The Ribosomal Ribonucleic Acid of *Agrobacterium tumefaciens*

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The 23S rRNA of *Agrobacterium tumefaciens* contains at least two nicks which result in the formation of RNA components with mol.wts. of 0.52×10^6 and 0.48×10^6 . Thus under the usual conditions of extraction and analysis, no 23S rRNA was recovered from the bacterium. The experiments show that 23S rRNA is synthesized as a continuous chain, in which one or two nicks are formed almost immediately near the ends of the molecule and an additional nick in the middle at a later time.

The study of the RNA of the large subunit of the ribosome in many species of bacteria, plants and animals has had a history confused by the inability to distinguish between degradation of RNA during extraction and the presence of breaks in the polynucleotide chain which may occur *in vivo*. There have indeed been numerous reports that the 23S RNA in bacteria (Midgley, 1965; Pace 1973) or the 25S RNA of the lower animals (Loening, 1973) is not a single chain, but is nicked, giving rise to two molecules approximately the size of the 16S or 18S RNA. One might wonder, even, whether the well-known experiments of Yankofsky & Spiegelman (1963), which demonstrate that the 16S and 23S rRNA species in bacteria have distinct sequences, were carried out on *Bacillus megaterium* instead of *Escherichia coli*, not as the authors state 'to alleviate somewhat the current monotony of molecular biology', but because the 23S RNA of *E. coli* is indeed frequently nicked in half and contaminates the 16S fractions. The recognition that such contamination might interfere with competitive-hybridization experiments of this sort has led to the practice of extracting rRNA from the previously separated ribosomal subunits (Brown & Weber, 1968). More recently, it has become clear that the larger rRNA (23S, 25S or 28S) is readily nicked in the middle by nucleases during the extraction procedure. It is also becoming clear, however, that in many species, this RNA is nicked *in vivo* and is held together by its secondary structure in suitable ionic conditions. For example, among the lower animals the large RNA of *Tetrahymena* (Bostock *et al.*, 1971) or *Drosophila* (C. Pelling, personal communication; Rubinstein & Clever, 1971) and of blue-green algae (Szalay *et al.*, 1972; Grierson & Smith, 1973) and *Amoeba* (Loening *et al.*, 1972; Stevens & Pachler, 1972) appears as two pieces similar in size to 18S RNA under conditions which open the secondary structure. In the cases quoted, it was suggested that this nick

in the chain occurs *in vivo* and is not an artifact of extraction.

Among the prokaryotes, it was reported that the 23S RNA of *Rhodopseudomonas* (Marrs & Kaplan, 1970) and of chloroplasts (Leaver & Ingle, 1971) was obtained in low yield; the normal conditions of isolation and analysis allow the fragments to fall apart. We report here the properties of the 23S RNA of *Agrobacterium tumefaciens*, which represents an extreme case among the prokaryotes in that almost no 23S RNA can be extracted when normal salt concentrations are used (Grienberger & Simon, 1972).

Materials and Methods

Bacteria

Agrobacterium tumefaciens strain B6, kindly given by Dr. D. Butcher (ARC Laboratory for Plant Physiology, Cambridge, U.K.), was used throughout the experiments. Cultures were grown at 25°C in 0.8% tryptone broth (Oxoid Ltd., London E.C.4, U.K.) containing 0.01 M-MgSO₄. Cultures were shaken overnight; they were then in exponential growth phase and were used for nucleic acid extraction.

Radioactive labelling

The cells were collected by centrifugation at 15000g for 5 min at 25°C. They were washed once in water and resuspended in a defined medium containing 0.01 M-Tris-HCl, pH 7.6, 3.0 g of (NH₄)₂SO₄, 0.15 g of NaCl, 0.01 g of CaCl₂, 0.5 g of MgSO₄, and 20 g of glucose per litre. The cells were labelled by addition of 1 mCi of [³²P]phosphoric acid in 0.1 ml (The Radiochemical Centre, Amersham, Bucks., U.K.) per 2 ml of medium, and incubation was continued for the times indicated in the legends to the Figures. The times of incubation required were long (30–60 min), probably because of the 'step-down'

effect on the cells and because of washing and transfer to the defined medium. The cells were collected in a cooled ($\sim 4^{\circ}\text{C}$) centrifuge in ice-cooled tubes and nucleic acids were extracted as follows.

