genes are under the control of the *lpp-lac* hybrid promoter (Masui *et al.*, 1983).

For DNA sequencing, a 570 bp fragment (*SalI*–*BstXI*) from pIN-SP97 was cloned into the *SalI* and *KpnI* sites of bluescript KS. DNA was isolated and sequenced with Sequenase according to the recommendation of the manufacturer (USB).

Purification of RNAaseIII-G97E and RNAaseIII. *E. coli* A6008 *rnc-14::ΔTn10* harbouring plasmid pIN-pPY or pIN-SP97 was grown in 2 litres of M9-glucose medium (Miller, 1972) supplemented with 20 µg tryptophan ml⁻¹, 50 µg ampicillin ml⁻¹ and 0.5% Casamino acids. At a concentration of 4×10^8 cells ml⁻¹, protein expression was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a concentration of 1 mM. Cultures were grown for an additional 6 h and then centrifuged, yielding 20 g cell pellets. The cells were lysed and the proteins were purified essentially according to March & Gonzalez (1990), including ammonium sulphate precipitation, a Sephadex G-100 column, a DEAE-Sepharose column and affinity chromatography on poly-rI–poly-rC agarose.

Results

Expression of wild-type and mutant RNAaseIII in E. coli and S. cerevisiae

Induction of expression of the *E. coli* RNAaseIII gene in *S. cerevisiae* which results in cell death has been used to isolate mutations localized within the RNAaseIII protein-coding region (Pines *et al.*, 1988). One of the mutant RNAaseIII genes, designated *rnc-97* (previously SP1), was selected for further analysis. This mutant gene was of particular interest since *S. cerevisiae* expressing the corresponding mutant protein (RNAaseIII-G97E) still exhibited high levels of dsRNAase activity (similar to that of cells expressing RNAaseIII); nevertheless, it was not lethal to the yeast cells (Pines *et al.*, 1988).

To investigate the nature of the *rnc-97* mutation and to facilitate purification of the protein, we cloned the mutant and wild-type genes from the respective yeast plasmids into an *E. coli* expression vector. DNA fragments (Pines *et al.*, 1988) from plasmids pPY2 (encoding wild-type RNAaseIII) and pSP97 (encoding RNAaseIII-G97E) were inserted into the appropriate sites of the *E. coli* expression vector pIN-III (Masui *et al.*, 1983). The resulting cloned genes (designated pIN-pPY and pIN-SP97 respectively) are under the control of the *lpp-lac* promoter; thus they are expressed at high levels in the presence of a *lac* inducer.

Since it is possible to detect a distinct phenotype of the *rnc-97* mutation in yeast, it was important to see if we could detect a phenotype of the mutation in *E. coli*. For this we have recently isolated and characterized a novel bacteriophage, *gyl*, whose propagation in *E. coli* depends on the expression of RNAaseIII in the cell (Davidov *et al.*, 1992). Phage *gyl* has a low efficiency of plating on *rnc*⁺ *E. coli* strains and a high efficiency of plating on *rnc*⁻ strains. The plating efficiency of *gyl* on *E. coli* BL107(pIN-pPY) (which encodes RNAaseIII) was 10–14 % of that of *gyl* on BL107(pIN-III) (which does not

encode RNAaseIII). In contrast, *gyl* retained 60 % of its plating efficiency on BL107(pIN-SP97) (which encodes RNAaseIII-G97E), indicating a fourfold effect of the *rnc-97* mutation on the capacity to reduce *gyl* propagation. In addition, the size of *gyl* plaques on BL107(pIN-SP97) was significantly larger than on BL107(pIN-pPY): approximately 1 mm for the former and 0.5 mm for the latter.

RNAaseIII expression in *E. coli* cells has been previously shown to affect the size of bacteriophage T7 plaques (Apirion & Watson, 1975; Nashimoto & Uchida, 1985). We found that T7 formed larger plaques on BL107(pIN-pPY) (7 mm diameter) than on BL107(pIN-SP97) (4 mm), which in turn were larger than T7 plaques formed on BL107(pIN-III) (3 mm).

