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Ribosomal Ribonucleic Acid and Ribosomal Precursor Ribonucleic Acid in *Anacystis nidulans*

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The RNA of the blue–green alga *Anacystis nidulans* contains three ribosomal RNA species with molecular weights of 0.56×10^6, 0.9×10^6, and 1.1×10^6 if the RNA is extracted in the absence of Mg^{2+}. The 0.9×10^6 mol.wt. rRNA is extremely slowly labelled in ^{32}P-incorporation experiments. This rRNA may be a cleavage product of the 1.1×10^6 mol.wt. rRNA from the ribosomes of cells in certain physiological states (e.g. light-deficiency during growth). The cleavage of the 1.1×10^6 mol.wt. rRNA during the extraction procedure can be prevented by the addition of 10mM-$MgCl_2$. ^{32}P-pulse-labelling studies demonstrate the rapid synthesis of two ribosomal precursor RNA species. One precursor RNA migrating slightly slower than the 1.1×10^6 mol.wt. rRNA appears much less stable than the other precursor RNA, which shows the electrophoretic behaviour of the 0.7×10^6 mol.wt. rRNA. Our observations support the close relationship between bacteria and blue–green algae also with respect to rRNA maturation. The conversion of the ribosomal precursor RNA species into 0.56×10^6- and 1.1×10^6-mol.wt. rRNA species requires Mg^{2+} in the incubation medium.

The 70S ribosomes of prokaryotic organisms contain two high-molecular-weight rRNA species of 23S and 16S corresponding to mol.wts. of 1.1×10^6 and 0.56×10^6 respectively. In bacteria these two rRNA species are synthesized via precursor molecules designated as 'p23' and 'p16' (Hecht & Woese, 1968; Adesnik & Levinthal, 1969; Dahlberg & Peacock, 1971). They have slightly higher molecular weights than the mature molecules, from which they are separable only by polyacrylamide-gel electrophoresis.

The 70S ribosomes, which contain 23S and 16S rRNA, exist also in the blue–green algae (Craig & Carr, 1968; Loening, 1968; Rodriguez-Lopez & Vazquez, 1968; Howland & Ramus, 1971), although these photo-autotrophic organisms have developed a cellular organization more complex than bacteria (Lang, 1968). The occurrence of macromolecular precursors for the rRNA in such cells has not been demonstrated previously.

In the present paper we show that the electrophoretic behaviour and the synthesis of ribosomal precursor RNA species in the blue–green alga *Anacystis nidulans* are similar to the ribosomal precursor RNAs in bacteria. We also report the occurrence of an additional rRNA with a mol.wt. of 0.9×10^6 (21S). Its presence is strictly dependent on the absence of Mg^{2+} in the extraction medium and seems to be related to the conditions of illumination during the growth of the cell cultures. The 21S rRNA is extremely slowly labelled, suggesting a product which appears during the development of the cells or because of aging processes of the ribosomes.

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Materials and Methods

Growth conditions of cells

Anacystis nidulans (strain Göttingen) was cultured in 4-litre flasks at 40°C with CO_2+air (4:96). Illumination was provided by cool white fluorescent lamps of approx. 6000 lux. The cells were grown in the medium described by Kratz & Myers (1955), consisting of (in 1 litre of tri-distilled water): 1g of $NaNO_3$; 1g of K_2HPO_4; 0.15g of $MgSO_4,7H_2O$; 10mg of $Ca(NO_3)_2,4H_2O$; 50mg of EDTA; 4mg of $Fe_2(SO_4)_3,6H_2O$; 8.8mg of $ZnSO_4,7H_2O$; 1.44mg of $MnCl_2,4H_2O$; 0.7mg of MoO_3; 1.57mg of $CuSO_4,5H_2O$; 0.5mg of $Co(NO_3)_2,6H_2O$. The pH of the growth medium was 7.8.

