Structure and Biosynthesis of the Ribosomal Ribonucleic Acids from the Oncogenic Bacterium *Agrobacterium tumefaciens*

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The rRNA of the oncogenic bacterium *Agrobacterium tumefaciens* was extracted by several methods and analysed by polyacrylamide-gel electrophoresis. The large rRNA of this bacterium is degraded *in vivo* during the maturation of the ribosome. The influence of Mg^{2+} and denaturation on degradation of 23S RNA was studied. In pulse and chase experiments, we identified two precursors of the rRNA with mol.wts. of 1.04×10^6 and 0.70×10^6 . From studies of the structure of the large rRNA, we propose that it could have arisen from a gene duplication. This structure is discussed in relation to a recent hypothesis involving such gene duplication as a means of origin of 23S rRNA.

The inoculation of the bacterium *Agrobacterium tumefaciens* into wounded susceptible plants induces appearance of a tumoral disease called 'crown gall'. Braun & Wood (1966) demonstrated that the action of ribonuclease during the induction of crown gall inhibits the initiation of the tumour.

Le Goff (1968) and Larsen (1971) have shown that the 23S RNA of *A. tumefaciens* is not found in sucrose-gradient centrifugation; this component becomes evident when a Mg^{2+} -containing buffer is used (Larsen, 1971). Using polyacrylamide-gel electrophoresis, which allows more accurate analysis, we found that *A. tumefaciens* RNA displays a very atypical pattern (Grienemberger & Simon, 1972). This behaviour seems very similar to that exhibited by *Rhodospseudomonas spheroides* (Marrs & Kaplan, 1970). Only these two species have been shown so far to have this peculiar rRNA pattern.

The possible dimerization of the small rRNA to produce the large one has been the subject of a long, and still open, controversy (see Nomura, 1970, for a review). Loening (1973) has suggested that the large rRNA could have originated from a duplication of the primitive gene for the small rRNA. The study of the structure of the *A. tumefaciens* rRNA, as well as the processing pattern of their precursors, has allowed us, and Schuch & Loening (1975), to suggest such a scheme of dimerization.

In a preliminary report (Grienemberger & Simon, 1972), we have indicated that the atypical behaviour of *A. tumefaciens* rRNA does not seem to be related to the oncogenic properties of this bacterium.

Materials and Methods

Materials

Most chemicals were obtained from E. Merck A.G., Darmstadt, Germany (pro-analysis grade);

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tri-isopropyl naphthalenesulphonate, polyvinylsulphonic acid, acrylamide and *NN'*-methylenebisacrylamide were obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A.; diethyl pyrocarbonate and *NNN'*-tetramethylethylenediamine were obtained from Fluka A.G., Buchs, Switzerland.

All radioactive products were from the C.E.A., Saclay, France. [^{32}P]P_i in sterile form without carrier has a specific radioactivity of 100 mCi/mmol. [3H]Uridine has a specific radioactivity of 20 Ci/mmol and 99% purity.

Media and growth of bacteria

Agrobacterium tumefaciens, strain B6, and *Escherichia coli*, strain K12, were grown on Nutrient Broth medium (8 g/l; Difco Laboratories Inc., Detroit, Mich., U.S.A.) in Erlenmeyer flasks incubated with shaking at 29°C. The doubling time under these conditions was 80 min for *A. tumefaciens* and 40 min for *E. coli*. For radioactive labelling, the bacteria were grown on a phosphate-limiting medium (Takahashi *et al.*, 1967), to which ^{32}P was added to a final concentration of 10 μ Ci/ml. When used, the radioactive uridine (10 μ Ci/ml) was directly added to the complete Takahashi *et al.* (1967) medium. The doubling time in the phosphate-limiting medium was 2 h for *A. tumefaciens*. For the pulse experiments, growth was stopped by pouring the bacteria on to crushed ice containing 0.01 M-Na₃N and centrifuging them immediately. For chase experiments, the phosphate concentration was increased to 0.1 M with non-radioactive potassium phosphate buffer (1 M, pH 7.0). After harvesting, the bacteria were rapidly washed in the cold (14°C) and immediately processed. They were never frozen nor stored for more than a few minutes.

