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PRECURSORS OF CHLOROPLAST RIBOSOMAL RNA IN *EUGLENA GRACILIS*

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Key Word Index—*Euglena gracilis*; Chlorophyceae; *r*RNA precursors; chloroplasts; hybridization; rifampicin.

Abstract—Incorporation of ^{32}P into mature chloroplast *r*RNA species of MW 1.1×10^6 and 0.56×10^6 has been followed in *Euglena gracilis* by pulse and pulse chase experiments. Mature *r*RNA species have precursors of MW $1.16 \times 10^6 \pm 0.01 \times 10^6$ and $0.64 \times 10^6 \pm 0.01 \times 10^6$ resp. These precursors have base composition and hydridization properties similar to those of the mature *r*RNA species. No evidence of a single common precursor to these molecules was found. Rifampicin did not affect the synthesis of chloroplast *r*RNA.

INTRODUCTION

In eukaryotic cells, the two large cytoplasmic *r*RNA molecules (usually of MW 1.3×10^6 and 0.7×10^6 in plants [1] arise from the processing of a common high MW transcription product [2, 3]. This is the case in the synthesis of cytoplasmic *r*RNA in the alga *Euglena gracilis* [4].

In prokaryotes, such as *E. coli*, no large common precursor of both the 1.1×10^6 and 0.56×10^6 MW *r*RNA species has been found, but rather two individual precursor molecules, each slightly larger than the final product [5]. In the blue–green alga *Anacystis nidulans* two RNA molecules of MW slightly higher than the mature *r*RNA molecules have been found [6] and it was suggested that they are *r*RNA precursors. Studies of the rates of synthesis of the precursors in bacteria have been interpreted as providing evidence both for [7] and against [8] a common precursor molecule in prokaryotes.

In organelles the pathways of *r*RNA synthesis are not so well defined. In mitochondria of *Neurospora crassa* the *r*RNA is processed from a single large precursor of MW 2.4×10^6 [9]. In chloroplast preparations from *Nicotiana rustica* three rapidly labelled RNA molecules were found and pulse chase and inhibitor experiments suggested that one of these was a precursor for the *r*RNA of MW 1.1×10^6 and the other two were precursors for the *r*RNA of MW 0.56×10^6 [10]. Experiments with *Chlamydomonas reinhardi* were interpreted in the same way [11].

The synthesis of several rapidly labelled chloroplast RNA molecules has been described in both isolated spinach chloroplasts and whole spinach leaves [12], and kinetic evidence indicates that two of these molecules are precursors of chloroplast RNA. In spinach leaf discs the synthesis of two RNA molecules was stimulated by exposing the discs to light, and kinetic evidence indicated that these molecules are precursors of chloroplast *r*RNA [13].

In this report two rapidly labelled RNA molecules are described in chloroplasts of *E. gracilis*. In addition to kinetic experiments, studies of their properties provide direct evidence that they are separate precursors for the two large chloroplast *r*RNA molecules. A brief report of this work has been made [14], and subsequently similar kinetic experiments have been reported [15, 16].

RESULTS

Incorporation of ^{32}P into RNA of whole cells

The pathway of ribosomal RNA synthesis in *E. gracilis* has been described [4], and my results are similar in essential respects although I note some differences from the MWs quoted [4]. The general pathway can be summarized by the experiment shown in Fig. 1a. An extract of total RNA from dark-grown cells was treated with DNAse and fractionated by electrophoresis. The UV absorbance trace of the gel shows a small amount of RNA with MW of 2.4×10^6 and the main *r*RNA peaks with MW of 1.4×10^6 and 0.9×10^6, as well as a third species which has a MW of 1.1×10^6 and is a breakdown product of the *r*RNA of MW 1.4×10^6 [18]. When dark-grown *E. gracilis* cells were exposed to ^{32}P for 20 min, radioactivity appeared in several RNA peaks as shown in the histogram in Fig. 1a. The first *r*RNA precursor to appear has a MW of $4{\cdot}2 \times 10^6$ and can only just be seen in this experiment as it is already being masked by other radioactive material. It is not associated with detectable amounts of UV absorbance though there is radioactivity associated with all the other RNA species shown in the absorbance trace. The species of MW 4.2×10^6 is processed to the mature *r*RNA of MW 0.9×10^6 and another precursor of MW 2.4×10^6 [4]. Radioactivity appears slightly later in the other mature *r*RNA of MW 1.4×10^6 because of the relatively large pool of its precursor (MW of 2.4×10^6) (Scott, unpublished results) which may be up to 3% of the total cellular RNA.

