Circular Chloroplast DNA from *Euglena gracilis*

(electron microscopy/density gradient centrifugation/mitochondrial DNA)

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ABSTRACT Chloroplast DNA of the protozoan flagellate, *Euglena gracilis*, exists as circular molecules, 40 μ m in contour length, as shown by electron microscopy and buoyant density analyses.

Cytochemical studies (1-3) and cell fractionation and buoyant density analyses (4-8) have shown that chloroplasts of microorganisms and of higher plants contain DNA distinct from that of the nuclear DNA of these organisms. Electron microscope examinations of DNA from chloroplast fractions of several species have failed to reveal circular molecules (9-13). However, in each of these studies, the possibility of contamination by nuclear DNA, mitochondrial DNA, or DNA of contaminating organisms was not adequately ruled out, thus negating the value of length measurements of linear molecules. The size and structure of chloroplast DNA have also been examined by its renaturation kinetics (8, 14) and sedimentation velocity (5), which have suggested minimal sizes for this DNA. Our results indicate that the chloroplast DNA of *Euglena gracilis* exists as circular molecules, 40 μ m in contour length.

MATERIALS AND METHODS

Culture conditions

Euglena gracilis Klebs, strain Z, cells were maintained autotrophically (15). Cells were harvested at 4°C, suspended in cold STE (0.37 M sucrose-10 mM Tris·HCl-50 mM EDTA, pH 7.6), and centrifuged at 3000 $\times g$ for 10 min. The pellets were suspended in 4 volumes of cold STE and used immediately for chloroplast isolation.

Cells of the permanently bleached *Euglena gracilis* strain W3 BUL and of strain Z were grown heterotrophically (15) for the isolation of mitochondrial fractions.

Isolation of chloroplasts

Euglena chloroplasts were isolated by a modification of the procedure of Brawerman and Eisenstadt (6). Autotrophically grown cells were broken by passage through a French pressure cell at 3000-3500 psi. The resulting suspension was centrifuged at 3000 $\times g$ for 10 min, the pellet was resuspended in 20 vol (all volumes are based on the original packed-cell volume) of STE and allowed to stand for 10 min. The upper 3/4 of the suspension was decanted through 10 layers of cheesecloth to remove unbroken cells and cell debris, and the solution remaining was discarded. The decanted suspension was cen-

trifuged at 3000 $\times g$ for 10 min and the pellet was suspended in 2 volumes of STE. 4 volumes of 2.2 M sucrose were added and the suspension was centrifuged at 13,000 $\times g$ for 30 min in the Sorvall HB-4 rotor. The green pad that layered on top of the sucrose solution was removed and suspended in STE and centrifuged at 3000 $\times g$ for 10 min; the pellet was suspended in STE. Another sucrose flotation was performed, and the floating pad was again suspended in STE and centrifuged at 3000 $\times g$ for 10 min. This pellet was washed two more times. These chloroplasts were suspended in 0.3 M KCl-0.05 M EDTA (pH 7.6) and centrifuged at 1000 $\times g$ for 5 min. This washing was repeated twice, and the pellet was finally suspended in an equal volume of 0.15 M NaCl-0.10 M EDTA-0.05 M Tris·HCl (pH 9.0) and stored at -20°C.

Isolation of mitochondria

Mitochondria were isolated from bleached cells, strain W3 BUL, by the method of Sharpless and Butow (16). Modifications included cell rupture by shaking with glass beads of 0.2-mm diameter for 5 sec and washing the mitochondria four times with buffer. Frequently, mitochondria were then suspended in an equal volume of 0.25 M sucrose-10 mM Tris·HCl-5 mM MgCl₂ (pH 7.4), and treated with 100 μ g/ml of pancreatic DNase for 30 min at 37°C. The suspension was centrifuged at 5000 $\times g$ for 15 min, and the pellet was washed three times with 0.25 M sucrose-10 mM Tris·HCl-10 mM EDTA (pH 7.4) to remove the enzyme. The mitochondria were then washed once with 0.15 M NaCl-0.10 M EDTA-0.05 M Na₂HPO₄ (pH 8.5), and portions were stored at -20°C before analysis in the electron microscope, while the majority of the pellet was suspended in 0.15 M NaCl-0.10 M EDTA-0.05 M Tris·HCl (pH 9.0) and frozen prior to DNA extraction.

