Changes in the Expression of the Chloroplast Genome of *Euglena gracilis* during Chloroplast Development†

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ABSTRACT: The transcription program from the chloroplast genome of *Euglena gracilis* Z during light-induced chloroplast development has been characterized by hybridization of total cell RNA to ^{3}H-labeled chloroplast DNA. Pancreatic DNase activated, purified *Euglena* chloroplast DNA was enzymatically labeled by *Escherichia coli* DNA polymerase I with [^{3}H]TTP as a substrate. The [^{3}H]DNA "hybridization probe" was characterized by the kinetics of its renaturation with purified chloroplast DNA, and the thermal stability of [^{3}H]DNA–DNA, and [^{3}H]DNA–RNA hybrids. The [^{3}H]DNA was hybridized in trace amounts to total cellular RNA extracted from *Euglena* cells 0, 4, 8, 12, 24, 48, and 72 h after the onset of chloroplast development. A large percentage (17%) of the chloroplast genome was found to be transcribed in dark adapted cells. Development is marked by an initial decrease in the fraction of the genome transcribed followed by an increase to 23% transcribed at the end of 72 h of light growth. Chloroplast RNA transcripts were also characterized by the kinetics of their hybridization to chloroplast DNA. The chloroplast specific RNA population is composed of three abundance classes, and the $R_0t_{1/2}$ for each class varies during the early stages of chloroplast development.

Euglena gracilis chloroplasts represent an ideal model system for studying the temporal control of RNA synthesis during a developmental process. *Euglena* is a rapidly growing eucaryote that is easily maintained axenically on simple media. When *Euglena* is grown photoautotrophically, chloroplasts comprise approximately half of the cell volume. *Euglena* also grows well in the dark in the presence of oxidizable substrates, but during dark growth chloroplasts loose their pigments, photosynthetic lamallae, and the ability to fix CO_2. When dark-adapted cells are shifted to light growth, chloroplast development begins, whether the cells are dividing or not. Development is complete after 72 h, with each cell having a full complement of mature chloroplasts (Ben-Shaul et al., 1964; Schiff et al., 1967). Dark-adapted *Euglena* cells retain significant amounts of chloroplast DNA, approximately 168 copies/cell when maintained on a heterotrophic medium (Rawson, 1975). This compares to 677 copies/cell of chloroplast DNA, or 3.1% of total cellular DNA, in light-grown cells maintained on an autotrophic medium (Rawson, 1975). The *Euglena* chloroplast DNA exists as a covalently closed, circular duplex molecule of molecular weight 92×10^6 (Manning and Richards, 1972). The chloroplast chromosome codes for chloroplast rRNA (Scott and Smillie, 1967; Stutz and Rawson, 1970), and possibly chloroplast transfer RNA (Goins et al., 1973). Although *Euglena* chloroplast coded proteins have not been described, the genome in principle could also code for 200–300 proteins of molecular weight 40 000.

The fact that the chloroplast genome is well characterized and that there is such a dramatic change in the structure and physiology of *Euglena* chloroplasts during light-induced development led us to examine the question of whether there is a parallel temporal program of RNA synthesis from the chloroplast genome. There have been previous attempts to study light-induced chloroplast RNA synthesis in *Euglena*. Zeldin and Schiff (1967) reported that ^{32}P is rapidly incorporated into RNA by cells undergoing chloroplast development. Brown and Haselkorn (1971) found that chloroplast rRNA increases in abundance during chloroplast greening, but other changes in the transcription of chloroplast DNA could not be detected.

We have undertaken a study of RNA transcription from the chloroplast genome of *Euglena gracilis* during chloroplast development, utilizing a hybridization "probe" to detect chloroplast specific RNA transcripts present as only a small fraction of total cellular RNA. We report the preparation and properties of a ^{3}H-labeled chloroplast DNA hybridization probe, and its use to measure changes in the percent of the genome expressed during a defined developmental process. We also report the detection of classes of chloroplast RNA differing in their relative abundance, and changes in these abundance classes during early developmental stages. There has been a preliminary report of this work (Chelm and Hallick, 1975).

Materials and Methods

Materials. Chemicals were obtained from the following sources: cesium chloride (optical grade) was from Calbiochem, [6-^{3}H]thymidine triphosphate was from New England Nuclear, deoxyribonucleotide triphosphates were a gift from Marvin Caruthers, sodium lauroyl sarcosinate and bovine serum albumin were from Sigma Chemical Co., calf thymus DNA was from Worthington Biochemical Corp., and Euglena Broth from Difco Laboratories. All salts and buffers were reagent grade.

S1 nuclease was prepared by the method of Sutton (1971). DNA polymerase I of *E. coli* was a gift from Marvin Caruthers. Pancreatic DNase I was obtained from Worthington Biochemical Corp.

Growth of Cells. Euglena gracilis Klebs, Strain Z cells, obtained from Oliver Richards, were axenically maintained at 28° in a heterotrophic medium (Euglena Broth). Etiolat-

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ed cultures were prepared by maintaining the cells in the same medium in a light-tight cabinet in a darkroom for greater than 12 generations.

