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Chloroplast RNA Populations in Dark-Grown, Light-Grown, and Greening *Euglena gracilis*

(polyacrylamide gel electrophoresis/DNA–RNA hybridization–competition)

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ABSTRACT **RNA preparations from dark-grown, light-grown, and greening *Euglena gracilis* have been compared by polyacrylamide gel electrophoresis and by hybridization to *Euglena* chloroplast DNA. Chloroplast ribosomal RNA is not detected in dark-grown cells; its abundance increases in greening cells over a 72 hr period until the concentration characteristic of light-grown cells is reached. Other RNA species complementary to chloroplast DNA are present in comparable abundance in light-grown, dark-grown, and greening cells.**

Euglena gracilis grown in the dark contains small proplastids (about 1 μm in diameter) with surrounding double membranes, but that lack inner structure (1). After transfer into the light, the components of the photosynthetic apparatus are synthesized and assembled. During a 72 hr period, chlorophyll content, the ability to fix carbon dioxide, and the internal plastid structure all develop to the levels of light-grown cells (1, 2).

We have previously identified, by polyacrylamide gel electrophoresis, short-lived RNA species synthesized in dark-grown *Euglena* as precursors to the cytoplasmic ribosomal RNA (3). With the techniques developed for this analysis, it became possible to ask whether any such species are synthesized as precursors to the chloroplast ribosomal RNA and whether discrete species are synthesized as part of the response of dark-grown *Euglena* to the light. However, we have been unable either to detect precursors to the chloroplast ribosomal RNA or to find discrete species other than ribosomal RNA that are preferentially synthesized in response to light. The failure to detect such species could be due to the large number of high molecular weight RNAs synthesized in the nucleus. We have, therefore, studied the synthesis of new chloroplast RNA species by DNA–RNA hybridization. This technique has defined in some detail the transcription program during the infection of *Escherichia coli* and *Bacillus subtilis* by bacteriophages T4 and SP01 (4, 5). However, we have found no differences in the populations of chloroplast DNA transcripts in dark-grown, light-grown, or greening *Euglena* by this technique. Chloroplast ribosomal RNA is the only chloroplast RNA species whose abundance can be shown to increase during the greening process.

MATERIALS AND METHODS

Euglena gracilis, strain Z, was grown and labeled with ^{32}P (3). The isolation of total cellular RNA, analysis of RNA on polyacrylamide gels, and DNA–RNA hybridization conditions were as described (3). Chloroplasts were isolated on Renografin gradients (Brown, R.D., and R. Haselkorn, manuscript in preparation) and chloroplast DNA was prepared as described (3).

Under the hybridization conditions used, some material in the RNA preparations adsorbed nonspecifically to nitrocellulose filters. This background ranged from 0.25–0.5% of the input radioactivity and masked the *bona fide* hybridization of RNA–DNA. We found that the contaminating material could be removed by centrifugation of the RNA in Cs_2SO_4 gradients. The following step-gradient was constructed: 3 ml of saturated Cs_2SO_4 was placed into a 1.3 × 5.1 cm cellulose-nitrate tube, followed by 2 ml of Cs_2SO_4 of density 1.560. Over this, a solution containing 1–5 mg of RNA in 0.1 × SSC* was layered. After centrifugation at 50,000 rpm in an SW 65 rotor at 25°C for 5 hr, the RNA was found in a tight band between the two Cs_2SO_4 layers. The contaminating material remains above the lighter Cs_2SO_4 solution. RNA was then withdrawn from the gradient with a Pasteur pipette and dialyzed against two 1-liter changes of 0.1 × SSC.

RESULTS

As has been demonstrated, to the limit of detection on polyacrylamide gels or sucrose gradients, no chloroplast ribosomal RNA is present in dark-grown *Euglena* (3, 6–8). Therefore, we first studied the time course of synthesis of this RNA during development of the photosynthetic apparatus in the light.

Figs. 1 and 2 illustrate the synthesis of ribosomal RNA after transfer of dark-grown *Euglena* into the light. In Fig. 1, the total RNA present at various times after the transfer is shown in the form of absorbance profiles across polyacrylamide gels. The analysis of the gels by autoradiography in Fig. 2 illustrates those species that have been labeled during a 2-hr pulse at each time point. Synthesis of chloroplast ribosomal RNA appears to begin immediately after transfer into the light, with a small amount of 0.55 RNA (RNA of molecular weight 5.5×10^5) detectable in the first 2 hr after transfer. The rate of synthesis increases slowly, until 24 hr after transfer. By this time the total RNA present in the culture appears very similar to that previously demonstrated in cells fully grown in the light (3).