Cell disrupture and RNA extraction

If not otherwise stated, the cells were harvested in the late exponential growth phase after 72h of cultivation. They were centrifuged for 10min at 0°C and 6000*g* and were washed twice with standard buffer (0.1M-NaCl; 0.05M-tris–HCl, pH7.8). The packed material was ground in a mortar with aluminium oxide (Alcoa A-305; Aluminum Company of America, Ark., U.S.A.) at 0°C for 15min. The nearly dry paste was suspended in 10vol. of standard buffer/g fresh wt. of cells. To investigate the effect of Mg^{2+} on the RNA profiles (see the Results and Discussion section), 1M-$MgCl_2$ was added to the standard buffer to give a final concentration of 0.01M-$MgCl_2$, or the addition was omitted. After removal of the aluminium oxide and the unbroken cells by centrifugation, the supernatant was extracted 4–5 times with water-saturated phenol

with stirring at 25°C. The nucleic acids in the buffer phase were precipitated by addition of 2.5vol. of ethanol at −10°C, then were dissolved in 10ml of 0.015M-sodium citrate+0.15M-NaCl before being reprecipitated and stored under ethanol. This procedure appears to extract DNA and RNA, including pulse-labelled RNA; we do not know, however, whether the extraction is complete, since some unbroken cells remain after grinding. The total RNA from freshly-harvested cells of *Penicillium cyclopium* and *Escherichia coli* were prepared in a similar way.

Polyacrylamide-gel electrophoresis and measurement of radioactivity

The nucleic acids were fractionated by polyacrylamide-gel electrophoresis by the method of Loening (1967). Total RNA (35μg) was layered on gels (9cm long) consisting of 2.4% acrylamide, 0.12% bis-acrylamide and 0.4% agarose. Electrophoresis was done for 3h at 20°C at 4.15mA/tube. The E_{265} of the gels was measured, then they were dried and scanned for radioactivity with a Berthold thin-layer scanner. In some cases the gels were sliced into 1mm discs, dried on filter discs (2.3cm diam.), and the radioactivities were determined in a Tri-Carb scintillation spectrometer in vials containing 13ml of scintillation fluid [4g of 2,5-diphenyloxazole/litre and 0.2g of 1,4-bis-(5-phenyloxazole-2-yl)benzene/litre in toluene]. Corrections for filter discs and gel material were made.

Chemicals

Incorporation studies were performed with carrier-free $Na_2H^{32}PO_4$ from Zentralinstitut für Kernforschung, Dresden-Rossendorf, German Democratic Republic. Acrylamide, bis-acrylamide and agarose were obtained from Serva Feinbiochemica G.m.b.H., Heidelberg, Germany.

Other details are given in the legends of the figures or are described by Szalay *et al.* (1972).

Results and Discussion

When the total nucleic acids extracted from light-grown, exponential-phase cells of *Anacystis nidulans* were fractionated by polyacrylamide-gel electrophoresis, there was a clear separation of the high-molecular-weight rRNA species. The extraction

