Volume 9 Number 7 1981 Nucleic Acids Research

The 3'-terminal region of bacterial 23S ribosomal RNA: structure and homology with the 3'-terminal region of eukaryotic 28S rRNA and with chloroplast 4.5S rRNA

Mohamed Ali Machatt, Jean-Pierre Ebel and Christiane Branlant+

Institut de Biologie Moléculaire et Cellulaire du CNRS, Laboratoire de Biochimie, 15, rue René Descartes, 67084 Strasbourg Cedex, France

Received 19 February 1981

ABSTRACT

The sequence of the 110 nucleotide fragment located at the 3'-end of *E.coli*, *P.vulgaris* and *A.punctata* 23S rRNAs has been determined. The homology between the *E.coli* and *P.vulgaris* fragments is 90%, whereas that between the *E.coli* and *A.punctata* fragments is only 60%. The three rRNA fragments have sequences compatible with a secondary structure consisting of two hairpins. Using chemical and enzymatic methods recently developed for the study of the secondary structure of RNA, we demonstrated that one of these hairpins and part of the other are actually present in the three 3'-terminal fragments in solution. This supports the existence of these two hairpins in the intact molecule. Indeed, results obtained upon limited digestion of intact 23S RNA with T_1 RNase were in good agreement with the existence of these two hairpins.

We observed that the primary structures of the 3'-terminal regions of *yeast* 26S rRNA and *X.laevis* 28S rRNA are both compatible with a secondary structure similar to that found at the 3'-end of bacterial 23S rRNAs. Furthermore, both *tobacco* and *wheat* chloroplast 4.5S rRNAs can also be folded in a similar way as the 3'-terminal region of bacterial 23S rRNA , the 3'-end of chloroplast 4.5S rRNAs being complementary to the 5'-end of chloroplast 23S rRNA. This strongly reinforces the hypothesis that chloroplast 4.5S rRNA originates from the 3'-end of bacterial 23S rRNA and suggests that this rRNA may be base-paired with the 5'-end of chloroplast 23S rRNA.

Invariant oligonucleotides are present at identical positions in the homologous secondary structures of *E.coli* 23S, *yeast* 26S , *X.laevis* 28S and *wheat* and *tobacco* 4.5S rRNAs. Surprisingly, the sequences of these oligonucleotides are not all conserved in the 3'-terminal regions of *A.punctata* or even *P.vulgaris* 23S rRNAs. Results obtained upon mild methylation of *E.coli* 50S subunits with dimethylsulfate strongly suggest that these invariant oligonucleotides are involved in RNA tertiary structure or in RNA-protein interactions.

INTRODUCTION

The 3'-terminal region of bacterial 16S ribosomal RNA has been shown to participate in mRNA selection (1-3). The question is open whether bacterial 23S ribosomal RNA also plays a functional role. A study of evolutionarily conserved sequences of this RNA region might be informative in this respect. Furthermore, determination of the primary structure of the 3'-terminal regions of different bacterial 23S rRNAs should be helpful in the determination of the secondary structure of this rRNA region. Indeed, the secondary structures of

tRNAs, 5S rRNAs and 16S rRNAs (4-7) were found to be conserved throughout bacterial evolution, with mutations balanced by compensatory mutations in order to maintain strand complementarity. Detection of such compensatory mutations has already been very useful in predicting 16S rRNA secondary structure (6,7). The same situation is expected for 23S RNA.

To study the 3'-terminal region of 23S RNA, we took advantage of the fact that this region is released as a fragment of 110-112 nucleotides in length (fragment 3') upon digestion of 50S ribosomal subunits with *Naja oxiana* RNase (8,9). In this way, we prepared the 23S RNA 3'-terminal regions from *E.coli MRE 600* and from two phylogenetically related bacterial species : *Proteus vulgaris* and *Aeromonas punctata*. We previously determined 70% of the nucleotide sequence of the *E.coli* fragment 3' (30). As there were differences between this sequence and the rDNA sequence established by Brosius et al. (10), we completed it and determined those of the *P.vulgaris* and *A.punctata* fragments 3'. Comparison of these sequences strongly suggested the existence of two hairpins in fragment 3'. Using both chemical (11) and enzymatic (12,13) methods recently developed for the study of RNA secondary structure, we checked if fragment 3' bears these two hairpins in solution as well as within 50S subunits.

As the 3'-terminal sequences of the large ribosomal rRNAs from *yeast* and *X.laevis* cytoplasmic ribosomes (14,15) and from mouse and human mitochondrial ribosomes (16,17) have recently been sequenced, we checked if these may have common structural features with the 3'-terminus of bacterial 23S rRNAs.

The ribosomes of flowering plants contain an additional small rRNA species designated 4.5SrRNA (18-21). On the basis of a sequence homology between *wheat* chloroplast 4.5SrRNA and *E.coli* 4.5SRNA, Wildeman and Nazar (22) suggested that these two 4.5S RNAs may have a common function. On the other hand, on the basis of sequence homology between *tobacco* chloroplast 4.5SrRNA (23,24) and the 3'-terminal region of *E.coli* 23S rRNA, Mac Kay (25) proposed that the two latter sequences are derived from a common ancestor and may be functionally equivalent. In order to gain more insight into this problem, we checked if chloroplast 4.5SrRNA and the 3'-terminal region of bacterial 23S rRNAs may have similar secondary structures.

MATERIALS AND METHODS

1. Preparation of 50S subunits. Three bacteria species were used : *E.coli MRE 600*, *Proteus vulgaris* (strain isolated by Dr. Monteil) and *Aeromonas punctata* (67.12 Institut Pasteur). *E.coli* and *P.vulgaris* were grown as previously described in the presence or absence of {^{32}P} orthophosphate (26). *A.punctata* was

grown in the same medium as for *E.coli* but at 30°C and for 3.5 hours. The 50S subunits were prepared from "tight couples 70S" according to Hapke and Noll. (27).

2. Methylation of 50S subunits with dimethylsulfate was achieved according to Peattie and Gilbert (11). 50S subunits (250 µg) were dissolved in 250 µl of buffer containing 10 mM $MgCl_2$ and 50 mM Na cacodylate pH 7.0.After addition of 8 µl of dimethylsulfate, the mixture was incubated for 12 min at 37°C. 75 µl of buffer containing 1M Tris acetate pH 7.5, 1 M 2-mercaptoethanol and 1.5 M Na acetate were added and the subunits were precipitated with 900 µl of ethanol. They were then dissolved in 200 µl Na cacodylate buffer containing 0.15M NaCl, reprecipitated and washed with ethanol.