It has been demonstrated that a minor breakdown of the 1.35 RNA occurs to yield an RNA species that migrates on polyacrylamide gels at the position of chloroplast ribosomal RNA of 1.1×10^6 molecular weight. A comparison of the total RNA present in dark-grown cells (Fig. 1*a*) with the RNA labeled during a 2-hr pulse in the dark (Fig. 2*a*) suggests that the cytoplasmic rRNA of molecular weight 1.1×10^6 might arise *in vivo* after the cytoplasmic RNA (1.35×10^6) has been in the cytoplasm for longer than this 2-hr period. The presence of cytoplasmic RNA (1.1×10^6) makes it difficult to observe the

* 0.15 M NaCl–0.015 Na_3 citrate.

appearance of chloroplast RNA (1.1×10^6). Most likely, the best estimate of the synthesis of chloroplast RNA of molecular weight 1.1×10^6 can be obtained from Fig. 2, which demonstrates no cytoplasmic RNA of this molecular weight labeled in dark-grown culture.

The large stimulation of ribosomal RNA synthesis by transfer into the light suggests a much greater need for protein synthesis in the developed chloroplast than in the proplastid. It is natural, then, to ask whether differences can be observed in the messenger RNA of light-grown and dark-grown cells. Fig. 3 illustrates the titration of chloroplast DNA with RNA isolated from cells labeled under various conditions. RNA from cells (either light-grown or dark-grown) that were labeled for 2 hr, whose label was then chased into stable species by growth in the absence of ^{32}P for two generations, saturates chloroplast DNA at low RNA concentrations. This is the expected result for species that are present in high concentration in the RNA, and is the result observed when chloroplast DNA is saturated with chloroplast ribosomal RNA (10). When cells have been labeled for 2 hr but not chased, the titration is very different; within the concentration range studied, no saturation is observed. Together, these results indicate the presence of unstable RNA complementary to chloroplast DNA. The failure to saturate chloroplast DNA with unstable RNA at these concentrations is easily understood. The RNA preparations isolated from whole cells contain only a small proportion of their label in chloroplast RNA species if the rate and frequency of transcription of nuclear and chloroplast DNA are the same. Therefore, at the RNA concentrations used, there is probably less chloroplast RNA present in the hybridization mixture than there is chloroplast DNA on the filter. Under these conditions, the relative amount of RNA hybridizing to a particular nucleotide sequence in DNA depends on the relative abundance of the corresponding transcripts. It will be demonstrated that this limitation does not affect the conclusions to be drawn from these experiments.

A widely used technique for testing the fidelity of hybridization has been to observe the release of RNA from hybrid by melting (11). When hybrids made with pulse-labeled RNA from light- or dark-grown cells are melted, the result in Fig.