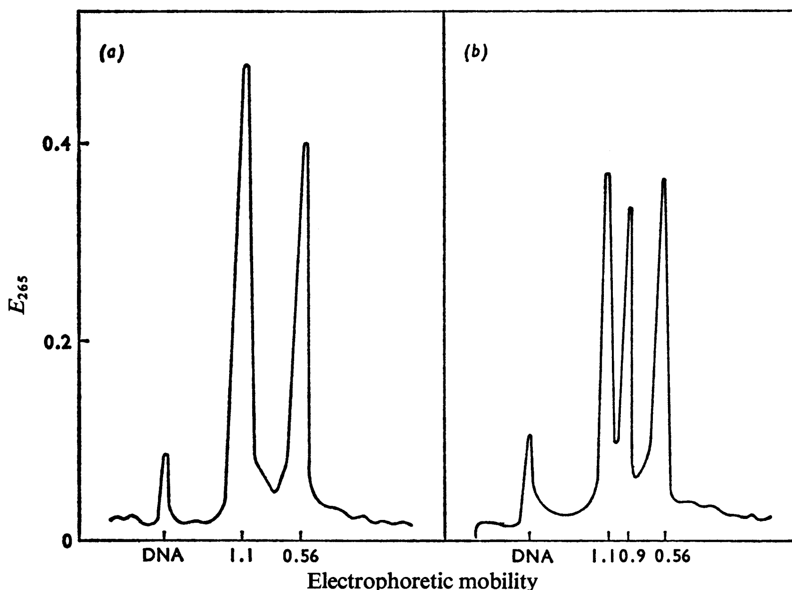

Fig. 1. *Comparison of Anacystis rRNA fractions extracted in the presence (a) and the absence (b) of* 10mM-$MgCl_2$ *after separation of the total nucleic acid fraction by polyacrylamide-gel electrophoresis*

Cells after 72h growth under optimum conditions were harvested by centrifugation for 10min at 0°C and 8000*g*, washed twice with buffer (0.1M-NaCl; 0.05M-tris–HCl, pH7.8) and the packed material was ground at 0°C with aluminium oxide [approx. 2g/g cell fresh wt.)]. The paste was resuspended in 10vol. of the buffer indicated above, but with (*a*) or without (*b*) 10mM-$MgCl_2$/g fresh wt. of cells. Extraction of the total nucleic acids and their separation by means of polyacrylamide-gel electrophoresis is described in the Materials and Methods section. In all cases electrophoresis was performed without Mg^{2+}. RNA components are referred to as $10^{-6}\times$mol.wt.

