Volume 9 Number 12 1981 Nucleic Acids Research

16S Ribosomal RNA of Escherichia coli contains a N^2-methylguanosine at 27 nucleotides from the 3' end

Rein Van Charldorp, Hans A.Heus and Peter H.Van Knippenberg

Department of Biochemistry, State University of Leiden, P.O.Box 9505, 2300 RA Leiden, The Netherlands

Received 24 April 1981

ABSTRACT

The 49 nucleotide fragment derived from the 3' end of 16S rRNA by cloacin DF_{13}, is not cleaved by ribonuclease T_1 at a guanosine residue that is present at 27 nucleotides from the 3' terminus (position 1515 in 16S rRNA). Analysis of the isolated nucleotide indicates that it is a modified G residue. *In vivo* labeling with (^{3}H)methionine shows that this G is methylated and co-chromatography with markers reveals that it is N^2-methylguanosine.

INTRODUCTION

The modified nucleotides in ribosomal RNA of *E. coli* were first identified by Fellner (1) in RNase T_1 oligonucleotides. When the complete nucleotide sequence of 16S rRNA was elucidated (2,3) allocation of the positions of the modified nucleotides was based on earlier results. Untill now 9 modified nucleotides have been localized in the 16S rRNA sequence. In several instances, however, it was not possible to determine the site of the modified nucleotide. Hence, there is still doubt on the actual number of modified nucleotides and of their position in the 16S rRNA. Of the three or four m^2G residues, for example, found by Fellner to be present per molecule 16S rRNA, only two have been placed in the final sequence, to wit at positions 965 and 1206, respectively (2).

One of the parts of 16S ribosomal RNA of *E. coli* that has been studied most, both with respect to function and to structure, concerns the 49 nucleotides near the 3' end (compare Steitz (4) for a review). The reason for this is that (*i*) a special function in initiation of protein synthesis has been assigned to this part of the RNA (5), and (*ii*) the bacteriocins colicin E_3 and cloacin DF_{13} cleave the 16S rRNA *in situ* at 49 nucleotides from the 3' end (6,7), making this fragment available for separate study (8). We report here a previously unidentified methylation of a guanosine residue

in this 49 nucleotide fragment i.e. at position 1515 of the 16S rRNA.

MATERIALS AND METHODS

Materials. *E. coli* strain PR_7 and its kasugamycin resistant derivative TPR_{201} were kindly provided by Dr. J.E. Davies (Univ. of Wisconsin, Madison, U.S.A.). Cloacin DF_{13} was a gift of Dr. F.K. De Graaf. Dr. C.J. Van der Laken generously provided methylated markers. The following were commercial products: (γ-^{32}P)ATP (Amersham, U.K.), L(methyl-^{3}H)methionine (Amersham, U.K.), RNase T_1 (Sankyo, Calbiochem.), RNase P_1 (Yamasa Shoyu Co. Ltd., Tokyo, Japan). Plastic backed polyethyleneimine plates (polygram Cc/300) were from Machery and Nagel, Düren, West-Germany.

Isolation of the cloacin fragments. The isolation of the cloacin fragments was according to the procedure described by Baan *et al.* (9). To check its intactness and purity each time after the final preparation the fragment was labeled with (γ-^{32}P)ATP and sequenced as described in (10).

Isolation of the (^{3}H) labeled cloacin fragments. Bacteria of the strains TPR_{201} and PR_7 were grown in 25 ml minimal media. Minimal media contains per liter deionized water: 0.2 gr $MgSO_4.7H_2O$, 2 gr citric acid, 10 gr K_2HPO_4, 3.5 gr $Na(NH_4)HPO_4.4H_2O$. This basic medium was supplemented with 50 ml 20% glucose, 50 mg L-leucine, 50 mg L(-) threonine, 5 mg thiamine, 62 mg methionine, 50 mg $MgSO_4.H_2O$ and 50 mg $FeSO_4.7H_2O$.

In the case of the strain TPR_{201} kasugamycin (100 mg/l) was added. A log-phase culture was washed in medium lacking methionine and used to inoculate 25 ml of medium containing 1 mCi (0.06 Ci/mmol) of L-(methyl-^{3}H)-methionine. The cells were harvested in late log-phase, washed and resuspended in standard buffer (10 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 60 mM NH_4Cl, 6 mM 2-mercaptoethanol). Using this method 90% of the label introduced in the growing culture was taken up by the bacteria. About 0.8% of the (^{3}H)-methyl label was recovered as methyl labeled ribosomal 16S RNA. The cloacin fragments were isolated as described before (9).

