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EVIDENCE FOR A NOVEL DNA COMPONENT IN CHLOROPLASTS OF *EUGLENA GRACILIS*

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1. Introduction

Chloroplasts from *Euglena gracilis* contain double stranded DNA having a mean buoyant density of 1.685 g/cc in neutral CsCl [1–3]. Manning and Richards [4] showed by electron microscopy that chloroplast DNA of this density occurs in circular form (up to 30% of a given DNA preparation), the circumference being 44.5 (± 0.6) μm. These authors also showed, using sucrose gradients, that the fastest sedimenting chloroplast DNA fraction had a mol. wt of 9.2×10^7; this value corresponds to the reported length of the circles.

An additional DNA component with a mean buoyant density of 1.700 g/cc was found to be associated with highly purified chloroplasts [5, 6]. It was not immediately clear as to whether this heavier DNA was a contaminant of unknown origin, or represented a second type of chloroplast 'chromosome' or was a G+C rich part of the main circular DNA. Most characteristic for this heavy DNA component was its high affinity for chloroplast rRNA as evidenced by DNA–RNA hybridization experiments [5]. Recently, Rawson and Haselkorn [7] working with highly purified total chloroplast DNA confirmed our observations and further showed that some 1.700 g/cc DNA could be generated from total chloroplast DNA by mechanical degradation. They inferred that the 1.700 g/cc DNA is a segment of the 44 μm long chromosome, carrying the chloroplast rRNA genes.

In order to get some further insight into the structure of *Euglena* chloroplast DNA and in particular to exploit its base compositional heterogeneity we ventured to isolate in preparative amounts either intact circular DNA or at least DNA of comparable high molecular weight. Unfortunately, in no case were we able to achieve this goal, however, using a method described in the following, we routinely obtained chloroplast DNA with a mean mol. wt of $2–3 \times 10^7$. Such chloroplast DNA when analyzed in CsCl-density gradients showed two DNA components. The respective density profiles had a main peak corresponding to a density of 1.685 g/cc and a shoulder corresponding to 1.692 g/cc. DNA with a density in the range of 1.692 to 1.690 g/cc in the literature is usually referred to as *Euglena* mitochondrial DNA [8, 9]. However, we show in the following that this 1.692 g/cc DNA is a genuine chloroplast DNA. In particular, evidence is given that the 1.692 g/cc DNA upon shearing yields 1.700 g/cc DNA and that it has a high affinity for chloroplast rRNA similar to the 1.700 g/cc DNA characterized earlier [5, 7].

2. Materials and methods

2.1. *DNA isolation*

Euglena gracilis Klebs (Z strain) cells were grown under autotrophic conditions, harvested, washed and stores at –60°C as reported earlier [10]. Chloroplasts were isolated from trypsinized cells and purified by flotation on gradients of Renografin [11]. Purified chloroplasts were lysed in 8 M urea and 5% sodium dodecyl sulfate in a buffer containing 0.1 M NaCl, 0.01 M Tris–HCl (pH 7.9), 1 mM sodium EDTA. The lysed chloroplasts were gently extracted with an equal volume of phenol–chloroform–isoamyl alcohol

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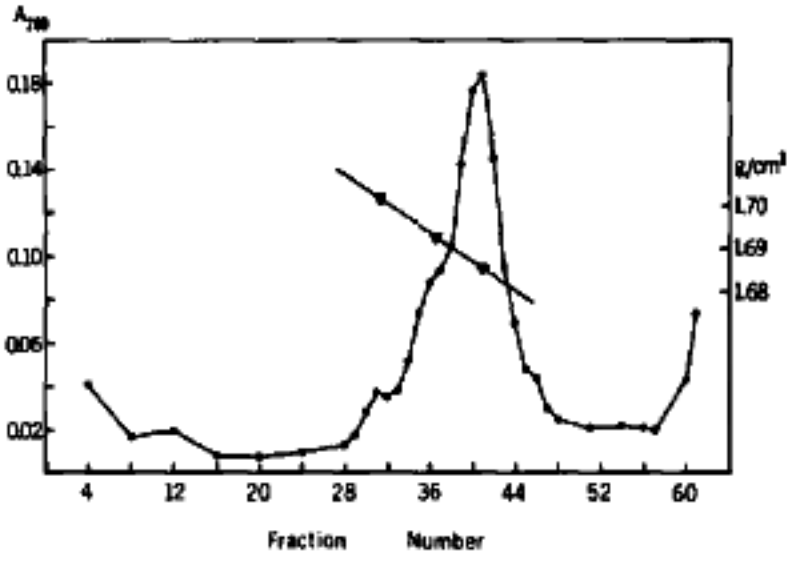