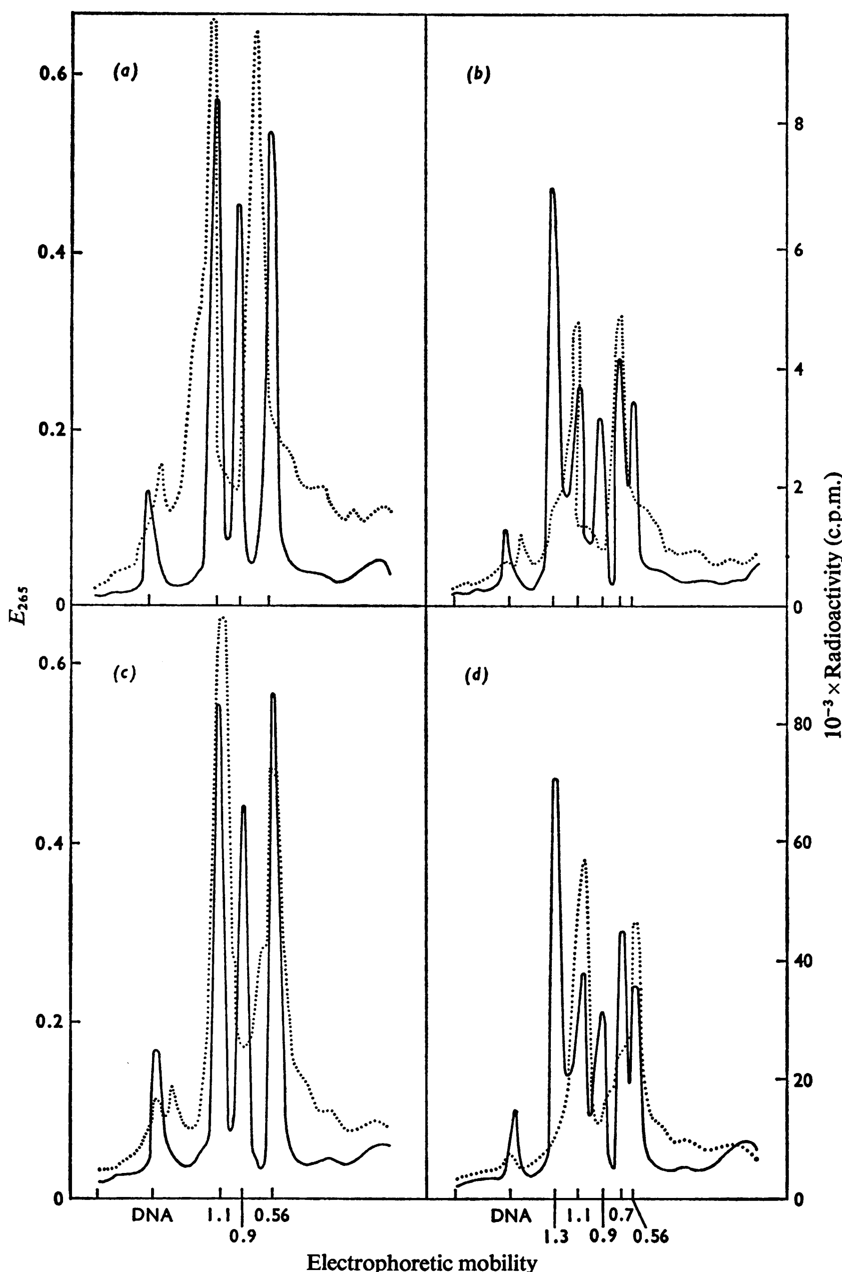

Fig. 2. *Distribution of* ^{32}P *in the rRNA fractions after* 3*min of pulse-labelling* (*a*, *b*) *and* 3*h of long-term labelling* (*c*, *d*) *of A. nidulans*

Cells of the stationary growth phase (72h) were harvested and incubated for 5h under optimum growth conditions but under phosphate deficiency. The cells (10^{11}/10ml) were supplied with 5mCi of $Na_2H^{32}PO_4$ for 3min or for 3h. The suspension of the labelled cells was poured on frozen (−30°C) 0.1M-potassium phosphate buffer, then the cells were ground and the RNA species extracted at 0°C. The RNA separation was performed by polyacrylamide-gel electrophoresis as described in the Materials and Methods section. The RNA extraction was done without Mg^{2+}. The 1.3×10^6- and 0.7×10^6-mol.wt. rRNA species were obtained after similar extraction of *Penicillium* cells and were used as marker RNA species in co-electrophoresis (*b*, *d*). ——, E_{265}; ·····, radioactivity. RNA components are referred to as $10^{-6}\times$mol.wt.

in the presence of 0.01M-$MgCl_2$ yielded two rRNA species with mol.wts. of 1.1×10^6 and 0.56×10^6 (Fig. 1*a*). The Mg^{2+} in the standard buffer could not be replaced by Na^+ or K^+. The absence of Mg^2 during the RNA extraction provided an additional third major rRNA with a calculated mol.wt. of 0.9×10^6 of 21S (Fig. 1*b*). The molecular weights of the rRNA species were determined relative to both *E. coli* and *Penicillium* rRNA species. The RNA extracted from *E. coli* under identical conditions contained no 21S RNA. However, rRNA of this size was reported to be a constituent of the cytoplasmic ribosomes of *Amoeba* and *Euglena* (Loening, 1968). The three rRNA fractions can also be found after sucrose-gradient centrifugation or chromatography on methylated-albumin–kieselguhr columns (Szalay *et al.*, 1972).

The appearance under certain conditions of three prominent high-molecular-weight rRNA species was a surprising observation which focused our interest on two questions. (*a*) What are the reasons for the formation of the 0.9×10^6 mol.wt. rRNA and in what way is it synthesized? (*b*) What are the ribosomal RNA precursors in *Anacystis*?