Figure 1a shows no evidence of the chloroplast *r*RNA species of MW 1.1×10^6 and 0.56×10^6 [17], but these can be seen in the UV absorbance trace of RNA extracted from autotrophically-grown cells of *E. gracilis*

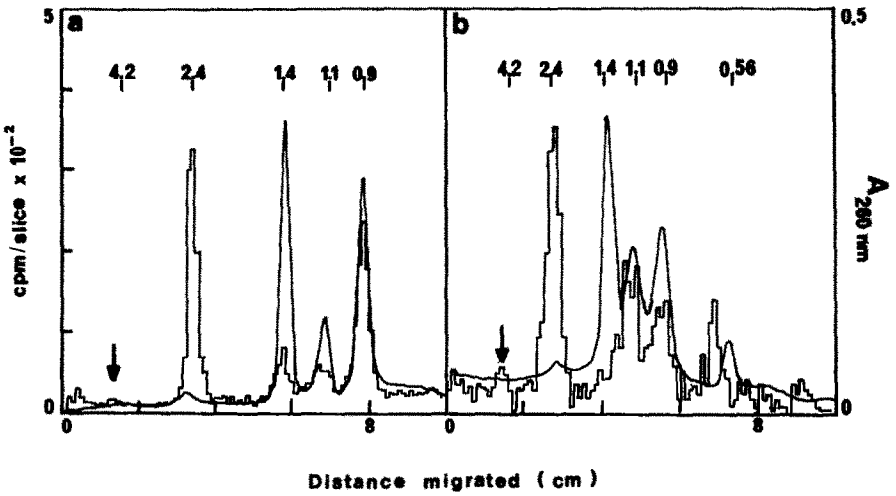

Fig. 1. Incorporation of ^{32}P in RNA of whole cells of *E. gracilis*. RNA was extracted from cultures of *E. gracilis*, which had been exposed to ^{32}P (2 μCi/ml) for 20 min, and fractionated by polyacrylamide gel electrophoresis as described in the text. The solid line shows the UV absorbance of the gels and the histogram the distribution of radioactivity along the gels. (a) RNA from dark-grown cells. (b) RNA from autotrophically-grown cells. The MW ($\times 10^{-6}$) of RNA species is also shown.

(Fig. 1b). Thus in this extract (Fig. 1b) the RNA peak of MW 1.1×10^6 has both a plastid and a cytoplasmic *r*RNA component. When these autotrophically-grown cells were exposed to ^{32}P for 20 min two ^{32}P-labelled RNA species which were not seen in dark-grown cells appeared, and are shown in the distribution of radioactivity in Fig. 1b. The first species has a slightly higher MW (see below) than the UV-absorbing peak containing the two *r*RNA species of MW 1.1×10^6 and results in a spreading of the radioactivity profile under the UV absorbancy profile. The second species has a MW higher than that of the chloroplast *r*RNA of MW 0.56×10^6.

Incorporation of ^{32}P into RNA of chloroplasts

In the same experiment as shown in Fig. 1b, chloroplasts were purified from the autotrophically-grown cells which had been incubated with ^{32}P for 20 min and the RNA, extracted from the chloroplasts, was fractionated by electrophoresis (Fig. 2a). The distribution of radioactivity along the gels is not coincident with the UV-absorbing peaks and forms two new distinct RNA species which are labelled with ^{32}P. At this stage there is little evidence of any incorporation of ^{32}P into mature chloroplast *r*RNA species. The mean of 10 determinations shows that the two new RNA species have MW of $1.16 \times 10^6 \pm 0.01 \times 10^6$ and $0.64 \times 10^6 \pm 0.01 \times 10^6$. After 2 hr incubation ^{32}P appears in the mature *r*RNA species as evidenced by the broadening of the radioactivity peak under the larger *r*RNA species (Fig. 2b), while for the smaller molecules the RNA of MW 0.64×10^6 and 0.56×10^6 are separated. After longer time periods ^{32}P accumulated in the mature *r*RNA species and the new RNA species could not be seen (Fig. 2c). The radioactivity and associated UV absorbing material at the top of the gels shown in Figs. 2c and 2d is DNA and could be removed by a brief DNAse treatment.