A greening culture was prepared by a modification of the method of Brown and Haselkorn (1971). A Virtis Model 43-100 20-l. fermenter, equipped with a Model 43-I light manifold with 12 fluorescent lights (6 Sylvania Grolites and 6 Vitalites), was installed in a darkroom. Fourteen liters of heterotrophic medium (Euglena Broth) was inoculated with dark-adapted *Euglena* to an initial cell density of 10^3 cells/ml. Growth was continued at 27° with aeration and mechanical stirring in the dark, except for occasional illumination by a 25-W safelight fitted with a Kodak safelight filter No. 8 (dark yellow), to a cell density of 1×10^6 cells/ml. The cells were harvested by centrifugation at 1500 rpm for 5 min in autoclaved, polycarbonate bottles in a Sorvall GC-3 rotor.

The cells were gently resuspended in autotrophic medium (Richards et al., 1971) to a cell density of 1×10^6 cells/ml. The light manifold was switched on to begin light-induced greening. The culture was maintained for 72 h at 27° with mechanical stirring and sparging with 5% CO_2 in air. The cell density was maintained at $0.7–1.0 \times 10^6$ cells/ml by dilution with fresh autotrophic medium. Cells from 3 l. of culture were harvested 0, 4, 8, 12, 24, 48, and 72 h after the onset of illumination by centrifugation at 1500 rpm for 5 min in a Sorvall GC-3 rotor. The cells were resuspended in 500 ml of cold NET[1] buffer (0.15 M NaCl, 0.1 M EDTA, and 0.05 M Tris-HCl (pH 9.0)) and recentrifuged as described above. The resulting cell paste was resuspended in 3 volumes of NET and stored at −80°.

Cellular chlorophyll content was determined by the method of Arnon (1949).

Preparation of RNA. RNA was purified from whole cells by a modification of the method of Glisin et al. (1974). Packed cells were thawed, collected by centrifugation, resuspended in 9 volumes of cold 4% sodium lauroyl sarcosinate and 0.1 *M* Tris-HCl (pH 8.0), and homogenized with 12 strokes of a motor driven Teflon pestle in a Potter-Elvehjem homogenizer. The homogenate was then sonicated at 0° with three bursts of 60 s each in an MSE sonicator. Self-digested Pronase was then added to a final concentration of 2 mg/ml and the solution was stirred at 4° for 4 h. One gram of cesium chloride per milliliter was then dissolved in the homogenate; 3.4 ml of this mixture was next layered over 1.2 ml of 5.25 M cesium chloride (ρ = 1.7070 g/ml). This was overlayered with 0.4 ml of 4% sodium lauroyl sarcosinate and Tris-HCl (pH 8.0). Centrifugation was carried out in a Spinco SW 50.1 rotor at 35000 rpm for 12 h at 25°. Following centrifugation the supernatant was decanted and the RNA pellet was redissolved in SSC. The RNA solution was extracted twice with chloroform–isoamyl alcohol (24:1) to remove any residual protein. RNA was collected by ethanol precipitation and redissolved in 0.1 mM NaEDTA (pH 8).

DNA was determined by the method of Burton (1968). RNA nucleotide concentration was estimated using the relationship $\epsilon_{260nm} = 1 \times 10^4\ M^{-1}\ cm^{-1}$.

Preparation of ^{3}H-Labeled Chloroplast DNA. *Euglena* chloroplast duplex DNA, free of nuclear DNA contamination, was a gift from Oliver C. Richards. The isolation and buoyant density analysis of the DNA is reported elsewhere (O. C. Richards, in preparation). Partial digestion of chloroplast DNA was achieved in a reaction containing 200 μg/ml of chloroplast DNA, 0.01 mg/ml of pancreatic DNase, 5 mM $MgCl_2$, 0.5 mg/ml of bovine serum albumin and 0.05 M Tris-HCl (pH 7.5) which was incubated for 15 min at 37°. The DNase was inactivated by incubation at 70° for 5 min. In vitro labeling of the DNA was accomplished by directly transfering 40 μl of this reaction mixture to a 200-μl reaction containing 0.2–1.0 unit of *E. coli* DNA polymerase I, 32 μM each of dATP, dCTP, dGTP, 33 μM [6-^{3}H]TTP (45.2 Ci/mmol), 7 mM $MgCl_2$, 32 μM 2-mercaptoethanol, and 0.07 M sodium glycine buffer (pH 9.2). After 120-min incubation at 30°, the mixture was passed through a Sephadex G-25 column previously equilibrated with 0.1 × SSC. The DNA containing fractions were pooled and the DNA was collected by ethanol precipitation. The resulting DNA pellet was redissolved in 0.1 mM NaEDTA (pH 8.0) and overlayered on 4.6 ml of 5–20% alkaline sucrose gradients containing 0.9 M NaCl and 0.1 M NaOH, and centrifuged in a Spinco SW 50.1 rotor at 40000 rpm for 24 hr at 4°. Gradients were fractionated dropwise from the bottom of the centrifuge tube. Approximately $\frac{2}{3}$ of the ^{3}H-labeled chloroplast DNA, representing components with sedimentation coefficients of greater than 5 S, was pooled, dialyzed extensively against 0.1 × SSC, and collected by ethanol precipitation. The resulting DNA pellet was redissolved in 0.1 mM NaEDTA (pH 8) and used for subsequent hybridization experiments. The specific activity of the radioactive *Euglena* chloroplast DNA was 5.4 Ci/g of DNA.