The pattern of synthesis of *Anacystis* rRNA is shown in Fig. 2. After pulse-labelling for 3min with ^{32}P, one main portion of radioactivity was located between the 0.56×10^6- and 0.9×10^6-mol.wt. rRNA species and another one migrated slightly slower than the 1.1×10^6 mol.wt. rRNA. Co-electrophoresis with marker rRNA from *Penicillium* (mol.wts. 1.3×10^6 and 0.7×10^6) showed coincidence of the ^{32}P distribution in the gels with the u.v.-absorption profile of the 0.7×10^6 mol.wt. marker RNA, but not with the 1.3×10^6 mol.wt. marker RNA (Figs. 2*b* and 2*d*).

These rapidly labelled fractions evidently represent two ribosomal precursor RNA species of *A. nidulans*. Their maturation to 1.1×10^6 and 0.56×10^6 mol.wt. rRNA species could be shown after 1h of ^{31}P-chase or after 3h of long-term incubation (Figs. 2*c* and 2*d*). Like the findings with *E. coli* (Adesnik & Levinthal, 1969; Dahlberg & Peacock, 1971), the slow-migrating ribosomal precursor RNA was less stable or was converted more rapidly than the ribosomal precursor RNA of 0.7×10^6 mol.wt.

The conversion of the ribosomal precursor RNA into mature rRNA seems to be dependent on the presence of Mg^{2+} (Table 1). *Anacystis* cells were pulse-labelled with ^{32}P for 5min, the radioactivity outside the cells was removed by centrifugation, then the pellet was divided into four equal portions and resuspended in growth medium (Kratz & Myers, 1955), but with 0.01g of K_2HPO_4/litre and with or without 10mM-$MgCl_2$. After 1h of incubation in light or darkness the total RNA of the samples was extracted, separated by polyacrylamide-gel electrophoresis and analysed for incorporation of ^{32}P into the rRNA species. The values of the specific radio-

Table 1. *Effect of Mg^{2+} and light on the distribution of ^{32}P in Anacystis rRNA species and ribosomal precursor RNA species during chase incubation after pulse-labelling*

Cells of the stationary growth phase were harvested and then grown for 5h under conditions of phosphate deficiency, then they were collected and labelled for 5min with 5mCi of $Na_2H^{32}PO_4/10^{11}$ cells in 10ml. After removal of the remaining ^{32}P, the cells were resuspended in growth medium (Kratz & Myers, 1955) containing 0.01g of K_2HPO_4/litre and incubated for 1h under the conditions indicated below. The ^{32}P distribution is expressed in relative values (c.p.m. at the peak maximum). No radioactivity was found in the 0.9×10^6 mol.wt. rRNA.

		Incubation conditions		^{32}P distribution in rRNA fractions			
Labelling conditions	Sample no.	$MgCl_2$ (10mM)	Illumination	Slow-migrating ribosomal precursor RNA	Fast-migrating ribosomal precursor RNA	1.1×10^6 mol.wt. RNA	0.56×10^6 mol.wt. RNA
5min pulse-labelling	0	+	+	4000	4800	Trace	
5min pulse followed by 1h incubation in new culture medium	1	−	−	4000	4800	Trace	
	2	+	−	0	0	7000	7000
	3	−	+	14000	15000	Trace	
	4	+	+	0	0	25000	23000

