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A Measurement of the Fraction of Chloroplast DNA Transcribed during Chloroplast Development in *Euglena gracilis*†

James R. Y. Rawson* and Cindy L. Boerma

ABSTRACT: The fraction of chloroplast DNA transcribed at different stages of chloroplast development in *Euglena gracilis* was measured by RNA–DNA hybridization. *Euglena* cells were grown in the dark in a heterotrophic medium to stationary phase and then transferred to the light. Chloroplast development was monitored by the increase in the cellular chlorophyll content in the absence of cell division. Total cell RNA was isolated at various stages of chloroplast development, and hybridized in a vast excess to [^{125}I]chloroplast DNA. The fraction of [^{125}I]chloroplast DNA in the form of a duplex was monitored by chromatography on hydroxylapatite columns. The amount of RNA–DNA hybrid in the duplex mixture was determined by correcting for the contribution of DNA–DNA renaturation under the same conditions. The fraction of chloroplast DNA transcribed was calculated by multiplying by two the amount of single-stranded DNA in the form of an RNA–DNA hybrid. Prior to the initiation of chloroplast development (i.e., in dark grown cells) the fraction of chloroplast DNA represented as RNA transcripts in the cell is 0.53. As chloroplast development proceeds, the fraction of the chloroplast DNA transcribed decreases to 0.47. Experiments in which mixtures of various RNA samples were hybridized to the chloroplast DNA indicate that there is a small portion of chloroplast DNA transcribed at later stages of chloroplast development which is not represented as transcripts at the onset of chloroplast development. Melting properties of the RNA–DNA hybrids show that the RNA–DNA duplexes are slightly less stable than renatured [^{125}I]chloroplast DNA.

The differentiation of proplastids into chloroplasts in the single cell alga *Euglena gracilis* offers a unique system for the study of transcriptional events during an intracellular developmental process. When *Euglena* is grown in the dark in a heterotrophic medium, it contains numerous proplastids which are the precursor bodies of chloroplasts. Illumination of these dark grown cells initiates chloroplast development, and within 48 hr a cell contains ten chloroplasts

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with the enzymes, the constituents of the photosynthetic electron transport and the membranes associated with photosynthesis.

Chloroplast development can best be resolved from chloroplast replication in nondividing cells (Schiff, 1970). If *Euglena* is grown in the dark in a heterotrophic medium to stationary phase and then transferred to the light, chloroplast development proceeds in the absence of cell division. Although a rapid increase in the cellular content of chloroplast RNA occurs during this process, hybridization competition experiments have failed to detect any new transcripts derived from the chloroplast genome (Brown and Haselkorn 1971).

This report described the measurement of the fraction of chloroplast DNA represented as RNA transcripts in *Euglena* during the maturation of proplastids into chloroplasts. Total cell RNA, isolated from cells at various stages of chloroplast development, was hybridized in a vast excess to small amounts of low molecular weight [^{125}I]chloroplast DNA, and the resulting duplex structures were separated from single-stranded chloroplast DNA on hydroxylapatite columns. The fraction of chloroplast DNA represented in the different RNA samples was calculated after correcting for DNA self-association. The fraction of chloroplast DNA represented as RNA transcripts in dark grown cells was 0.53 (7.2×10^4 nucleotide pairs). As chloroplast development proceeds, the fraction of chloroplast DNA transcribed rises slightly to 0.57 (7.8×10^4 nucleotide pairs) and then decreases to 0.47 (6.4×10^4 nucleotide pairs). Hybridization of mixtures of RNA samples obtained at different stages of chloroplast development indicates that a small fraction of the chloroplast DNA (0.04–0.05 or 6×10^3 nucleotide pairs) not transcribed in dark grown cells is transcribed at later stages of chloroplast development.

Materials and Methods

Cell Growth. Euglena gracilis var. Z cells were grown in the dark in a heterotrophic medium (*Euglena* Broth, Difco Laboratories, as described by Greenblatt and Schiff, 1959) containing glutamic acid and malic acid as carbon sources. When the cells reached stationary phase ($6–8 \times 10^6$ cells/ml), they were transferred to the light (2500 lux) and maintained with constant shaking for varying periods of time. Chloroplast development was monitored by measuring the chlorophyll content per cell (Wintermans and de Mots, 1965). Cell concentration was determined with a hemacytometer.