Nucleic acid extraction

The cells were resuspended in medium containing 0.05M-Tris-HCl, pH 7.8, 0.05M-NaCl, and 2% (w/v) sodium tri-isopropyl-naphthalenesulphonate (Parish & Kirby, 1966; Loening, 1969). The suspension was frozen and the cells were broken in an Eaton (1962) press. The frozen block of broken cells was thawed into a warm phenol-cresol-8-hydroxyquinoline mixture (Parish & Kirby, 1966). Deproteinization, treatment with deoxyribonuclease in 2-(*N*-morpholino)ethanesulphonic acid, adjusted to pH 7.0 with NaOH, at 0°C for 30 min, and reprecipitation of the RNA from sodium acetate-sodium dodecyl sulphate buffer, followed the procedures previously described (Loening, 1969).

For the extraction of RNA in the presence of Mg^{2+} , the above method was followed, except that the extraction buffer contained 0.03M-Tris-HCl, pH 7.8,

0.1mM-EDTA, 0.01M- MgSO_4 and 2% sodium tri-isopropyl-naphthalenesulphonate. All other buffers similarly contained 0.01M- Mg^{2+} throughout.

Polyacrylamide-gel electrophoresis

Gel electrophoresis was carried out as described previously (Loening, 1969). The E buffer used contained 30mM-Tris-HCl, 36mM- NaH_2PO_4 , 1mM-EDTA and 0.2% sodium dodecyl sulphate, at pH 7.8. This buffer had been designed to allow a suitable voltage and current for electrophoresis, and to maintain the secondary structure of RNA in a stable configuration. It was shown that the composition of this buffer was a reasonable compromise at which the molecular weights of most sorts of RNA could be determined from the electrophoretic mobility. For analysis in the presence of Mg^{2+} , the following buffer was used: 30mM-Tris, 16mM-HCl, 2mM- MgSO_4 and 0.1mM-EDTA, pH 8.1 at 25°C ; sodium dodecyl sulphate (0.1%) was added to the running buffer.

The 2.2 and 2.6% (w/v) polyacrylamide gels were run at room temperature (20° – 24°C) for the times indicated in the Figures. The gels were washed in

Fig. 1. Electrophoresis of ^{32}P -labelled *Agrobacterium* nucleic acids

Bacteria were incubated with ^{32}P for 45 min. Approx. $100\mu\text{g}$ of nucleic acid was applied to a 2.6% polyacrylamide gel of 9mm diameter. Electrophoresis was for 4h. After u.v. scanning, the gel was cut into 0.4mm slices and ^{32}P radioactivity determined. ---, E_{265} ; (histogram) ^{32}P radioactivity. The RNA species are identified by their molecular weights in millions. The amount of the 0.52×10^6 -mol.wt. component was exceptionally small in this sample. The dashed part of the E_{265} profile indicates that a background absorbance obtained from a blank gel has been subtracted; such absorbance is frequently found near the tops of the gels, and can be removed by prolonged washing after electrophoresis; it may be associated with storage of the gels before use, especially after polymerization with old ammonium persulphate.

water, and scanned in a Joyce-Loebl u.v. gel scanner at 265nm. For radioactivity counting, gels were frozen in solid CO₂ and sliced into 0.4mm slices with a Mickle gel slicer. The slices were dried on a film base, and radioactivity was determined in a low-background Geiger counter.

Two methods were used for 'melting' the RNA: (1) RNA solutions in E buffer with all components at half the normal concentration were heated in a water bath at 60°C for 2min, or (2) formamide was added to 60% (v/v) final concentration to RNA in

Fig. 3. Electrophoresis of *Agrobacterium* nucleic acids on the presence of Mg²⁺

RNA was isolated in the presence of Mg²⁺, and separated in low-salt buffer containing 10mM-Mg²⁺ on 2.6% gels for 5.5h. The broadness of the bands is a common feature with RNA, which was extracted throughout in the presence of Mg²⁺.

solution in the same buffer at room temperature. Formamide (50–100 μl) was layered on the surface of the gels during the 30min pre-run.

For further study, RNA was eluted from gel slices, cut 0.8mm thick, by shaking the slices in 2ml of 0.6M-lithium acetate, pH 6, containing 0.5% sodium dodecyl sulphate. Elution was continued for at least 6h at 4°C or at room temperature. The RNA was recovered from the eluate by centrifugation at 54000g at 10°C for 12h. The pellet (which was, of course, invisible in the case of traces of labelled RNA) was dissolved in E buffer of half normal concentration, 50 μl of buffer being used for labelled RNA and larger volumes for unlabelled RNA.