Mitochondria were also prepared from cells containing chloroplasts (strain Z) by the procedure of Krawiec and Eisenstadt (17), through the washes of the crude mitochondrial pellet. These mitochondria were then treated with DNase, washed, and suspended in 0.15 M NaCl-0.10 M EDTA-0.05 M Tris·HCl (pH 9.0).

Isolation of DNA

E. gracilis chloroplast or mitochondrial DNA, *Escherichia coli* bromouracil-labeled hybrid DNA, and *Micrococcus luteus* DNA were prepared as described (15).

Some preparations of chloroplast DNA for examination by electron microscopy were modified to minimize shearing action. A solution (2 ml) of 0.79 M sucrose-0.15 M NaCl-0.10

Abbreviations: SDS, sodium dodecyl sulfate; STE, 0.37 M sucrose-10 mM Tris·HCl-50 mM EDTA (pH 7.6).

FIG. 1. Electron micrograph of a rotary-shadowed circular molecule of DNA from a SDS lysate of a chloroplast fraction isolated from cells of *Euglena gracilis*, strain Z. The molecule is 40.5 μm in total length. $\times 49,000$; the line represents 1 μm .

M EDTA–0.05 M Tris·HCl (pH 9.0) was added to 2 ml of thawed, packed chloroplasts. SDS (sodium dodecyl sulfate, 0.5%), pronase (1 mg/ml), and sodium deoxycholate (1%) were added and the mixture was gently rocked overnight at 4°C. NaClO_4 (5 M) was added to a final concentration of 1 M, and the solution was rocked for 30 min at 4°C with an equal volume of chloroform–isoamyl alcohol 24:1. The mixture was centrifuged at $10,000 \times g$ for 10 min at 4°C. The aqueous phase was removed, reextracted with chloroform–isoamyl alcohol, and centrifuged. The aqueous phase was dialyzed overnight against 0.15 M NaCl–15 mM sodium citrate–0.5 mM EDTA (pH 8.0) in the cold, and then prepared directly for electron microscopy.

Analytical density gradient centrifugation in CsCl

Density gradient centrifugations were performed in the Spinco model E ultracentrifuge (15), using *M. luteus* DNA and/or bromouracil-labeled hybrid DNA from *E. coli* as

markers. The density of the DNA was determined by the method of Schildkraut, Marmur, and Doty (18).

Electron microscopy

(a) *Sections*: Pellets of chloroplasts or mitochondria were fixed (19) and picked up on uncoated 400-mesh copper grids. (b) *Protein monolayers*: Whole cells or samples of chloroplasts, or of mitochondria, were digested with 1 mg/ml of pronase freed of DNase in 0.25% SDS–15 mM NaCl–10 mM EDTA for 4 hr at 37°C. Chloroplasts were also lysed with 2% SDS in 15 mM NaCl–10 mM EDTA for 30 min. Approximately 10 μl of the digest or lysate was added to 0.1 ml of 1 M ammonium acetate that contained 0.05% cytochrome *c* and 0.5% formaldehyde. This solution was spread onto a hypophase of 0.3 M ammonium acetate that contained 0.5% formaldehyde. The surface film was picked up on carbon-coated formvar films supported on 100-mesh

Fig. 2. Frequency distributions of the lengths of circular DNA molecules from whole cells and from a chloroplast fraction of *E. gracilis*, strain Z. The DNA was prepared directly for electron microscopy after the treatments indicated. The mean and standard error of each sample is given.

copper grids and shadowed (19). All grids were examined in a Hitachi 7S electron microscope.

In order to estimate the amount (percentage of total length) of DNA in the form of circles in preparations of chloroplast DNA, grids were chosen on which the DNA was in low enough concentration so that the molecules did not overlap each other. Grid squares were methodically searched from one side to the other and *all* of the molecules seen, both circular and linear, were photographed at an original magnification calibrated with a diffraction grating replica (2160 lines/mm) to be 11,600. Measurements of molecules were made on positive prints, at a magnification of 60,400, with a map measure.

RESULTS

When a chloroplast fraction from strain Z cells was lysed with 2% SDS and the DNA was examined in the electron microscope, 34% of this DNA was in the form of circular molecules with an average contour length of 40.3 μm (Figs. 1, 2, and 3). The remaining DNA was in the form of linear molecules that ranged in length from less than 1 to 43 μm (Fig. 3).