RNA Isolation. Total cell RNA was isolated from 500-ml cultures of *Euglena* by a modified procedure of Rawson (1975a). Cells (7×10^{10}) were suspended in 40 ml of 1 × SSC[1] and 2% dodecyl sulfate. The viscosity of the suspension was decreased by passing the cell suspension through a French press at 15000 psi. The lysate was deproteinized at room temperature first with a mixture of phenol, cresol (10% v/v), and 8-hydroxyquinoline (0.1% w/v) saturated with 1 × SSC, and then with chloroform–isoamyl alcohol (24:1). The RNA was precipitated from the aqueous phase by adding 1/10 vol of 3 *M* sodium acetate and 2 vol of ethanol and resuspended in 20 ml of 0.01 *M* Tris-HCl (pH 8.0), 0.15 *M* NaCl, and 0.001 *M* $MgCl_2$. Five hundred micrograms of electrophoretically purified DNase I (Worthington, DPFF 54N332) was added, and the mixture was incubated for 30 min at room temperature. The nuclease reaction was stopped by adding 1 ml of 20% sarkosyl and 1 ml of 0.2 *M* EDTA (pH 8.0). One gram of CsCl was added to each milliliter of RNA (final ρ = 1.650 g/cm^3), and the RNA pelleted in a Beckman SW 50.1 rotor at 30000 rpm for 20 hr. The RNA pellets were resuspended in 10 ml of water and dialyzed against water, and the final RNA samples were stored as ethanol precipitates at 4°C.

In Vitro Labeling of Chloroplast DNA. Chloroplast DNA was isolated from chloroplasts which had been purified on renografin gradients (Rawson and Haselkorn, 1973). The chloroplast DNA was labeled in vitro with [^{125}I]iodine (Oroz and Wetmur, 1974). The DNA was sonicated in 1 *M* NaCl using a micro-tip on a Labsonic sonicator. Sonication was at 75 W for a total of 6 min during which time the solution was not permitted to rise above 10°C. The DNA was dialyzed extensively against water, denatured by boiling at 100°C for 5 min, and cooled to 4°C. A 2.0-ml reaction mixture of 1×10^{-5} *M* KI, 0.10 *M*/0.04 *M* sodium acetate–acetic acid (pH 5.0), 2.25 mCi/ml of carrier free [^{125}I]iodine (New England Nuclear) in the form of NaI, 15.0 μg/ml of chloroplast DNA, and 5.0×10^{-4} *M* $TlCl_3$ was incubated at 60°C for 30 min and then cooled to 4°C. The iodinated DNA was separated from the reactants on a 1 × 27 cm Sephadex G-25 column (equilibrated and eluted with water). The labeled DNA in the void volume was dialyzed at 60°C for 5 hr against 1 l. of 0.4 *M* NaCl, 0.015 *M* NaH_2PO_4, and 0.002 *M* EDTA (adjusted to pH 6.0 with NaOH) to remove an unstable side product of the iodination reaction (Getz et al., 1972). The resulting [^{125}I]DNA was then dialyzed at 4°C overnight against 2 l. of water. The DNA was concentrated by precipitation with 1/10 vol of 3 *M* sodium acetate and 2 vol of ethanol. The DNA precipitate was resuspended in 1.0 ml of H_2O and stored at 4°C. At optimal settings on a Packard TriCarb liquid scintillation counter (Model 3320) the [^{125}I]chloroplast DNA had a specific activity of $3–4 \times 10^6$ cpm/μg. The single-stranded molecular weight of the DNA was 100000 (Rawson, 1975a).