Purification of mutant and wild-type RNAaseIII

Plasmids pIN-pPY and pIN-SP97 were transformed into the *E. coli rnc*⁻ strain A6008 (which contains a *ΔTn10* disruption of the *rnc* gene: Altuvia *et al.*, 1991; Takiff *et al.*, 1989) and then used to express and purify RNAaseIII and RNAaseIII-G97E. The procedure, based on the work of March & Gonzalez (1990), included chromatography employing Sephadex G-100, DEAE-Sepharose and poly-rI–poly-rC agarose. The proteins recovered at various stages of the purification process and subjected to PAGE are shown in Fig. 1(a,b). The position of RNAaseIII in the gel (arrow) was determined by Western blot analysis using RNAaseIII polyclonal antibodies (Fig. 1c). Approximately 2.5 mg of RNAaseIII and 1.5 mg of RNAaseIII-G97E were obtained. Following electrophoretic separation of 5 µg of the material from the final purification step, no additional bands were detected (Fig. 1b). The purified protein can digest *S. cerevisiae* L killer dsRNA and introduce specific cleavages into defined RNAaseIII single-stranded RNA substrates as described in the following sections. The electrophoretic mobilities of RNAaseIII and RNAaseIII-G97E on SDS-PAGE gels were identical, indicating that the *rnc-97* mutation has not significantly altered the molecular mass of the mutant protein.

DNA sequence analysis of the rnc-97 mutation and determination of the pI of RNAaseIII-G97E.

We have previously shown that the *rnc-97* mutation is located within an 850 bp sequence which includes the RNAaseIII protein-coding sequence (Pines *et al.*, 1988). In this study we reciprocally exchanged a *SalI*–*BstXI* 570 bp sequence between pPY2 (which encodes RNAaseIII) and pSP97 (which encodes RNAaseIII-G97E). By examining the lethality of the resulting hybrid genes in

Fig. 1. (a) Purification of RNAaseIII and RNAaseIII-G97E. Samples containing RNAaseIII encoded by pIN-pPY (lanes 2-4) and RNAaseIII-G97E encoded by pIN-SP97 (lanes 5-7) from different stages of the purification were separated by 17.5% SDS-PAGE and stained with Coomassie blue. Lanes 2 and 5 contain samples after ammonium sulphate precipitation; lanes 3 and 6 contain samples after Sephadex G-100 chromatography; lanes 4 and 7 contain samples after DEAE-Sepharose ion-exchange chromatography. The molecular masses of the markers in lane 1, indicated by dots, from top to bottom are: 66, 45, 36, 29, 24, 20 and 14 kDa. The arrow indicates the position of the RNAaseIII and RNAaseIII-G97E monomers. (b) Samples of RNAaseIII-G97E (lane 1) and RNAaseIII (lane 2) from the last step of purification (poly-rI-poly-rC agarose affinity chromatography) were subjected to PAGE as above. (c) Immunoblot detection of purified RNAaseIII (lane 1) and RNAaseIII-G97E (lane 2). Samples were subjected to SDS-PAGE followed by immunoblotting using rabbit anti-RNAaseIII antibodies. The large arrow shows the position of monomeric RNAaseIII and RNAaseIII-G97E; the small arrow shows the position of a minor aggregated species of RNAaseIII.

Fig. 2. Isoelectric focussing. Cell extracts were prepared from *rnc*-disrupted *E. coli* strains A6008(pIN-SP97) (lane 1) and A6008(pIN-pPY) (lane 2) which were induced for the expression of RNAaseIII-G97E and RNAaseIII respectively. Lane 3 contains pI markers. The arrows show the positions of the electrophoresis origin (top) RNAaseIII (middle) and RNAaseIII-G97E (bottom).

yeast, it was possible to determine that the *rnc-97* mutation is located within the 570 bp sequence. DNA sequence analysis of this 570 bp fragment revealed a single substitution of G:C to A:T, 290 bp from the A of the first methionine codon. This changes glycine 97 of RNAaseIII to glutamic acid.

