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Identification and analysis of the *rnc* gene for RNase III in *Rhodobacter capsulatus*

Reinhard Rauhut, Andreas Jäger, Christian Conrad and Gabriele Klug*

Institut für Mikrobiologie und Molekularbiologie der Justus Liebig Universität Giessen, Frankfurter Straße 107, 35392 Giessen, Germany

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ABSTRACT

The large subunit ribosomal RNA of the purple bacterium *Rhodobacter capsulatus* shows fragmentation into pieces of 14 and 16S, both fragments forming the functional equivalent of intact 23S rRNA. An RNA-processing step removes an extra stem–loop structure from the 23S rRNA [Kordes,E., Jock,S., Fritsch,J., Bosch,F. and Klug,G. (1994) *J. Bacteriol.*, 176, 1121–1127]. Taking advantage of the fragmentation deficient mutant strain Fm65, we used genetic complementation to find the mutated gene responsible for this aberration. It was identified as the *Rhodobacter* homologue to *rnc* from *Escherichia coli* encoding endoribonuclease III (RNase III). The predicted protein has 226 amino acids with a molecular weight of 25.5 kDa. It shares high homology with other known RNase III enzymes over the full length. In particular it shows the double-stranded RNA-binding domain (dsRBD) motif essential for binding of dsRNA substrates. The Fm65 mutant has a frame shift mutation resulting in complete loss of the dsRBD rendering the enzyme inactive. The cloned *Rhodobacter* enzyme can substitute RNase III activity in an RNase III deficient *E.coli* strain. Contrary to *E.coli,* the *Rhodobacter rnc* is in one operon together with the *lep* gene encoding the leader peptidase.

INTRODUCTION

The three ribosomal rRNA species in prokaryotes arise from co-transcriptional processing of a single large (30S) primary RNA transcript, which may also contain tRNA sequences. At least three endoribonucleases are involved in the processing of this precursor in *Escherichia coli*: RNase III, RNase E and RNase P (1). Although it is known that RNase III recognizes and cleaves double-stranded RNA regions, detailed sequence and structural requirements of the cleavage site are only poorly understood (2). The enzyme initiates maturation of rRNA from 30S precursor rRNA by introducing cleavages in two double-stranded regions of the primary rRNA transcript ultimately leading to the 16S and 23S rRNAs. Maturation of 16S and 23S RNA can take place in the absence of RNase III (3), but the process is rather inefficient, and RNase III mutants are impeded in growth. In addition to the regular task of removing internal transcribed spacers separating the functionally independent ribosomal rRNA species, RNase III has also been implicated in the removal of intervening sequences (IVS) from 16 and 23S rRNAs. These IVS usually have the secondary structure of a hairpin loop (4). They interrupt the normally linear continuity of the large or small rRNA and are post-transcriptionally removed without religation of the resulting fragments. The processing thus generates fragmented rRNAs. The functional equivalent of a continuous prokaryotic rRNA is then held together by secondary and tertiary interactions of these RNA fragments which constitute the basic unit of the RNA molecule. Despite the crucial role of rRNA in the protein synthesis machinery, it is not necessary for proper function that rRNA is a single continuous polynucleotide. Fragmented rRNA is a widespread biological phenomenon observed for large and small subunit rRNAs of prokaryotic, eukaryotic and organellar origin (5–7). Recent results from *Salmonella* suggest a possible biological role for fragmentation in this bacterium. During late logarithmic growth phase, *Salmonella* strains with fragmented rRNA degrade rRNA and ribosomes more rapidly than *E.coli* with continuous rRNA (8). This may provide *Salmonella* with a selective advantage under this particular physiological condition.

We recently identified and analyzed the rRNA site which is processed in 23S rRNA of the purple bacterium *Rhodobacter capsulatus* (9). An IVS of ~100 nucleotides (nt) is removed by an RNase III like activity, yielding a fragmented 23S rRNA of 14 and 16S. The cleavage sites are located in an extra stem–loop which is deleted by this processing step. The insertion position is almost identical to the positions of IVS found in two other members of this subdivision, *Salmonella* spp. and *Campylobacter* spp. (5,7). In this paper we provide direct evidence that it is indeed the *rnc* gene encoding RNase III which confers normal processing (i.e. fragmentation) to the fragmentation-deficient *Rhodobacter* mutant Fm65.