RNA extraction

Unless otherwise stated, the RNA was extracted by a modification of the Click & Hackett (1966)

method. The bacteria were suspended in acetate buffer (0.1 M-sodium acetate-0.1 M-NaCl, pH 7.0) containing two ribonuclease inhibitors: Macaloid (Omya, Paris, France; 1 mg/ml final concn.) (Stanley & Bock, 1965) and polyvinylsulphonic acid (10 µg/ml). The cell suspension was passed through a chilled Aminco French press at 10^8 Pa (15000 lb/in²). Sodium dodecyl sulphate (1%) and 2 vol. of acetate-buffer-saturated phenol were added to the lysate. In appropriate experiments, the extraction buffer also contained 0.01 M-EDTA or magnesium acetate at the concentration quoted in the Results section. After the aqueous phase had been collected, the nucleic acids were extracted twice with buffer-saturated phenol and the final aqueous phase was precipitated overnight with 2 vol. of ethanol. The precipitated RNA was dissolved in 0.1 M-sodium acetate-0.5% sodium dodecyl sulphate, pH 5.0, and reprecipitated. This step was repeated two or three times to eliminate the remaining phenol.

After centrifugation, the RNA pellet was dissolved in 0.01 M-NaCl-0.03 M-MgCl₂-0.01 M-Tris-HCl, pH 7.0, and treated with ribonuclease-free deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo., U.S.A.; 10 µg/ml) for 30 min at 5°C. The RNA was precipitated again and could be stored under ethanol at -20°C for at least 6 months without any noticeable degradation.

Ribosome preparation

The bacteria were suspended in 0.03 M-NH₄Cl-0.01 M-MgCl₂-0.006 M-2-mercaptoethanol-0.01 M-Tris, pH 7.5, containing Macaloid (1 mg/ml), polyvinylsulphonic acid (10 µg/ml) and ribonuclease-free deoxyribonuclease (25 µg/ml). The bacteria were broken as in the RNA extraction. After centrifugation of the lysate at 25000g for 30 min in the SS34 rotor (Sorvall), the supernatant was collected and a crude ribosomal pellet was obtained by centrifugation at 105000g for 2 h. The pellet was suspended in the same buffer after a further cycle of low- and high-speed centrifugation and dialysed against this buffer in the cold for 3 h. For dissociation of the subunits, the concentration of Mg²⁺ was lowered to 0.3 mM. The subunits were separated by sucrose-density-gradient centrifugation. For this, 5-20% (w/v) linear sucrose gradients (ribonuclease-free sucrose; Mann Research Laboratories, New York, N.Y., U.S.A.) in the same buffer were overlaid with 15 E₂₆₀ units of ribosome solution (0.2 ml), and then centrifuged in a SW 27 rotor (Spinco) at 25000 rev./min for 12 h at 5°C. Gradients were collected by pumping upward from the bottom of the tube through a needle and the absorption profile was monitored at 257 nm with an LKB Uvicord densitometer and a Bryans (Micham, Surrey, U.K.) recorder.

Gel electrophoresis

The polyacrylamide-gel-electrophoretic analysis was performed essentially as described by Loening (1967), except for the following points. The gels were allowed to polymerize in quartz tubes (Heraeus, Orsay, France; 10 cm long × 0.7 cm internal diam.). For radioactivity studies, Perspex tubes with the same dimensions were used. The gels were scanned with a Polyfrac U.V. scanner (Joyce-Loebl). Running buffers were: E acetate buffer (Loening, 1967) for experiments in the presence of EDTA, and E buffer containing 2 mM-magnesium acetate and 0.1 mM-EDTA (Loening, 1969) for electrophoresis in the presence of magnesium. All buffers contained 0.1% sodium dodecyl sulphate. Pre-electrophoresis was for 45 min and the electrophoresis was continued at 5 mA/tube, 6-7 V/cm. Other details are indicated in the Figures. Gels containing radioactive label were washed in water, frozen on solid CO₂, and sliced in 1 mm fractions with a Mickle gel slicer. The correspondence between radioactivity and E₂₅₇ profile was established by ink markers in the gel. ³²P-labelled slices were counted for radioactivity with a low-background (1-2 c.p.m.) counter (Tracerlab). When [³H]uridine and ³²P were both present in the RNA, the slices were incubated overnight in 1 ml of 10% (v/v) NH₃ until dry, then mixed in scintillation vials with 10 ml of the counting mixture consisting of naphthalene (100 g/l), PPO (2,5-diphenyloxazole) (7 g/l) and dimethyl-POPOP [1,4-bis-(4-methyl-5-phenyloxazolyl)benzene] in dioxan and counted for radioactivity in a Packard Tri-Carb scintillation counter. The contribution of ³²P to the ³H channel was 6%.