Total cellular extracts of *E. coli* strains harbouring pIN-pPY and pIN-SP97 were subjected to analytical isoelectric focusing on polyacrylamide gels (Fig. 2). The migration positions in the gel of RNAaseIII (middle arrow) and RNAaseIII-G97E (bottom arrow) were determined by Western blot analysis using RNAaseIII polyclonal antibodies (not shown). The apparent pI of the enzymes when compared with the pI markers (lane 3) is approximately 7.30 for RNAaseIII (lane 2) and 6.85 for RNAaseIII-G97E (lane 1). Substitution of a glycine by a glutamic acid is expected to lower the pI of RNAaseIII. The theoretical pI of the RNAaseIII, calculated from its amino acid composition using the simplified procedure described by Sillero & Ribeiro

Fig. 3. Cleavage of yeast killer dsRNA by RNAaseIII and RNAaseIII-G97E. Total RNA from *S. cerevisiae* was treated with various amounts of purified RNAaseIII (lanes 3, 5 and 7) and RNAaseIII-G97E (lanes 4, 6 and 8); 100 ng (lanes 3 and 4), 25 ng (lanes 5 and 6) and 10 ng (lanes 7 and 8) of each purified protein was used in the assay. The RNA was separated by agarose gel electrophoresis and was stained with ethidium bromide. Lane 2 contains undigested RNA and lane 1 contains bacteriophage λ DNA cleaved with *Hind*III (fragments of the latter are indicated by dots). The positions of A, the electrophoresis origin; B, chromosomal DNA; C, yeast L dsRNA; D, yeast M dsRNA; and E, ribosomal RNA are indicated on the right.

(1989), is 6.80. According to this calculation, substitution of one glycine residue by a glutamic acid residue in RNAaseIII-G97E would give a pI of 6.55. Disagreement between experimental and theoretical pI values is a common finding, and can be explained by the degree and type of folding of the proteins, which can affect the binding of ions and carrier ampholytes to the proteins and can affect the pK values of their specific groups.

Activity of RNAaseIII-G97E and RNAaseIII

RNAaseIII exhibits two kinds of ribonuclease activity: (a) nonspecific activity towards dsRNA of sufficient length (see Introduction), and (b) cleavage of single-stranded RNAs which form certain double-stranded structures that are specific substrates of the enzyme. It was of interest to determine whether the *rnc*-97 mutation affects one of these activities more than the other, thus

Fig. 4. Cleavage of single-stranded RNAaseIII RNA substrates. The activity of purified RNAaseIII and RNAaseIII-G97E was examined on *in vitro* transcripts containing RNAaseIII-cleavage sites of the *rnc* operon (a), the T7 R0.3 gene (b) and the T7 R1.1 gene (c). Following the enzymic reaction, samples were separated on polyacrylamide gels which were stained with ethidium bromide. (a) Lane 1, untreated *rnc* RNA; lane 2, *rnc* RNA treated with 100 ng RNAaseIII; lane 3, *rnc* RNA treated with 200 ng RNAaseIII-G97E. Arrows (from top to bottom) indicate positions of: plasmid pAR2656 DNA; the 375 nucleotide uncut transcript; the 208 nucleotide fragment; the 84 nucleotide fragment; the 83 nucleotide fragment. (b) Lane 1, T7 R0.3 RNA treated with 300 ng RNAaseIII-G97E; lane 2, T7 R0.3 RNA treated with 200 ng RNAaseIII; lane 3, untreated T7 R0.3 RNA. Arrows show the positions of the untreated (top) and cleaved (bottom) transcripts. (c) Lane 1, T7 R1.1 RNA treated with 300 ng of RNAaseIII-G97E; lane 2, T7 R1.1 RNA treated with 200 ng RNAaseIII; lane 3, untreated T7 R1.1 RNA. Arrows show the positions of the untreated (top) and cleaved (bottom) transcripts.

producing a difference between the specificities of the wild-type and mutant enzymes. This in turn could be responsible for the observed phenotypes in yeast and *E. coli*. We chose to examine the activities described above in the same way; the RNA substrates were incubated with various concentrations of the enzymes and then the cleavage products were analysed by gel electrophoresis.