DNA–RNA Hybridization. Nucleic acid hybridization reactions and detection of hybrids were performed using a modification of the procedure of Leong et al. (1972). Total cellular RNA from different stages of chloroplast development was hybridized in vast excess to ^{3}H-labeled *Euglena* chloroplast DNA. Individual reactions contained heat-denatured [^{3}H]DNA, 0.5 μM in nucleotides (specific activity = 5.4 Ci/g of DNA), RNA, 6 mM in nucleotides, 0.3 M NaCl, 1 mM EDTA, and 0.025 M Tris-HCl (pH 7.4). Five-microliter reactions were incubated at 68° for from 4 min to 30 h.

Each hybridization reaction was diluted with 2.1 ml of 0.03 M sodium acetate, 0.3 M NaCl, 3 mM $ZnCl_2$, and 10 μg/ml of denatured calf thymus DNA (pH 4.5) (S1 buffer). DNA–RNA hybrids were detected by their insensitivity to the single-strand specific S1 nuclease from *Aspergillus oryzae*. S1 nuclease was added to a 1-ml aliquot of the diluted hybridization reaction, and the resulting mixture was incubated at 50° for 2 h. Following incubation, bovine serum albumin (150 μg) and denatured calf thymus DNA (25 μg) were added and nondigested material was precipitated by the addition of 1 ml of ice-cold 10% trichloroacetic acid. The filters were washed five times with 1 M HCl and 0.1 M tetrasodium pyrophosphate and twice with ethanol, and dried. A second 1-ml aliquot of each diluted hybridization mixture, serving as a non-S1 digested control, was directly Cl_3CCOOH precipitated. The precipitate was collected on GF/C filters as described above. Radioactivity on the filters was determined in 5 ml of Omnifluor (New England Nuclear) liquid scintillation cocktail in a Packard TriCarb Model 3310 counter. The fraction of the DNA hybridized is expressed as the radioactivity in the S1 digested aliquot divided by the radioactivity in the non-S1 digested aliquot from the same diluted hybridization mixture.

[1] Abbreviations used are: NET buffer, 0.15 M NaCl–0.1 M EDTA–0.05 M Tris-HCl (pH 9.0); SSC, 0.15 M NaCl–0.015 *M* sodium citrate.

Table I: Cellular Chlorophyll Content of *Euglena gracilis* during Light-Induced Chloroplast Development.[a]

Time after Light Exposure (hours)	Chlorophyll Content ($\mu g/10^6$ cells)	Time after Light Exposure (hours)	Chlorophyll Content ($\mu g/10^6$ cells)
0	<0.058	24	2.0
4	0.069	48	4.9
8	0.24	72	10.0
12	0.83		

[a] The cells were dark adapted in a heterotrophic medium, and then switched to an autotrophic medium for development studies, as described in Materials and Methods. Chlorophyll was determined by the method of Arnon (1949). The typical chlorophyll absorption spectrum was not evident in the 0-h sample.

DNA-DNA Hybridization. DNA-DNA hybridization was carried out with nonradioactive DNA in vast molar excess over ^{3}H-labeled *Euglena* chloroplast DNA. The procedure was essentially the same as the RNA-DNA hybridization previously described, except that nonradioactive DNA replaced total cell RNA. *E. coli* DNA was isolated by the procedure of Marmur (1961). Prior to use, DNA samples were sheared by three passes through a French pressure cell at 12 000 psi. The resulting fragment size was 500–600 base pairs, as determined by the method of Vinograd et al. (1963).

Thermal Denaturation of Nucleic Acid Hybrids. Thermal denaturation profiles for DNA-DNA and DNA-RNA hybrids were determined using chromatography on hydroxylapatite as described by Kohne and Britten (1971).

Results

Chlorophyll Synthesis during Chloroplast Development. Cellular chlorophyll content was measured during the light-induced chloroplast maturation process. The cells which had been dark adapted on a heterotrophic medium for greater than 12 generations showed a chlorophyll content of less than 0.058 $\mu g/10^6$ cells (Table I). When the cells were transferred to an autotrophic medium and exposed to light, there was a dramatic increase in cellular chlorophyll content (Table I), reaching a value of 10 $\mu g/10^6$ cells after 72 h of continuous illumination.

^{3}H-Labeled Chloroplast DNA Hybridization "Probe" Preparation. The specific activity of the ^{3}H-labeled chloroplast DNA prepared by the action of DNA polymerase I of *E. coli* on chloroplast DNA randomly nicked with pancreatic DNase was 5.4 $\mu Ci/\mu g$ of DNA, or 1.8 $mCi/\mu mol$ of DNA nucleotide. The radioactive substrate for DNA labeling was [^{3}H]TTP, specific activity 45.2 $mCi/\mu mol$. Since the chloroplast DNA of *Euglena gracilis* has an A-T base pair content of 75 mol % (Brawerman and Eisenstadt, 1964), the specific activity of thymine bases in the DNA is 4.8 $mCi/\mu mol$, representing an 11% substitution during the DNA polymerase reaction.