In some experiments a ^{32}P RNA molecule of MW 2.4×10^6 was observed in chloroplast preparations (Fig. 2d). The appearance of this species was also associated with the appearance of RNA of MW 0.9×10^6 both in the UV and radioactivity profiles of the gel. Comparison of these two RNA molecules with Fig. 1a suggests that they may well be cytoplasmic RNA contaminants of the chloroplast preparations and after isolation of these two components they could not be separated from the cytoplasmic RNA species by gel electrophoresis. In the experiment in Fig. 2d, 0.5 mm slices were cut from the gel and show the separation of the new ^{32}P–RNA species and the mature *r*RNA very clearly.

Autotrophically-grown cells of *E. gracilis* were incubated with ^{32}P (3 μCi/ml) for 15 min and then a 100-fold excess of unlabelled KH_2PO_4 was added to the culture to a concentration of 3.6 g/l. At the beginning of the chase period (Fig. 3a) the incorporation of ^{32}P into the new RNA species can be seen and after 2 hr (Fig. 3b) ^{32}P is evident in both the new RNA species and the mature *r*RNA species. By 4 hr almost all of the radioactivity is in the mature *r*RNA species and during the rest of the experiment the samples taken at 7, 11 and 24 hr (Figs 3d, e, f) show a slow decrease in the activity in the mature *r*RNA species.

These experiments suggest that there is a precursor product relationship between the RNA of MW 1.16×10^6 and 1.1×10^6 and also between the RNA of MW 0.64×10^6 and 0.56×10^6. The next experiments in this report are concerned with characterizing the precursor molecules.

Base compositions

The new chloroplast RNA molecules were isolated after 20 min incubation of the cells with ^{32}P as shown in Fig. 2a, and their base composition measured together with the base composition of steady-state labelled *r*RNA from chloroplasts; the results are shown in Table 1. The G + C content of all the plastid *r*RNA species is low and corresponds to the low G + C content of chloroplast ribosomal DNA cistrons [18]. The base ratios of the new RNA species are very similar to those of their corresponding *r*RNA species and allow for the loss of some bases during the processing of this precursor *r*RNA