Digestion of yeast killer dsRNA is presented in Fig. 3. Equivalent amounts of RNAaseIII and RNAaseIII-G97E were incubated with this dsRNA under standard assay conditions (see Methods). We estimate that the wild-type enzyme has two- to threefold more activity than the mutant enzyme (Fig. 3).

Cleavage of single-stranded RNA is presented in Fig. 4. Since it was possible that the *rnc*-97 mutation may alter the specificity of the enzyme, we examined a number of single-stranded RNA substrates. This included *in vitro*

runoff transcripts encoding RNAaseIII cleavage sites from the *rnc* operon (Fig. 4a) and from the bacteriophage T7 R0.3 (Fig. 4b), R1.1 (Fig. 4c) and R18.5 (not shown) genes. In all cases, the wild-type and the mutant enzymes introduced cleavages at identical sites as determined by the mobility of their respective cleavage fragments on polyacrylamide gels. When comparing the activity of similar amounts of these RNAaseIII proteins, we again found (as for the activity on dsRNA) that the wild-type enzyme was about twofold more active than the mutant enzyme. This is demonstrated in Fig. 4 by the full cleavage of RNA substrates by RNAaseIII versus partial cleavage by an equivalent amount of RNAaseIII-G97E (compare lanes 2 and 3 in Fig. 4a, lanes 1 and 2 in Fig. 4b and lanes 1 and 2 in Fig. 4c). Thus, we did not detect differences in the specificity of the two RNAases, but rather a small difference in the level of their activity.

Factors affecting the activity of RNAaseIII-G97E

Taking into consideration the composition of the RNAaseIII activity buffer and the conditions of the enzyme assay, we decided to examine the effects of temperature, pH, ionic strength and concentration of Mg^{2+} ions on RNAaseIII-G97E activity (dsRNAase and cleavage of the bacteriophage T7 R1.1 site). Whereas we found no differential effect of temperature (at 25, 30, 37, 42 and 50 °C) or ionic strength (at 120, 100, 50, 10, and 2 mM-KaCl) on the activity of RNAaseIII-G97E versus RNAaseIII, there was a detectable difference between the enzymes with respect to pH. At pH 5.8 RNAaseIII-G97E had about half the activity exhibited by RNAaseIII. Even though this differential effect of pH on the wild-type and mutant enzymes was reproducible, it was rather subtle. It is important to note that in all the experiments described here we compared the effect of the various factors on amounts of RNAaseIII and RNAaseIII-G97E which correspond to equivalent levels of dsRNAase activity under standard conditions.

A striking effect on RNAaseIII-G97E activity was discovered upon limitation of Mg^{2+} ions in the reaction buffer. At the standard concentration of Mg^{2+} in the assay (10 mM), both enzymes exhibited dsRNAase activity towards yeast L dsRNA (Fig. 5a, lanes 3 and 4). However, with 1 mM- Mg^{2+} in the assay buffer, RNAaseIII exhibited dsRNAase activity whereas the mutant enzyme did not (compare lanes 5 and 6). Even when the concentration of RNAaseIII-G97E was increased two, four and eightfold (lanes 8, 10 and 12 respectively) we could not detect L dsRNA degradation. Thus, RNAaseIII-G97E is sensitive to a reduction in Mg^{2+} concentration; under such conditions, we estimate that there is at least a 20-fold difference between the activities of