The sensitivity of ^{3}H-labeled chloroplast DNA to S1 nuclease was tested by incubation of DNA samples with S1 nuclease followed by Cl_3CCOOH precipitation of the nondigested DNA, as described above. Prior to the alkaline sucrose gradient purification step, the [^{3}H]DNA was greater than 99% insensitive to S1-nuclease. Following heat denaturation, or denaturation in an alkaline sucrose gradient, the denaturated [^{3}H]DNA was greater than 95% sensitive to S1 nuclease digestion. No appreciable nondenaturable [^{3}H]DNA was detected.

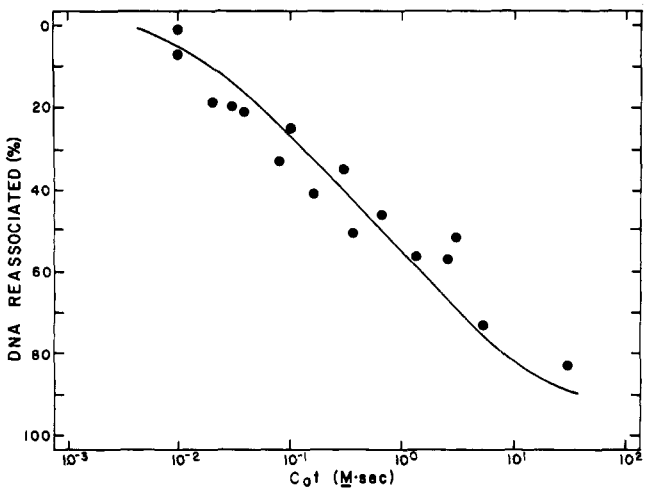

FIGURE 1: Hybridization of [^{3}H]chloroplast DNA to excess, nonradioactive, sheared chloroplast DNA. Samples (5 μl) were incubated at 68° in 0.3 M NaCl-0.001 M EDTA-0.025 M Tris-HCl (pH 7.4) for varying lengths of time as described in the text.

Properties of the ^{3}H-Labeled Chloroplast DNA. An important criterion for the fidelity of copying during the in vitro DNA labeling reaction is the ability of ^{3}H-labeled chloroplast DNA to renature with excess nonradioactive chloroplast DNA. The [^{3}H]DNA was found to specifically renature with *Euglena* chloroplast DNA. The results of the renaturation of ^{3}H-labeled chloroplast DNA with a 120-fold excess of sheared, nonradioactive chloroplast DNA are shown in Figure 1. Greater than 80% of [^{3}H]DNA renatures at C_0t values above 10 M s. The $C_0t_{1/2}$ value of approximately 0.5 M s is consistent with the expected value for a DNA sample with a genome the size of chloroplast DNA, 139 kilobase pairs (Britten and Kohne, 1968). There was no measurable hybridization of the [^{3}H]DNA when incubated with calf thymus DNA to C_0t = 4700 M s, or with *E. coli* RNA when incubated to R_0t = 870 M s. A sensitive criteria for the fidelity of base pairing of the hybridization probe to *Euglena* chloroplast DNA, or to chloroplast transcribed RNAs is provided by their thermal stabilities. The thermal stability of DNA-DNA hybrids formed after incubation to C_0t = 4.12 M s was determined using thermal elution chromatography on hydroxylapatite. The data are shown in Figure 2. The mean thermal stability is at 82°. The thermal stability profile is biphasic, as has been observed for the thermal denaturation profile of native *Euglena* chloroplast DNA (Brawerman and Eisenstadt, 1964). The thermal stability of DNA-RNA hybrids was also determined (Figure 2). DNA-RNA hybrids were formed as described above, after incubation of [^{3}H]DNA with *Euglena* whole cell RNA to an R_0t = 710 M s. The mean thermal stability is 78° (Figure 2). The fact that there is approximately a 4° lowering of the thermal stability of RNA-DNA hybrids as compared to the homologous DNA-DNA duplex has been previously observed (Kohne, 1968; Brown and Church, 1971; Davidson and Hough, 1971; Firtel, 1972). Furthermore the mean thermal stability of 78° is similar to temperatures observed by others for RNA-DNA hybrid dissociation under similar conditions (Grouse et al., 1972; Brown and Church, 1971; Hahn and Laird; 1971). These authors have argued that the hybrids dissociate at a temperature consistent with that of well-matched duplexes.