Hybridization of [^{125}I]Chloroplast DNA to RNA. Hybridization of a vast excess of RNA to [^{125}I]chloroplast DNA was carried out in liquid at 60°C in 0.48 *M* sodium phosphate (pH 6.8). Appropriate amounts (7–8 mg) of the ethanol precipitates of the various RNA samples were centrifuged at 10000 rpm to recover the RNA. The pellets were dried and resuspended in 0.5 ml of 0.48 *M* sodium phosphate (pH 6.8); 5–10 μl of [^{125}I]chloroplast DNA was added to the RNA solution to make the final DNA concentration 0.05 μg/ml; 50-μl samples of the reaction mixture were sealed in capillary tubes. The reassociation reaction was started by boiling the capillary tubes for 5 min and immediately transferring them to a 60°C bath. At various times the samples were diluted into 0.12 *M* sodium phosphate (pH 6.8) and transferred to a 60°C jacketed column containing pretreated hydroxylapatite (Rawson, 1975b). Single-stranded and double-stranded structures were eluted with 10 times the bed volume of 0.12 and 0.48 *M* sodium phosphate (pH 6.8), respectively. The single-stranded and double-stranded fractions were adjusted to 10% Cl_3CCOOH and collected on Millipore filters. The filters were dried and placed in toluene–Omnifluor (New England Nuclear) scintillation fluid and counted in a Packard Tri-Carb liquid scintillation counter. The proportion of double-stranded DNA to single-stranded DNA plus double-strand-

[1] Abbreviations used are: 1 × SSC, 0.15 *M* NaCl–0.015 *M* sodium citrate; EDTA, ethylenediaminetetraacetic acid; T_m, the temperature required for 50% thermal denaturation.

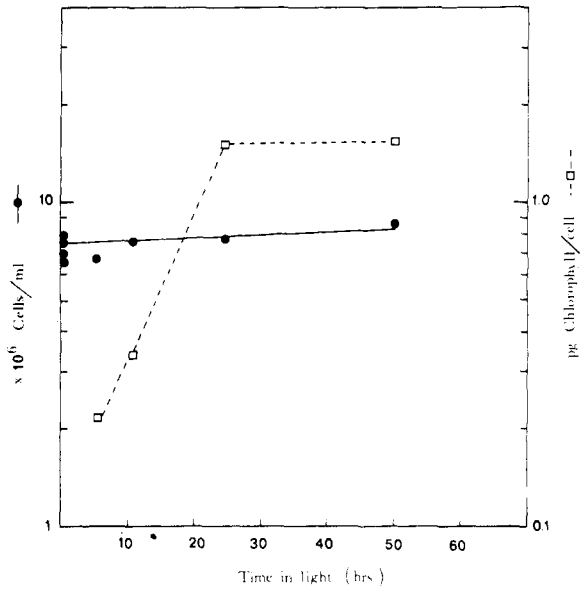

FIGURE 1: Growth curve for *E. gracilis* cells during chloroplast development. One-liter flasks containing 500 ml of heterotrophic medium were grown in the dark with constant shaking. When the cells reached stationary phase (7–8 × 10^6 cells/ml), they were illuminated for various periods of time. At those times indicated, a sample of cells was removed for a cell count and a chlorophyll determination. The remainder of the cells was collected by centrifugation and frozen at −20°C until RNA extraction.