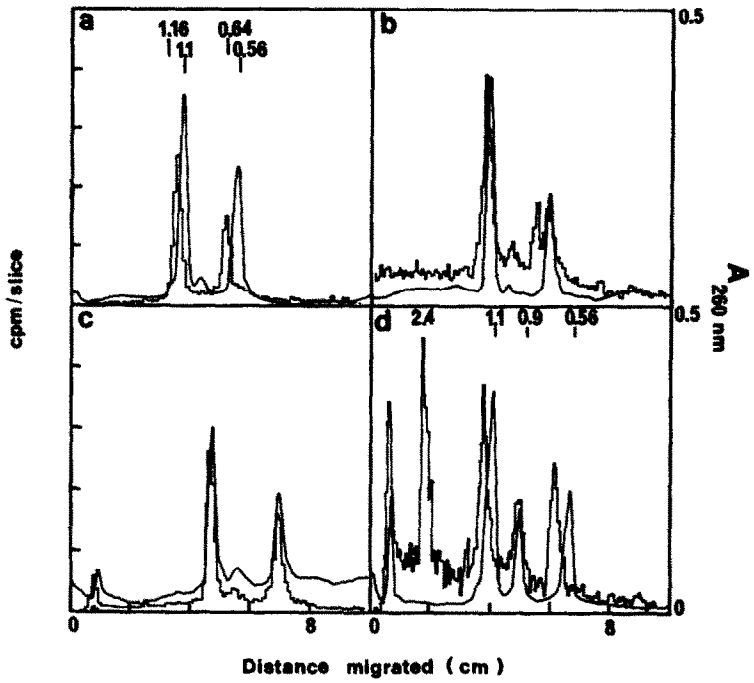

Fig. 2. Incorporation of ^{32}P into RNA from *E. gracilis* chloroplasts. RNA was isolated from chloroplasts of *E. gracilis* and fractionated by polyacrylamide gel electrophoresis. The solid line shows the UV absorbance of the gel while the histogram shows the distribution of radioactivity along the gel. The chloroplasts were isolated from autotrophically growing cells which had been exposed to ^{32}P (2 μCi/ml) for (a) 20 min; (b) 2 hr; (c) 12 hr (a, b and c are from the same experiment; (d) 20 min in a separate experiment. The scales on the y axis are (a), (b) and (d) 0–5000 cpm, (c) 0–30000 cpm.

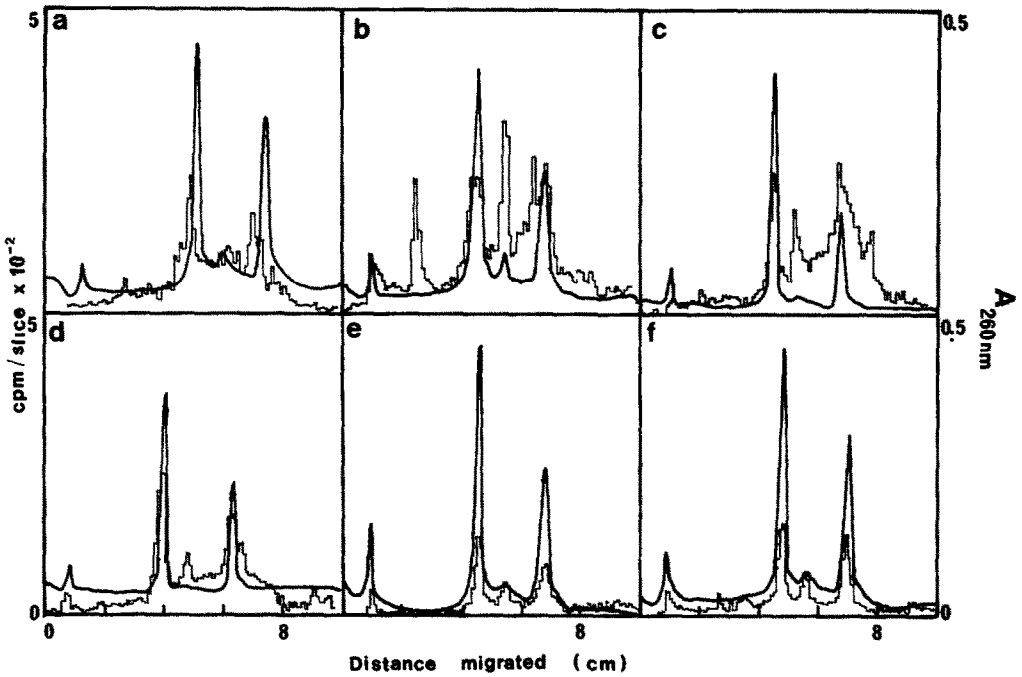

Fig. 3. Incorporation of ^{32}P into RNA from *E. gracilis* chloroplasts. After *E. gracilis* had been incubated for 15 min with ^{32}P (2 μCi/ml), KH_2PO_4) (3.6 g/l.) was added to the medium and RNA isolated from the chloroplasts at varying times after the addition. The solid line represents A at 260 nm and the histogram distribution of radioactivity along the gel. After the addition of KH_2PO_4 to the culture, the chloroplasts were isolated at (a) 0 hr, (b) 2 hr, (c) 4 hr, (d) 7 hr, (e) 12 hr, and (f) 24 hr.