DNA obtained from whole cells that contained chloroplasts (strain Z) showed minor peaks with buoyant densities of 1.690 and 1.685 g/cm^3 in CsCl equilibrium density gradients (Fig. 4), due to mitochondrial and chloroplast DNA, respectively (6, 20, 21). DNA obtained from the same chloroplast fraction used for electron microscopy formed a band in a CsCl gradient with a main peak at 1.685 g/cm^3 (Fig. 4). Two lesser components at densities of 1.690 and 1.699 g/cm^3 were visible, but together accounted for no more than 20% of the total DNA in the chloroplast fraction. Thus, the amount (34%) of DNA in the form of 40- μm circles makes it unlikely that this DNA represents one of the lesser components. When the chloroplast fraction was initially treated with DNase (2 $\mu\text{g}/\text{ml}$ for 1 hr at 37°C), the characteristic DNA filaments could not be detected in preparations for the electron microscope, and the ultraviolet-absorbing band at 1.685 g/cm^3 was not found upon buoyant density analysis.

Fig. 3. Frequency distributions, expressed as the percentage of the total length of DNA examined, of the lengths of circular and linear DNA molecules from a chloroplast fraction of *E. gracilis*, strain Z. The chloroplasts were lysed with 2% SDS and the lysate was spread directly. The total length of DNA represented in the histogram is 1368 μm , of which 34% was in the form of circles.

In thin sections of the chloroplast fraction examined in the electron microscope, only intact chloroplasts and broken membranes were seen (Fig. 5). Bacteria or other contaminating organisms were not found.

In the circular DNA molecules of 40 μm originate from chloroplasts, then they should be demonstrable in whole-cell lysates of cells that contain chloroplasts (strain Z), but not in whole-cell lysates of cells that contain neither chloroplasts nor chloroplast DNA (strain W3 BUL, Fig. 6). Satisfactory

Fig. 4. Densitometer tracings of photographs after equilibrium density gradient centrifugation in CsCl of whole-cell DNA (44 μg), of DNA (2 μg) isolated from a chloroplast fraction, and of DNA (3 μg) isolated from a mitochondrial fraction (treated with DNase) from *E. gracilis* cells that contained chloroplasts. *M. luteus* DNA ($\rho = 1.731 \text{ g}/\text{cm}^3$) and *E. coli* bromouracil-labeled DNA ($\rho = 1.754 \text{ g}/\text{cm}^3$) served as references.

FIG. 5. A thin section of a pellet of a chloroplast fraction isolated from cells of *E. gracilis*, strain Z. The fraction consists mainly of chloroplasts and broken membranes. $\times 11,000$; line represents $1\ \mu\text{m}$.

rotary-shadowed preparations of DNA from whole cells could not be made by lysis with SDS alone, but were obtained by digestion with pronase and SDS. In preparations from cells containing chloroplasts, circular DNA molecules with an average contour length of $47.2\ \mu\text{m}$ (Fig. 2) were found (albeit rarely). No circular molecules were found in lysates of cells lacking chloroplasts. The observed circular molecules from digests of strain Z were 17% longer than the circular molecules obtained from a chloroplast fraction of these cells. However, when DNA from the chloroplast fraction was digested with pronase and SDS, the circular molecules were found to have an average contour length of $44.0\ \mu\text{m}$. When DNA was prepared from the chloroplast fraction by a method that included pronase digestion, but was followed by deproteinization with chloroform-isoamyl alcohol, the circular molecules had an average contour length of $40.1\ \mu\text{m}$ (Fig. 2), a value very similar to that found for circular molecules from SDS lysates of the chloroplast fraction. These results suggest that the presence of pronase results in a transitory increase in length of the DNA molecules. They also suggest that the circular DNA molecules found in

FIG. 6. Densitometer tracings of photographs after equilibrium density gradient centrifugation in CsCl of whole-cell DNA ($44\ \mu\text{g}$), of DNA ($1\ \mu\text{g}$) isolated from a mitochondrial fraction treated with DNase, and of DNA ($4\ \mu\text{g}$) from a mitochondrial fraction not treated with DNase from *E. gracilis* cells lacking chloroplasts. *M. luteus* DNA ($\rho = 1.731\ \text{g/cm}^3$) and *E. coli* bromouracil-labeled hybrid DNA ($\rho = 1.754\ \text{g/cm}^3$) served as references.

whole-cell lysates of cells containing chloroplasts were the same as those found in lysates of chloroplast fractions.