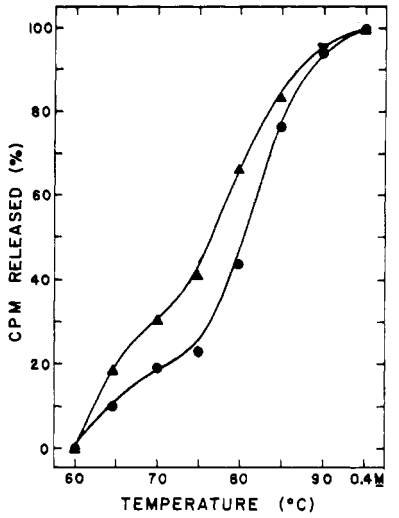

FIGURE 2: Thermal stability of DNA-DNA and DNA-RNA hybrids. RNA extracted from dark adapted *Euglena* cells, and purified, sheared *Euglena* chloroplast DNA were hybridized to ^{3}H-labeled chloroplast DNA to R_0t = 710 M s and C_0t = 4.1 M s, respectively, as described in Materials and Methods. The double-strand nucleic acid hybrids were collected on 1-ml hydroxylapatite columns in 0.12 M sodium phosphate and 0.4% sodium dodecyl sulfate (pH 6.8). Nonhybridized material was not retained on the column. Thermal stability of the hybrids was determined by raising the temperature of the column and buffer in 5° increments for 5-min equilibration periods, and subsequently eluting any material which had denatured with 2.0 ml of 0.12 M sodium phosphate and 0.4% sodium dodecyl sulfate (pH 6.8). One-milliliter aliquots of the eluent were mixed with 4.0 ml of RPI 3a70 scintillation cocktail. Radioactivity was estimated in a Packard Tri-Carb Model 3310 scintillation counter. (●) DNA-DNA hybrid; (▲) DNA-RNA hybrid.

Chloroplast RNA Synthesis during Chloroplast Development. To determine the amount of the chloroplast genome which is expressed as RNA transcripts during different stages of chloroplast development, RNA-driven saturation experiments were carried out. RNA isolated from *Euglena gracilis* following light-induced development was hybridized at an RNA/DNA ratio of 12000:1 to ^{3}H-labeled chloroplast DNA. RNA was extracted at individual developmental stages from whole cells rather than from purified chloroplasts since chloroplast isolation could lead to loss of chloroplast RNA transcripts. The amount of the chloroplast DNA transcribed is taken as the fraction of chloroplast DNA resistant to digestion by the single strand specific S1-nuclease from *A. oryzae* at the completion of an RNA-driven reaction. Experiments on the kinetics of annealing, described below, showed that the reactions were complete at R_0t = 700 M s. All reactions were carried out at constant [^{3}H]DNA and RNA concentration, and incubation time to ensure that the DNA-DNA renaturation contribution to DNA hybrid formation is constant. Therefore changes in the amount of DNA as hybrid reflect real differences in the chloroplast RNA population present in developing cells.

The results on chloroplast RNA synthesis during chloroplast development are summarized in Table II. There are several significant features of these data. First, there is a high percentage of transcription of chloroplast DNA at all developmental stages. If we correct for the approximately 10% of [^{3}H]DNA reannealed as a DNA-DNA duplex, the range of the amount of chloroplast DNA transcribed into RNA is from 12 to 23% of the double strand molecular weight of the chloroplast DNA. If we assume that only one strand of the DNA duplex is transcribed into RNA, perhaps a questionable assumption, 24–46% of the genomic information is present as RNA transcript during light-induced development in an autotrophic medium, which potentially could code for 34–64 genes of 1000 bases/gene.

Table II: Percentage of the Chloroplast Genome Transcribed during Light-Induced Chloroplast Development.[a]

Hours after the Onset of Development	RNA Concn (mg/ml)	Hours of Incubation	No. of Determinations	% DNA[b] in Hybrid
0	2	29	8	26.9 ± 0.4
4	2	29	9	21.3 ± 1.7
8	2	29	8	22.9 ± 0.7
12	2	29	6	24.4 ± 0.8
24	2	29	6	26.4 ± 1.0
48	2	29	6	31.3 ± 1.7
72	2	29	6	32.6 ± 0.8
None	0	29	6	10.2 ± 1.4

[a] Hybridization of ^{3}H-labeled chloroplast DNA to RNA from *Euglena* at various stages of chloroplast development. [b] Values are for percent DNA hybridized at the completion of an RNA-excess reaction are reported as the average ± SEM. The 0- and 4-h values are significantly different, $p < 0.05$. The 4- and 72-h values are significantly different, $p < 0.01$.

The second feature of the data in Table II is the high percentage of the chloroplast DNA transcribed in dark-adapted cells, 26.8% of DNA as hybrid at 0 h. Even though the dark-adapted cells have lost their photosynthetic pigment and lamallae, and are incapable of photosynthesizing, the chloroplast DNA in the proplastids is extensively transcribed.

Finally, there are changes in the percent of the chloroplast genome expressed during the process of chloroplast development. From the onset of greening (0 h) to the period 4 h after the onset of illumination, there is a significant decrease of 5.6% in the percent chloroplast DNA as DNA-RNA hybrid. This change could be related to the fact that the cells were dark adapted in a heterotrophic media, and switched to a autotrophic media with the onset of illumination, or it could be due to some fundamental aspect of information usage during chloroplast development. Following this initial decrease, there is a subsequent increase in the percentage of the chromosome expressed as RNA. From 4 h of development, at which time the cells cannot evolve O_2, fix CO_2, or photophosphorylate, until 72 h of light-induced development, when chloroplasts are mature, there is an 11.3% increase in the [^{3}H]DNA as DNA-RNA hybrid. This change represents a gradual, temporal increase spread over the entire time of development. These data indicate that the fully developed cells must contain chloroplast RNA sequences not present in either dark-adapted or early greening cells, and that these new RNA sequences may account for more than 20% of the information content of the chloroplast DNA.