3. Digestion of 50S subunits with *Naja oxiana* RNase (8). Intact or methylated 50S subunits were dissolved at 1 mg/ml in buffer containing 10 mM $MgCl_2$, 350mM KCl and 10 mM Tris-HCl pH 7.5. Digestion was for 4 hours at 0°C with 8U of *Naja oxiana* RNase per 100 µg of subunits. The reaction was stopped by phenolic extraction in the presence of Na dodecylsulfate. The extracted RNA was precipitated twice and washed with ethanol. The resulting fragments were fractionated by electrophoresis on 6 % polyacrylamide gel.

4. Limited digestion of 23S RNA. 23S RNA was obtained from 50S subunits by extraction with phenol. It was then partially digested with T_1 RNase for 30 min at 0°C at an enzyme/substrate ratio between 1/10 and 1/100 (w/w) in buffer containing 350 mM KCl, 10 mM $MgCl_2$ and 10 mM Tris-HCl pH 7.5, or at an enzyme/substrate ratio of 1/1000 (w/w) in 10 mM Tris-HCl pH 7.5. The resulting fragments were fractionated by electrophoresis on 8 % polyacrylamide gel.

5. End-labeling of RNA fragments. 3'-end labeling was achieved according to England and Uhlenbeck (28), overnight at 4°C or for 1 hour at 37°C. 5'-end labeling was performed with $\{\gamma\text{-}^{32}P\}$ATP and polynucleotide kinase.

6. Chemical digestions of 3'-end labeled RNA fragments. For nucleotide sequence analysis, digestions were performed according to Peattie (29).

For detection of higher-order structure, digestions were performed according to Peattie and Gilbert (11). Guanines and cytosines were methylated with dimethylsulfate. Adenines were carbethoxylated with diethylpyrocarbonate. Both reactions were performed under mild conditions preserving the RNA structure. Strand scission was induced by Na borohydride and aniline at m^7G, by hydrazine and aniline at m^3C and by aniline at modified A residues.

7. Enzymatic digestions of end-labeled RNA fragments.

For nucleotide sequence analysis, digestions were performed as previously described (30).

For secondary structure analysis, digestions were performed with T_1, S_1 and *Naja oxiana* RNases under the conditions in which RNA structure is maintained as previously described (13).

8. Polyacrylamide gel electrophoresis. Both enzymatic and chemical digests of end-labeled fragments were fractionated by electrophoresis on polyacrylamide slab gels (0.5X300X400 mm or 0.5X300X900mm) in Tris-borate buffer pH 8.3.

RESULTS

1. Isolation of 23S RNA 3'-terminal fragments. Using the conditions described in Methods, *E.coli* ribosomal 50S subunits were reproducibly digested with *Naja oxiana* RNase (8). Fingerprint analysis of the released fragments had revealed that one of them (fragment 3') is about 110 nucleotide lond and corresponds to the 3'-end of 23S RNA (9). *P.vulgaris* and *A.punctata* 50S subunits were digested under the same conditions and the digests were fractionated together with the *E.coli* digest on a 6% polyacrylamide gel. Very similar patterns of fragment fractionation were obtained for *E.coli* and *P.vulgaris* whereas the pattern obtained for *A.punctata* was different (Fig. 1). Nevertheless, 50S subunits from both *A.punctata* and *P.vulgaris* released a fragment having the same electrophoretic mobility as fragment 3' from *E.coli*.

2. Sequence analysis of *E.coli*, *P.vulgaris* and *A.punctata* 23S RNA 3'-terminal fragments. *P.vulgaris* and *A.punctata* 23S RNA fragments having the same electrophoretic mobility as *E.coli* fragment 3' together with fragment 3' itself were labeled at the 3'-terminus by ligation with {5'-^{32}P} pCp. Their primary structures were determined by the chemical method for RNA sequencing. The results are summarised in Figure 2.

Concerning the *E.coli* fragment 3' the results obtained complete those previously published (30) and confirm the existence of sequence differencies between *E.coli MRE 600* 23S RNA and the *E.coli K12* rrnB operon (10). The sequence A-C-U-C-C-C-U-G-A-G-A-G-Up in *E.coli MRE 600* 23S RNA is replaced by A-C-C-C-U-U-U-A-A-G-G-G-Up in the *E.coli K12* rrnB operon (nucleotides 2992-2804). It is noteworthy that Bram et al. (31), who determined the sequence of the 3'-part of the 23S RNA genes of the rrnX and rrnD operons obtained the same sequences as in *E.coli MRE 600* 23S RNA.

The homology displayed by the sequences of the *E.coli*, *P.vulgaris* and

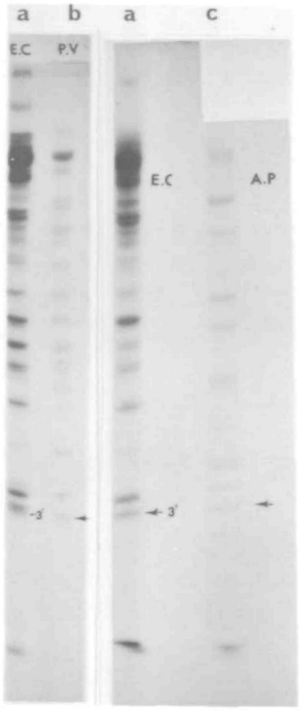

Figure 1 : Polyacrylamide gel electrophoresis of *Naja oxiana* RNase digests from *E.coli* (a), *P.vulgaris* (b) and *A.punctata* (c) 50S subunits.

The digestions were performed as described in Methods. The digests were fractionated on 6% polyacrylamide gels. Fragment 3' corresponding to the 3'-terminal 112 nucleotides of *E.coli* is indicated.

A.punctata fragments (Fig. 2) confirms the fact that the two latter fragments correspond to the 3'-terminal regions of *P.vulgaris* and *A.punctata* 23S RNA, respectively. Nevertheless, it should be pointed out that, whereas the degree of homology is very strong between the *E.coli* and *P.vulgaris* fragments (90%), it is only 60% between the *E.coli* and *A.punctata* fragments.

3. Secondary structure study of bacterial 23S RNA 3'-terminal region.

3.1 Model binding. Previous results suggested that nucleotides 3 to 10 starting from the 3'-end of 23S RNA are base-paired with the 8 nucleotides located at the 5'-end of the molecule (32), the two 3'-terminal uridines being free of base-pairing. The 3' and 5' ends of *P.vulgaris* 23S RNA display the same complementarity (unpublished results). But in this case only one unpaired 3'-terminal uridine is present. In *A.punctata* 23S RNA there are no free 3'-terminal uridine. As no sequence work has been performed on the 5'-end region of this RNA, it is not possible to know whether base complementarity exists between the 3' and the 5' regions.