DNA was prepared from lysates of mitochondrial fractions (DNase treated) of cells containing chloroplasts. Only linear molecules were found. They ranged in length from 1 to $19\ \mu\text{m}$, and had a mean length of $1.3\ \mu\text{m}$. Similarly, in a preparation of DNA from mitochondrial fractions of cells lacking chloroplasts, only linear molecules were found. Buoyant density analyses of the DNA from these mitochondrial fractions (Figs. 4 and 6) yielded single bands with a density of $1.690\ \text{g/cm}^3$. The mitochondrial fractions consisted mainly of mitochondria, although some remnants of other cell organelles were present. Bacteria were not seen in any of the preparations.

In order to test whether the DNase treatment of mitochondria could be responsible for the failure to find circular molecules, DNA was prepared from both strains from mitochondrial fractions that had not been treated with DNase. No circular molecule was found; more than 1000 molecules were examined from each preparation. Buoyant density analysis of DNA extracted from these fractions indicated that as much as 40% of the DNA was of mitochondrial origin (Fig. 6).

Digests of whole cells containing chloroplasts revealed rare circular molecules, with an average contour length of $3.6\ \mu\text{m}$ (standard error = $\pm 0.03\ \mu\text{m}$; $n = 10$). Molecules that apparently consisted of 2, 3, or 4 interlocked circles

of 3.6 μm (each) were also seen. Similar circles of 3.6 μm were not found either in lysates of cells lacking chloroplasts or in lysates of chloroplasts.

DISCUSSION

Our results indicate that at least one-third of the DNA of chloroplasts of *Euglena gracilis* is in the form of circular molecules of about 40 μm in contour length. Since none of the linear molecules in the same preparation were longer than the longest circle, our finding is consistent with the view that all of the chloroplast DNA is present as circular molecules of 40 μm *in situ*. The mass per unit length of deproteinized DNA prepared by the protein monolayer technique has been shown to be influenced by the salt concentration of the hypophase and spreading solutions (22–26). Taking these findings into consideration, and using a hypophase of 0.3 M ammonium acetate–0.5% formaldehyde, we have assumed a value of 2.07×10^6 daltons per micrometer for the deproteinized chloroplast DNA. (It is recognized that an internal standard was not used and there may be some error in this value.) A molecule of 40 μm length would have a molecular weight of 8.3×10^7 . Since deproteinized DNA from a chloroplast fraction has the same contour length as DNA prepared directly after lysis of chloroplasts with SDS alone (Fig. 2), it appears these DNA molecules have the same molecular configuration. However, preparation of the DNA by spreading directly after digestion with pronase in the presence of SDS yielded circular DNA molecules with a mean contour length as much as 17% greater than that of molecules of deproteinized DNA or of DNA prepared by lysis of chloroplasts with SDS.

It seems unlikely from our data that the continuity of the circular molecules is dependent on non-DNA links. If such links existed, they must be stable to detergent, pronase, and chloroform–isoamyl alcohol, and they must be relatively short and/or have a diameter close to that of the DNA double helix.

From a study of the rate of renaturation of chloroplast DNA of *Euglena gracilis*, Stutz (14) concluded that the kinetic complexity of this DNA was 1.8×10^8 daltons, corresponding to a unique sequence length of 87 μm . If a correction is made to the data of Stutz for the low guanine plus cytosine content of this chloroplast DNA, from the considerations of Wetmur and Davidson (27), a unique sequence length of 43.5 μm (9×10^7 daltons) is obtained. This, together with our data, indicates that the total genetic information carried by chloroplast DNA of *E. gracilis* is accommodated by the length of the circular molecule.

A DNA molecule of 40 μm could code for the amino acid sequences of about 280 polypeptides with an average molecular weight of 20,000 (assuming an average molecular weight for an amino acid of 135). Estimates of the weight of DNA per chloroplast of *E. gracilis* range from 1.2×10^{-15} g (7) to 1×10^{-14} g (6), which correspond to lengths of DNA of 360 and 2900 μm , respectively. Each chloroplast could, therefore, carry between 9 and 72 copies of its own genetic information.

Noncircular mitochondrial DNA molecules have been shown to be heterogeneous in length by electron microscopy

(19, 28–30). The DNA molecules of different size may be a true representation of the lengths of molecules *in situ*, but could also represent the products of nuclease activity and/or small amounts of nuclear DNA contamination. Heterogeneity of length has also been observed for *E. gracilis* mitochondrial DNA in this report. Correcting for the apparently greater length of DNA molecules prepared for electron microscopy after digestion by pronase–SDS, the longest molecule we found in the mitochondrial DNA preparation (19 μm) could carry about 3 times the genetic information of a single DNA circle from animal mitochondria. Alternatively, as has been suggested (19), the longer molecules may comprise tandem repeats of a shorter sequence.

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