The Sites of Transcription and Translation for Euglena Chloroplastic Aminoacyl-tRNA Synthetases
(organelles/chloroplast isolation/streptomycin/cycloheximide)

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ABSTRACT We find that cycloheximide completely blocks the light-induced appearance of Euglena chloroplastic aminoacyl-tRNA synthetases in dark-grown cells of Euglena gracilis var. bacillaris. Streptomycin, on the other hand, has no effect on the light-induction of these organellar enzymes. These observations, together with the finding that an aplanthidic mutant (strain W4BUL, which has neither significant plastid structure nor detectable chloroplast DNA) contains low levels of the chloroplastic synthetases, indicate that the chloroplastic synthetases are transcriptional products of nuclear genes and are translated on cytoplasmic ribosomes prior to compartmentalization within the chloroplasts.

The chloroplastic aminoacyl-tRNA synthetases (EC 6.1.1.17) of Euglena have been shown to be quantitatively under the control of light (1) in that exposure of dark-grown cells to light results in a marked increase in the level of these organellar-specific enzymes. In the present report, we have taken advantage of this light inducibility as well as of the techniques developed by Schiff et al. (2, 3). These techniques permit the study of chloroplast development in the absence of such complicating factors as cell growth and cell division in order to determine the site of translation of chloroplastic synthetases. More specifically, we examined the effect of streptomycin (4–6) and cycloheximide (7), which block protein synthesis on organelle (68S) and cytoplasmic (87S) ribosomes, respectively, on the light induction of chloroplastic synthetases in “resting” or nondividing dark-grown Euglena.

We observe that, while cycloheximide completely blocks induction, streptomycin has no effect upon induction of chloroplast synthetases. In addition, we find as indicated previously (1), that low levels of the chloroplast synthetases are present in strain W4BUL (3, 8–10), a mutant strain that lacks both detectable chloroplast DNA and chloroplast structure. Thus we interpret our results to indicate that the chloroplast-specific aminoacyl-tRNA synthetases are translated on cytoplasmic ribosomes from transcriptional products of the nuclear genome, and are subsequently compartmentalized within the organelle, as has also been suggested for the NADP+-dependent dehydrogenase and alkaline DNase of Euglena (11–13).

Abbreviation: Synphen, chloroplast phenylalanyl-tRNA synthetase.
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MATERIALS AND METHODS

Strains and Growth Conditions. Dark-grown Euglena gracilis Klebs var. bacillaris Pringsheim was grown, rested, and illuminated as described previously (2), except that dark-grown cells were grown in Hutner’s pH 3.5 medium and were transferred directly to pH 6.8 resting medium. For studying the effects of inhibitors of protein synthesis, cycloheximide (11) was added to a final concentration of 15 μg/ml immediately before exposure to light, or streptomycin sulfate (4) was added to a final concentration of 0.5 mg/ml 10 hr before exposure of the resting cells to light. For preparation of chloroplasts and tRNA, cells were grown as previously described by Goins et al. (14), except that the cells were bubbled gently with air instead of CO2.

Preparation of tRNA. Light-grown Euglena cells in the logarithmic phase of growth were harvested in a Sharples centrifuge at 4°C, and tRNA was prepared essentially by the methods of Holley et al. (15) as described by Fairfield and Barnett (16).

Preparation of Aminoacyl-tRNA Synthetases. Soluble protein fractions containing synthetase activities were prepared according to the methods of Barnett et al. (17).

Assays. The acylation reaction mixture contained (in addition to enzyme) per ml: 50 μmoles of Bicine [N,N-bis(2-hydroxyethyl)glycine] buffer (pH 8.5), 10 μmoles of magnesium acetate, 5 μmoles of mercaptoethanol, 1.0 μmole of ATP, 6 μCi of [14C]amino acid, 500 pmoles of each of the other unlabeled amino acids, and 20 A200 units of tRNA. Reactions were performed at 30°C, and for the assay of phenylalanyl-tRNA synthetase activity, the reaction was terminated after 5 min; for valyl-tRNA synthetase activity, the reaction was terminated after 20 min. Under these conditions aminoacyl-tRNA formation is linearly proportional to enzyme concentration. Radioactive aminoacyl-tRNA was determined by a modification (18) of the filter-paper-disc method of Bollum (19).