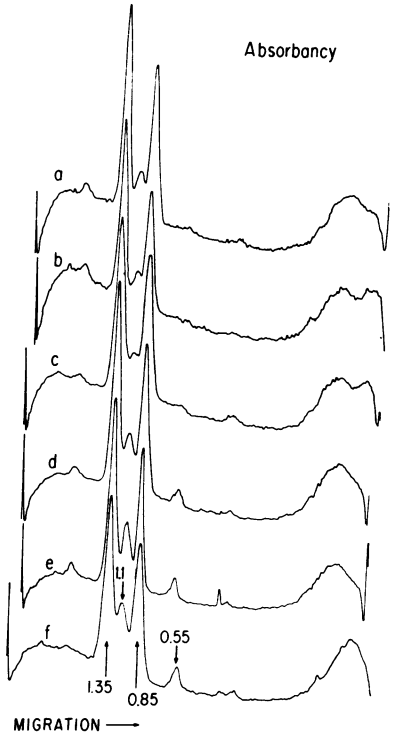

FIG. 1. Polyacrylamide gel electrophoresis of *Euglena* RNA isolated during a shift from growth in the dark to growth in the light. Cells initially at a density of 1.5×10^6 per ml were diluted over the course of the experiment so that their density remained in the range of $1–2 \times 10^6$ per ml. 2 hr before the isolation of a cell sample, 10 μCi/ml of ^{32}P was added to an aliquot of cells. After isolation of a sample of cells grown in the dark, the remainder of the culture was illuminated with 350 cd of light. Samples of the culture were removed after (*a*) 0 hr, (*b*) 2 hr, (*c*) 10 hr, (*d*) 26 hr, (*e*) 50 hr, and (*f*) 74 hr in the light. RNA was isolated, and 20–25 μg of each sample was electrophoresed for 4 hr at 10 mA/gel. After electrophoresis, gels were scanned at 265 nm in a Gilford spectrophotometer with a linear-transport accessory. The RNA species designated 1.35 and 0.85 are cytoplasmic ribosomal RNA components, while those designated 1.1 and 0.55 are chloroplast ribosomal RNA components (3). The designations refer to molecular weight (e.g., the 1.35 component has a molecular weight of 1.35×10^6, etc.).

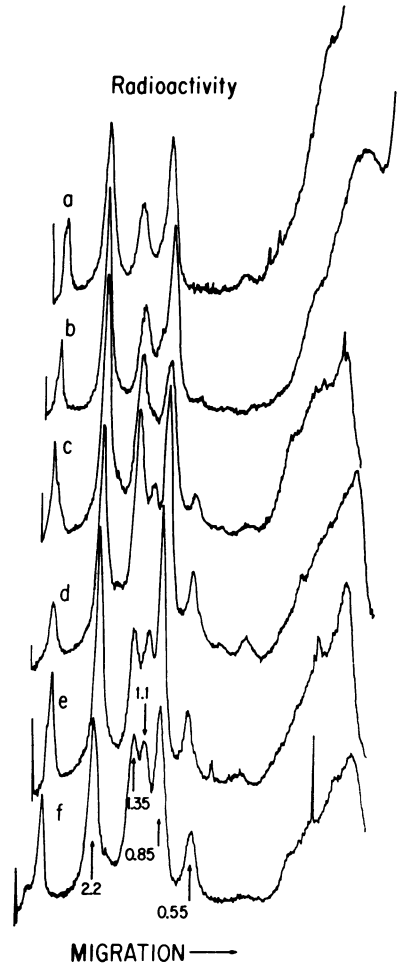

FIG. 2. Polyacrylamide gel electrophoresis of RNA isolated during a shift from growth in the dark to growth in the light. Autoradiograms of the gels described in the legend to Fig. 1 were prepared and analyzed (3). RNA indicated as 2.2 is a precursor, of molecular weight 2.2×10^6, to the cytoplasmic ribosomal RNA (3).

4 is obtained. This result is unusual in that a significant proportion of the "hybrid" is released at temperatures below 50°C. The remainder of the RNA melts in a quite reasonable manner. The distribution of the melting is broad, but at least a part of this range must be due to variations of nucleotide composition of different DNA–RNA hybrid species. The material that melts at low temperature does not adsorb to filters that lack DNA. This material might consist of short oligoribonucleotide or oligodeoxyribonucleotide segments. In the experiments that follow, all filters were treated at 50°C in 1 × SSC to remove this contaminant. This treatment removed 20–40% of the initial "hybrid" in different experiments.

An analysis of pulse-labeled RNA preparations by hybridization to chloroplast DNA, and competition with homologous and heterologous unlabeled RNA preparations, is shown in Fig. 5. All of the species labeled in the light are completely competed against by RNA isolated from either light- or dark-grown cells. Similarly all of the species labeled in the dark are competed against by either unlabeled RNA preparation. A third competition experiment, illustrated in Fig. 5, is the competition of each of these labeled preparations by unlabeled RNA isolated from *Euglena* strain ZHB. This strain lacks chloroplast DNA and, presumably, also lacks chloroplast DNA transcripts. Strain ZHB was produced by growth of *Euglena* at 34°C by H. Lyman (State University of New York at Stony Brook), who generously provided it.