Digestion of *E.coli* 23S RNA at position 2793 by *Naja oxiana* RNase indicates that the nucleotides around this position belong to a structured RNA region (33). The sequence 2791-2795 displays a complementarity with the sequence 2801-2805. Observations made during 23S RNA sequence analysis strongly suggest that the corresponding base-pairing exists in large 23S RNA fragments and even that it is stable in the presence of 8 M urea. Indeed, when 23S RNA fragments containing the sequence 2791 to 2805 were 5'-end labeled and partially digested with RNases in the presence of 8 M urea and that the resulting products were fractionated on polyacrylamide gel containing 8 M urea, we observed very low yields of cleavage at guanines 2791, 2801 and 2803 whereas guanine 2799 was deeply digested (Fig. 3). In addition, the bands corresponding to

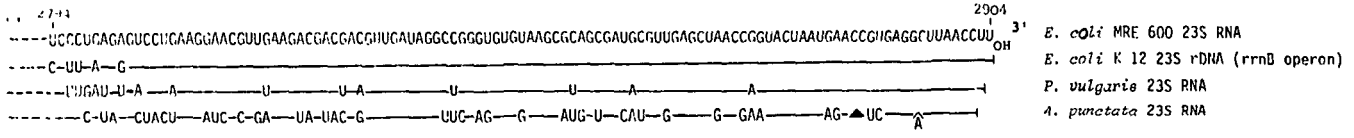

Figure 2 : Sequence of the 3'-terminal 110 nucleotides of *E. coli* MRE 600, *P. vulgaris* and *A. punctata* 23S RNAs.

The nucleotide sequence of the *E. coli* MRE 600 23S RNA fragment is given, only the differences in the *P. vulgaris* and *A. punctata* fragments are indicated. The sequence found for the corresponding rDNA region of the *E. coli* K12 rrnB operon (9) is also indicated. The numbering adopted for *E. coli* 23S RNA is that of Brosius et al. (9). Hyphens have been omitted for reasons of space.

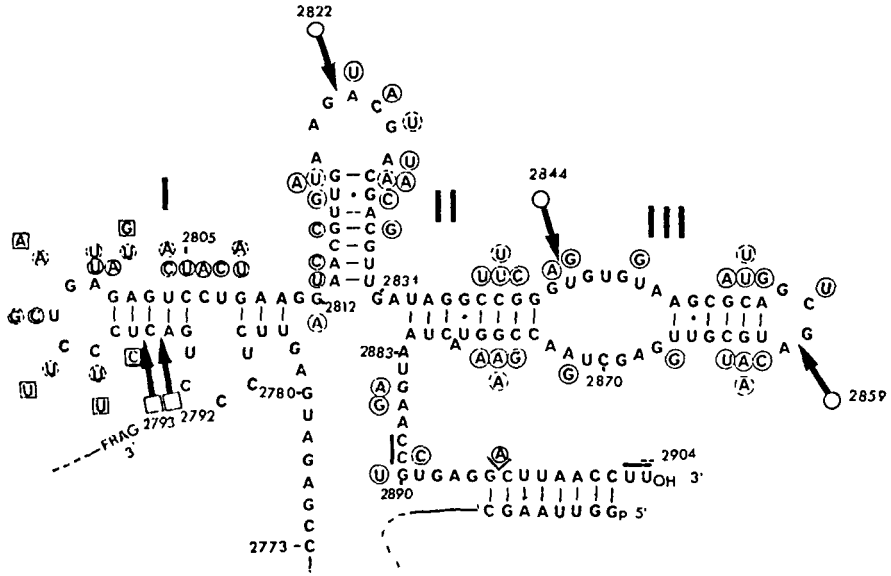

Figure 3 : Possible secondary structures of *E. coli*, *P. vulgaris* and *A. punctata* 23S RNA 3'-terminal regions

The nucleotide sequence of the *E. coli* 3'-terminal region is given. Only the differences in the *P. vulgaris* ◌ and *A. punctata* ○ fragments 3' and in the *E. coli* K12 rrnB operon □ are indicated. The arrows indicate: □→ the positions of *Naja oxiana* RNase digestion of 23S RNA within 50S subunits, ○→ the positions of T_1 RNase cleavage of 23S RNA in solution.

cleavage at positions 2803, 2804 and 2805 were compressed on the sequencing gel. Both observations are well explained by the existence of the base-pairing between regions 2791-2795 and 2801-2805 (upper stem of the hairpin denoted I in Figure 3).

Naja oxiana nuclease probably recognizes this base-paired region and cleaves *E.coli* 23S RNA at positions 2792 and 2793. Since similar cleavage positions were observed in *P.vulgaris* and *A.punctata* 23S RNAs, these RNAs are expected to bear a similar base-paired region. The presence of the corresponding complementarity could not be demonstrated, since the sequences established for these two 23S RNAs begin at the 5'-end of the loop of hairpin I.

As mentioned above, sequence differences were observed between the *E.coli MRE 600* 23S RNA and the *E.coli K12* rrnB operon in the region 2793-2802. It can be seen that three of the differences are located in the loop of hairpin I whereas the two others are located in the stem of this hairpin and they compensate for each other in maintaining the complementarity (Fig. 3). This observation is an additional argument for the existence of the upper stem of hairpin I. We have no experimental data concerning the lower stem of this hairpin, it has been proposed as a tentative.

On the assumption that hairpin I exists and that the 3' and 5' extremities of 23S RNA are base-paired to each other, we looked for the possible secondary structure in the region 2806-2894. Two hairpins II and III could be formulated in the three 23S RNAs studied (Fig. 3), their existence is strongly supported by compensatory sequences.

3.2 Experimental verification of the model

3.2.1 Study of the 3'-terminal RNA region within 50S subunits. The 3'-terminal region of 23S RNA is strongly resistant to ribonuclease attack within 50S subunits (34). No T_1 RNase cleavage was observed undermild conditions and only positions 2793 and 2792 were cleaved by *Naja oxiana* RNase. Therefore approaches based on enzymatic digestion of the subunits were not suitable to determine the secondary structure of this region.

Peattie and Gilbert (11) recently developped a chemical method in order to detect higher order structures in RNA. We applied it to the study of 23S RNA within 50S subunits. In this method, dimethylsulfate is used to sense secondary structure at the level of cytosine and tertiary structure at the level of guanine. The reagent methylates the N-3 of cytosine and the N-7 of guanine if these N-moieties are not hydrogen-bonded. Diethylpyrocarbonate is used to detect the stacking of adenine.