In *E.coli* the *rnc* gene, encoding RNase III, is the first of three genes in the multifunctional *rnc* operon: *rnc*, *era* and *recO* (10,11) (Fig. 1). The *rnc* gene overlaps by two nucleotides with the *era* gene which codes for a GTP-binding protein (12). The expression of the two genes is translationally coupled (13). The *rnc* operon is preceded by the *lep* operon comprising the *lepA* and *lep* genes.

The cloned and sequenced gene for *Rhodobacter* RNase III codes for a predicted protein of 226 amino acids with a monomer molecular weight of 25.5 kDa. We show that the *R.capsulatus* *rnc* operon structure deviates significantly from the structural

* To whom correspondence should be addressed

organization in *E.coli*. The *rnc* gene forms an operon with the upstream *lep* gene and possibly with the downstream *era* gene. *Lep* and *rnc* are transcribed from the same promoter upstream of *lep*. *Rnc* shows an autoregulatory effect when differently sized promoter regions are used during *lacZ*-fusion experiments.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Rhodobacter capsulatus strains used in this study were wild-type strains 37b4 (DSM938), B10 (14) and the chemical mutant Fm65 (15). Strains were grown in a minimal malate salt medium (16). During growth of plasmid carrying strains, tetracycline was added at a final concentration of 1 µg/ml.

In vitro cleavage of RNase III substrates with *R.capsulatus* RNase III

As substrate we used the well studied phage T7 R1.1 substrate, an RNase III processing signal upstream of the 1.1 gene of phage T7 (4,17). This ~60 nt long RNA hairpin consists of two presumably double-helical RNA stems separated by an internal loop which bears the single-cleavage site. This RNA has been cloned under T7 control. The construct (pET-4) was kindly provided by J.J. Dunn (Brookhaven National Laboratory). *In vitro* transcriptions were performed in a 10 µl volume, containing T7 buffer (40 mM Tris–HCl pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT), 1 µg pET-4 template linearized with *Nci*I, 20 µg RNasin (Promega), rNTPs (0.5 mM each, except for rUTP at 0.1 mM), 75 U T7 RNA polymerase (NEB) and 20 µCi [α-^{32}P]rUTP (specific activity 3000 Ci/mmol). The reaction mixture was phenol extracted and stored in ethanol at –20°C. Prior to use, ~2000 c.p.m. of substrate was spun down and dissolved in 2 µl 5× RNase III buffer (100 mM Tris–HCl pH 7.6, 50 mM MgOAc, 0.65 M NH_4Cl, 25% glycerol). With water and enzyme added a 10 µl reaction is incubated at 32°C for 1 min. Reaction products are analyzed on a 10% PAA–7 M urea gel. RNase III cleaves the 115 nt pET-4 transcript into fragments of 86 and 29 nt.

RNase III-containing fractions from *R.capsulatus* and *E.coli*

Frozen *Rhodobacter* or *E.coli* cells (~1 g) were pulverized in liquid nitrogen using a mortar and pestle. The powder was dissolved in 6 ml of extraction buffer containing 50 mM Tris–HCl pH 7.9, 0.25 M KCl, 2 mM EDTA, 1 mM β-mercaptoethanol, 0.1 mM DTT and 0.5 mM PMSF. The extract was centrifuged for 45 min at 150 000 *g* and the supernatant used for enzymatic tests.

Cloning of the *Rhodobacter rnc* gene in a prokaryotic overexpression vector

Primers with *Eco*RI and *Hin*dIII restriction site tags, respectively and matching the exact 5′- and 3′-ends of the sequenced *rnc* gene from *Rhodobacter capsulatus* were used during polymerase chain reaction (PCR) with *R.capsulatus* chromosomal DNA as template. The amplified gene was cut with *Eco*RI/*Hin*dIII to generate the termini necessary for cloning it into the IPTG inducible prokaryotic overexpression vector pKK223-3 (Pharmacia). Primers used: antisense primer 5′-CTGGGG**AAGCTT**TCATGCGCCTCTCCAA-3′, sense primer 5′-GCTCGG**GATTC**ATGAAAGTTGCGGCAGAC-3′. *Hin*dIII and *Eco*RI sites respectively are in bold face immediately adjacent to the start and stop codons (underlined).