Isolation of Chloroplasts. Cells in the logarithmic phase of growth were harvested in a Sharples centrifuge. The cells (about 600 g) were washed once in 3:5:1 buffer (20) (sorbitol, 3% w/w; sucrose, 5% w/w; Ficoll, 1% w/w) by centrifugation, then resuspended in five volumes (w/w) of 3:5:1 buffer, and broken at 2000 lb/inch² by passage through a Gaulin press and filtered through glass wool on a Buchner funnel.
Fig. 1. Hydroxyapatite chromatography of Euglena phenylalanyl-tRNA synthetase. Soluble protein (about 50 mg), prepared from light-grown cells as previously described (1), was applied to a 1 × 20-cm column of hydroxyapatite, and 10-ml fractions were eluted with a linear 1-liter gradient of 0.0005–0.25 M potassium phosphate (pH 7.0) containing 0.01 M 2-mercaptoethanol and 10% glycerol. Aliquots (0.023 ml) were assayed, as described in Methods, for phenylalanyl-tRNA formation in a total reaction volume of 0.125 ml. In all figures where factors are given on the ordinates, the convention is that the experimental data have been multiplied by that factor to obtain the numbers on the ordinate.

The resulting filtrate was centrifuged for 5 min at 500 × g, and the pellet was resuspended in 2.5 volumes of 3:5:1 buffer, passed through the Gaulin press again, and centrifuged at 500 × g. The combined 500 × g supernatants were then centrifuged at 2500 × g for 10 min in a Sorvall RC2B centrifuge (GSA head). The 2500 × g pellet was resuspended in 2500 ml of 6:5:2% (w/v) sorbitol:sucrose:Ficoll buffer and loaded on a K-11 zonal rotor with a K-10 core (spinning at 6000 rpm) at 100 ml/min. The chloroplasts which were thus trapped were spun (7 hr) to equilibrium at 35,000 rpm on a 5.5-liter linear gradient from 6:6:6% (w/v) sorbitol:sucrose:Ficoll to 14:14:14% (w/v) supported on a 1.2-liter cushion of 55% (w/v) sucrose. [The chloroplasts band at approximately 10:10:10% (w/v) sorbitol:sucrose:Ficoll.] The gradient was unloaded from the bottom and 300-ml fractions were collected; those containing chloroplasts were diluted 1:1 with 3:5:1 buffer and the plastids were collected by centrifugation.

RESULTS AND DISCUSSION

In a previous report (1), we identified the chloroplast phenylalanyl-tRNA synthetase (SynPhe) as the more rapidly eluting of the two major SynPhe activities observed upon hydroxyapatite chromatography of whole-cell extracts of light-grown Euglena B (see Fig. 1). We have since found (Fig. 2a), however, that through the use of considerably more shallow gradients this “chloroplastic” SynPhe activity can be resolved into two chromatographically different enzymes, of which the major is the chloroplastic species shown in Fig. 2b. The smaller, most rapidly eluting activity has been found to be the mitochondrial SynPhe. It should also be noted that the cytoplasmic SynPhe is not eluted from hydroxyapatite under the conditions used in Fig. 2 and consequently is not observed.

In Fig. 3 it may be seen that exposure of dark-grown resting cells to light results in a rapid increase in the level of SynPhe. The kinetics of induction appear in a more quantitative
Fig. 4. Effect of streptomycin and cycloheximide on the light-induction of chloroplastic phenylalanyl-trNA synthetase. Dark-grown resting cells were grown as described in Methods and exposed to light in the presence of 0.5 mg/ml of streptomycin (O); in the presence of 15 µg/ml of cycloheximide (Δ); or in the absence of antibiotic (●). Phenylalanyl-tRNA synthetase levels were determined by hydroxyapatite chromatography as described in Methods and in Fig. 3. Cell number and total extractable soluble protein per cell remained constant throughout the course of the experiment. One synthetase unit is defined as that amount of enzymatic activity that forms 1 pmole of aminoacyl-tRNA per mg of soluble protein in 1 min under the conditions described in Methods and Fig. 1.

fashion in Fig. 4, in which the effects of streptomycin and cycloheximide upon enzyme induction are also shown. It is apparent from these observations that streptomycin has no effect upon induction, whereas cycloheximide completely blocks the light-induced appearance of this chloroplastic enzyme.