The *E.coli* 50S subunits were treated with these reagents in the mild condi-

tions described in Methods. They were then digested with *Naja oxiana* nuclease. In the case of dimethylsulfate treatment for time durations less than 15 min the pattern of fractionation was identical to that of untreated 50S subunits. This was not the case for the diethylpyrocarbonate treatment. Thus, we only studied fragment 3' obtained after modification of 50S subunits with dimethylsulfate. Cleavage of the phosphodiester bonds at methylated cytosines and guanines was performed as described in Methods and the resulting products were analyzed on polyacrylamide gel in parallel with digests of fragment 3' prepared as for RNA sequencing (Fig. 4a).

Under our conditions of dimethylsulfate treatment only cytosine 2858 and a limited number of guanine residues from fragment 3' were methylated (Fig. 4b). The yield of cytosine 2858 methylation was low. Therefore, this approache essentially indicates the availability of guanines N-7 and thus provides little

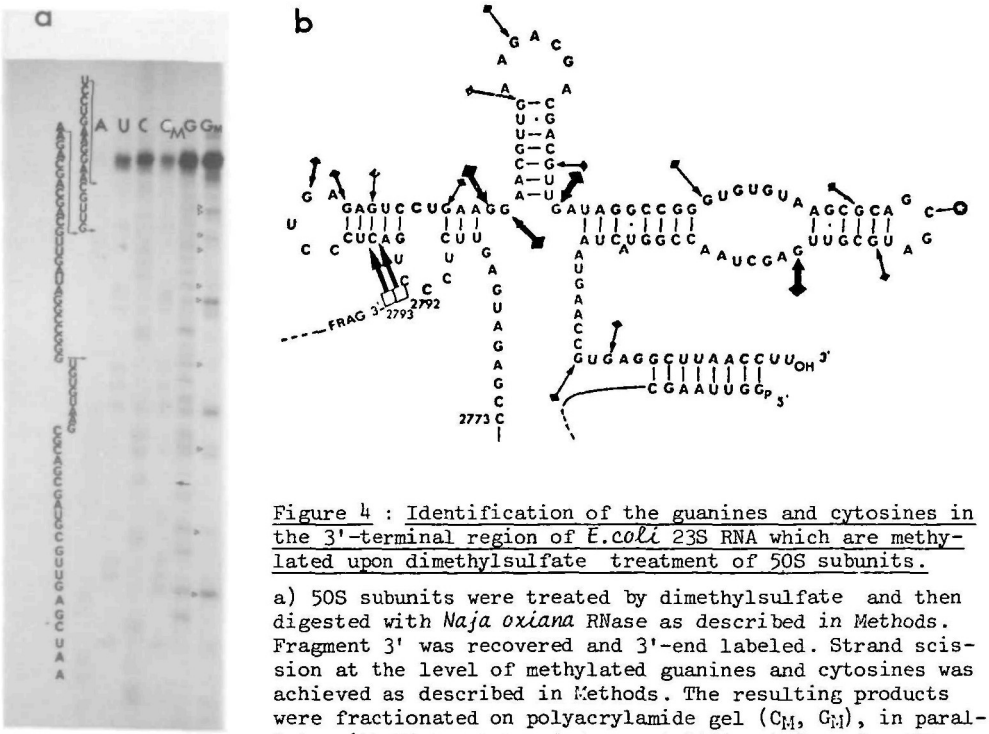

Figure 4 : Identification of the guanines and cytosines in the 3'-terminal region of *E.coli* 23S RNA which are methylated upon dimethylsulfate treatment of 50S subunits.

a) 50S subunits were treated by dimethylsulfate and then digested with *Naja oxiana* RNase as described in Methods. Fragment 3' was recovered and 3'-end labeled. Strand scission at the level of methylated guanines and cytosines was achieved as described in Methods. The resulting products were fractionated on polyacrylamide gel (C_M, G_M), in parallel, with 3'-end labeled fragment 3'digested as for RNA sequencing (A,U,C,G). The bands corresponding to the methylated cytosine → and methylated guanines ▷ are indicated.
b) Schematic representation of the results ■→methylated G, ●→methylated C.

information on fragment 3' secondary structure. These results will be discussed below in the paragraph dealing with tertiary interactions and RNA protein interactions at the 3'-end of 23S RNA.

3.2.2 Study of the 3'-terminal RNA region within 23S RNA. Limited digestion of 23S RNA with T_1 RNase gave information on the guanine residues in the single - stranded regions of this RNA. The partial digestion products were labeled at their 5'-terminus and sequenced by the enzymatic method for RNA sequencing. Three cleavage positions were identified in the 3'-terminal region (Fig. 3). The existence of the three cuts is in good agreement with the model proposed in Figure 3 , as they are all located in single-stranded regions.

3.2.3 Study of fragment 3' in solution. As *Naja oxiana* digestion of isolated 23S RNA yielded a large number of fragments, it was not possible to use this procedure to split chemically modified 23S RNA as in the case of 50S subunits. Therefore we studied isolated fragment 3'. This fragment contains neither the 5'-end of 23S RNA nor the trinucleotide 2791-2793 which is supposed to be base-paired with fragment 3' sequences 2896-2904 and 2803-2805, respectively. Therefore, this fragment is not likely to have the same structure in solution as within 23S RNA. However, fragment 3' in solution may contain hairpins II and III.

The study of fragment 3' was made with both the enzymatic and chemical methods for secondary structure study. The chemical method has been described above. Fragment 3' from *E.coli* 23S RNA, labeled at the 3'-terminus, was modified with dimethylsulfate and diethylpyrocarbonate under the same conditions as for 50S subunits. After strand scission, the products were analyzed on polyacrylamide gel . In this case, almost all cytosine residues in single-stranded regions were methylated. All guanine residues were modified, a difference was only observed in the yield of methylation, guanine 2822 beeing highly methylated. Almost all adenine residues were also modified with dielthylpyrocarbonate adenines 2821 and 2823 in the loop of hairpin II being modified at a higher yield.

The enzymatic method for secondary structure determination is based on the fact that single-stranded RNA regions are more sensitive to T_1 and S_1 nuclease attack, whereas base-paired RNA regions are more sensitive to *Naja oxiana* RNase digestion (33). Fragment 3' from *A.punctata* labeled at the 3'-terminus was digested with these three nucleases under conditions preserving RNA structure. The digests were fractionated on polyacrylamide gel in parallel with digests performed with T_1 and U_2 RNases in denaturing conditions. The ladder was obtained by random degradation in boiling water (Fig. 5a).