Determination of molecular weight

Molecular-weight determination of the different RNA species was carried out by co-electrophoresis with *E. coli* rRNA species as markers, assuming the rRNA species of this bacterium to have mol.wts. of 1.07×10^6 and 0.56×10^6 (Loening, 1968; Stanley & Bock, 1965).

Results

Atypical behaviour of *A. tumefaciens* rRNA

The electrophoretic analysis of the total RNA of *A. tumefaciens* shows four RNA species whose mol.wts are 0.95×10^6 , 0.56×10^6 , 0.52×10^6 and 0.47×10^6 (Figs. 1a and 1b). All peaks are ribonuclease-sensitive. The migration pattern of *E. coli* rRNA is shown in Fig. 1(c). There is a great discrepancy between the migration patterns of the RNA of these two bacteria. The RNA extracted from a crude ribosomal fraction of *A. tumefaciens* displays the same pattern. The large rRNA of *A. tumefaciens* cannot be found and seems to have been degraded. When the extraction was performed by the method of

Fig. 1. Electrophoresis of *A. tumefaciens* RNA in EDTA gels

RNA was prepared from *A. tumefaciens* and *E. coli* by the acetate-sodium dodecyl sulphate procedure. All samples were applied in 10 μ l. (a) 10 μ g of *A. tumefaciens* RNA; (b) 10 μ g of *A. tumefaciens* RNA and 5 μ g of *E. coli* RNA; (c) 5 μ g of *E. coli* RNA. Electrophoresis was carried out in 2.8% gels for 3 h at 50V in EDTA buffer.

Kirby (1965) or by the diethyl pyrocarbonate method of Solymosy *et al.* (1968) there were no noticeable differences. No changes were found in this migration pattern when other ribonuclease inhibitors (polyvinyl sulphate, Macaloid, tri-isopropyl naphthalenesulphonate and diethyl pyrocarbonate) or different breakage methods (manual breakage with mortar and pestle, or sonication) were used.

To eliminate every possibility of an extraction artifact and/or ribonuclease action, *E. coli* and

A. tumefaciens cultures were labelled respectively with [3 H]uridine and 32 P for three generations. The cultures were then mixed and pelleted. The RNA of the mixed pellet was extracted by the acetate-phenol method and analysed by polyacrylamide-gel electrophoresis. The radioactivity pattern obtained is shown in Fig. 2. *E. coli* RNA (3 H-labelled) shows a classical profile, in contrast with the *A. tumefaciens* RNA, which shows the same pattern of migration as in Fig. 1(a). This degradation seems then to be a characteristic of *A. tumefaciens* RNA and not to be due to an artifact of preparation.

The distribution of these RNA species was studied by extraction and separation of ribosomal subunits of *A. tumefaciens*. The RNA from each subunit was analysed by polyacrylamide-gel electrophoresis. Fig. 3 shows that the large subunit contains RNA species of mol.wts. 0.95×10^6 , 0.56×10^6 , 0.52×10^6 and 0.47×10^6 , whereas the small subunit contains RNA species of mol.wts. 0.56×10^6 and 0.47×10^6 . Although cross-contamination between subunits obviously occurred during purification, we can reasonably assume from the relative importance of each RNA peak that the large subunit contains RNA species of mol.wts. 0.95×10^6 , 0.52×10^6 and 0.47×10^6 whereas the small one contains only an RNA of mol.wt. 0.56×10^6 .

Fig. 2. Electrophoresis of RNA obtained by extraction of mixed *A. tumefaciens* and *E. coli* cultures

Cultures of *E. coli* and *A. tumefaciens* were mixed after labelling with [3 H]uridine and 32 P respectively. The RNA was extracted by the acetate-sodium dodecyl sulphate method. Electrophoresis was carried out in 2.8% gels at 50V for 2.5 h in EDTA buffer. Slicing and counting was as described in the text. $\bullet$, 3 H radioactivity; ∇ , 32 P radioactivity.