Construction of translational *lacZ* fusions

In order to fuse the 5′ part of the *rnc* gene (amino acids 1–71) and differently sized upstream fragments to the *lacZ* gene of the pPHU plasmids (18), a set of DNA fragments was amplified by PCR. We used primer Prfm7.5- (5′-GACT**GGATCC**GCGCGGCC-3′), which anneals to the sequence around the *Bam*HI site located within the *rnc* gene, and one of the following primers: uplepEco1800 (5′-CCAGCAAATCC**GAATTC**GGC) annealing to positions +5 to +24, uplepEco1850 (5′-GCTTGCTT**GAATTC**CCCCCCG) annealing to positions –61 to –41, uplepEco2000 (5′-GCCGG**GAATTC**ATCTGACCGGC) annealing to positions –194 to –178, and uplepEco2120 (5′-GCGCC**GAATTC**GCGGGCACC) annealing to positions –328 to –309. Positions are given with respect to the start of the *lep* gene. All upstream primers contain an internal *Eco*RI site (bold letters). The resulting PCR products were cut with *Eco*RI and *Pvu*II and ligated into the *Eco*RI and *Sca*I sites of plasmid pPHU234.

Construction of plasmids for complementation tests

Our original plasmid isolate pRK2fm1, with a 6.5 kb *Eco*RI insert, was able to restore 23S rRNA fragmentation in the fragmentation deficient *R.capsulatus* mutant Fm65 (9). pRK4fmH4 has the 4 kb *Hin*dIII fragment of pRK2fm1 cloned into the *Hin*dIII site of plasmid pRK415 (19), with *lep* being transcribed in the inverse direction of the plasmid's *lac* promoter. To test which upstream sequences are required, plasmids pRK4fmHP2.3 and pRK4fmP1.7 have the *Hin*dIII–*Pst*I fragments of plasmid pRK4fmH4 cloned into pRK415 (Fig. 1). To test whether *lep* and *rnc* are transcribed from the same promoter a series of plasmids was constructed containing different sequence extensions upstream of the *lep* gene. To this end we amplified differently sized fragments by PCR using plasmid pRK4fmH4 as a template. As primers we used Prfm7.5- and the *Eco*RI-tagged uplepEco primers as described for the construction of the *lacZ* fusions. The resulting DNA fragments were cut with *Eco*RI and *Bam*HI which results in two fragments, since the amplified DNA fragments contain two *Bam*HI sites. The two fragments were religated and in a second step ligated to the large fragment that arises from *Eco*RI/*Bam*HI digestion of pRK4fmH4. Clones that contained the internal *Bam*HI fragment in the correct orientation were transferred into mutant strain Fm65 by conjugation and the rRNA patterns were analyzed. Construct pRK4fmHB1.9 contains the 1.9 kb *Bam*HI–*Pst*I fragment from pRK4fmH4.

Analysis of rRNA patterns on agarose gels

Bacteria from 20 ml of a semiaerobic culture were harvested and resuspended in 125 µl buffer A containing 0.3 M saccharose, 10 mM sodium acetate pH 7.6 and 125 µl buffer B containing 2% SDS, 10 mM sodium acetate pH 7.6. After three hot phenol (65°C) extractions, total RNA was precipitated with ethanol and the remaining DNA was digested with 80 U RNase-free DNase I (BRL). RNA was ethanol precipitated and stored in 50 µl 20 mM sodium phosphate pH 6.5, 1 mM EDTA at –20°C. Electrophoresis