Hybridization Kinetics. In order to further characterize the changes in the chloroplast specific RNA during the early stages of chloroplast development, we have investigated the kinetics of hybridization of total cell RNA to ^{3}H-labeled chloroplast DNA. When RNA is in excess of DNA, the rate of hybridization is dependent on the kinetic complexity of the RNA (Laird and McCarthy, 1968; Bishop, 1969) and the frequency of any given sequence in the total RNA population (Bishop et al., 1974). RNA populations

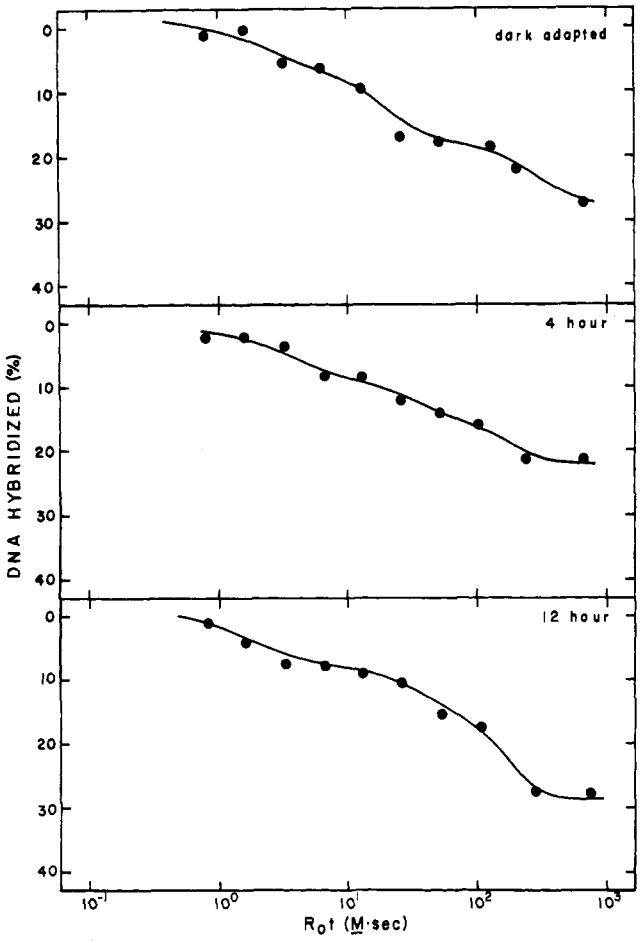

FIGURE 3: Hybridization of ^{3}H-labeled chloroplast DNA with RNA from *Euglena* cells at different stages of chloroplast development. Total cellular *Euglena* RNA was hybridized in excess to ^{3}H-labeled chloroplast DNA for varying lengths of time as described in Materials and Methods. The values reported are the averages of three determinations. Data are shown for dark-adapted cells (top panel), and for cells following 4 h (middle panel) and 12 h (lower panel) of light-induced development.

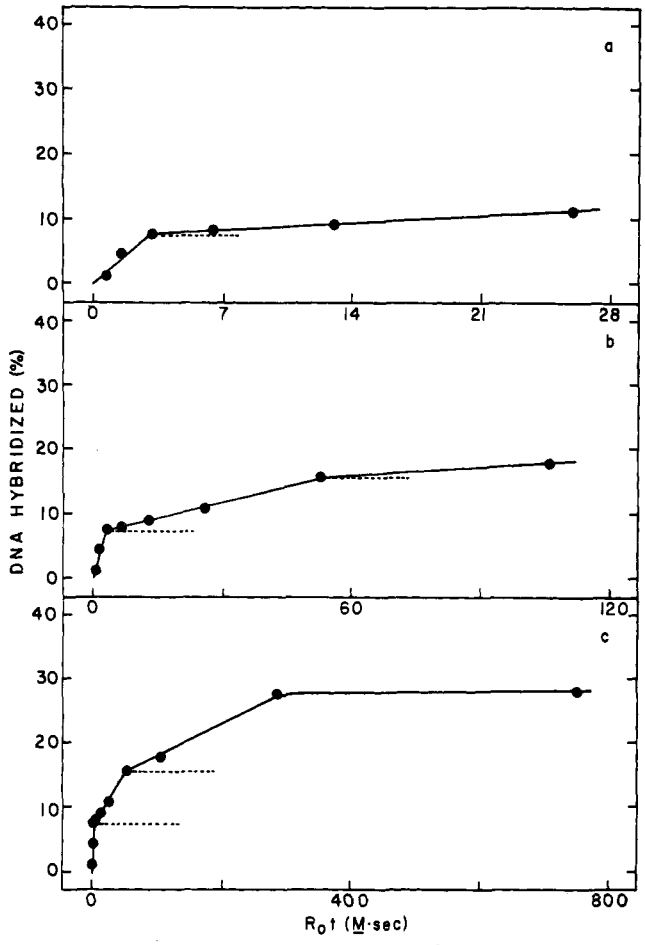

FIGURE 4: Linear plots of the hybridization of ^{3}H-labeled chloroplast DNA to RNA from *Euglena* cells following 12 h of light-induced development. The data are taken from Figure 3, lower panel. Dotted lines indicate inflections between transitions.

characterized by the criterion of hybridization kinetics have been shown to exist as defined RNA classes whose individual sequences differ in their relative abundance (Frenkel and Roizman, 1972; Bishop et al., 1974; Levy and McCarthy, 1975).