Nucleotide analysis of the cloacin fragments on polyethyleneimine plates. 0.1 A_{260} unit of the cloacin fragment was dissolved in 10 µl buffer (100 mM Tris-HCl pH 9.0, 20 mM $MgCl_2$, 10 mM DTT) to which was added 1 µl T_4 polynucleotide phosphokinase (0.25 units μl^{-1}), 4 µl 10 mM ATP and 5 µl H_2O. The mixture was incubated at 37°C for 45 min. After the addition of 0.05 volume 3 M ammonium acetate it was precipitated with 3 volumes of ethanol. The pellet was suspended in 10 µl deionized formamide and incubated at 100°C

for 10 min. After another precipitation with 3 volumes of ethanol and 0.05 volume 3 M ammonium acetate the pellet was resuspended in 10 µl buffer (20 mM Tris-HCl pH 8.0, 20 mM $MgCl_2$, 12 mM mercaptoethanol) to which was added 2 µl T_4 polynucleotide phosphokinase (2 units μl^{-1}) and 8 µl H_2O. The mixture was added to lyophilised γ-(^{32}P)ATP (20 µCi, 3000 Ci/mmol) and incubated at 37°C for 30 min. An aliquot was taken and treated in the presence of 10 µg carrier RNA with 2 µl RNase P_1 (0.1 ng/ml) for 1 h at 37°C. The rest of the sample was brought onto a 20% acrylamide slab gel containing 7 M urea after addition of 10 µl formamide dye mix (11) and electrophoresed for 20 h at 400 volt. The gel was autoradiographed and a ladder pattern was visible. Adjacent bands were selected from the gel, cut out and eluted with 1 M NaCl. After several precipitations with 3 volumes of ethanol, the RNA was taken up in a small volume of sterilized water to which 10 µg carrier RNA was added and incubated with 2 µl RNase P_1 (0.1 ng/ml). The resultant 5' ^{32}P-labeled nucleotides were fractionated by chromatography on polyethyleneimine plates.

Plastic backed, 20 x 20 cm polyethyleneimine plates were first washed with 2 M formic acid-pyridine pH 2.2 and with water. After drying they were kept at 4°C. After the sample was brought onto the plates they were first developed with H_2O, then with 0.5% formic acid. The third step was run with 0.15 M Li^+ formate adjusted to pH 3.0.

Nucleotide analysis of the (^{3}H) labeled cloacin fragments on polyethyleneimine plates. The (^{3}H) labeled cloacin fragments were treated as above except that in the second kinase treatment non-radioactive ATP was used. Segments of 5 mm of the polyethyleneimine plates were cut and treated with triethylamine carbonate whereafter the radioactivity was determined by liquid scintillation counting.

RESULTS

The sequence of the cloacin fragment shown in Figure 1, is drawn in a hairpin structure as previously established by physical methods (8). For simplicity the nucleotides are numbered from the 5' end of the fragment and number 1 of this fragment corresponds to number 1493 in the complete sequence of 16S RNA. Figure 2, lane 2, displays the autoradiograph of a gel of a partial RNase T_1 digest of the 49 nucleotide fragment labeled with (^{32}P) at its 5' terminus. All the radioactive fragments must bear a G-residue at the 3' end and by comparison with a gel pattern of a partial alkaline digest of the same (^{32}P) labeled fragment (lane 3), the positions of the G-residues

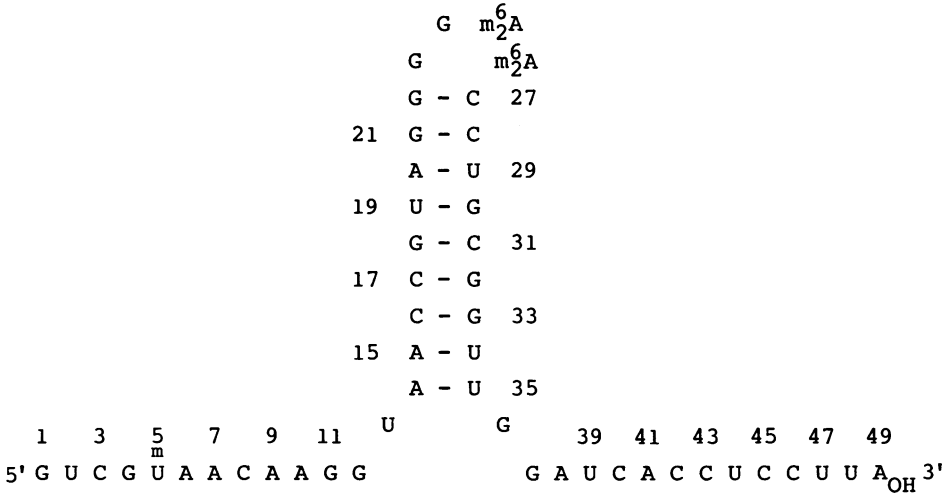

Figure 1. The reported nucleotide sequence of the 3' end of 16S rRNA from *E. coli* arranged in a hairpin structure (8). The numbering system is from 5' to 3' to facilitate description of the methods.