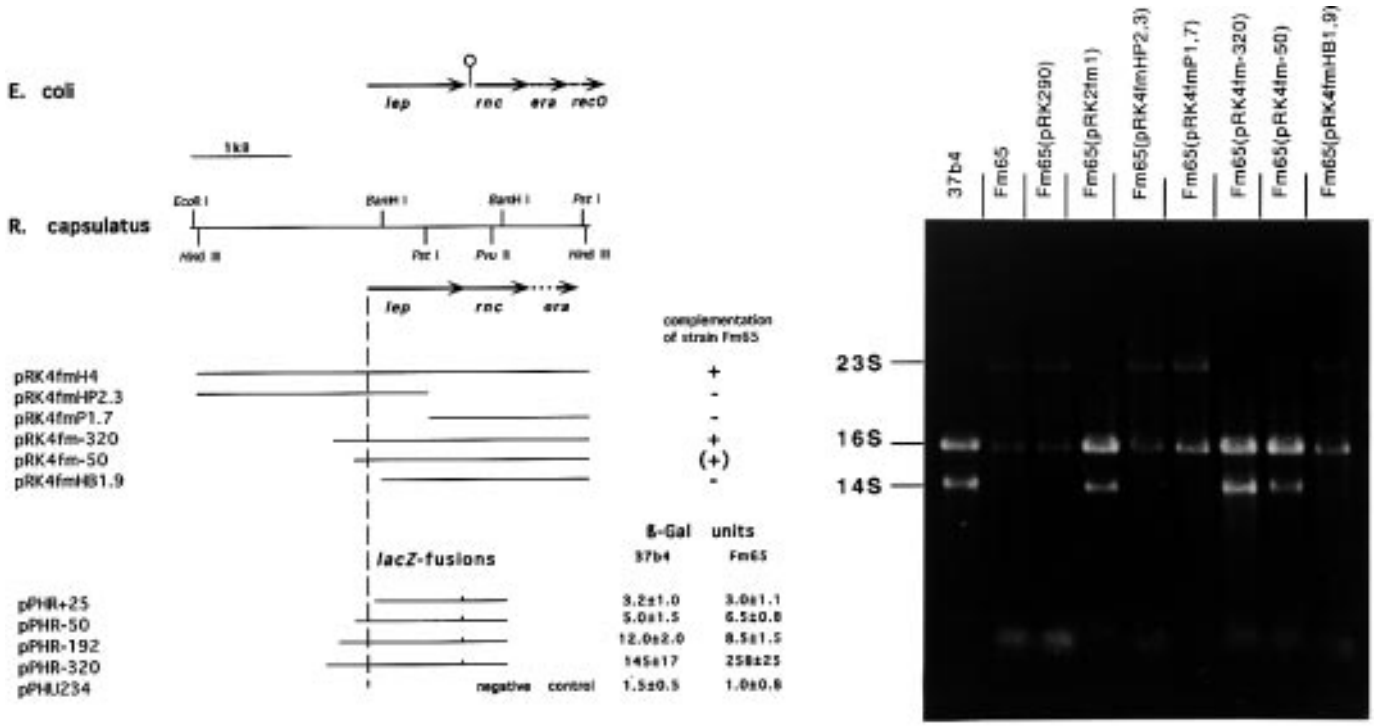

Figure 1. (**A**) Organization of the *rnc* operon in *R.capsulatus*. The scheme shows the organization of the *lepB–rnc–era* operon and the location of restriction sites mentioned in the text. Relative positions of constructs used for complementation studies and of the *lacZ*-fusion constructs are shown. For comparison the structural organization of the *rnc* operon in *E.coli* is given. The hairpin indicates the structure implicated in autoregulation in *E.coli*. (**B**) *In vivo* pattern of ribosomal RNAs from *Rhodobacter* wild-type strain 37b4 and mutant strains Fm65 harbouring various plasmid constructs for complementation. pRK290, the parental vector of pRK2fm1, does not carry any *Rhodobacter* sequences (29). RNA was run on a 1% gel as described in Material and Methods and subsequently stained with ethidium bromide. The positions of the 14 and 16S rRNA species are marked.

with 10 µg RNA per lane was done on 1% formaldehyde agarose gels.

DNA sequencing and amplification of DNA

We used double-stranded templates for sequencing (pGEM-derived plasmids). Double-stranded plasmid DNA was isolated using the Qiagene midiprep kit. 3–5 µg DNA were used per sequence reaction using the USB or the Pharmacia sequencing kits. Sequence analyses were performed using DNA Star software.