Fig. 5. Requirement of Mg^{2+} -ions for the activity of RNAaseIII-G97E and RNAaseIII. (a) Cleavage of yeast L dsRNA. The activity of RNAaseIII (lanes 3, 5, 7, 9, 11, 13) and RNAaseIII-G97E (lanes 4, 6, 8, 10, 12, 14) was examined in the presence of 10 mM (lanes 3, 4); 1 mM (lanes 5–12); and 0.1 mM (lanes 13 and 14) $MgCl_2$. The amount of RNAaseIII used was 100 ng for samples in lanes 3, 5 and 13, which was increased two, four and eight fold in lanes 7, 9 and 11, respectively. The amount of RNAaseIII-G97E used was 200 ng in lanes 4, 6 and 14, which was increased two, four and eight fold in lanes 8, 10 and 12, respectively. Lane 2 contains uncleaved yeast RNA and lane 1 contains bacteriophage λ DNA cleaved with *Hind*III. Assay conditions and electrophoresis were as described for Fig 3. Arrows show the positions of yeast L dsRNA (bottom) and chromosomal DNA (top). (b) Cleavage of single-stranded RNA. The *in vitro* transcript from plasmid pET-4 encoding the bacteriophage T7 R1.1 RNAaseIII cleavage site was treated with 100 ng RNAaseIII (lanes 2, 4, 6 and 8) and 200 ng RNAaseIII-G97E (lanes 3, 5, 7 and 9) in the presence of 10 mM (lanes 2 and 3), 1 mM (lanes 4 and 5), 0.1 mM (lanes 6 and 7) and 0.01 mM- $MgCl_2$ (lanes 8 and 9). Lane 1 contains undigested RNA. Assay and electrophoresis conditions were as described for Fig. 4. Arrows show the positions of the uncleaved (top) and cleaved (bottom) transcript.

the wild-type and mutant enzymes. As previously determined (Robertson *et al.*, 1968), wild-type RNAaseIII also requires Mg^{2+} for its activity; however, only when the Mg^{2+} concentration was reduced to 0.1 mM did we detect a reduction in dsRNA degradation by RNAaseIII (Fig. 5a, lane 13).

The sensitivity to Mg^{2+} limitation was also reflected in the capacity of RNAaseIII-G97E to cleave a single-stranded RNA substrate. As shown in Fig. 5(b), both the wild-type and mutant enzymes cleaved RNA containing the T7 R1.1 cleavage site when 10 mM- Mg^{2+} was present

in the assay buffer (lanes 2 and 3). With 1 mM-Mg²⁺ in the assay, RNAaseIII-G97E exhibited no cleavage of R1-1 RNA (lane 5) whereas RNAaseIII fully cleaved this substrate (lane 4). Furthermore, RNAaseIII could partially cleave this site in the presence of 0.1 mM-Mg²⁺ (lane 6).

Discussion

In this study we refer to two phenotypes of RNAaseIII expression: the lethal expression of RNAaseIII in the lower eukaryote *S. cerevisiae* (Pines *et al.*, 1988) and the capacity of RNAaseIII expression in the prokaryote *E. coli* to reduce the efficiency of propagation of bacteriophage ϕ 1 (Davidov *et al.*, 1992). A single point mutation substituting glycine 97 by a glutamic acid residue eliminates the lethal phenotype of the mutant RNAaseIII (RNAaseIII-G97E) in yeast and reduces fourfold the effect of its expression on ϕ 1 propagation in *E. coli*. This phenomenon can be accounted for in two ways: (a) the mutation alters the substrate specificity of RNAaseIII so that it does not recognize certain substrates *in vivo*; (b) the mutation reduces or eliminates the activity of RNAaseIII so that it cannot efficiently cleave its substrates *in vivo*. Our data do not support the more attractive notion that the rnc-97 mutation causes a change in substrate specificity of RNAaseIII-G97E. All four single-stranded RNAaseIII substrates that were examined appeared to be cleaved identically by RNAaseIII and RNAaseIII-G97E. However, we have not ruled out the possibility that there are RNAaseIII sites which are not recognized by RNAaseIII-G97E, although this seems unlikely.