Fig. 3. Comparison of rRNA species from the ribosomal subunits of *A. tumefaciens*

A. tumefaciens ribosomes were extracted as described in the Materials and Methods section. Subunits were separated by sucrose-density-gradient centrifugation in low-Mg²⁺ buffer. The RNA of three identical gradients was extracted from these subunits. They were analysed in 2.8% gels at 50V for 3h in EDTA buffer. (a) RNA from the large subunit; (b) RNA from the small subunit.

Effect of Mg²⁺

The RNA obtained by extraction in the presence of Mg²⁺ and electrophoresed on EDTA-polyacrylamide gels shows the same profile as in Fig. 1(a). It is necessary to have Mg²⁺ in all solutions during the extraction and the electrophoresis. We have done such experiments in the presence of 5mM-magnesium acetate, with an EDTA:Mg ratio of 1:20 in the electrophoresis buffer as described by Loening (1969). The gel pattern of this experiment is shown in Fig. 4(b). Loening (1969) has shown discontinuities in the relation of mobility to molecular weight during electrophoresis in Mg²⁺ buffer. This technique cannot therefore be used directly for the determination of molecular weight. The tentative molecular weights of the RNA species shown in Fig. 4(b) are 1.04×10^6 , 0.92×10^6 , 0.56×10^6 and 0.50×10^6 . Owing to this approximation, and the folding of the RNA in Mg²⁺-containing buffers, the RNA species

of mol.wts. 0.92×10^6 , 0.56×10^6 and 0.50×10^6 can be compared with those found in EDTA buffer (mol.wts. respectively 0.95×10^6 , 0.56×10^6 and 0.52×10^6 or 0.47×10^6). Different concentrations of Mg²⁺ ions in the extraction buffer (5mM, 10mM, 20mM) were used and Figs. 4(b), 4(c) and 4(d) respectively show the patterns of migration obtained for these three extractions. There is an enhancement of the high-molecular-weight RNA species when the Mg²⁺ concentration is as high as 20mM. It is noticeable also that in the presence of Mg²⁺ in each step of the

Fig. 4. Effect of Mg²⁺ on the migration pattern of *A. tumefaciens* rRNA

A. tumefaciens RNA was extracted by the acetate-sodium dodecyl sulphate procedure, by using different concentrations of Mg²⁺. All solutions for the precipitation and purification of the RNA contained 5mM-magnesium acetate. Electrophoresis was carried out in 2.8% gels at 50V for 3.5h in Mg²⁺-containing buffer. (a) EDTA extraction; (b) 5mM-Mg²⁺ extraction; (c) 10mM-Mg²⁺ extraction; (d) 20mM-Mg²⁺ extraction.

experiment, a peak of 1.04×10^6 -mol.wt. RNA appears. The electrophoretic analysis (in a buffer containing Mg^{2+}) of an extraction with EDTA is shown in Fig. 4(a): there is no 1.04×10^6 -mol.wt. RNA in this case and a great lowering in the amount of 0.95×10^6 -mol.wt. RNA. The high-molecular-weight RNA species of *A. tumefaciens* are partially stabilized by Mg^{2+} . It seems that the hypothetical large RNA is cut into a few pieces. These breaks might be hidden by Mg^{2+} interaction with the double-stranded portions of the molecule. This hypothesis could be tested by denaturing these double-stranded regions with reagents that weaken the hydrogen bonds in the EDTA system.

Action of denaturing agent

RNA from *A. tumefaciens* was treated with dimethyl sulphoxide and heat to weaken the hydrogen

Fig. 5. Electrophoresis of *A. tumefaciens* RNA treated by heat and dimethyl sulphoxide

A. tumefaciens RNA was extracted and treated as follows. (a) RNA was dissolved in sodium dodecyl sulphate-acetate buffer, made 80% (v/v) in dimethyl sulphoxide and incubated for 1 h at 37°C. The RNA was then cooled and precipitated with cold ethanol at -20°C. (b) The RNA in the same buffer was heated at 70°C for 3 min. The same precipitation procedure was followed. Electrophoresis was carried out in 2.8% gels at 50 V for 3 h in EDTA buffer.