can be identified. All G's expected are found except number 23. Since the DNA corresponding to the entire 16S rRNA has been sequenced (2), there is no doubt that the transcript must have a G at this position. For some unknown reason the RNase T_1 might fail to hydrolyze the phosphodiester bond between G23 and G24 because of the presence of the dimethylated A's. Therefore the same RNA fragment was isolated from a kasugamycin resistant strain of *E. coli* (TPR_{201}), that specifically lacks methylation of these adenines (12). Figure 2 (lane 5) shows, however, that in the RNase T_1 digest of this fragment, G23 is also missing from the gel pattern.

In order to identify the nucleotide at position 23 further, a slight modification of the method described by Stanley and Vassilenko (13) was used. The 49 nucleotide fragment from the mutant strain, which has a free 5' hydroxyl group was first treated with polynucleotide kinase and non-radioactive ATP. Then the fragment was mildly subjected to formamide treatment under conditions that on the average less than one phosphodiester bond per molecule was hydrolyzed, followed by labeling with γ-(^{32}P)ATP and polynucleotide kinase. Since there is an unlabeled 5' phosphate on the original fragment after the first kinase treatment, only the 3' parts of the hydrolyzed fragment will have a 5' radioactive phosphate. Fractionation of the mixture on a denaturing polyacrylamide gel gives a ladder pattern, where

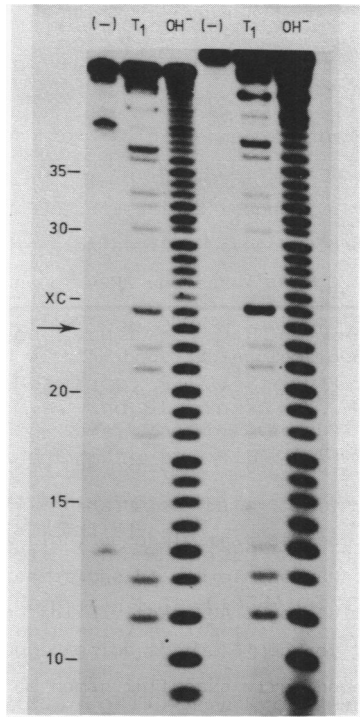

Figure 2. Autoradiogram of a sequencing gel using the 49 nucleotide fragments derived from the 3' end of 16S rRNA. The purified labeled fragments were treated with RNase T_1 (lanes 2, 5) and alkali (lanes 3, 6) according to the procedure described by Donis-Keller *et al.* (11).
Lanes 1, 2, 3: fragments derived from the strain PR_7; lanes 4, 5, 6: fragments derived from the strain TPR_{201}. The arrow indicates the place of the missing nucleotide at position 23. XC: xylene cyanol FF. The electrophoresis was for 16 h at 400 volt. For explanation concerning the differences in migration between wild type and mutant fragment compare Van Charldorp *et al.* (10).

each band contains the same 3' end. The nucleotide on the 5' side of each band within the ladder may be determined by elution of the material and subsequent analysis after complete hydrolysis with the enzyme RNase P_1. After this treatment each band will give (^{32}P)5'X3'OH, where X stands for the nucleoside corresponding to the position in the fragment. The RNase P_1 digest of each band was chromatographed on polyethyleneimine plates and the chromatograms were autoradiographed (Figure 3). A control sample, containing a P_1 digest of a formamide treated γ-(^{32}P)ATP labeled RNA was also analyzed on these plates, providing a reference for the position of the 4

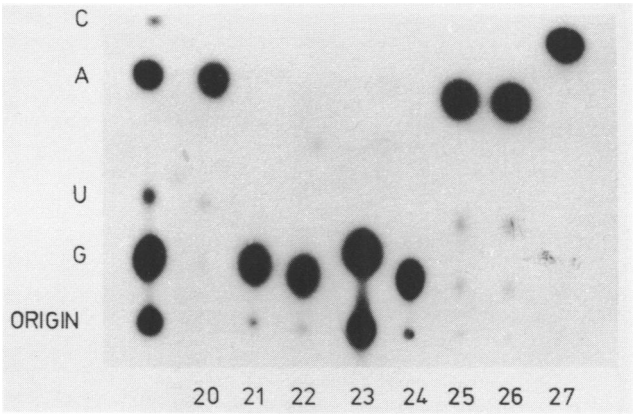

Figure 3. Nucleotide analysis on a polyethyleneimine plate. The material is derived from the purified 49 nucleotide fragment of the 3' end of 16S rRNA of ribosomes from the strain TPR_{201}. The most leftward lane shows the mobility of the four common nucleotides. The numbering beneath the figure refers to the position in the fragment (compare Figure 1).

common nucleotides. In this figure we see that the nucleoside phosphate from band 23 has an increased mobility as compared to (^{32}P)G and that it neither contains, U, A or C.