Fig. 1. Preparative CsCl-density gradient of *Euglena* chloroplast DNA. Approximately 50 μg of chloroplast DNA were equilibrated in a 8 ml preparative CsCl-density gradient. Initial density was 1.687 g/cc. Centrifugation was carried out in a Beckman type 40 rotor, at 33 000 rev/min, 20°C, 60 hr. The gradient was monitored by collecting 4 drop fractions (approx. 0.15 ml), adjusting the fractions to 0.5 ml with 1 × SSC and measuring the absorbance (260 nm). The fractions 27–37 and 38–48 were pooled separately, and dialyzed extensively in the cold against 1 × SSC containing 10^{-4} M EDTA. The dialysates were used for subsequent analytical work without further treatment.

(25:24:1, v/v/v) for 15 min at room temperature. The aqueous phase was chromatographed through a Sephadex G-50 (fine grade) column (1.5 × 30 cm) equilibrated with 1 × SSC (0.15 M NaCl, 0.015 M sodium citrate). The DNA fractions were pooled and further purified on a preparative CsCl-density gradient (see legend, fig. 1).

2.2. *Preparation of chloroplast* [^3H] *rRNA*

Highly purified chloroplast rRNA [10] (1 mg) was labeled with 5 mCi of [^{3}H] dimethylsulfate (New Wngland Nuclear, 385 mCi/mM) according to Smith et al. [12]. This in vitro labeling seems not to interfere with the specificity of the subsequent hybridization reaction [13]. Prior to use, the labeled rRNA was filtered three times through nitrocellulose filters. The final product had a specific activity of 5595 cpm/μg RNA.

3. Results and discussion

In fig. 1, a preparative CsCl-density gradient of total

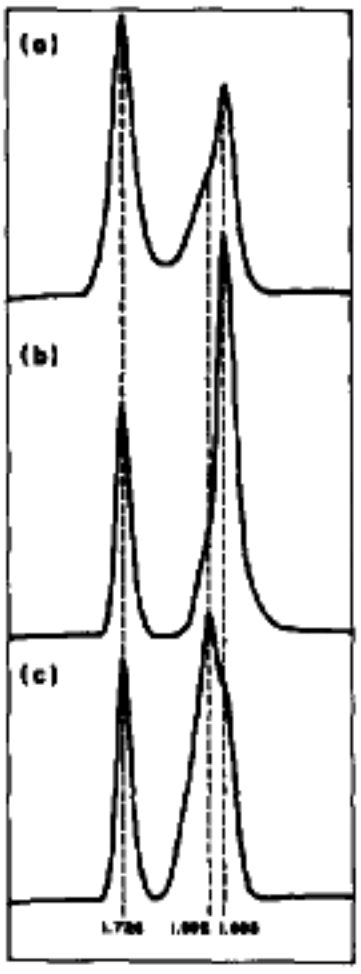

Fig. 2. Densitometer tracings of photographs from analytical CsCl equilibrium-density gradients of chloroplast DNA. *Euglena* chloroplast DNA (2–3 μg) with an average molecular weight = 2×10^7 was loaded on the gradients. Profile: (a) total DNA; (b) DNA from fractions 38–48 (fig. 1); (c) DNA from fractions 27–37 (fig. 1). Centrifugation was carried out in a Beckman Model E An-D rotor at 44,770 rev/min 20°C, 20 hr. *Pseudomonas aeruginosa* DNA (density 1.726 g/cc) is used as a density marker.