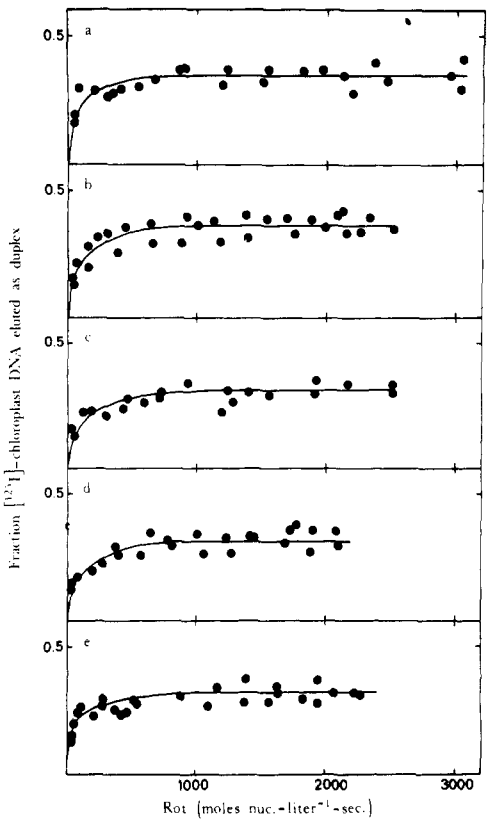

FIGURE 2: Hybridization of [^{125}I]chloroplast DNA to total cell RNA from *Euglena* cells at different stages of chloroplast development. Total cell RNA was isolated from cells at different stages of chloroplast development as indicated in Figure 1. Fifty microliters of [^{125}I]chloroplast DNA (0.05 μg/ml) with a specific activity of 3.9 × 10^6 cpm/μg was hybridized in 0.48 *M* sodium phosphate (pH 6.8) with 8–10 mg/ml of total cell RNA. The fraction of [^{125}I]chloroplast DNA in a duplex was determined by chromatography on hydroxylapatite columns. The R_0t values were calculated from the initial RNA concentrations of the individual RNA samples (moles of nucleotide per liter) × time (sec) and corrected for the increased rate of reassociation in 0.48 *M* sodium phosphate (pH 6.8) relative to 0.12 *M* sodium phosphate (pH 6.8). Curves a–e represent the hybridization of RNA samples isolated from dark grown cells illuminated for 0, 5.25, 10.75, 24.75, and 50.00 hr, respectively.

ed DNA was determined from the Cl_3CCOOH precipitable counts.

The extent of duplex formation was monitored as a function of the product of the initial RNA concentration (R_0, moles of nucleotide liter^{-1}) × time (t, sec). The R_0t values were corrected for the relative increase in the rate of reassociation in 0.48 *M* sodium phosphate to that in 0.12 *M* sodium phosphate (Britten et al., 1974).

Melting Curve of Hybrids. The T_m values of [^{125}I]chloroplast DNA–DNA and [^{125}I]chloroplast DNA–RNA duplexes were determined by melting them off hydroxylapatite columns. Renatured RNA–DNA hybrids or renatured DNA–DNA duplexes were adsorbed on hydroxylapatite in 0.12 *M* sodium phosphate (pH 6.8) at 60°C. Any remaining single-stranded DNA was eluted with 0.12 *M* sodium phosphate (pH 6.8) at 60°C. The temperature of the column was then raised in increments, allowed to reach temperature equilibrium for several minutes, and washed with 0.12 *M* sodium phosphate (pH 6.8). To assure that the [^{125}I]chloroplast DNA had been completely melted, the hydroxylapatite was washed finally at 100°C with 0.48 *M* sodium phosphate (pH 6.8). The fraction of [^{125}I]DNA eluted from the columns at various temperatures was determined by Cl_3CCOOH precipitation of the samples onto Millipore filters which were then counted in a Packard Tri-Carb scintillation counter.

Results

Chloroplast Development. The fraction of chloroplast DNA transcribed during chloroplast development was monitored in nondividing *Euglena* cells. *Euglena* cells were grown in the dark to stationary phase and then transferred to the light. Synthesis of chlorophyll was used as the means of quantitating chloroplast development. Cell concentration and cell chlorophyll content were determined when cells were collected for RNA isolation.

Figure 1 shows a growth curve of the cells and the chlorophyll content per cell when RNA was isolated for the hybridization experiments. The time for cell replication in this medium was normally 12 hr, but under these conditions there was only a 10% increase in cell concentration during the ensuing chloroplast development. Chlorophyll synthesis began immediately after the cells were placed in the light and was complete after 25 hr of illumination. Chlorophyll determinations of cells grown in the dark showed that there was less than 1.2 × 10^{-3} pg of chlorophyll per cell.

Hybridization of [^{125}I]Chloroplast DNA with RNA. The fraction of chloroplast DNA transcribed at various stages of chloroplast development was determined by hybridizing in liquid a vast excess of RNA to [^{125}I]chloroplast DNA and analyzing the resulting duplexes on hydroxylapatite columns. Figure 2 shows the kinetics of hybridization of total cell RNA, obtained from cells at various stages of chloroplast development, to [^{125}I]chloroplast DNA. The hybridization reaction was followed to R_0t values sufficiently great to assure that all regions on the chloroplast DNA which had been transcribed had ample time to form RNA–DNA hybrids.