For amplification of DNA fragments by PCR 50 pmol of plasmid template, 100 µmol of each primer and 1 U Vent polymerase (NEB) were used. After an initial denaturation step for 3 min at 96°C, 30 cycles were run as follows: 96°C for 45 s, annealing for 45 s, extension at 74°C for 20–90 s. The annealing temperature was chosen 5–8°C below the calculated melting temperature for the primer–DNA hybrid. To exclude PCR artifacts affecting β-galactosidase measurements, two PCR reactions for each fragment and two cloning experiments were carried out independently for each construct.

RESULTS

Cloning and sequence analysis of the *rnc* gene of *R.capsulatus*

We have previously described the *R.capsulatus* mutant strain Fm65, which does not process its 23S rRNA and suggested that this strain has only reduced RNase III activity (9). We described the isolation of plasmid pRK2fm1 from a genomic library that restored 23S rRNA processing after transfer into strain Fm65. Plasmid pRK2fm1 harbours a 6.8 kb *Eco*RI fragment of *R.capsulatus* DNA. Several subclones of plasmid pRK2fm1 were tested for complementation of strain Fm65. A 4 kb *Hin*dIII fragment was sufficient to restore 23S rRNA cleavage, whereas the 2.3 kb *Hin*dIII–*Pst*I and the 1.7 kb *Pst*I fragments were not (Fig. 1). We concluded that the cleavage at the *Pst*I site had disrupted a DNA sequence that is required for 23S rRNA cleavage. DNA sequencing around this *Pst*I site revealed a strong homology to bacterial and eukaryotic leader peptidases (Klug *et al.*, submitted for publication). Further sequencing resulted in the identification of the *rnc* gene which overlaps the preceding *lep* gene by 2 nt. A good Shine–Dalgarno sequence is not found at the start of the *rnc* gene. The 3′-end of the *rnc* gene overlaps a third open reading frame (ORF) by 2 nt. The derived amino acid sequence of the first 300 nt of this ORF shows strong homology to the *era* gene product of *E.coli* (not shown). The DNA sequence data reported here have been assigned EMBL Nucleotide Sequence Database accession number Z68305.

The percent similarity of the *Rhodobacter* enzyme with homologous bacterial RNase III lies in the range of 33–63%, whereas the similarity of the yeast enzyme with the bacterial proteins rests at ~20%. The C-terminal sequence implicated in double-stranded RNA binding is indicated in Figure 2 together with the consensus sequence for this type of protein domain. Strong homologies with known *rnc* sequences are also found in the N-terminal half of the protein. Particularly striking is the presence of an 11 amino acid

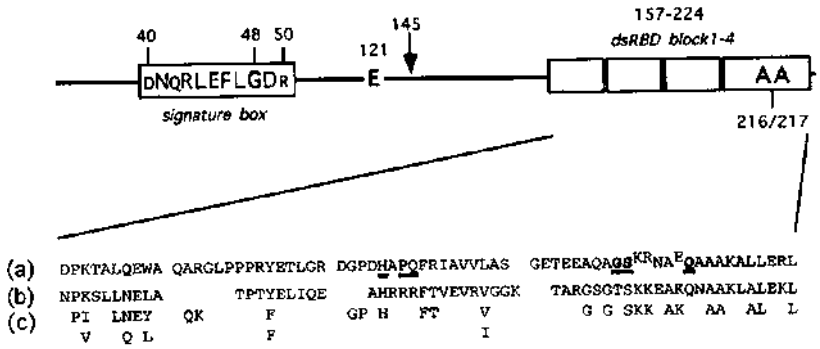

Figure 2. Schematic representation of the RNase III from *Rhodobacter*. The upper panel shows the position of the signature box conserved in eukaryotic and prokaryotic RNases III (small capitals signify semi-conserved positions) and the position of the dsRBD. Bold letters signify positions of proven catalytic importance (see text). The block scheme for the dsRBD is adapted from (25). The arrow marks the position of the frame shift in *Rhodobacter* Fm65. The lower part shows the dsRBD of *Rhodobacter* (a) together with the dsRBD consensus sequence from refs 24 and 25 (b), (c). A recently proposed cluster of (semi)conserved amino acids and amino acids which can act as hydrogen bond donors in the RNase III dsRBD is indicated in (a): semiconserved positive charges are underlined, strongly conserved charges are given in superscript, conserved H-bond donors are bold and underlined (27).

signature sequence at positions 40–50 of the *R.capsulatus* sequence, which is also present in all other known *rnc* genes (Fig. 2).