bonds (Wagner *et al.*, 1967). After treatment, the RNA species were immediately precipitated by cold ethanol. All electrophoretic analyses were performed in EDTA buffer. Figs. 5(a) and 5(b) show the migration pattern obtained with dimethyl sulphoxide and heat (70°C) treatments. The usual migration pattern was obtained after treatments at 30°, 40° or 50°C. The denaturation of the RNA begins at 50°C and is completed at 70°C. The heat treatment induces aggregates (mol.wts. 0.99×10^6 , 1.07×10^6 , 1.13×10^6 ; Fig. 5b) (see Wagner *et al.*, 1967). The common feature between the two treatments is the disappearance of the 0.52×10^6 -mol.wt. RNA and the relative enhancement of 0.47×10^6 -mol.wt. RNA. The dimethyl sulphoxide treatment eliminates the 0.95×10^6 -mol.wt. RNA. Calculation of the area under the two peaks in Fig. 5(a) shows that there are two molecules of 0.47×10^6 -mol.wt. RNA for one molecule of 0.56×10^6 -mol.wt. RNA. It seems then that the large rRNA of *A. tumefaciens* contains two molecules of 0.47×10^6 -mol.wt. RNA.

One could hypothesize that the 0.52×10^6 -mol.wt. RNA is denatured into the 0.47×10^6 -mol.wt. RNA and liberates a small RNA whose molecular weight should be approx. 0.05×10^6 . Fig. 6(a) shows the results of electrophoresis of EDTA-extracted RNA in 7.5% polyacrylamide gels with EDTA buffer. It shows the two classical low-molecular-weight RNA species, 5S RNA and tRNA. Dimethyl sulphoxide-treated RNA species were run in identical gels and a new RNA peak heavier than the 5S RNA appeared, whose mol.wt. was 48000. This value fits well with the RNA portion hypothesized above and involved in the mol.wt. transition 0.52×10^6 – 0.47×10^6 (Fig. 6b).

Biosynthesis of the rRNA

We have utilized pulse-labelling to study the biosynthesis of the rRNA of *A. tumefaciens*. First, a pulse of 5 min with ^{32}P , followed by short chase time, was carried out, in order to investigate the existence of precursors. Secondly, the same pulse (with longer chase times) was used to check the possible precursor-product relationship, which especially may exist for the large rRNA precursor. The pulse-labelling and chase procedures are described in the Materials and Methods section. The extraction and the electrophoretic analysis were done in EDTA buffer. Fig. 7(a) shows that after a 5 min pulse and a 1 min chase, the ^{32}P label is essentially present in two peaks whose mol.wts. are 1.04×10^6 and 0.70×10^6 . A small peak is also noticeable with a mol.wt. of 0.56×10^6 . After 3 min chase there is an increase in the radioactivity of 0.56×10^6 -mol.wt. RNA and the appearance of a peak at 0.47×10^6 mol.wt. (Fig. 7b). A very short pulse (1 min) without chase shows that

Fig. 6. Electrophoresis of *A. tumefaciens* low-molecular-weight RNA treated by dimethyl sulphoxide

EDTA-extracted RNA was treated as in Fig. 5. (a) Untreated RNA; (b) dimethyl sulphoxide-treated RNA. Electrophoresis was carried out in 7.5% gels at 50V for 2.5h in EDTA buffer.

there is only one peak at 0.70×10^6 mol.wt. above the high background (result not shown).

We have performed a second set of experiments with longer chase-times (10 and 20min). Fig. 8(a) (10min chase) shows that the label in the two precursor peaks has decreased and that there is a great increase in 0.56×10^6 - and 0.47×10^6 -mol.wt. RNA. The confirmation of this change is shown in Fig. 8(b) after 20min chase. During all experiments of pulse labelling, no radioactivity was detected in the 0.52×10^6 -mol.wt. peak.

Discussion

It is not possible to demonstrate the existence of a large rRNA in mature ribosomes of *Agrobacterium tumefaciens* (as also found by Loening in Ingle *et al.*, 1970). Extraction of the RNA from the ribosomal subunits shows that the large ribosomal subunit contains three high-molecular-weight RNA species (mol.wts. 0.95×10^6 , 0.52×10^6 and 0.47×10^6), whereas the small subunit contains only a 0.56×10^6 -mol.wt. RNA. The simultaneous extraction of *A. tumefaciens*

and *E. coli* RNA shows that this situation does not originate from artifacts arising during the preparation procedure. Other methods were used by Le Goff (1968), Larsen (1971) and Schuch & Loening (1975) without significant differences. The large rRNA of *A. tumefaciens* is then degraded and contains very specifically located breaks, since the products of the split migrate in gel electrophoresis as monodisperse peaks.