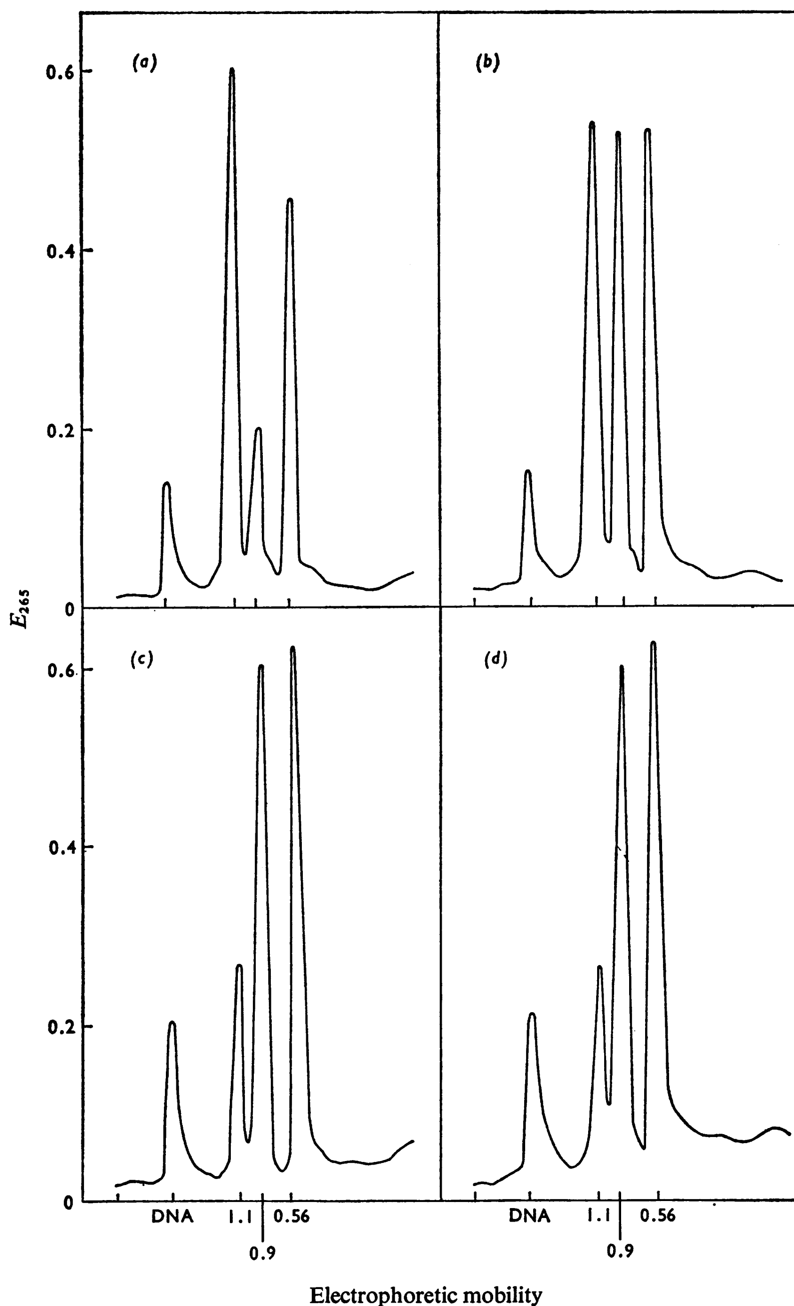

Fig. 3. *Correlation between the appearance of* 0.9×10^6 *mol.wt. rRNA and culture conditions in A. nidulans*

The RNA fractions were extracted without Mg^{2+} and were separated by polyacrylamide-gel electrophoresis from cells of the following cultures: (*a*) 10h in light (early exponential growth phase); (*b*) 25h in light (late exponential growth phase); (*c*) 72h in light (stationary phase); (*d*) 10h in light, followed by 10h in darkness. RNA components are referred to as $10^{-6}\times$mol.wt.

activities (c.p.m./mg of RNA) of the peak maxima were compared with the results obtained from the rRNA species extracted immediately after 5min of pulse-labelling.

It is obvious from Table 1 that the samples incubated without Mg^{2+} (samples 1 and 3) showed no shift of ^{32}P from the gel regions of the ribosomal precursor RNA to the 1.1×10^6- and 0.56×10^6-mol.wt. rRNA species. The presence of Mg^{2+} during the incubation caused such a shift (samples 2 and 4). This maturation process is independent of the illumination. However, light always stimulated the rates of synthesis of both precursor and mature rRNA (samples 3 and 4) compared with the samples incubated in darkness (samples 1 and 2).