We have not only determined the sequence of the *rnc* gene isolated from wild-type strain B10, but have also amplified and sequenced this DNA region from mutant strain Fm65 and its parental wild-type strain 37b4. The nucleotide sequences of wild-type strains B10 and 37b4 differ in 30 positions (4.4 % of the total number of nucleotides). Most of these nucleotide exchanges have no effect on the amino acid composition. The amino acid sequence of the wild-type strains 37b4 and B10 differs at only six positions. Five of these amino acid exchanges are at positions that are not homologous to any of the other known *rnc* sequences. Instead of a histidine at position 62 of RNase III from strain B10, which is also present in the *E.coli* protein, the RNase III of strain 37b4 contains an aspartate at this position, that is not found in any other RNase III protein.

The *rnc* sequence of mutant strain Fm65 has the G nucleotide in codon 145 missing when compared with the wild-type sequence. The missing G residue causes a reading frame shift and extension of translation for an additional 91 amino acids beyond the regular stop codon. The mutant protein, as predicted from the DNA sequence, comprises 319 amino acids and translates well into the downstream *era* gene. Since this frame shift occurs in the middle of the RNase III gene, the complete C-terminal half comprising the full double-stranded RNA binding domain is not functional, rendering the enzyme inactive.

The *rnc* gene and the *lep* gene encoding signal peptidase form an operon

The *lep* gene and the *rnc* gene of *R.capsulatus* overlap by two nucleotides suggesting that both genes form an operon and are translationally coupled. Using Northern blot analysis it was not possible to detect an mRNA species that is clearly homologous to both genes (data not shown). The 1.7 kb *Hin*dIII–*Pst* I fragment derived from plasmid pRK2fm1 was not sufficient to reconstitute 23S rRNA processing in strain Fm65, although this fragment comprises the complete *rnc* gene and an additional 430 nt of upstream sequence. Although there is no *lepA* homologue upstream of *lep*, we could not exclude the possibility of other upstream genes that are also part of an operon including *lep* and *rnc*. We therefore deleted sequences upstream of *lep* to define the DNA sequences sufficient to restore 23S rRNA cleavage in strain Fm65. The construct pRK4fmHB1.9, which has all sequences 5′ to the Lep start and a small part of the 5′ coding region of *lep* deleted, shows no complementation. Plasmids pRKfm-50 and pRKfm-320 carry 50 and 320 nt upstream of the Lep start, respectively. We transferred the plasmids into strain Fm65 and analyzed total RNA isolated from the transconjugants on formaldehyde–agarose gels. Fm65(pRKfm-320) shows normal cleavage of 23S rRNA, suggesting that all sequences required for *rnc* transcription are included within the 320 nt upstream of the Lep start. Fm65(pRKfm-50) shows only partial cleavage of 23S rRNA (Fig. 1). We therefore conclude that the promoter for *rnc* transcription is localized ~50 nt upstream of the Lep start.

In order to study the expression of *rnc* we analyzed three constructs with different extensions 5′ of the Lep start during *lacZ* fusion experiments (Fig. 1). In all three clones, the *rnc* gene was translationally fused to *lacZ*, allowing direct measurement of the RNase III transcription rate by β-galactosidase activity. The *lacZ* fusions confirm our complementation experiments, showing that *rnc* transcription requires an intact promoter region upstream from *lep* between positions –50 and –320.

The *rnc* gene of *R.capsulatus* shows autoregulation

The *rnc* gene of *E.coli* shows autoregulation through RNase III recognition and cleavage of a stem–loop structure in the 5′ untranslated region of this gene. Cleavage at this position decreases the *rnc* messenger concentration in the cell by a factor of six with concomitant decrease of rnc protein by a factor of three (20).