Since methylation of the base moiety is the most common modification, the kasugamycin resistant mutant of *E. coli* (TPR_{201}) and its parental strain (PR_7) were grown in the presence of methyl (^{3}H)methionine in order to detect methylation of the nucleotides. The 49 nucleotide fragments were isolated from the cultures, degraded as above and treated with polynucleotide kinase and non-radioactive ATP. A control sample from unlabeled mutant bacteria was processed as in the control of Figure 3. The reference and the control samples were, after treatment with RNase P_1, analyzed on polyethyleneimine plates. The part of the plate where the references were run, was cut in segments and treated with triethylamine carbonate, whereafter the radioactivity was determined by liquid scintillation counting. Figure 4 displays the distribution of the (^{3}H)radioactivity of both fragments. The wild type fragment (Figure 4A) contains a highly (^{3}H) labeled region which is lacking in the mutant fragment (Figure 4B). This represents the dimethylated adenines as is clear from Figure 4C. In this latter experiment, the 49 nucleotide fragment was isolated from 30S particles, derived from the kasugamycin resistant mutant. These particles were specifically methylated *in situ* with purified methylase and (methyl-^{3}H)AdoMet (14). By this a "pseudo wild type"

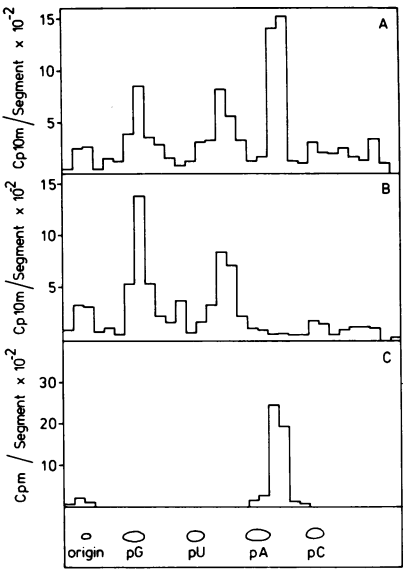

Figure 4. Analysis of nucleotides of (^{3}H) labeled cloacin fragments on polyethyleneimine plates. (A) Fragment derived from the strain PR_7. (B) Fragment derived from the strain TPR_{201}. (C) Fragment derived from 30S particles from the *E. coli* strain TPR_{201} which were methylated with methylase and (methyl-^{3}H)AdoMet as described before (10).

fragment is made with a (^{3}H) label on the adenines number 25 and 26. The other two regions of (^{3}H) radioactivity are present in both mutant and wild type. The radioactivity moving at a position between the U and A marker almost certainly corresponds to the methylated U residue at position 5 in the fragment (3). The (^{3}H) label between the G and U marker then must correspond to the modified G at position 23. A similar chromatographic separation in the presence of unlabeled modified markers reveals that the modification is m^2G. Together with the m^2G's at position 965 and 1206, this would account for the total number of three of these modified bases found previously (1).

DISCUSSION

The nucleotide sequence of the RNA of small ribosomal subunits is remarkably conserved at the 3' end. In all instances a similar hairpin as in Figure 1, containing 2 dimethylated adenines in the loop, can be constructed. However, although position 23 is a G residue in 16S RNA of *E. coli* (2,

3), *Zea mays* chloroplasts (15) and in 13S RNA of hamster mitochondria (16), it is a U residue in 18S RNA from hen reticulocyte, mouse sarcoma, rabbit reticulocyte, barley embryo (17), rat liver (18), *Bombyx mori* (19), *Drosophila melanogaster* (20) and *Saccharomyces cerivisiae* (21). As shown here this G residue is methylated in *E. coli*. Inspection of RNase T_1 digestion pattern of the 13S RNA of hamster mitochondria in the paper of Bear and Dubin (16) suggests that this G residue may be modified in mitochondria as well. The sequence of *Zea mays* chloroplast 16S rRNA was deduced from the DNA sequence of the gene so that in this case the situation is not clear.

ACKNOWLEDGEMENTS

The authors are indebted to Drs. L. Bosch and J. Van Duin for critical reading the manuscript. This work was sponsored in part by the Netherlands Foundation for Chemical Research (S.O.N.) with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

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