The results of both enzymatic (Fig. 5b) and chemical methods are

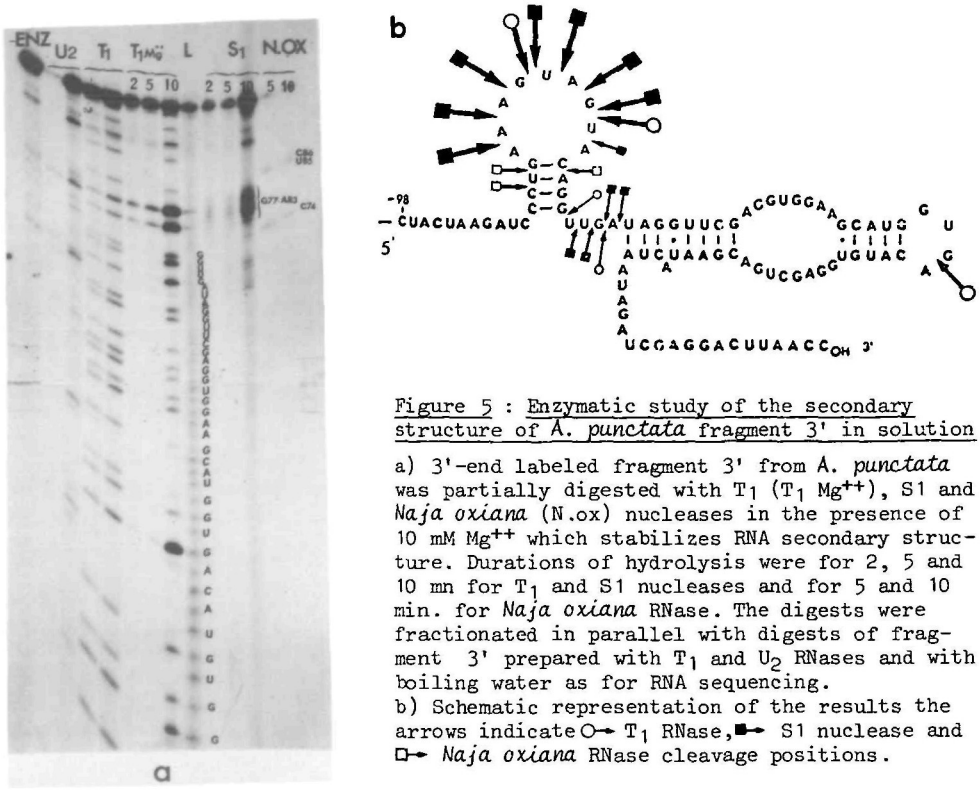

Figure 5 : Enzymatic study of the secondary structure of *A. punctata* fragment 3' in solution

a) 3'-end labeled fragment 3' from *A. punctata* was partially digested with T_1 (T_1 Mg^{++}), S1 and *Naja oxiana* (N.ox) nucleases in the presence of 10 mM Mg^{++} which stabilizes RNA secondary structure. Durations of hydrolysis were for 2, 5 and 10 mn for T_1 and S1 nucleases and for 5 and 10 min. for *Naja oxiana* RNase. The digests were fractionated in parallel with digests of fragment 3' prepared with T_1 and U_2 RNases and with boiling water as for RNA sequencing.
b) Schematic representation of the results the arrows indicate ○→ T_1 RNase, ■→ S1 nuclease and □→ *Naja oxiana* RNase cleavage positions.

in good agreement. They reveal that hairpin II and the upper stem of hairpin III are present in fragments 3' from both *E.coli* and *A.punctata* 23S RNAs. The situation is less clear in the case of the lower stem of hairpin III : two of the cytosines located in this stem were methylated with dimethylsulfate, suggesting structural rearrangements in the isolated fragment . Nevertheless, the results obtained provide additional arguments in favour of the existence of hairpins II and III in intact bacterial 23S RNAs. It should be noted that the segment 2883-2894, which is single-stranded in Figure 3 might be base-paired with a segment of 23S RNA exterior to the 3'-terminal region.

3.3 Comparison with the 3'-terminal regions of yeast 26S rRNA, *Xenopus laevis* 28S rRNA and mouse and human mitochondrial 16S rRNAs. Veldman et al.(14) have observed sequence homologies among the 3'-terminal regions of *E.coli* 23S rRNA, yeast 26S rRNA and *X.laevis* 28S rRNA.

Hall and Maden (35) recently sequenced the 5'-terminal region of *X.laevis* 28S rRNA. This sequence reveals that the complementarity found between the two

ends of *E.coli* 23S rRNA does not exist in the case of 28S rRNA. Nevertheless, we observed that the 20 nucleotides at the 3'-end of the molecule contain two pentanucleotides displaying a complementarity with two pentanucleotides located within the 100 nucleotides at the 5'-end of the molecule (Fig. 6 a). On the basis of the primary structure, hairpins similar to bacterial hairpins I, II

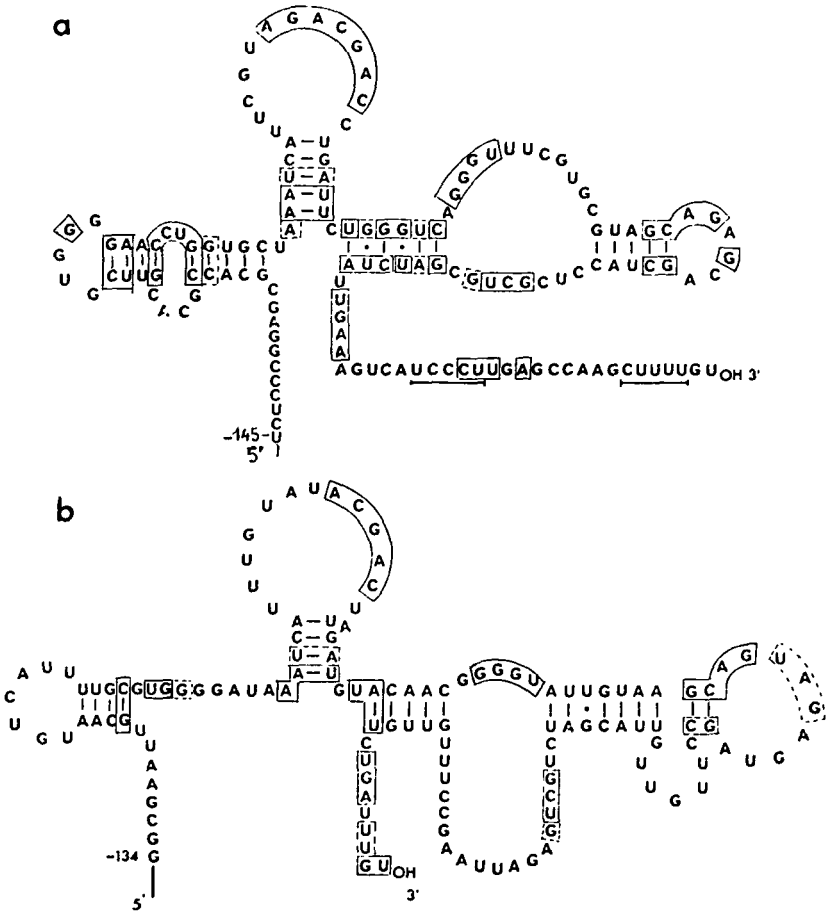

Figure 6 : Possible secondary structure of a) the 3'-terminal region of *X. laevis* 28 rRNA and b) the 3'-terminal region of yeast 26S rRNA.