Our previous results indicated that extracts of yeast cells, expressing wild-type RNAaseIII and mutant RNAaseIII-G97E exhibit similar levels of dsRNAase activity, as determined under standard assay conditions. In this study, we have shown that the purified proteins exhibit about a twofold difference in the magnitude of dsRNAase activity and of the cleavage of single-stranded RNA substrates (under standard assay conditions). It is obvious that the clear-cut phenotype of the rnc-97 mutation in yeast and in *E. coli* cannot be attributed to such small differences between the activities of the wild-type and the mutant enzymes. However, we can identify assay conditions in which the activities of the mutant and wild-type enzymes differ by a factor of 20 or more. This provides the basis for explaining, at the molecular level, the effects of the rnc-97 mutation on the enzyme which result in its phenotypes *in vivo*. Thus, a low concentration of Mg²⁺ ions (and a nonoptimal-physiological pH) in the yeast cell may result in an essentially inactive RNAaseIII-G97E versus an active RNAaseIII *in vivo*. Consistent with this notion are reports indicating that the in-

tracellular free Mg²⁺ concentration in higher eukaryotic cells is approximately 0.5 mM (Maguire, 1990). Free Mg²⁺ concentrations in prokaryotes have not been reported, although a free concentration of 1–2 mM has been estimated (Snively, 1990). Hence, the Mg²⁺ concentration inside cells relevant to enzyme modulation is in the order of 0.1–1.0 mM (Maguire, 1990). For an enzymic system or cellular process to be modulated by Mg²⁺ it should be sensitive to Mg²⁺ concentrations within this range. In fact, it is within this range that we observe a strong effect on RNAaseIII-G97E activity *in vitro*.

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References

- ALTUVIA, S., KORNITZER, D., KOBİ, S. & OPPENHEIM, A. B. (1991). Functional and structural elements of the mRNA of the *cIII* gene of bacteriophage lambda. *Journal of Molecular Biology* **218**, 723–733.
- APIRION, D. & WATSON, N. (1975). Mapping and characterization of a mutation in *Escherichia coli* that reduces the level of ribonuclease III specific for double-stranded ribonucleic acid. *Journal of Bacteriology* **124**, 317–324.
- BARDWELL, J. C. A., REGNIER, P., CHEN, S., NAKAMURA, Y., GRUNBERG-MANAGO, M. & COURT, D. L. (1989). Autoregulation of RNaseIII operon by mRNA processing. *EMBO Journal* **8**, 3401–3407.
- DAVIDOV, Y., ZIVION, G. & PINES, O. (1992). Ribonuclease III reduces the efficiency of bacteriophage ϕ 1 propagation in *E. coli*. *Current Microbiology* **24**, 63–66.
- DUNN, J. J. (1976). RNaseIII cleavage of single-stranded RNA. *Journal of Biological Chemistry* **251**, 3807–3814.
- DUNN, J. J. & STUDIER, F. W. (1973). T7 early RNAs and *Escherichia coli* ribosomal RNAs are cut from large precursor RNAs *in vivo* by ribonuclease III. *Proceedings of the National Academy of Sciences of the United States of America* **70**, 3296–3300.
- DUNN, J. J. & STUDIER, F. W. (1975). Effect of RNaseIII cleavage on translation of bacteriophage T7 messenger RNAs. *Journal of Molecular Biology* **99**, 487–499.
- KINDLER, P., KEIL, T. U. & HOFFSCHNEIDER, P. H. (1973). Isolation and characterization of a ribonuclease III deficient mutant of *Escherichia coli*. *Molecular and General Genetics* **126**, 53–69.
- KING, T. C., SIRDESHMUKH, R. & SCHLESSINGER, D. (1986). Nucleolytic processing of ribonucleic acid transcripts in prokaryotes. *Microbiological Reviews* **50**, 428–451.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MAGUIRE, M. E. (1990). Magnesium, a regulated and regulatory cation. In *Metal Ions in Biological Systems*, vol. 26, pp. 135–153. Edited by H. Sigel & A. Sigel. New York: Dekker.
- MANIATIS, T., FRITSCH, E. F. & SAMBROOK, J. (1982). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- MARCH, P. E. & GONZALEZ, M. A. (1990). Characterization of the biochemical properties of recombinant ribonuclease III. *Nucleic Acids Research* **18**, 3293–3298.
- MARCH, P. E., AHNN, T. & INOUE, M. (1985). The DNA sequence of the gene *rnc* encoding ribonuclease III of *Escherichia coli*. *Nucleic Acids Research* **13**, 4677–4685.
- MASUI, Y., COLEMAN, J. & INOUE, M. (1983). Multipurpose expression cloning vehicles in *E. coli*. In *Experimental Manipulation of Gene Expression*, pp. 15–32. Edited by M. Inouye. New York: Academic Press.