Results

A scan of pulse-labelled RNA analysed by electrophoresis in E buffer is shown in Fig. 1. To prevent any possibility of breakdown during enzyme treatment, this sample was not treated with deoxyribonuclease. The E_{265} profile shows the DNA near the origin, the 16S RNA (mol.wt. 0.56×10^6), and a smaller molecule of approx. mol.wt. 0.48×10^6 . There is no apparent 23S RNA. (The approximate positions of 23S and 16S RNA were obtained from parallel gels of *E. coli* RNA and are indicated with arrows.) In some preparations a small but variable amount of RNA with a mol.wt. of about 0.95×10^6 – 1.0×10^6 was found; traces of this are apparent in Fig. 1 at 3.2cm and rather more in Fig. 5(a) at 2.3cm. There is also a small amount of a RNA component between those of mol.wts. 0.56×10^6 and 0.48×10^6 . Its mol.wt. is about 0.52×10^6 . The amount of this component is variable in different preparations. The profile

Fig. 2. Electrophoresis of rRNA from *Agrobacterium* isolated from ribosomal subunits

Ribosomal subunits were isolated by sucrose-gradient centrifugation in 0.3M-KCl–0.01M-Tris–HCl (pH 7.4)–1mM-mercaptoethanol (W. Schuch, unpublished work). RNA was extracted with phenol from the pooled fractions of the 30S and 50S subunits. (a) RNA from the 30S subunit; (b) RNA from the 50S subunit. The small amount of 16S rRNA in the 50S subunit is due to some cross-contamination by 30S subunits. Electrophoresis was on 2.6% gels for 3h.

Fig. 4. Electrophoresis of isolated 23S RNA from *Agrobacterium*

^{32}P -labelled RNA of *Agrobacterium* was separated as indicated in Fig. 1. Gel slices containing the ^{32}P -labelled 23S RNA were pooled, and the RNA was eluted and pelleted as described in the text. (a) ^{32}P -labelled 23S RNA was electrophoresed with *E. coli* rRNA as a marker; (b) ^{32}P -labelled 23S RNA was 'melted' by the addition of formamide to 60% (v/v) and separated with *E. coli* rRNA as a marker. Electrophoresis was on 2.6% gels for 3 h. $\cdots$, E_{265} of *E. coli* rRNA. —, ^{32}P in 23S *Agrobacterium* RNA.

of radioactivity shows that two components, in addition to those seen in the E_{265} profile, are labelled. The larger of these has mol.wt. 1.1×10^6 , and we interpret this to be the pulse-labelled 23S RNA. The other component has mol.wt. approx. 0.7×10^6 , which we interpret tentatively as the precursor to the

16S rRNA (W. Schuch & U. E. Loening, unpublished work). With longer times of labelling, the relative proportions of both 1.1×10^6 - and 0.7×10^6 -components decrease, whereas those of the others increase. These results indicate that newly synthesized 23S RNA is a single polynucleotide chain, and that this

Fig. 5. Effect of 'melting' on the total RNA of *Agrobacterium*

(a) (·····, right-hand scale) RNA, treated with deoxyribonuclease, was fractionated by electrophoresis on a 2.2% gel for 2 h. (b) (—, left-hand scale) A sample identical with that in (a) was dissolved in E buffer at half normal concentration, heated to 60°C in water for 2 min and cooled quickly. Electrophoresis was as in (a).

molecule has a short half-life and breaks down into smaller components, one of which is likely to be of mol.wt. 0.48×10^6 .

RNA was extracted from separated 50S and 30S subunits. The results (Fig. 2) confirm that the 0.52×10^6 - and 0.48×10^6 -mol.wt. components occur in the 50S ribosomal subunit of this bacterium.

Extraction and electrophoresis of RNA in the presence of Mg^{2+} preserves the secondary structure of the 23S RNA, as shown in Fig. 3. Although the

resolution is poor under these conditions, it is clear that the two rRNA species are obtained in approximately the expected proportions.