The nucleotide sequences are from Sollner-Webb and Reeder (15) and Veldman et al. (14), respectively. Homologies with the *E. coli* 23S rRNA 3'-terminal region are indicated ▭ identical nucleotides at identical positions, ⊏⊐ mutations of the transition type. In the case of *X. laevis* 28 rRNA the pentanucleotides displaying a complementarity with pentanucleotides located in the 5'-terminal region of the molecule are underlined.

and III can be formulated at the 3'-end of *X.laevis* 28S rRNA (Fig. 6a). Furthermore, in these structures the oligonucleotides whose sequences are conserved from *E.coli* 23S rRNA to *X.laevis* 28S rRNA are located at identical positions.

On the basis of yeast 26S rRNA 3'-terminal sequence, hairpins similar to bacterial hairpins I, II and III can also be formulated in this rRNA. Though the hairpin corresponding to bacterial hairpin III is somewhat larger (Fig.6b), the homologous oligonucleotides are located at similar positions.

No significant homology was found between the 3'-terminal sequences of bacterial 23S rRNA and those of mouse and human mitochondrial 16S rRNAs (16,17). We did not find any significant homology at the level of secondary structure either. We only observed that, as is the case for 23S rRNA, the postulated 5' and 3' ends of human mit 16S rRNA display a complementarity. Such a complementarity is, however, not observed in the case of mouse mit 16S rRNA.

3.4 Comparison with chloroplast 4.5S rRNA. As mentioned above, it was of interest to know wether chloroplast 4.5S rRNA has a secondary structure similar to that of the 3'-terminal region of bacterial 23S rRNA or to that of *E.coli* 4.5S RNA. Tobacco and wheat chloroplast 4.5S rRNAs have been recently sequenced (22-24). On the basis of these sequences both rRNAs can be folded in similar way as the 3'-terminal region of bacterial 23S rRNA (Fig. 7a and b), but not in similar way as *E.coli* 4.5S RNA. Furthermore, we observed a strong complementarity between the 5'-terminal sequence of *Euglena gracilis* chloroplast 23S rRNA (36) and the 3'-end of both tobacco and wheat 4.5S rRNAs (Fig. 7a and b). This observation strongly suggests that the 3'-terminus of chloroplast 4.5S rRNA and the 5'-terminus of chloroplast 23S rRNA are base-paired as is the case for the two ends of *E.coli* 23S rRNA.

Wildeman and Nazar (22) estimated the secondary structure of wheat chloroplast 4.5S rRNA in solution. They found all the base-pairs of hairpins II and III, plus a certain number of additional base-pairs. The additional base-pairing they proposed involving the two ends of the molecule is considerably less stable than the possible base-pairing between the 3'-end of 4.5 rRNA and the 5'-end of 23S rRNA. So that this base-pairing is unlikely to occur within the ribosomes. On the other hand, the 5'-end of 4.5S rRNA may be base-paired with a segment in the 3'-terminal region of chloroplast 23S rRNA, since its counterpart in *E.coli* 23S rRNA is probably base-paired with segment 2783-2785. The other additional complementary base-pairs considered by Wildeman and Nazar are not complementary in tobacco chloroplast 4.5S rRNA or in bacterial 23S rRNA. Therefore, from a phylogenetic point of view, the corresponding base-

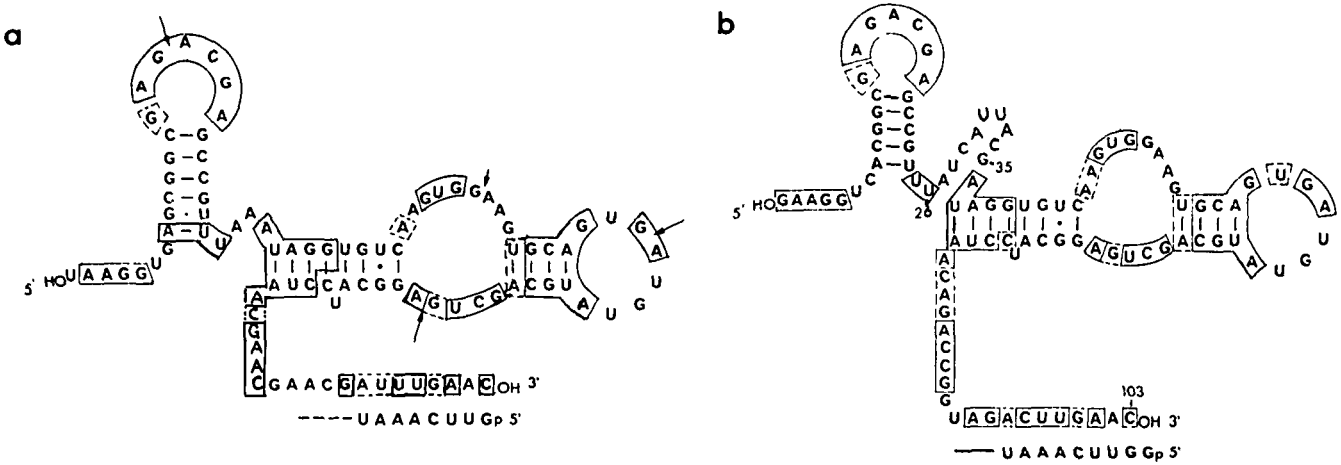

Figure 7 : Possible secondary structure of wheat (a) and tobacco (b) chloroplast 4.5S rRNAs within chloroplast 50S subunits. The nucleotide sequences are from Wildeman and Nazar (22) and Takaiwa and Sugiura (23). The complementarity existing between the 3'-terminus of these two 4.5S rRNAs and the 5'-terminus of chloroplast 23S rRNA from *Euglena gracilis* (36) is shown. The arrows in the structure of wheat 4.5S rRNA indicate the positions of partial T_1 RNase digestion of this rRNA in solution, according to Wildeman and Nazar (22). The homology with *E. coli* 23S rRNA 3'-terminal region is represented as in Figure 6.