DNA isolated from renografin-purified chloroplasts is shown. A main component with a mean density of 1.685 g/cc, a major shoulder of 1.692 g/cc and a minor peak in the range of 1.700 g/cc is discernible. The average molecular weight of this DNA preparation is 2×10^7. Obviously, even at such a high molecular weight level, there is a considerable heterogeneity in terms of buoyant densities suggesting that at least two populations of DNA molecules occur differing in average G+C content. This and similar type gradients were fractionated and fractions were pooled in such a way as to enrich for the heavy component (1.692 g/cc) (fractions 27–37) and for the light component (1.685 g/cc) (fractions 38–48). From parallel gradients DNA corresponding to fractions 27–48 was pooled (total chloroplast DNA). The pooled DNA samples were analyzed in analytical gradients.

In fig. 2, the buoyant density profiles (neutral CsCl) of the three different samples of chloroplast DNA are displayed. Profile (a) was obtained with total chloroplast DNA. The profile shows a main band (1.685 g/cc) and a shoulder (1.692 g/cc) as anticipated from the preparative profile. Profile (b) represents the light DNA component. More than 90% of the DNA equilibrate at 1.685 g/cc and a slight shoulder is visible in the range of 1.692 g/cc. Profile (c) represents the heavy fraction. The main band has an average density of 1.692 g/cc but a substantial shoulder remains in the range of 1.685 g/cc. While we obtained relatively pure 1.685 g/cc, the heavier fraction is strongly mixed with lighter DNA.

Each of the three samples was fragmented to an average molecular weight of 4×10^6, and the density profiles of the sheared DNA samples are given in fig. 3. Total chloroplast DNA, profile (a) shows a somewhat broadened main peak (1.685 g/cc) and a distinct minor peak at a density of 1.701 g/cc. The 1.692 g/cc component is not discernible anymore. Profile (b) representing the light chloroplast DNA has also a main 1.685 g/cc peak plus a small distinct peak with a mean density in the range of 1.702–1.703 g/cc. The 1.692 shoulder from the previous pattern (fig. 2, profile (b)) has disappeared. Profile (c) representing the heavy fraction, is clearly bi-modal. The two approximately equal peaks equilibrate at 1.701 g/cc and 1.687 g/cc. The 1.692 g/cc band is no longer present. This shearing experiment can be interpreted only in such a way that the 1.692 g/cc DNA upon degradation to an average molecular weight of 4×10^6 yields two DNA populations, one with density in the range of 1.701 g/cc, the other with a density in the range of 1.687 g/cc.

We had reported earlier that the 1.700 g/cc DNA carries the genes for chloroplast 23 S–16 S rRNA [5]. Depending on the amount of heavy DNA component present, a DNA preparation would hybridize up to 6% with rRNA. We repeated the hybridization experiments using [^{3}H] rRNA and either total chloroplast DNA, or the heavy or light DNA component. The hybridization experiments were done as reported earlier [5] and for each DNA sample a saturation curve was established. The hybridization plateaux were reached at a rRNA–DNA (μg/μg) ratio of 0.4 : 0.5 and remained constant up to a ratio of 14 (J.P. Vandrey, Doctoral Thesis, Northwestern University, 1973). The values for total