Precisely the same is true for SynVaL. Fig. 5 shows the two SynVaL activities that are resolved by hydroxyapatite chromatography of whole-cell extracts from light-grown cells, as well as the identification of SynVaL as the more rapidly eluting of the two. Both the light-inducible nature of SynVaL and the effects of streptomycin and cycloheximide on its induction may be seen in Fig. 6. As with SynChl induction of SynVaL is unaffected by streptomycin but is completely inhibited by cycloheximide. We have also found the same to be true for the chloroplastic lysyl- and leucyl-tRNA synthetases.

It should be emphasized that the conditions of streptomycin treatment used here are the same as those which inhibit the light-induced formation of certain other Euglena chloroplastic enzymes (5, 12) such as ribulose diphosphate carboxylase and cytochrome 552, which are apparently translated on chloroplastic ribosomes. The formation of chlorophyll and carotenoids and the appearance of photosynthetic competence are also streptomycin-sensitive (see refs. 5 and 12, and Table 1). Thus it appears from our data that the chloroplast aminoacyl-tRNA synthetases are not translated on plastidic ribosomes and are in fact of cytoplasmic origin.

During the course of these experiments, a report appeared by Parthier et al. (21) which also takes advantage of the light-inducible nature of Euglena chloroplastic aminoacyl-tRNA synthetases and the selective inhibitory action of chloramphenicol and cycloheximide on translation on 70S- and 80S-type ribosomes, respectively. In contrast to our own data, however, their results may be interpreted to indicate that the chloroplastic synthetases are translated within the plastid rather than the cytoplasm.

More specifically, these authors report that chloramphenicol inhibits the light induction of certain chloroplastic synthetases by about 50%. In addition, they found that cycloheximide actually stimulates the induction of certain synthetases by about 100%. The actual basis for the discrepancies between our observations is not completely clear. Several differences in experimental protocol warrant attention, however: (i) In the experiments of Parthier et al. (21), quantitation (i.e., level of synthetase induction by light) is accomplished by determining the level of acylation (by whole cell extracts) of a heterologous tRNA preparation from Anaaytis. The tacit assumption is made that only the chloroplastic synthetases are capable of acylating Anaaytis tRNA and that cytoplasmic synthetase cannot catalyze these reactions. This assumption remains to be experimentally verified and is in fact partially negated by their data, which show that Anaaytis tRNA is in part acylated by all chromatographic species of SynChl. (ii) Induction in the present investigation has been quantitated by organelle isolation and chromatographic identification of the chloroplastic synthetases using homologous tRNA's. Thus we feel that we can unambiguously follow the kinetics of appearance of the chloroplastic synthetases. (iii) We have chosen to utilize nondividing or resting cells for examination of the effects of antibiotics on synthetase induction. This system has the advantage of permitting one to study the enzymes involved in chloroplast development and their genesis in the absence of cell division. (iv) The level of cycloheximide used by Parthier et al. (21) was 4 µg/ml, in contrast to the 15 µg/ml used in our studies. Smillie et al. (7) have shown that, for as yet unknown reasons, low levels (2-5 µg/ml) of cycloheximide actually accelerate Euglena chloroplast development. Thus, it is not clear what the

| Table 1. Effect of streptomycin on chlorophyll in nondividing cells of Euglena |
|-----------------------------|-----------------------------|
| Exposure to light (hr) | Chlorophyll (pg/cell) | Exposure to light (hr) | Chlorophyll (pg/cell) |
|-----------------------------|-----------------------------|
| 0 | 0.000 | 0 | 0.000 |
| 12 | 0.141 | 11 | 0.144 |
| 24 | 0.578 | 23 | 0.243 |
| 37 | 2.141 | 34 | 0.467 |
| 48 | 3.245 | 49 | 0.735 |
| 63 | 4.674 | 73 | 0.963 |