Despite the different organization of the *lep–rnc* region in *R.capsulatus* which lacks a 5′ untranslated region immediately preceding *rnc*, we could not exclude autoregulation of the *rnc* gene. In order to test this possibility the set of *lacZ*-fusion constructs with the *rnc* gene translationally fused to *lacZ* and expressing the *lep* promoter activity was transferred into wild-type strain 37b4 as well as into the mutant Fm65. Cultures of both transconjugants were grown to identical optical density and β-galactosidase activity was determined. In both strains a region between –50 and –320 nucleotides preceding the *lep* gene is required for full promoter activity in the expression of *Rhodobacter*

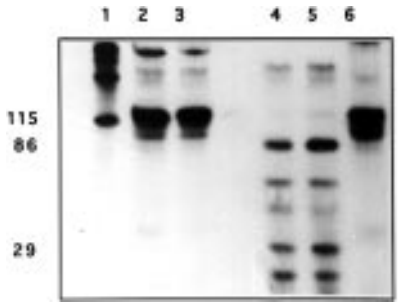

Figure 3. RNase III *in vitro* assays using extracts from various cell materials: lane 1, RNA standard; lanes 2 and 3, fragmentation-deficient *Rhodobacter* mutant Fm65; lane 4, induced *E.coli* strain BL 321 (RNase III$^-$) carrying the *rnc* gene from *Rhodobacter* on a plasmid; lane 5, *E.coli* strain BL322 (RNase III$^+$); lane 6, uninduced *E.coli* strain BL 321 (RNase III$^-$). The T7-transcript of pET-4 used as a substrate to screen for RNase III activity is 115 nt long. A single RNase III cleavage produces the 86 and 29 nt bands, respectively. Additional bands are due to other RNase activities present in crude extracts.

rnc. Interestingly the pPHR-320 construct exhibits a significant difference in measured β-gal units for the two *Rhodobacter* strains (factor 1.7) (Fig. 1). For this construct the presence or absence of intact rnc protein obviously makes a difference in the expression of *rnc*.

Rhodobacter capsulatus RNase III functions in *E.coli*

Further proof that the enzymatic activity responsible for rRNA fragmentation in *R.capsulatus* is an RNase III stems from an experiment in which the *rnc* gene from *R.capsulatus* was cloned in the IPTG-inducible plasmid pKK223-3 (Pharmacia) and transferred into the RNase III deficient *E.coli* strain BL321. Figure 3 shows that RNase III activity in *E.coli* BL321(RNase III$^-$) is restored after induction. The *Rhodobacter* enzyme thus can functionally replace the *E.coli* enzyme in a reaction using the phage R1.1 model substrate.

DISCUSSION

We have shown that RNase III is the enzymatic activity responsible for removing an extra stem–loop from 23S rRNA in *R.capsulatus*. Since intact 23S rRNA is not observed in *Rhodobacter*, it seems that all four rRNA operons show this particular type of processing. The predicted rnc protein in *Rhodobacter* has a molecular weight of ~25.5 kDa which corresponds to the molecular weight of the α-monomer of rnc in *E.coli* (25 kDa). The *Rhodobacter* enzyme not only structurally resembles the *E.coli* enzyme but can also functionally replace it in the cleavage of an RNase III substrate from phage (Fig. 3).

RNase III, an endoribonuclease that cleaves specifically double-stranded RNA structures, is an ubiquitous component of the prokaryotic and eukaryotic RNA processing machinery. The enzyme has been implicated in the processing of polycistronic transcripts in phages, the processing of rRNA-precursors, mRNA- and snRNA-metabolism (2,21,22). Other known ribonucleases III include four prokaryotic sequences and the eukaryotic homologue from *S.pombe*. Two functional aspects have to be addressed in RNase III. First, the enzyme has to recognize and bind the double-stranded RNA substrate, and subsequently the scissile internucleotide bond has to be cleaved in the active center. The *Rhodobacter* enzyme shows extended sequence homologies with the other bacterial RNase III proteins. An 11 nt long signature sequence in the N-terminal part of the protein is strongly conserved, even across the prokaryote–eukaryote boundary (Fig. 2). This sequence is possibly involved in substrate recognition, is part of the active site, or supports the quaternary structures of the *rnc* dimer. The Gly 44 in *E.coli* and the corresponding Gly 178 in *S.pombe* have been demonstrated to be of catalytic importance in both organisms (22,23). This position is conserved in all known RNase III sequences including the *Rhodobacter* sequence (Gly 48). Another position conserved in all sequences is the *R.capsulatus* Glu121 (*E.coli* Glu117 and *S.pombe* Glu251, respectively). Mutagenesis of this residue in *E.coli* and *S.pombe* has shown that it is possibly involved in catalysis (2,22).