Table 1. Properties of chloroplast RNA species

MW ($\times 10^{-6}$)	Base Composition (mol %) C	A	G	U
1.16 ± 0.01 (10)	12.2 ± 1.0	33.1 ± 0.4	30.1 ± 2.0	24.6 ± 1.6 (4)
1.1	14.3 ± 0.5	31.8 ± 0.4	27.6 ± 0.5	26.3 ± 0.5 (9)
0.64 ± 0.01 (10)	16.0 ± 0.2	29.7 ± 0.9	29.4 ± 0.2	24.5 ± 0.6 (4)
0.56	16.2 ± 0.5	29.5 ± 0.7	27.9 ± 0.5	26.1 ± 0.4 (13)

The figures shown are the means of the measurements together with the standard error. The figure in brackets is the number of measurements.

to *r*RNA, with the exception of the low cytosine content of the RNA of MW 1.16 × 10^6 which has 40 fewer cytosine residues than the mature *r*RNA of MW 1.1 × 10^6. The background of heterogeneous radioactivity in the gels (Fig. 2a) suggests that the purified molecules may be contaminated by up to 10% of ill-defined RNA species and this may introduce errors in the base ratio analysis. The base composition of these four RNA species does not correspond to the base composition of the bulk of the DNA of either nuclei or chloroplasts, emphasizing the close relationship between the new RNA species and the mature chloroplast *r*RNA.

Hybridization experiments

The new RNA species did not form measurable amounts of hybrid with DNA from dark-grown cells but did hybridize with chloroplast DNA preparations. Table 2 shows that the hybrids between the new RNA species and chloroplast DNA are only competed for by the homologous *r*RNA from chloroplasts and not by the *r*RNA from dark-grown cells. These experiments are not performed under ideal conditions since preparations of the new ^{32}P RNA species are contaminated with unlabelled *r*RNA. Nevertheless the trends are clear.

The cistrons for cytoplasmic *r*RNA in *E. gracilis* have a density of 1.717 g cm^{-3} on a CsCl gradient [18] compared with the average density of nuclear DNA of 1.707 g cm^{-3}, while the cistrons for chloroplast *r*RNA have a density of 1.696 g cm^{-1} compared with the main band of chloroplast DNA which has a density of 1.686 g cm^{-1} [18, 19]. Figure 4a shows that the two new RNA species also anneal to chloroplast DNA with a mean density of 1.696 g cm^{-3}.

In Fig. 4a the chloroplast DNA preparation contains about equal amounts of chloroplast and nuclear DNA, but Fig. 4b shows a preparation from which the nuclear DNA has been removed by preparative CsCl gradient fractionation. This procedure also selectively removes some of the chloroplast *r*RNA cistrons [18, 19], but it ensures that no cytoplasmic *r*RNA cistrons are in the preparation. The RNA of MW 2.4 × 10^6 found in the chloroplast preparation (Fig. 2d) did not show a specific peak of hybridization with DNA fractions from the CsCl gradient (Fig. 4b) although it did hybridize with DNA of a mean density of 1.717 g cm^{-3} isolated from dark-grown cells of *E. gracilis* (Fig. 4c). In contrast, the RNA of MW 1.16 × 10^6 isolated from the preparation shown in Fig. 2d hybridized with DNA of mean density 1.696 g cm^{-3} isolated from chloroplasts (Fig. 4b), but showed no peak of hybridization with DNA isolated from dark-grown cells. These results suggest that the RNA of MW 2.4 × 10^6 found in some chloroplast preparations is not a precursor of chloroplast *r*RNA, but the precursor of the cytoplasmic *r*RNA of MW 1.4 × 10^6 shown in Fig. 1a.

The effect of rifampicin on chloroplast rRNA synthesis

In the alga *Chlamydomonas reinhardii*, rifampicin inhibits both the formation of chloroplast *r*RNA *in vivo* and the activity of RNA polymerase [11, 20]. In *E. gracilis* the incorporation of ^{32}P into an RNA fraction was inhibited by 20 μg/ml of rifampicin in 1% DMSO and it was suggested that this also was an effect on chloroplast *r*RNA synthesis [21]. In *Nicotiana rustica* leaves rifampicin also inhibited chloroplast *r*RNA synthesis [10].