To determine whether the 1.1×10^6 -mol.wt. pulse-labelled 23S RNA shown in Fig. 1 contains any hidden breaks, this component was recovered from gel slices of a similar preparation and re-electrophoresed with *E. coli* rRNA as a marker. Fig. 4(a) shows that this labelled 23S RNA has survived the manipulations involved, and confirms that its molecular

weight is identical with that of *E. coli* 23S rRNA. In Fig. 4(b), a duplicate sample was treated with 60% formamide and analysed with *E. coli* rRNA as in Fig. 4(a). This reveals at least two nicks in the molecule, which yield components with mol.wts. 0.95×10^6 and 0.48×10^6 . Fig. 5, in which the total nucleic acids of *Agrobacterium tumefaciens* were 'melted' by heating to 60°C, also suggests that there are a few components smaller than that of mol.wt. 0.48×10^6 , but the latter appears to be the major breakdown product of the 1.1×10^6 -mol.wt. rRNA. The 0.52×10^6 -mol.wt. component disappears after 'melting'. Most of its breakdown products are thus to be found in the 0.48×10^6 -mol.wt. peak.

Discussion

These results confirm that most of the 23S rRNA of *Agrobacterium tumefaciens* is nicked *in vivo*, probably in at least two places.

The simplest interpretation is that the molecule is nicked near each end and in the middle. The nicks near the ends are made first, as revealed by the formation of the 0.95×10^6 -mol.wt. RNA and by its apparent stability under these conditions of 'melting'. The nick in the middle is formed later and results in the formation of two equal pieces of mol.wt. 0.48×10^6 . The 0.52×10^6 -mol.wt. RNA component would be half of the complete 23S RNA molecule, and includes the 0.48×10^6 -mol.wt. component, with a small piece (about 0.05×10^6 mol.wt.) from one end. The latter is removed on melting, when the 0.52×10^6 -mol.wt species loses RNA to give that of mol.wt. 0.48×10^6 .

These determinations of molecular weights are not sufficiently exact to confirm this interpretation. It is, however, more difficult to fit the data into a model in which the initial nick is at one end only.

It should be noted that the pulse-labelled 23S RNA appears to be intact in E buffer, whereas a proportion of it releases the 0.48×10^6 -mol.wt. component on 'melting'. This behaviour is in contrast with that of the bulk of the 23S RNA, which falls apart in E buffer. This indicates that some conformational changes occur in the 23S RNA after the nicks have been made. Such structural features seem

to survive the deproteinization and purification of the RNA.

We conclude that *Agrobacterium* represents one of the most extreme cases in which the rRNA is nicked, and in which the secondary structure fails to maintain the apparent integrity of the molecule at usual salt concentrations. Similar work was carried out at the same time by Grienberger & Simon (1975).

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References

- Bostock, C., Prescott, D. & Lauth, M. (1971) *Exp. Cell Res.* **66**, 260-262
- Brown, D. D. & Weber, C. S. (1968) *J. Mol. Biol.* **34**, 681-697
- Eaton, N. R. (1962) *J. Bacteriol.* **83**, 1359-1360
- Grienberger, J. M. & Simon, D. (1972) *C. R. Acad. Sci. Ser. D* **274**, 1399-1402
- Grienberger, J. M. & Simon, D. (1975) *Biochem. J.* **149**, 7-14
- Grierson, D. & Smith, H. (1973) *Eur. J. Biochem.* **36**, 280-285
- Leaver, C. J. & Ingle, J. (1971) *Biochem. J.* **123**, 235-243
- Loening, U. E. (1969) *Biochem. J.* **113**, 131-138
- Loening, U. E. (1973) *Biochem. Soc. Symp.* **37**, 95-104
- Loening, U. E., Grierson, D., Rogers, M. E. & Sartirana, M. L. (1972) *FEBS Symp.* **23**, 395-405
- Marrs, B. & Kaplan, S. (1970) *J. Mol. Biol.* **49**, 297-317
- Midgley, J. E. M. (1965) *Biochim. Biophys. Acta* **108**, 348-354
- Pace, N. R. (1973) *Bacteriol. Rev.* **37**, 562-603
- Parish, J. H. & Kirby, K. S. (1966) *Biochim. Biophys. Acta* **129**, 554-562
- Rubinstein, L. & Clever, U. (1971) *Biochim. Biophys. Acta* **246**, 517-529
- Stevens, A. R. & Pachler, P. F. (1972) *J. Mol. Biol.* **62**, 225-237
- Szalay, A., Munsche, D., Wollgiehn, R. & Parthier, B. (1972) *Biochem. J.* **129**, 135-140
- Yankofsky, S. A. & Spiegelman, S. (1963) *Proc. Natl. Acad. Sci. U.S.A.* **49**, 538-544