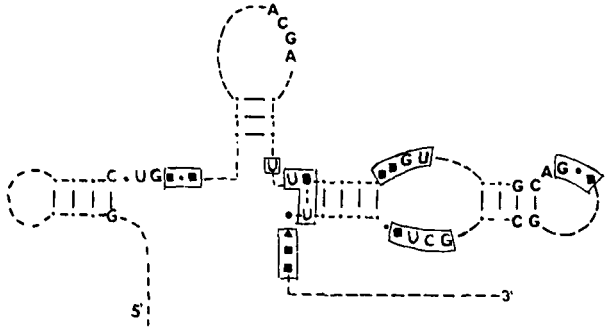

Figure 8 : Invariant and semi-invariant nucleotides in the postulated structures of *E. coli* 23S rRNA, yeast 26S rRNA and *X. laevis* 28S rRNA 3'-terminal regions and in the structures of wheat and tobacco 4.5S rRNAs. Those nucleotides which are found in the 3'-terminal regions of *P. vulgaris* and *A. punctata* 23S rRNA 3'-terminal regions have been surrounded by a line.

pairingsare unlikely to occur, and indeed they are rather unstable.

Thus, our observations based on comparison of sequence potential secondary structures are supported by the experimental results of Wildeman and Nazar. As proposed by Mac Kay chloroplast 4.5S rRNA corresponds to the 3'-terminal region of bacterial 23S rRNA. The homology between these two RNAs is not restricted to 4.5S rRNA fragment 35-103 as suggested by this author. Rather, it extends up to the 5'-end of the 4.5S rRNA molecule, fragment 1-26 corresponding to a part of hairpin I and to hairpin II. This is also true for wheat chloroplast 4.5S rRNA and also for Duckweed 4.5S rRNA, which has been tentatively sequenced by Wildeman and Nazar on the basis of its T_1 and pancreatic RNase digestion products. This, therefore, seems to be a general rule for 4.5S rRNAs. Nevertheless, 4.5S rRNAs from different flowering plants display differences. For instance, compared to Duckweed and wheat 4.5S rRNAs, tobacco 4.5S rRNA contains an additional sequence between hairpins II and III. This sequence is self complementary and may be folded in a small hairpin.

As shown in Figures 3, 7 a and 7b, the 3'-end of *E.coli* 23S rRNA and tobacco and wheat 4.5S rRNAs contain a series of common oligonucleotides which are located at identical positions in their secondary structures. Some of these oligonucleotides are also found at similar positions in the putative secondary structures of yeast 26S rRNA and *X.laevis* 28S rRNA 3'-terminal regions (Fig. 6a, 6b and 8).

DISCUSSION

We determined the nucleotide sequence of the 110 nucleotide fragment located at the 3'-end of *E.coli*, *P.vulgaris* and *A.punctata* 23S RNAs. A strong sequence conservation is observed between the *P.vulgaris* and *E.coli* fragments (90%) . The conservation is not as strong between the *A.punctata* and *E.coli* fragments (60%). This is surprising since the *Aeromonas* have been classified close to *P.vulgaris* and *E.coli* in the bacteria classification established by Fox et al. (37) on the basis of 16S rRNA comparisons. Our results are nevertheless in agreement with the phylogenetic relationships they proposed : namely, *Escherichia* and *Proteus* having a common ancestor which itself has a common ancestor with *Aeromonas*.

In spite of the great difference in their nucleotide sequences, the three 3'-terminal fragments studied could be folded in the same structure made of two hairpins (II and III). The presence of hairpin II and of part of hairpin III in fragment 3' in solution strongly reinforces the idea that these two hairpins exist in bacterial 23S rRNA. Indeed, guanines 2822, 2844 and 2859

located in loops of hairpins II and III are the only ones to be cleaved upon mild T_1 RNase digestion of 23S RNA.

Tobacco chloroplast 4.5S rRNA, the 3'-end of yeast 26S rRNA and that of *X.laevis* 28S rRNA were found to display sequence homology with *E.coli* 23S rRNA 3'-terminal region (25,14). The degree of homology between this latter 23S rRNA region and the two 4.5S rRNAs is high (about 65%). It is higher than that observed between the 3'-terminal regions of *E.coli* and *A.punctata* 23S rRNAs (60%). The homology between the 3'-terminal region of *E.coli* 23S rRNA and the 3'-terminal regions of the two cytoplasmic rRNAs, is smaller (50% and 40% for *X.laevis* 28S rRNA and yeast 26S rRNA, respectively). In spite, of the numerous differences in their primary structures these prokaryotic and eukaryotic rRNA regions potentially have a very similar secondary structure. This observation strongly suggests the existence at the 3'-end of the large cytoplasmic rRNAs and in chloroplast 4.5S rRNA of a structure similar to that found at the 3'-end of bacterial 23S rRNA. Furthermore, it supports the idea that chloroplast 4.5S rRNAs and the 3'-terminal region of bacterial 23S rRNA have a common ancestor and equivalent functions.

A similar structure was not detected in either mouse or human mitochondrial 16S rRNAs. However, the presence of a small size rRNA, similar to chloroplast 4.5S rRNA in mouse and human mitochondria is not excluded.

The 3'-terminal region of *E.coli* 23S rRNA, the two chloroplast 4.5S rRNAs and the 3'-terminal regions of the two cytoplasmic rRNAs, not only have secondary structures with the same general shape. They also bear common oligonucleotides located at identical positions in their secondary structures. It is surprising that the sequence of these oligonucleotides is not entirely conserved in the 3'-terminal region of *A.punctata* 23S rRNA, and even in that of *P.vulgaris* 23S rRNA which is 90% homologous to the 3'-terminal region of *E.coli* 23S rRNA. One explanation could be that in respect to these oligonucleotides *P.vulgaris* and *A.punctata* 23S rRNAs have diverged from their ancestor. Another alternative could be that a convergent evolution has lead to these oligonucleotides in different ribosomes species. Finally, a third possibility is that the ancestor of cytoplasmic and chloroplastic ribosomes was closer to *E.coli* ribosomes than to *P.vulgaris* or *A.punctata* ribosomes. Whatever is the answer, one point is obvious. Since these oligonucleotides are found in *E.coli*, chloroplast and cytoplasmic ribosomes, they should have an important role : they may be involved in RNA tertiary structure, in RNA-protein interactions or in ribosome ligand binding.

The third alternative is not very likely, since the 3'-terminal region of

E.coli 23S rRNA is not exposed within the 50S subunits. Indeed, this region is not cleaved upon T_1 RNase digestion of the subunits. The degree of methylation of its guanine residues upon dimethylsulfate treatment of the subunits is smaller than that observed for other 23S rRNA regions, in particular, the 5'-terminal one (unpublished results). Finally, previous results have demonstrated that this 3'-terminal region belongs to a compact area of the 50S subunits (38). Only the extreme 3'-end seems to be prominant at the surface of the subunit, since, it was labeled upon incubation of the 50S subunits with {5'-^{32}P} pCp and T_4 RNA ligase.