It is well known that the large rRNA from many species is easily degraded, either after standard extraction or after denaturing treatments. Such a specific breakage was found in rRNA of *Ilyanassa obsoleta* (Koser & Collier, 1971), silkworm (Applebaum *et al.*, 1966), *Amoeba* (Loening, 1968; Stevens & Pachler, 1972), *Drosophila* (Greenberg, 1969), pea (Higo *et al.*, 1971) and *Euglena* (Rawson *et al.*, 1971). *A. tumefaciens* and *Rhodospseudomonas spheroides* (Marrs & Kaplan, 1970) are the only bacteria to show such a situation.

Fig. 7. Electrophoresis of pulse-labelled rRNA in *A. tumefaciens*

Bacteria were grown in low-phosphate medium with $10 \mu\text{Ci}$ of ^{32}P /ml for 5min and chased with a 1000-fold excess of unlabelled phosphate. The RNA was extracted by the acetate-sodium dodecyl sulphate method with EDTA and analysed on polyacrylamide gels. (a) 1 min chase; (b) 3 min chase. Electrophoresis was carried out in 2.8% gels at 50V for 2.5h in EDTA buffer. Arrows, from left to right, indicate the position of 0.95×10^6 , 0.56×10^6 , 0.52×10^6 and 0.47×10^6 -mol.wt. RNA on the E_{257} profile.

Fig. 8. Processing of rRNA precursors in *A. tumefaciens*

The same pulse was given as in Fig. 7 but with different chases: (a) 10 min; (b) 20 min. Electrophoresis was carried out in 2.8% gels at 50V for 3 h in EDTA buffer.

Fig. 9. Tentative structure of the large rRNA of *A. tumefaciens*

This Figure shows how the four RNA species demonstrated in the 1.04×10^6 -mol.wt. RNA of *A. tumefaciens* could be arranged. Arrows indicate the nicks occurring in the molecule.

This was also demonstrated in chloroplast rRNA (Loening & Ingle, 1967). Leaver & Ingle (1971) have shown that the constant presence of Mg^{2+} during the extraction and analysis of rRNA permits the isolation of this RNA in primary form. It was claimed by Larsen (1971) that the constant presence of Mg^{2+} permits the isolation of a 23S RNA from *A. tumefaciens*. Using this method, we were only able to obtain partial stabilization, as shown by the large increase in 1.04×10^6 and 0.95×10^6 -mol.wt. RNA species.

Because of the lack of complete stabilization of the large rRNA, we cannot be sure that the 1.04×10^6 -mol.wt. RNA is in fact the mature large RNA. This means that the nicks are located in portions of the molecule which are poor in double-stranded regions. We find two degradation products on electrophoresis in the presence of Mg^{2+} , namely RNA species of mol.wt. 0.95×10^6 and 0.50×10^6 . The latter is probably equivalent to the 0.52×10^6 -mol.wt. RNA found in electrophoresis in the presence of EDTA. Owing to the difficulties of molecular-weight determination in Mg^{2+} -containing buffer, we have not tried to get more accurate data for these RNA species.