Table I: Fraction of Chloroplast DNA Represented as RNA Transcripts during Chloroplast Development.[a]

Length of Light Exposure (hr)	Fraction Total [^{125}I] Chloroplast DNA in Form of Duplex (RNA–DNA and DNA–DNA)[b]	Fraction [^{125}I]-Chloroplast DNA in Form of RNA–DNA Hybrid	Fraction Chloroplast DNA Transcribed
0	0.343	0.265	0.530
5.25	0.364	0.286	0.572
10.75	0.311	0.233	0.466
24.75	0.310	0.232	0.464
50.00	0.317	0.239	0.478

[a] The fraction of chloroplast DNA transcribed during chloroplast development was measured by RNA–DNA hybridization (Figure 1). The extent of DNA–DNA reassociation for comparable periods of time was determined separately (Figure 2). The fraction of chloroplast DNA transcribed is equal to [(fraction total [^{125}I] chloroplast DNA in the form of a duplex) – (fraction [^{125}I] chloroplast DNA reassociated during times similar to that used for hybridization)] × 2. [b] 0.078 of the [^{125}I] chloroplast DNA had reassociated during the time needed to form these duplexes.

Table II: Fraction of Chloroplast DNA Represented in Mixtures of Different RNAs[a]

Time Illuminated (hr)	0	4	17.0	25.5	41.5	49.5
0	0.454	0.488	0.468	0.502	0.504	0.490
4		0.424	0.468	0.456	0.486	0.472
17.0			0.364	0.494	0.468	0.462
25.5				0.324	0.460	0.506
41.5					0.364	0.472
49.5						0.344

[a] Cells were grown to stationary phase in the dark and transferred to the light. Total cell RNA was isolated from cells which had been illuminated for 0, 4, 17, 25.5, 41.5, and 49.5 hr. Individual samples of RNA (mg/ml) in 0.48 *M* sodium phosphate (pH 6.8) were prepared each containing 0.05 μg/ml of [^{125}I] chloroplast DNA; 50 μl samples of equal volumes of two different RNA samples were hybridized at 60°C for 5 hr. The resulting duplexes were analyzed on hydroxylapatite columns, and the fraction of the [^{125}I] chloroplast DNA represented in the RNA transcripts was calculated. Individual samples of RNA were also hybridized to the [^{125}I] chloroplast DNA. The data in the table are an average of three individual determinations for each mixture. The R_0t values for these hybridizations corrected for high salt was 300–430 mol of nucleotide $l.^{-1}$ sec.

The fraction of [^{125}I]chloroplast DNA eluted from the hydroxylapatite columns as double-stranded structures represents both RNA–DNA and DNA–DNA duplexes. The extent of DNA–DNA reassociation in these reactions was calculated by renaturing [^{125}I]chloroplast DNA in the absence of RNA at concentrations and time periods comparable to those used for the RNA–DNA hybridization. The fraction of [^{125}I]chloroplast DNA renatured as a function of C_0t (the initial DNA concentration, C_0, × time, t, in moles of nucleotide liter^{-1} sec) found during the RNA–DNA hybridization was 0.078 of the total DNA in the reaction mixture. If the reassociation of [^{125}I]chloroplast DNA is followed to completion, the $C_0t_{1/2}$ of the reaction is 0.32 (not shown). The fraction of chloroplast DNA in an RNA–DNA hybrid is equal to the difference between the labeled DNA in the total duplex and that attributable to DNA–DNA renaturation in the absence of RNA. If one assumes only asymmetrical transcription of the chloroplast DNA, then the fraction of the chloroplast genome transcribed will be equal to two times the fraction of [^{125}I]chloroplast DNA in the RNA–DNA hybrids. Table I summarizes the information derived from Figures 1 and 2. It shows the length of time the cells were exposed to light before RNA was isolated, the fraction of total [^{125}I]chloroplast DNA in the form of a duplex and an RNA–DNA hybrid, and the fraction of chloroplast DNA transcribed.