As indicated in Fig. 2, under our conditions the 0.9×10^6mol.wt. rRNA is neither pulse-labelled nor radioactive in long-term or chase experiments of up to 3h duration. Consequently, during this time 0.9×10^6mol.wt. rRNA was not formed *de novo*, nor was there a conversion of another newly synthesized high-molecular-weight rRNA into 0.9×10^6mol.wt. rRNA. After 24h of incubation, however, the differences of labelling have disappeared. The appearance of the 0.9×10^6mol.wt. rRNA seemed to be controlled by the growth conditions of the cell cultures (Fig. 3). The portion of this rRNA was small in the early exponential phase at sufficient illumination. It increased with the age of the *Anacystis* culture and/or (because of the higher cell density) with decreasing light intensity. In any case the presence of the 0.9×10^6mol.wt. rRNA was linked with a decrease in the amount of 1.1×10^6mol.wt. rRNA relative to the 0.56×10^6mol.wt. rRNA, which was found to be a very stable component.

This observation led us to the assumption of a partial conversion of the 1.1×10^6mol.wt. rRNA fraction into 0.9×10^6mol.wt. rRNA plus a smaller RNA fragment. Indeed, we could detect RNA of approx. 0.2×10^6mol.wt., which contained no ^{32}P label under conditions yielding non-labelled 0.9×10^6mol.wt. rRNA. Although we were unable to convert isolated 1.1×10^6mol.wt. rRNA into 0.9×10^6mol.wt. rRNA *in vitro* by dialysis against Mg^{2+}-free medium (Szalay *et al.*, 1972), in support of the idea that the degradation of the 1.1×10^6mol.wt. rRNA was not caused by the removal of Mg^{2+} *per se*, this experiment could not exclude that a cleavage of the molecule occurred during the disintegration of the cells caused by nucleolytic activity. Mg^{2+} would then play a stabilizing role during the isolation procedure. A similar effect was reported for the integrity of the 1.1×10^6mol.wt. rRNA in chloroplasts (Leaver & Ingle, 1971).

We explain our results as follows, in accordance with findings about the formation of 0.9×10^6mol.wt. rRNA in *Amoeba* (U. E. Loening, personal communication). *Anacystis* cells grown under optimum conditions (early-exponential phase) contain ribosomes with a rather stable 1.1×10^6mol.wt. rRNA. If the cells encounter sub-optimum growth conditions (limited light and nutrition), the status or the conformation of this rRNA is changed in the ribosomes, which makes it less protected against nucleolytic attack during the preparation steps. The sensitive site of the molecule might be stabilized by Mg^{2+}, thus preventing the appearance of the 0.9×10^6mol.wt. rRNA. (Alternatively, the cells under sub-optimum conditions may produce, as a consequence of aging, more sensitive ribosomes or subunits susceptible to RNAase attack. Our experiments have not been done with synchronous cultures. The use of such cultures should clarify this alternative.)

In conclusion, the appearance of the 0.9×10^6mol.wt. rRNA seems to be a consequence of a maturation process that affects only the 1.1×10^6mol.wt. rRNA. We do not suggest that the 21S RNA occurs in the *Anacystis* ribosome *in vivo*. Our results also provide evidence concerning analogies in synthesis and maturation of ribosomal RNA species between bacteria and blue-green algae. Moreover, there are indications that the synthesis of ribosomal precursor RNA in the chloroplasts of higher plants (R. Wollgiehn & D. Munsche, unpublished work) proceeds in a manner very similar or identical with that in *A. nidulans*. On the other hand, after pulse-labelling we observed a small RNA fraction migrating in the gels not much further than DNA (Fig. 2). We have not investigated the nature of this RNA, but have shown the existence of a DNA–RNA complex, with a rapidly labelled RNA moiety, in *A. nidulans* (Szalay *et al.*, 1972). It remains an open question whether *Anacystis* contains an initial ribosomal precursor RNA, presumably polycistronic, with a higher mol.wt. than the value of approx. 1.3×10^6 and 0.7×10^6 reported in this paper.

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