Denaturation of *A. tumefaciens* RNA by dimethyl sulphoxide and heat shows that the 0.95×10^6 - and 0.52×10^6 -mol.wt. RNA species contain nicks. Analysis of the low-molecular-weight RNA species shows the simultaneous appearance of a 0.048×10^6 -mol.wt. RNA. We can then assume that the denaturation process induces the two following transitions: one molecule of mol.wt. 0.95×10^6 is split into two molecules of mol.wt. 0.47×10^6 , and one molecule of 0.52×10^6 -mol.wt. RNA is cut into portions of mol.wt. 0.47×10^6 and 0.048×10^6 . The large ribosomal subunit contains at least two molecules of 0.47×10^6 -mol.wt. RNA and one molecule of 0.048×10^6 -mol.wt. RNA. The mature large rRNA of *A. tumefaciens* would have a minimum mol.wt. of 1.00×10^6 . To give the mol.wt. 1.04×10^6 as found in Mg^{2+} -containing buffer, another molecule of $0.04\text{--}0.05 \times 10^6$ mol.wt. would be needed. Formamide denaturation (Schuch & Loening, 1975) of the isolated 1.04×10^6 -mol.wt. RNA shows only 0.47×10^6 -mol.wt. RNA as the degradation product in the high-molecular-weight range. So the chain contains two portions of 0.47×10^6 -mol.wt. RNA and two portions of 0.048×10^6 -mol.wt. RNA. Addition of all these molecules gives a mol.wt. of 1.04×10^6 , which fits well with the size of the precursor. We can construct a symmetrical structure for this RNA as shown in Fig. 9. According to this scheme, the large rRNA contains three nicks. The masking of one of these nicks allows, as shown in Fig. 9, the appearance of peaks at mol.wts. 0.95×10^6 and 0.52×10^6 , which must be considered as aggregation products.

It is known that the primary products of transcription of bacterial ribosomal cistrons are two RNA species slightly larger than the mature rRNA. They can be separated from the final rRNA by polyacrylamide-gel electrophoresis, especially for the 0.56×10^6 mol.wt.-RNA precursor (Hecht & Woese, 1968). In their work on the rRNA of *Rhodospseudomonas spheroides*, Marrs & Kaplan (1970) detected a 1.1×10^6 -mol.wt. precursor RNA, although they could not find any RNA of this size in the large ribosomal subunit. The structural similarity existing between the rRNA of *A. tumefaciens* and *Rhodospseudomonas spheroides* suggests that such precursors

can occur in *A. tumefaciens*. We have studied the synthesis of *A. tumefaciens* RNA to confirm the possible existence of a 1.04×10^6 -mol.wt. RNA. By pulse labelling we found two precursors of mol.wts. 1.04×10^6 and 0.70×10^6 . These molecules are similar to those found in *Bacillus subtilis* (Hecht & Woese, 1968) and *E. coli* (Adesnik & Levinthal, 1969). It is reasonable to believe that they represent the precursor form of the RNA contained in the large and the small ribosomal subunit respectively. The precursor of the small rRNA has mol.wt. 0.70×10^6 . This is a high value, compared with 0.61×10^6 found in *E. coli* (Adesnik & Levinthal, 1969). There must be 350 nucleotides in excess in *A. tumefaciens*, which are split out during the maturation. It is of interest to know the significance of such an excess. Our interest was focused on the large rRNA precursor, of mol.wt. 1.04×10^6 . This RNA has a very high specific radioactivity and we think that the presence of such a peak demonstrates the non-artificial origin of the degradation of *A. tumefaciens* rRNA. Its molecular weight is the same as that of the hypothetical RNA contained in the large ribosomal subunit. After chase, this molecule disappears and the label appears in 0.47×10^6 -mol.wt. RNA, whereas, as in *E. coli*, the 0.70×10^6 -mol.wt. precursor gives a mature form of 0.56×10^6 -mol.wt. RNA. We were not able to detect, in many experiments, any labelling that involves the 0.52×10^6 -mol.wt. RNA. This RNA seems to be a late-forming species. It must involve an aggregation in our conditions for electrophoresis (ionic strength; Mg^{2+}).

The degradation of *A. tumefaciens* rRNA is induced during the biosynthesis and the maturation of the ribosome. This maturation process could be achieved *in vivo* by a ribonuclease carried by the ribosome and active only in cellular conditions.

After a general analysis of the ribosomal structure, Loening (1973) concluded that the large rRNA arises from a first gene duplication. This gene duplication would have involved the original small rRNA. With time, these two structures have evolved separately, but there remain fragile links in the molecule at the point where the gene duplication occurred. Our tentative structure for the large rRNA of *A. tumefaciens*, as shown in Fig. 9, can be explained by such a process. The large rRNA would have originated from a gene duplication, whereas the original ribosomal gene would have an approx. mol.wt. of 0.52×10^6 . To what extent the evolution of this structure has induced divergence in the sequence of these two parts remains to be studied. We have no evidence for the origin of the gene duplication from the small ribosomal subunit. Demonstration of such an origin necessitates further

studies including competition-hybridization experiments between 0.56×10^6 - and 0.47×10^6 -mol.wt. rRNA.

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