We also find evidence for different abundance classes of RNA transcribed from *Euglena* chloroplast DNA. The hybridization of the ^{3}H-labeled chloroplast DNA to RNA from *Euglena* in the early stages of development is shown in Figure 3. Data are presented for RNA from dark-adapted cells, and for 4 and 12 h after the onset of light-induced development as semilogarithmic plots (Birnsteil et al., 1972). Although the inflections are somewhat obscure on semilogarithmic plots, in each case the data are most consistent with three RNA abundance classes.

To better analyze the different RNA abundance classes, the data were replotted as linear-linear plots (Bishop et al., 1974). Each curve was found to have three inflections, as illustrated in Figure 4 for the RNA from cells at 12 h of light-induced development, the three transitions having mid points ($R_0t_{1/2}$ in M s) of 1.6, 33, and 160. A summary of the kinetically determined constants for all three RNA samples is presented in Table III.

Table III also contains estimates of the fraction of the total RNA population represented by each abundance class.

Table III: Analysis of Hybridization Kinetics of RNA from the Early Stages of *Euglena* Chloroplast Development.

Hours of Chloroplast Development	Transition	Obsd $R_0t_{1/2}$ (M s)	Percent of the Chloroplast Genome	Calcd $R_0t_{1/2}$ (M s)[a]	Percent of the RNA Population[b]
0	1	2.5	6	0.011	0.4
	2	16	12	0.023	0.1
	3	250	10	0.019	0.008
4	1	4.0	8	0.015	0.4
	2	22	7	0.013	0.06
	3	170	7	0.013	0.008
12	1	1.6	7	0.013	0.8
	2	33	10	0.019	0.06
	3	160	10	0.019	0.01

[a] Calculated using $R_0t_{1/2} = 3.3 \times 10^{-3}$ M sec for the hybridization of 1.6×10^6 daltons of RNA and 92×10^6 daltons as the chemical complexity of the chloroplast genome as described in the text. [b] Fraction of the RNA Population = (calcd $R_0t_{1/2}$)/(obsd $R_0t_{1/2}$).

In order to make these calculations, we have used the hybridization of an excess of *Euglena* chloroplast rRNA to purified *Euglena* chloroplast rDNA as a hybridization standard. When purified chloroplast rRNA is hybridized to homologous [^{3}H]DNA, the $R_0t_{1/2}$ is 3.3×10^{-3} M s (B. Chelm and R. B. Hallick, unpublished observations). Since

the molecular weight of the chloroplast rRNAs is 1.6×10^6 (Rawson and Stutz, 1969), we take 3.3×10^{-3} M s to be the $R_0t_{1/2}$ of an RNA of this molecular weight under our reaction conditions, in good agreement with previously reported values (Bishop et al., 1974; Levy and McCarthy, 1975). From the fraction of the chloroplast DNA reacting with each RNA class in column 4, Table III, and the molecular weight of the chloroplast DNA, 92×10^6 (Manning and Richards, 1972), we calculate the $R_0t_{1/2}$ for each RNA class, if pure, reacting with its homologous DNA (column 5, Table III). The fraction of the total cellular RNA represented by each abundance class is therefore given as the calculated $R_0t_{1/2}$/obsd $R_0t_{1/2}$ in column 6, Table III.

The data indicate that abundance classes 1 and 3, the most and least abundant classes, respectively, begin increasing as cellular components after 4 h of chloroplast development. The intermediate abundance class, on the other hand, decreases as a cellular component with the onset of chloroplast development.

Discussion

We have made use of RNA driven hybridization reactions with ^{3}H-labeled *Euglena* chloroplast DNA, labeled in vitro to high specific activity, to investigate the transcription of the chloroplast genome of *Euglena* during light-induced chloroplast development. In vitro DNA labeling was achieved by the action of *E. coli* DNA polymerase I on pancreatic DNase activated chloroplast DNA, the "nick-translation" reaction. Because of the high amount of base substitution during this reaction, it seemed necessary to test the hybridization properties of the [^{3}H]DNA before attempting to detect chloroplast transcripts.

One class of side products that can arise during in vitro DNA synthesis by DNA polymerase I is rapidly renaturable, hairpin-like structures, attributed to a template switch during DNA replication (Kornberg, 1974). If rapidly renaturable DNA were present, it would appear as a duplex structure in our S1 nuclease assay. However, we find heat-denatured, rapidly cooled [^{3}H]DNA to be completely digested by S1 nuclease, suggesting that no appreciable amount of hairpin structures are present in the hybridization "probe". The fidelity of base pairing of the [^{3}H]DNA hybridization probe was judged satisfactory by several criteria. The renaturation reaction requires homologous *Euglena* RNA or chloroplast DNA, whereas nonhomologous nucleic acids do not anneal to the probe. In renaturation with excess nonradioactive chloroplast DNA, the observed $C_0t_{1/2}$ is 0.5 M s, consistent with the known genetic complexity of chloroplast DNA. Finally, the mean thermal stability of [^{3}H]DNA–DNA, and [^{3}H]DNA–RNA duplexes, of 82 and 78°, respectively, is in good agreement with previously reported values.