Table 2. Hybridization of chloroplast *r*RNA precursor

Precursor	Competitor	cpm in Hybrid
1.16	—	205 ± 19 (4)
	1.1	88 ± 17 (4)
	0.56	169 ± 15 (4)
	1.4 + 0.9	207 ± 23 (4)
0.64	—	200 ± 7 (4)
	1.1	169 ± 7 (4)
	0·56	127 ± 2 (4)
	1.4 + 0.9	186 ± 3 (4)

Chloroplast DNA (12.9 μg/filter) was hybridized with precursor ribosomal RNA (RNA of MW 1.16 × 10^6 3300 cpm/ml and RNA of MW 0.64 × 10^6 2700 cpm/ml) at an RNA concentration of 6 μg/ml for 2 hr at 70° in 6 × SSC [15]. Competing RNA species were added at a concentration of 6 μg/ml.

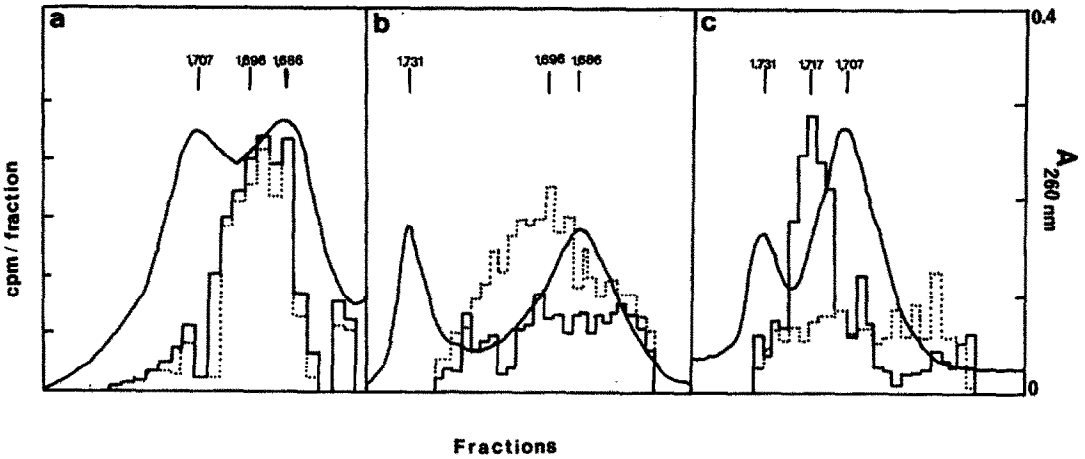

Fig. 4. Hybridization of chloroplast RNA species with *E. gracilis* DNA. DNA from *E. gracilis* was fractioned on a CsCl gradient and successive fractions from the gradient halved and each hybridized against a different RNA species as described in the text. The continuous solid line shows the distribution of DNA in the gradients and the histograms the distribution of radioactivity in the hybrids. *Micrococcus lysodeikticus* DNA ($\rho = 1.731\,g\,cm^{-3}$) was used as a marker in the CsCl gradients. (a) total DNA extracted from *E. gracilis* chloroplasts hybridized with RNA from the experiment shown in Fig. 2a, ----- 1.16×10^6 RNA, —— 0.64×10^6 RNA. (b) RNA of mean density $1.686\,g/cm^{-1}$ purified from *E. gracilis* chloroplasts hybridized with RNA from the experiment shown in Fig. 2d, ----- 1.16×10^6 RNA, —— 2.4×10^6 RNA. (c) DNA from dark-grown cells hybridized with RNA from the experiment shown in Fig. 2d, ----- 1.16×10^6 RNA, —— 2.4×10^6 RNA. The scale on the y axis in Fig. 4a is 0–300 cpm, and in Figs 4b and 4c 0–50 cpm.