RNA from strain ZHB competes with a fraction of the RNA hybridized to chloroplast DNA. This apparently anomalous competition may be explained as a consequence of heterologous hybridization. That is, an RNA preparation may contain

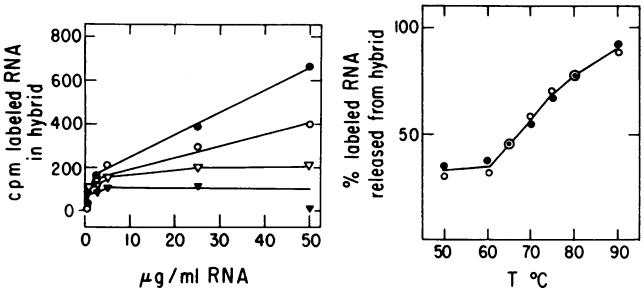

FIG. 3 (Left). Hybridization of total cellular RNA with chloroplast DNA. Filters containing 1 μg of adsorbed chloroplast DNA were annealed at 70°C for 16 hr with increasing concentrations of RNA containing 2–5000 cpm of ^{32}P per μg. Backgrounds were 0.05–0.2% of the input ^{32}P. RNA was labeled 2 hr in the light (●–●); 2 hr in the dark (O–O); 2 hr in the light followed by 24 hr of growth in the light in the absence of ^{32}P (▼–▼); or 2 hr in the dark followed by 24 hr of growth in the dark in the absence of ^{32}P (▽–▽). The initial cell concentration was 1.0×10^6/ml.

FIG. 4 (Right). Removal of RNA from DNA–RNA hybrids by melting. Cells at an initial concentration of 1.7×10^6 were labeled with 10 μCi/ml of ^{32}P for 2 hr. RNA was isolated and hybrids were prepared with 25 μg/ml of labeled RNA annealed with 1 μg of chloroplast DNA per filter. After the standard washing procedure and RNase treatment (3), the filters were dried and counted. The filters were then removed from scintillator fluid, dried, and heated to 50°C in 2 ml of 1 × SSC before being dried and counted again. This procedure was repeated, with the temperature being raised in 5°C intervals. (O–O) RNA labeled in the light; (●–●) RNA labeled in the dark.

labeled molecules that are nuclear DNA transcripts, but are sufficiently similar to chloroplast DNA to anneal with it. These sequences would then be competed against by identical unlabeled sequences in ZHB-RNA. In various RNA preparations, the fraction of hybrid competed by ZHB-RNA range from 10 to 50%. The reason for this variation is not known. It is presumed that at least that fraction of hybrid which is not competed against by ZHB-RNA corresponds to true chloroplast DNA transcripts. Most of these transcripts of chloroplast DNA are present in comparable abundance in light-grown and dark-grown cells.

The remaining condition under which the synthesis of new species of RNA might be expected to be observed is that of the shift from dark growth to light growth. RNA was isolated after cultures were labeled for 2-hr periods at various times after transfer from dark to light. The results of the competition for hydridization of these RNAs by unlabeled RNA isolated from dark-grown cells are shown in Fig. 6. In these experiments, 250 μg of unlabeled ZHB-RNA was added to each hybridization mixture to eliminate the heterologous hybridization described above. At no time after the transfer from dark to light growth is there significant transcription of chloroplast DNA sequences whose transcripts are not already present in the dark.

DISCUSSION

Two principal observations are reported in this work. First, the synthesis of chloroplast ribosomal RNA in *Euglena*, after transfer from dark to light, appears to follow roughly the same time course as that reported for the development of the photosynthetic apparatus (1, 2). Second, there is no measurable difference in the transcription of chloroplast DNA during the light-induced development of the chloroplast, other than the increase in chloroplast ribosomal RNA synthesis.

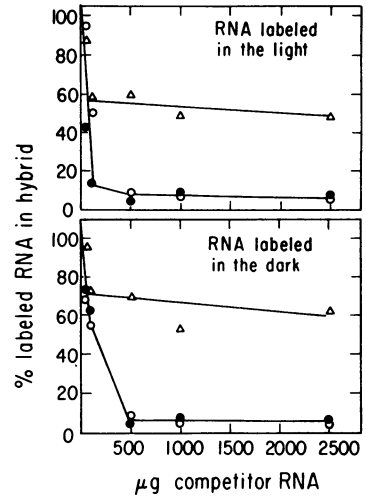

FIG. 5. Competition of labeled RNA preparations by homologous and heterologous RNA. Filters containing 1 μg of chloroplast DNA were annealed with 25 μg of RNA (about 500,000 cpm) labeled and isolated as described in the legend to Fig. 4, together with increasing amounts of unlabeled RNA isolated from light-grown (O–O) or dark-grown (●–●) wild-type cells or light-grown *Euglena gracilis* strain ZHB (▽–▽). Filters were then washed, treated with RNase, and incubated at 50°C for 10 min in 1 × SSC before being dried and counted. Filters annealed in the absence of competitor contained 3000 cpm in hybrid.