Dark-grown resting cells were grown and exposed to light in the presence of 0.5 mg/ml of streptomycin as described in Methods. Chlorophyll was determined as discussed in ref. 3.
Fig. 5. Hydroxyapatite chromatography of *Euglena* valyl-tRNA synthetases. Soluble proteins (1) (about 50 mg) from (a) light-grown whole cells and (b) isolated chloroplasts (see Methods) were chromatographed and assayed as described in Figs. 1 and 2 by use of a potassium phosphate gradient of from 0.0005 M to 0.25 M.

Fig. 6. Effect of streptomycin and cycloheximide on the induction of chloroplastic valyl-tRNA synthetase by light. The experimental protocol was exactly as that described for phenylalanyl-tRNA synthetase in Fig. 3 except that a potassium phosphate gradient of from 0.0005 M to 0.25 M was used. Cell number and total extractable soluble protein per cell remained constant throughout the course of the experiment. One enzyme unit is defined as that amount of enzymatic activity that forms 1 pmole of aminoacyl-tRNA in 1 min/mg of soluble protein under the conditions described in Methods and Fig. 1.

Fig. 7. Hydroxyapatite chromatography of the phenylalanyl-tRNA synthetases of an aplastic mutant (W3BUL) of *Euglena*. Chromatography of about 50 mg of soluble protein (1) from light-grown cells of W3BUL was performed as described in Figs. 1 and 2. Fractions were assayed with (a) 20 A₄₅₀ units/ml of light-grown whole-cell tRNA of *Euglena* B and (b) 20 A₄₅₀ units/ml of "cytoplasmic" tRNA from light-grown *Euglena* B cells. "Cytoplasmic" tRNA was prepared by disrupting cells as described for chloroplast isolation in Methods and centrifuging immediately at 20,000 × g for 30 min. After centrifugation, "cytoplasmic" tRNA is isolated from the supernatant as described in Methods.
stimulation of induction in Parthier's study reflects, nor is it apparent why 1 mg/ml of chloramphenicol did not more effectively block synthetase induction if in fact chloroplastic synthetases are translated on chloroplastic ribosomes. This concentration of chloramphenicol caused about 80% inhibition of chlorophyll formation during light-induced chloroplast development in Euglena (22). Nevertheless, it is clear in the current study that cycloheximide (15 \mu g/ml) completely blocks synthetase induction, whereas streptomycin has no effect at concentrations and conditions that prevent synthesis of other Euglena chloroplastic components such as chlorophyll (Table 1), ribulose diphosphate carboxylase, and cytochrome 552 (5, 12).

In a previous report (1), we presented evidence that certain chloroplastic synthetases are present in an aplanctic mutant of Euglena B, \( W_b \) BUL (3, 8-10). This was interpreted to indicate a nuclear genetic origin for these enzymes, since \( W_b \) BUL lacks both detectable chloroplast DNA and significant plastid structure. Further evidence that \( W_b \) BUL does indeed contain low levels (compared to wild-type) of Syn\( ^{P} \) and Syn\( ^{C} \) is shown in Figs. 7 and 8 (compare with Figs. 2 and 5). In the case of phenylalanine, we have used both "whole cell" tRNA from light-grown cells and "cytoplasmic" tRNA, and in both instances the presence of the chloroplastic Syn\( ^{P} \) is unambiguous, as is the presence of Syn\( ^{C} \) in \( W_b \) BUL.

Thus we interpret the two lines of evidence presented in this report to suggest that localization of synthetases within Euglena chloroplasts reflects intracellular compartmentalization of nuclear-coded, cytoplasmically translated proteins rather than either compartmentalization of a nuclear-derived messenger RNA in the organelle for translation or a purely chloroplastic origin for the synthetases.

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