Rifampicin did not affect the growth of cells or synthesis of chlorophyll in *E. gracilis* in the presence or absence of DMSO (Table 3). In the experiment shown in Table 3 ^{32}P was added to the culture 20 min after addition of the inhibitor and after 20 hr the RNA was isolated and fractionated by electrophoresis. In the gel patterns (not shown) there were no obvious differences between the treatments shown in Table 3; the sp. act. of the *r*RNA of MW 1.4×10^6 (cytoplasmic) and 0.56×10^6 (chloroplast) were determined and are shown in Table 3. The sp. act. vary from treatment to treatment over a two-fold range, probably because of the difficulties in controlling the separate cultures. But the ratio of sp. act. of the two *r*RNA species is constant throughout the experiment (Table 3), suggesting that rifampicin does not have a specific effect on chloroplast *r*RNA synthesis in *E. gracilis*. In work with Dr. R. E. Munns we have not found an effect of rifampicin on chloroplast RNA polymerase activity in *E. gracilis* or chloroplast development in general. Rifampicin did not affect chloroplast RNA synthesis in *E. gracilis* in another recent study [16]. It appears, that despite the earlier interpretation [21] rifampicin at these concentrations does not have an effect on chloroplast development in *E. gracilis*.

DISCUSSION

The study of *r*RNA synthesis in organelles is complicated by the difficulties involved in separating pure organelles and subsequently in isolating organelle RNA which is neither degraded nor contaminated with cytoplasmic *r*RNA. These difficulties are overcome in *E. gracilis* by the use of dark-grown cells, which allows the definition of cytoplasmic *r*RNA and its precursors, and by the purity and stability of chloroplast RNA prepared

Table 3. The effect of rifampicin on chloroplast *r*RNA synthesis

DMSO (%)	Rifampicin (μg/ml)	Cell No. ($\times 10^{-6}$/ml)	Chlorophyll (mg/10^9 cells)	Specific activities (cpm/μg) 1.4	0.56	$\frac{1.4}{0.56}$
—	—	0.84	5.5	124	88	1.4
1	—	0.77	5.2	94	73	1.3
1	20	0.86	5.4	103	80	1.3
1	200	0.79	6.0	56	46	1.2
	20	0.82	5.7	100	81	1.2
	200	0.71	5.9	64	56	1.1

Autotrophically-grown cells of *E. gracilis* (0.43×10^6/ml) were incubated with rifampicin and DMSO as indicated in the Table, and after 20 min 0.7 μCi/ml of ^{32}P was added to each culture. After a further 20 hr the cell density, chlorophyll content, and sp. act. of the *r*RNA molecules of MW 1.4×10^6 and 0.56×10^6 were determined.

from autotrophically-grown cells. The experiments in Figs. 1 and 2 show the existence of two new RNA species in *E. gracilis* chloroplasts and the time course and pulse chase experiments (Fig. 2 and 3) suggest a precursor-product relationship between the two new RNA species and the mature *r*RNA species.

The base ratio analyses and the hybridization data shown here could be obtained if there were significant amounts of ^{32}P in the mature *r*RNA species. It is apparent that after incubation of the cells with ^{32}P for 20 min, the amount of ^{32}P in the mature *r*RNA species is less than 10% of the amount in the new RNA species, although the exact amount cannot be determined from these experiments. This amount of ^{32}P in the mature *r*RNA species could not explain these results. Therefore the base ratio analyses and the hybridization data can best be explained by a precursor product relationship between the RNA species of MW 1.16×10^6 and 1.1×10^6 and between the species of MW 0.64×10^6 and 0.56×10^6.

In a study of *r*RNA synthesis in chloroplasts of *Nicotiana rustica* Munsche and Wollgiehn [10] found two new RNA species which could be precursors of the chloroplast *r*RNA of MW 0.56×10^6 while in *E. gracilis* only one is found. This may represent a species difference, and in other respects the results presented in the first part of this paper are similar to those obtained in the *N. rustica* system. In spinach chloroplasts, Hartley and Ellis [12], found the new RNA molecules of MW 1.2×10^6 and 0.65×10^6 and the kinetics of labelling of these two species was consistent with their being *r*RNA precursors. In addition they showed the synthesis of an RNA species of MW 2.4×10^6 in an *in vitro* preparation of chloroplasts but did not suggest a function for this molecule. Detchon and Possingham [13] also suggest that there is only one precursor for each of the mature chloroplast *r*RNA molecules in spinach. The results presented here show that similar RNA molecules, and labelling patterns are found in *E. gracilis*, an algal species. In addition the experiments reported here provide direct evidence in the form of base ratio analyses and hybridization experiments of the relationship between the precursor *r*RNA molecules and the mature *r*RNA.