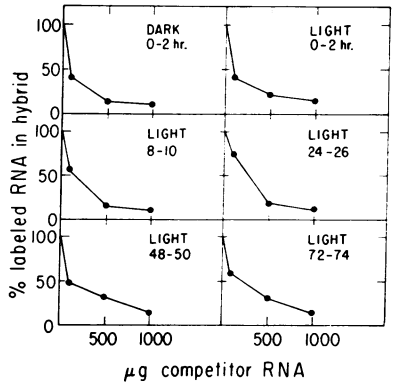

FIG. 6. Hybridization and competition of RNA labeled after a shift from growth in the dark to growth in the light. RNA preparations were identical to those described in the legend to Fig. 1. Filters containing 1 μg of adsorbed chloroplast DNA were annealed with 25 μg of each labeled RNA (3–500,000 cpm), 250 μg of unlabeled RNA from strain ZHB, and different amounts of unlabeled RNA from dark-grown wild-type cells. Filters annealed in the absence of competitor contained 1500–2500 cpm in hybrid.

While the first of these results is straightforward and requires little comment, the second must be examined more carefully for its possible limitations. Our hybridization–competition experiments were done with RNA concentrations for which DNA was in excess of most RNA species in the absence of competitor. The extent of competition by excess unlabeled RNA is determined by the fraction of radioactivity in RNA species that are common to the labeled and unlabeled RNA (not by the fraction of genes whose transcription is common to the labeled and unlabeled RNA). The competition of all hybridized RNA by excess unlabeled RNA, however, establishes the identity of the RNA species in labeled and unlabeled preparations and, thus, the identity of the genes transcribed to form those RNA populations.

The problems associated with heterologous hybridization should not be overlooked. RNA from *Euglena gracilis* strain ZHB, which lacks chloroplast DNA, will compete with a significant fraction of the RNA hybridized to chloroplast DNA. This competition is not unexpected: at the concentrations of RNA used in this experiment, purified cytoplasmic ribosomal RNA (known to be nuclear DNA transcripts) will saturate about 3% of the chloroplast genome (Rawson, J. R., and R. D. Brown, unpublished results). It is quite likely that this hybrid is competed with by ZHB-RNA. It cannot be ruled out, however, that some of the remaining hybrid observed is also heterologous and is due to labeled RNA species from wild-type cells that are not present in ZHB cells. Also, the labeled RNA contains a saturating amount of cytoplasmic ribosomal RNA and, thus, the true chloroplast RNA species that hybridize to the same parts of the chloroplast genome as the heterologous RNA species are probably ignored.

With these limitations in mind, possible models for the unction of the chloroplast genome may be considered. A model in which chloropoast development is determined by the control of transcription would have the following properties. The chloroplast genes responsible for most or all of the chloroplast transcripts code for components of the chloroplast whose synthesis is not regulated by light, and are independent of the developmental state of the chloroplast. Possibly the proteins coded by these genes include chloroplast DNA and RNA polymerases, ribosomal proteins, ribosomal translation factors, and those components of the membrane that are present in the proplastid. The only genes in chloroplast DNA whose transcription is known to be regulated by light are those for chloroplast ribosomal RNA, which comprise 1% of the chloroplast DNA (10). Other genes for those chloroplast components induced by light and characteristic of the developed chloroplast would then be contained in the nucleus. Finally, a prediction that can be made from this model is that chloroplast ribosomes are present in dark-grown cells to translate those chloroplast DNA transcripts necessary for the maintenance of chloroplast integrity. The new chloroplast ribosomes synthesized in response to light must then be involved in translating nuclear messages.

The existence of translational control would modify this model considerably. If control were at the level of translation, then any or all of the photosynthetic chloroplast components could be coded for by the chloroplast genome, with translation lacking in cells growing in the dark. The initial slopes of the hybridization–competition experiments (Fig. 5) indicate, however, that the abundance of chloroplast transcripts is similar in the light and in the dark. If control is at the level of translation, then it is necessary to postulate that chloroplast message is continuously transcribed at the same rate in the dark and in the light and that the stability of this message is independent of its translation.

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