When cells contain no detectable amounts of chlorophyll, a large fraction of the chloroplast genome (0.53) is represented as RNA transcripts. Immediately after transfer of the cells to light, a small increase (0.53–0.57) in transcription of the chloroplast genome occurs. Eleven hours after illumination of the cells, at which time slightly less than half of the final cellular chlorophyll has been synthesized, 46% of the chloroplast DNA is represented as RNA transcripts. This value remains relatively constant for the remaining time required for full chloroplast development.

An important question is whether all of the DNA sequences transcribed at later stages of chloroplast development are also represented in the transcripts at the onset of chloroplast development. RNA samples derived from a different batch of cells, but in which chloroplast development proceeded similarly to that described above, were used in the following mixing experiments. Six samples of RNA were isolated from cells illuminated for various time periods. First, each RNA sample was individually hybridized to the [^{125}I]chloroplast DNA, and then mixtures of the various RNA samples were hybridized to the same DNA. The proportion of [^{125}I]chloroplast DNA eluted as a duplex from hydroxylapatite columns was determined, and the fraction of the chloroplast genome represented as transcripts in the various RNA mixtures was calculated. Table II shows the results of these experiments and indicates that a small number of new sequences of chloroplast DNA are expressed after chloroplast development has begun. RNA samples at later stages of chloroplast development (25–50 hr in the light) contain transcripts which are derived from a small fraction of the chloroplast DNA (0.05) not previously transcribed.

Melting Properties of [^{125}I]Chloroplast DNA Duplexes. Determination of T_m values is a useful procedure to determine the fidelity of nucleic acid duplexes (Britten et al., 1974). Quantitation of the depression in T_m values of reassociated DNA–DNA and RNA–DNA duplexes is a good means of determining the extent of mismatch in the duplex.

The T_m of high molecular weight native chloroplast DNA melted in 0.12 *M* sodium phosphate is 79°C. Lowering the molecular weight of the native DNA to 300–400 base pairs results in a depression of the T_m to 76°C (not shown). When [^{125}I]chloroplast DNA, with a molecular weight of 100000, is allowed to reassociate to more than 80% completion, the T_m of the resulting duplex is 73.0°C or 6°C below the T_m of native DNA (Figure 3). The T_m of an RNA–DNA duplex monitored in the same fashion is 67.5°C or 5.5°C lower than a pure DNA–DNA reassociated duplex (Figure 3). The melting of both the reassociated DNA–DNA and RNA–DNA structures is cooperative, as would be expected for well-matched structures. The RNA–DNA hybrids are stable to RNase and show only a slightly lower T_m (66°, not shown).

Discussion

A lack of knowledge of the cellular location of all the

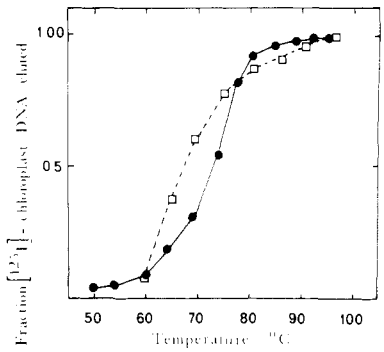

FIGURE 3: Hydroxylapatite melting curve of renatured [^{125}I]chloroplast DNA and an RNA-DNA hybrid. Renatured DNA-DNA or RNA-DNA duplexes were adsorbed on hydroxylapatite in 0.12 *M* sodium phosphate (pH 6.8) at 60°C and washed extensively with 0.12 *M* sodium phosphate (pH 6.8) at 50°C. The temperature of the column was then raised in increments, equilibrated at each temperature for 5 min, and washed with 0.12 *M* sodium phosphate buffer. After the final elution with 0.12 *M* sodium phosphate (pH 6.8), at greater than 97°C, the columns were washed with 0.48 *M* sodium phosphate (pH 6.8) to assure that all labeled DNA had been melted off the column. In all cases, greater than 99.9% of the DNA melted off the column in 0.12 *M* sodium phosphate buffer. The T_m of the reassociated DNA-DNA was 73.0°C. The T_m of the RNA-DNA hybrid was 67.5°C. (●—●) DNA-DNA melt; (□- -□) RNA-DNA melt.

transcripts derived from the chloroplast DNA requires that total cell RNA be used for hybridization to the [^{125}I]chloroplast DNA. The specificity of the hybridization of only chloroplast DNA transcripts to the chloroplast DNA has previously been demonstrated (Rawson, 1975a). Total cell RNA, derived from a heat-bleached mutant of *Euglena* which completely lacks chloroplast DNA (J. R. Y. Rawson, unpublished results), does not form RNA-DNA hybrids with [^{125}I]chloroplast DNA.