The studies discussed here, including my own, provide no direct evidence for the existence of a common large precursor to the two chloroplast *r*RNA species. If there is such a molecule it may not have been detected either because of a short half life or because it is partly processed before completion.

An association between the chloroplast membranes and the precursor *r*RNA species has been reported [10] and it was suggested this association might be related to an association of the transcription of chloroplast DNA with chloroplast membranes. In unpublished experiments in which *E. gracilis* chloroplasts were extracted with successively higher concentrations of detergents I have found no clear evidence on this point. These experiments also provided negative evidence for the absence of a large common precursor to chloroplast *r*RNA.

In the four prokaryotic types of systems which have been studied, bacteria, blue–green algae, chloroplasts and mitochondria only in the mitochondria of *Neurospora crassa* [9] has a large precursor molecule been found. While the two mature chloroplast *r*RNA species of MW 1.1×10^6 and 0.56×10^6 are transcribed from separate DNA sequences clustered in the same part of the chloroplast DNA [18], identification of the primary transcription products of these sequences is needed, to determine if they are transcribed separately or as a unit.

EXPERIMENTAL

Euglena gracilis strain Z was grown autotrophically [17] except that the concentration of KH_2PO_4 in the medium was reduced to 36 mg/l. The cells were grown at a light intensity of 4000 lx to a density of 1.8 to 2×10^6 cells/ml before adding ^{32}P (Pi in dil HCl, 10–25 Ci/mg P, A.A.E.C., Sydney). Under the culture conditions employed, cell growth was then entering the stationary phase and the incorporation of ^{32}P into RNA was slowed and consequently the synthesis and processing of *r*RNA precursors was easier to follow, particularly when the experiments involved preparation of chloroplasts. After 24 hr the cell number was 3.0 to 3.5×10^6/ml. Chloroplasts were prepared from these cells as described in ref. [22]. RNA was extracted from whole cells or chloroplasts suspended in 5–10 vol of 0.1 M Tris pH 8, 0.2 M NaCl, 0.002 M $MgCl_2$, and 0.2 mM EDTA. SDS was added to 2% and the aq phase extracted once (twice for whole cells) with 80% pHOH containing 10% M-cresol and 0.1% 8-hydroxyquinoline. The RNA was precipitated from the aq phase by the addition of 2 vol of 95% EtOH containing 0.01 M Mg acetate and 0.2% SDS. Before electrophoresis, RNA extracted from whole cells was treated with DNAse (electrophoretically purified, Worthington Biochemicals), 5 μg/ml for 10 min at 0°. The RNA was fractionated by polyacrylamide gel electrophoresis using *E. coli* *r*RNA as a standard, that from whole cells in a buffer system containing 2 mM Mg acetate [23], and that from chloroplasts as described in ref. [24]. After the A of the gels was scanned at 260 nm, Indian ink markers were injected into the gels at the UV-absorbing peaks and the gels scanned again. Gels were sliced and the slices dried and counted in a fluor containing 0.2 g of *p*-bis-2,5-diphenyloxazole and 3 g 2,5-diphenyloxazole in 1 l. of toluene. The distribution of radioactivity in the gels was plotted directly onto the A traces. The slices containing Indian ink were used to line up the absorbancy and radioactivity profiles and in this way the error involved in aligning the radioactivity histograms and the A traces was less than 0.5 mm. Chloroplast DNA was prepared and fractionated and hybridization reactions were measured as described in ref. [18]. RNA was recovered from polyacrylamide gels by eluting the centre portions of the required peaks [18] and the base ratios were determined after hydrolysis of the RNA and high voltage electrophoresis [25].

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