- MILLER, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- NASHIMOTO, H. & UCHIDA, H. (1985). DNA sequencing of the *E. coli* ribonuclease III gene and its mutations. *Molecular and General Genetics* **201**, 25–29.
- PINES, O., YOON, H. J. & INOUE, M. (1988). Expression of double-stranded RNA-specific RNaseIII of *Escherichia coli* is lethal to *Saccharomyces cerevisiae*. *Journal of Bacteriology* **170**, 2989–2993.
- PORTIER, C., DONDON, L., GRUNBERG-MANAGO, M. & REGNIER, P. (1987). The first step in the functional activation of the *Escherichia coli* polynucleotide phosphorylase messenger is a ribonuclease III processing at the 5' end. *EMBO Journal* **6**, 2165–2170.
- ROBERTSON, H. D. & DUNN, J. J. (1975). Ribonucleic acid processing activity of *Escherichia coli* ribonuclease III cleavage sites. *Journal of Biological Chemistry* **250**, 3050–3056.
- ROBERTSON, H. D., WEBSTER, R. E. & ZINDER, N. D. (1968). Purification and properties of ribonuclease III from *Escherichia coli*. *Journal of Biological Chemistry* **243**, 82–91.
- ROSENBERG, A. H., LADE, B. N., CHUI, D., LIN, S., DUNN, J. J. & STUDIER, F. W. (1987). Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* **56**, 125–135.
- SHONBERGER, O., HIRST, T. R. & PINES, O. (1991). Targeting and assembly of an oligomeric bacterial enterotoxin in the endoplasmic reticulum of *Saccharomyces cerevisiae*. *Molecular Microbiology* **5**, 2663–2671.
- SILLERO, A. & RIBEIRO, J. H. (1989). Isoelectric points of proteins: theoretical determination. *Analytical Biochemistry* **179**, 319–325.
- SNAVELY, M. D. (1990). Magnesium transport in prokaryotic cells. In *Metal Ions in Biological Systems*, vol. 26, pp. 155–175. Edited by H. Sigel & A. Sigel. New York: Dekker.
- STUDIER, F. W. (1975). Genetic mapping of a mutation that causes ribonuclease III deficiency in *Escherichia coli*. *Journal of Bacteriology* **124**, 307–316.
- STUDIER, F. W. & DUNN, J. J. (1983). Complete nucleotide sequence of bacteriophage T7 DNA and the location of T7 genetic elements. *Journal of Molecular Biology* **166**, 477–535.
- TAKIFF, H. E., CHEN, S. M. & COURT, D. L. (1989). Genetic analysis of the *rnc* operon of *Escherichia coli*. *Journal of Bacteriology* **171**, 2581–2590.
- TOWBIN, H., STAEBELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350–4354.
- WATSON, N. & APIRION, D. (1985). Molecular cloning of the gene for the RNA-processing enzyme RNaseIII of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 849–853.