Involvement of the invariant oligonucleotides in RNA tertiary structure or in RNA-protein interactions is strongly supported by the results we obtained upon dimethylsulfate treatment of the *E.coli* 50S subunits. Indeed, the guanine residues located in the single-stranded invariant oligonucleotides are not methylated, also those guanine residues located in the base-paired invariant oligonucleotides are only methylated to a small extent. It should be noted that these guanine residues are, on the contrary, all methylated in the 3'-terminal region of *E.coli* 23S rRNA in solution and with the same yield as the other guanine residues. Obviously, more RNA tertiary interactions are expected to be present in the 3'-terminal region within the 50S subunits than in solution, but this is probably not sufficient to account for the difference of methylation observed. Some of the protected guanine residues probably interact with proteins.

The sequence conservation in hairpins II and III is striking in view of the great variability in hairpin I. This hairpin represents a "hot spot" of mutation in 23S rRNA. The insertion which has separated the 4.5S rRNA from the 23S rRNA in chloroplast rDNA occured in this area.

ACKNOWLEDGMENTS

We are grateful to Drs. P. Monteil and P. Carbon for their generous gifts of the strain of *P.vulgaris* and of *Naja oxiana* RNase, respectively. The excellent technical assistance of B. Muller is acknowledged. Dr. R.E. Rhoads is thanked for careful reading of the manuscript. This work was supported by grants from the "Centre National de la Recherche Scientifique" and the "Délégation Générale à la Recherche Scientifique et Technique".

+To whom all correspondence should be sent

REFERENCES

1. Shine, J. and Dalgarno, L., (1974), Proc. Nat. Acad. Sci. US, 71, 1342-1346.

2. Steitz, J.A. and Jakes, K., (1975), Proc. Nat. Acad. Sci. US, 72, 4734-4738.
3. Dunn, J.J., Buzash-Pollert, E. and Studier, W.F., (1978), Proc. Nat. Acad. Sci. US, 75, 2741-2745.
4. Gauss , D. and Sprinzl, M., (1980), Nucl. Acids Res., 9, r1-r23.
5. Hori, M. and Osawa, S., (1979), Proc. Nat. Acad. Sci. US, 76, 381-385.
6. Woese, C.R., Magrum, L.J., Gupta, R., Siegel, R.B., Stahl, D.A., Kop, J., Crawford, N., Brosius, J., Gutell, R., Hogan, J.J. and Noller, H.F., (1980), Nucl. Acids Res., 8, 3275-3293.
7. Stiegler, P., Carbon, P., Zuker, M., Ebel, J.P. and Ehresmann, C., (1980), C.R. Acad. Sci. Paris, 291, série D, 937-940.
8. Vassilenko, S. and Babkina, V., (1965), Biokhimiya, 30, 705-712.
9. Vassilenko, V., Ebel, J.P. and Branlant, C., unpublished results.
10. Brosius, J., Dull, T. and Noller, H., (1980), Proc. Nat. Acad. Sci. US, 77, 201-204.
11. Peattie, D. and Gilbert, W., (1980), Proc. Nat. Acad. Sci. US, 77, 4679-4682.
12. Wrede, P., Wurtz, R., Vournakis, J. and Rich, A., (1979), Febs Lett., 107, 177-181.
13. Branlant, C., Krol, A., Ebel, J.P., Gallinaro, H., Lazar, E. and Jacob, M., (1981), Nucl. Acids Res., in press.
14. Veldman, G., Klootwijk, J., de Jonge, P., Leer, R. and Planta, R., (1980), Nucl. Acids Res., 8, 5179-5192.
15. Sollner-Webb, B. and Reeder, R.H., (1979), Cell, 18, 485-499.
16. Van Etten, R., Walberg, M. and Clayton, D., (1980), Cell, 22, 157-170.
17. Eperon, I., Anderson, S. and Nierlich, D., (1980), Nature, 286, 460-466.
18. Whitfeld, P., Leaver, C., Bottomley, W. and Atchison, B., (1978), Biochem. J., 175, 1103-1112.
19. Bowman, C. and Dyer, T., (1979), Biochem. J., 183, 605-613.
20. Hartley, M., (1979), Eur. J. Biochem. 96, 311-320.
21. Takaiwa, F. and Sugiura, M., (1980), Gene, 10, 95-103.
22. Wildeman, A. and Nazar, R., (1980), J. Biol. Chem., 255, 11896-11900.
23. Takaiwa, F. and Sugiura, M., (1980), Mol. Gen. Genet., 180, 1-4.
24. Takaiwa, F. and Sugiura, M., (1980), Nucl. Acids Res., 8, 4125-4129.
25. Mac Kay, R., (1981), Febs Lett., 123, 17-19.
26. Branlant, C., Krol, A., Machatt, A. and Ebel, J.P., (1981), Nucl. Acids Res. 9, 293-308.
27. Hapke, B. and Noll, H., (1976), J. Mol. Biol., 105, 97-109.
28. England, T. and Uhlenbeck, O., (1978), Nature, 275, 560-561.
29. Peattie, D., (1979), 76, 1760-1764.
30. Branlant, C., Krol, A., Machatt, M. and Ebel, J.P., (1979), Febs Lett., 107, 177-181.
31. Bram, R., Young, R. and Steitz, J., (1980), Cell, 19, 393-401.
32. Branlant, C., Sriwidada, J., Krol, A. and Ebel, J.P., (1976), Nucl. Acids Res., 3, 1671-1687.
33. Vassilenko, S. and Ryte, V., (1975), Biokhimiya, 40, 578-582.
34. Branlant, C., Sriwidada, J., Krol, A. and Ebel, J.P., (1977), Nucl. Acids Res., 4, 4323-4346.
35. Hall, L. and Maden, E., (1980), Nucl. Acids Res., 8, 5993-5996.
36. Orozco, E., Rushlow, K., Dodd, J., Hallick, R., (1980), J. Biol. Chem., 255, 10997-11011.
37. Fox, G., Stackebrandt, E., Hespell, R., Gibso, J., Maniloff, J., Dyer, T., Wolfe, R., Balch, W., Tanne, R., Magrum, L., Zablen, L., Blakemore, R., Gupta, R., Bonen, L., Lewis, B., Stahl, D., Luehrsen, K., Chen, K. and Woese, C., (1980), Science, 209, 457-463.
38. Branlant, C., Krol, A., Sriwidada, J. and Ebel, J.P., (1977), J. Mol. Biol. 116, 443-467.