With the availability of a chloroplast DNA hybridization probe, we have undertaken a study of RNA transcription from the chloroplast genome. One interesting finding of this work is that a large fraction of the genome is transcribed in dark-adapted cells, when the presence of chloroplasts is apparently gratuitous. We find that greater than 20% of the chloroplast genome is transcribed in the dark, that the transcripts occur in three abundance classes, and that they account for approximately 0.8% of the total cellular RNA. There have been several previous attempts to detect *Euglena* chloroplast transcripts and in particular chloroplast rRNA in dark-adapted cells (Brown and Haselkorn, 1971; Portier and Nigon, 1968; Heizmann, 1970). Using the criteria of RNA size determined by gel electrophoresis, no chloroplast rRNA could be detected (Brown and Haselkorn, 1971). The possibility exists that rRNA sequences are present in dark-adapted cells, but that they cannot be detected by gel electrophoresis or other methods based on RNA size. We are currently testing for rRNA sequences by another method, hybridizing cellular RNA from dark-adapted cells to a purified chloroplast rDNA hybridization probe.

Although the function of chloroplast RNA in dark-adapted cells remains obscure, there are several biochemical capabilities of proplastids that could potentially require RNA synthesis, including chloroplast DNA replication, RNA transcription, and plastid membrane biosynthesis. If proplastids are competent in protein synthesis, ribosomal and transfer RNAs would likely be synthesized.

A second finding of this work is that there are significant changes in RNA synthesis from the chloroplast genome during light-induced chloroplast development. We find an initial decrease in the fraction of the chloroplast genome transcribed during the first 4 h of light growth. There is almost no accumulation of chlorophyll during this period (Table I) and no O_2 evolution or CO_2 fixation (Stern et al., 1964). This period is followed by an increase in the fraction of the genome transcribed to a maximum value 72 h after the onset of development. These new RNA sequences begin appearing during the period when morphological changes in the plastid structure are significant (Ben-Shaul et al., 1974) and when the cells become competent in O_2 evolution and CO_2 fixation (Stern et al., 1964), suggesting that chloroplast RNA synthesis may be required for these aspects of chloroplast development.

A final observation of this study is that the chloroplast specific RNA population is composed of three abundance classes. The relative molar concentrations and the fraction of genome represented by each class changes during the early stages of development. Previous studies have shown that poly(A) containing RNA of HeLa cells (Bishop et al., 1974) and *Drosophilia melanogaster* (Levy and McCarthy, 1975) are grouped in three distinct abundance classes. Furthermore, distinct abundance classes of viral messenger RNA have been described in a virus infected human cell line. Frenkel and Roizman (1972) in studying the transcription program in human epidermoid carcinoma cells find that the viral messenger RNA occurs in two abundance classes, and that both the fraction of the viral chromosome transcribed, and the $R_0t_{1/2}$ for each abundance class vary with time after the cells are exposed to virus. In this example, the viral chromosome is similar to the *Euglena* chloroplast chromosome in that both are double-strand DNA molecules, of approximately 10^8 molecular weight and lacking any appreciable repetitive sequences.

These experiments also demonstrate that the *Euglena* chloroplast chromosome is especially amenable to further study on the temporal control of RNA synthesis in a eucaryotic cell. Changes in RNA synthesis occur during a well-defined developmental process. The chloroplast DNA is well characterized, and its relatively small size (92×10^6) lends itself to further characterization, perhaps utilizing restriction nucleases to isolated defined regions of the chromosome. Although the kinetics of renaturation suggest that the DNA is entirely unique sequence (Stutz, 1970), RNA transcripts exist within the cell in different abundance classes, paralleling the situation of nuclear messenger RNA. Finally, we have previously reported the isolation

and purification of the *Euglena* chloroplast chromosome in a transcriptionally active state (Hallick and Rutter, 1973), suggesting that it may be possible to study control of transcription in a purified in vitro system.

Added in Proof

While this manuscript was in preparation, we learned of a similar study by Rawson and Boerma (1976). Similar experimental techniques were used in both studies, although Rawson and Boerma employed a ^{125}I-labeled chloroplast hybridization probe, and did not examine hybridization kinetics at low R_0t values. We are in agreement that a large fraction of the chloroplast genome is transcribed at all development stages, including dark-adapted cells. We report measurements for the fraction of single-strand chloroplast DNA as an RNA–DNA hybrid at saturations that range from 0.12 to 0.23, and describe an initial decrease, followed by an increase in the fraction genome expressed during development. Both differences are statistically significant. Rawson and Boerma find that the fraction of single-strand chloroplast DNA as DNA–RNA hybrid ranges from 0.22 to 0.26, and suggest that there may be a decrease in the fraction genome expressed during development. The major difference in the experimental design of the two studies is the culture conditions during development. We employed a photoautotrophic medium and maintained the cells in exponential growth throughout the experiment, whereas Rawson and Boerma chose a heterotrophic medium and maintained the cells in stationary phase. Therefore the quantitative differences in the fraction of the chloroplast genome expressed are most likely due to the different culture conditions.

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