Binding of the dsRNA substrate is facilitated through the dsRNA-binding-domain (dsRBD) located in the N-terminal half of the protein. The dsRBD motif is one of at least five known RNA-binding-motifs (24). dsRNases are only a subset of all known proteins carrying the dsRBD motif, which is also found in other dsRNA binding proteins of diverse cellular functions, like for example RNA helicases. The dsRBD is a protein module of ~70 amino acids (24–26). Several copies of the dsRBD can be present in one protein. The *Rhodobacter* enzyme has a high degree of similarity with the consensus sequence of dsRBD motifs derived from a large set of dsRNA binding proteins (24–26) (Fig. 2). Though it is obvious that recognition of a double-stranded RNA plays an important role in fragmentation, the question remains open what particularly distinguishes the extra stem–loop of the cleavage site from the plethora of similar secondary structures in rRNA. While a dsRBD alone is not sufficient for catalysis, in *S.pombe pac1* (coding for RNase III) a mutation which completely removes the dsRBD results in an inactive RNase III. This resembles the situation in our Fm65 mutant which has a frame shift after amino acid 144, rendering the mutant enzyme inactive through loss of the complete dsRBD region. In Pac1 it was also shown that partial loss (deletion of block 4) of the dsRBD results in an inactive enzyme. In particular the two alanines 346 and 347 in pac1 are of catalytic importance (22). These two alanines are also conserved in *Rhodobacter* RNase III (positions 216 and 217). Recently the structure of the dsRBD in *E.coli* RNase III has been described in more detail using NMR techniques (27). The structure was described as a three stranded anti-parallel β-sheet backed against two helices. A very similar structure has been described for the staufen dsRBD (28). A cluster of (semi)conserved charged amino acids and amino acids which can act as a hydrogen bond donor has been proposed on the basis of structure analysis of the rnc dsRBD and dsRBD alignments. This cluster located on the surface of the dsRBD could act as a docking site for dsRNA substrates. Most of the amino acids implicated in this cluster, or at least their chemical characters, are also well conserved in the *Rhodobacter* enzyme (Fig. 2).

Our *in vivo* data show that 50 nt upstream of the *lep* start are sufficient to allow for transcription of *rnc* messenger to an extent which at least partially restores fragmentation of 23S rRNA. This is in good agreement with the transcription start of the *lep–rnc* messenger as determined by *lep–lacZ* fusions and primer extension experiments (Klug *et al*., manuscript submitted). Extending a *lep–lacZ* fusion from 50 to 320 nt upstream of *lep* does not result in a significantly higher transcription rate. Instead, *lep–rnc–lacZ* fusions show considerable increase in activity when the upstream *lep* sequence is extended. Both results show

that this effect must depend on sequences well downstream of the transcription start. In *E.coli* the amount of *rnc* messenger, and depending on it the amount of rnc protein, is subject to an autoregulatory mechanism which involves cleavage by RNase III of a stem–loop immediately upstream of the *rnc* start (20). Our results from the *lacZ*-fusion experiments in wild-type *Rhodobacter* and Fm65 mutant show a significant down regulation of messenger concentration in the presence of intact rnc protein. This requires regulatory mechanisms different from *E.coli* without a stem–loop immediately upstream of *rnc*. Since *lep* and *rnc* form an overlapping transcriptional unit in *Rhodobacter*, a regulatory site could be located in the upstream non-coding region of the *lep* gene. Regulation possibly involves mechanisms like the one described for *E.coli* polynucleotide phosphorylase, Pnp. It has been shown for *pnp* transcripts that processing of an RNase III site upstream of the coding region of Pnp creates a signal which unmasks an RNase E site inside the coding region immediately downstream of the translation start. This results in decreased stability of the messenger through the action of RNase E and possibly other nucleases (20,21). Knowledge of RNase III action thus adds important aspects to the understanding of the mechanisms of mRNA stability in prokaryotes.

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