Saturation of those regions of chloroplast DNA represented in the various RNA preparations has been assured by the fact that the R_0t values used for these hybridizations are sufficiently great to assure that all of the DNA fragments had ample opportunity for multiple collisions with complementary RNA molecules. The relatively low kinetic complexity (approximately 1.36×10^5 base pairs) of the chloroplast DNA and the very high R_0t values used for these experiments argue strongly for having saturated all those regions of chloroplast DNA represented in the various RNA samples. Since the average size of the DNA (300 nucleotides) used for the hybridization is considerably smaller than the average size of a structural gene, it is expected that the chloroplast DNA in the form of an RNA-DNA hybrid will be completely covered with RNA.

The thermal stability of the RNA-DNA hybrids and the cooperative melting patterns are a good indication of the specificity of these duplexes. The 6°C decrease in the T_m of the reassociated [^{125}I]chloroplast DNA indicates that faithful duplexes were formed after renaturation. A 3°C drop in the T_m of reassociated low molecular weight DNA (Britten et al., 1974) as well as a 2–3°C drop for reassociated iodinated DNA (Oroz and Wetmur, 1974) accounts for the observed 6°C drop of the reassociated DNA-DNA duplex from that of native DNA. We have not seen any evidence for the unspecific binding of cellular RNA reported by Brown and Haselkorn (1971).

The complexity of chloroplast DNA is 1.36×10^5 nucleotide pairs (Manning et al., 1971). When the cells are grown in the dark, 53% (7.2×10^4 nucleotide pairs) of the chloroplast DNA is transcribed. Five hours after illumination of the cells the fraction of chloroplast DNA transcribed rises to 0.57 (7.8×10^4 nucleotide pairs). At completion of chloroplast development, 47% of the chloroplast DNA (6.4×10^4 nucleotide pairs) is represented as RNA transcripts in the cell. The hybridization of various mixtures of RNA to the chloroplast DNA indicates that at later stages of chloroplast development, although there is less of the chloroplast DNA transcribed, these sequences are not all the same as those transcribed at the onset of chloroplast development. The conclusions drawn from the hybridization of various mixtures of RNA suffer from the somewhat lower R_0t values used compared to those for measuring the absolute fraction of chloroplast development. The absolute differences in the fraction of chloroplast DNA in the form of an RNA-DNA hybrid with the different mixtures of RNA may change slightly at higher R_0t values, but the qualitative observation that different regions of chloroplast DNA are being transcribed at later stages of chloroplast development will probably still hold.

If it is assumed that the average molecular weight of a protein within a cell is 40000 (Galau and Britten, 1974), then the number of nucleotide pairs of DNA required to code for this protein is 1000. The total complexity of the chloroplast DNA ever represented as RNA transcripts is 8.5×10^4 nucleotide pairs of which 1.08×10^4 nucleotide pairs (Rawson, 1975a) may be structural RNA transcripts (rRNA and tRNA). Therefore, the maximum complexity of the chloroplast DNA which may be represented as proteins is 7.4×10^4 nucleotide pairs or enough DNA to code for approximately 74 proteins.

The fraction of chloroplast DNA transcribed during chloroplast development is substantially greater than in exponentially growing cells containing mature chloroplasts (Rawson, 1975a). Measurements of the fraction of chloroplast DNA transcribed in dividing cells in a heterotrophic medium during chloroplast development have shown slightly different values (unpublished results), but the overall scheme of more chloroplast DNA being transcribed in the dark than at later stages of organelle development in the light remains the same.

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