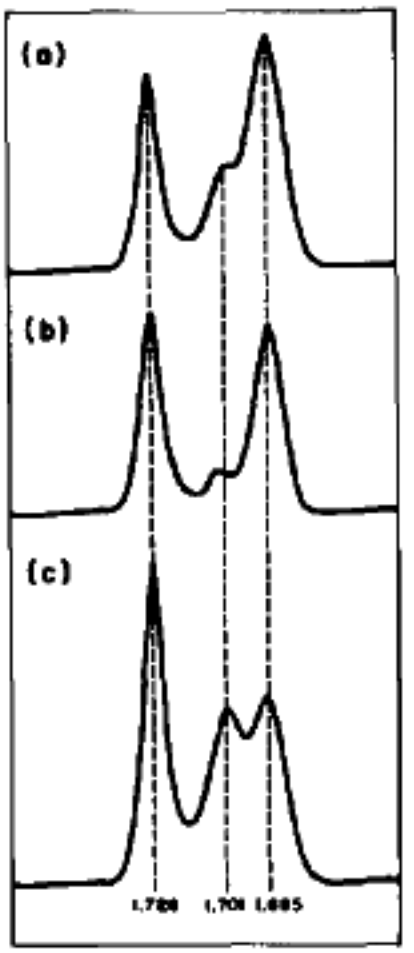

Fig. 3. Densitometer tracings of photographs from analytical equilibrium density gradients of sheared chloroplast DNA. Chloroplast DNA was forced through a 27 gauge needle four times prior to centrifugation (average mol. wt. of DNA was 4×10^6). Profile: (a) total chloroplast DNA; (b) chloroplast DNA from fractions 38–48 (fig. 1); (c) chloroplast DNA from fractions 27–37 (fig. 1). Centrifugation conditions and marker DNA as mentioned in legend to fig. 2.

chloroplast DNA, heavy fraction DNA and light fraction DNA were 2.8%, 6.9% and 1.1%, respectively. These hybridizations clearly show that the heavy component carries the majority of the rRNA genes which is in line with earlier reports [5, 7]. In this particular case, estimating the 1.692 g/cc DNA to represent about 25–30% of the total DNA (see fig. 2, profile (a)), we calculate that more than 70% of the ribosomal DNA has banded within the heavy fraction. Incidentally, the 1.1% hybridization found for the light fraction matches the earliest reported data [14, 15] and can be explained by the DNA purification procedures applied in those experiments.

Total chloroplast DNA hybridized to 2.8%. This is significantly higher than the 1.9% reported by Rawson and Haselkorn [7]. The only major difference in the two experiments is the starting cell material.

Rawson and Haselkorn used cells grown up to the stationary phase, while we harvested the cells in the exponential growth phase. As shall be shown elsewhere (Vandrey, J.P. and Stutz, E., to be published), the amount of 1.692 g/cc DNA fluctuates with the life cycle of the *Euglena* cells, indicating that the 1.692 g/cc DNA may at certain stages of chloroplast development replicate at a different rate than the 1.685 g/cc DNA component. This does not exclude the 1.692 g/cc DNA from being an integral part of the main DNA component (44 μm circle) at the stationary phase. In view of this situation, differences in hybridization values (rRNA6total DNA) are expected. However, the hybridization figures reported earlier from this laboratory with total chloroplast DNA [5] are most likely too high, due either to selective loss of lighter DNA or enrichment of heavier DNA during the purification procedure.

The 1.692 g/cc DNA is not mitochondrial DNA. *Euglena* mitochondrial DNA has quite different physical and chemical properties as shall be shown elsewhere (Fonty, G., Crouse, E.J., Stutz, E. and Bernardi G., to be published). Also, we have measured the hybridization capacity of highly purified mitochondrial DNA with chloroplast rRNA. Mitochondrial DNA (1.689 g/cc) hybridized with chloroplast rRNA to 0.12%, well below the 6.9% found for the 1.692 g/cc chloroplast DNA (Crouse, E.J. and Stutz, E., to be published).

Acknowledgements

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References

[1] Brawerman, G. and Eisenstadt, J.M. (1964) Biochim. Biophys. Acta 91, 477–485.
[2] Edelman, M., Cowan, C.A., Epstein, H.T. and Schiff, J.A. (1964) Proc. Natl. Acad. Sci. U.S. 52, 1214–1219.
[3] Ray, D.S. and Hanawalt, P.C. (1964) J. Mol. Biol. 9, 812–824.
[4] Manning, J.E. and Richards, O.C. (1972) Biochim. Biophys. Acta 259, 285–296.
[5] Stutz, E. and Vandrey, J.P. (1971) FEBS Letters 17, 277–280.
[6] Manning, J.E. and Richards, O.C. (1972) Biochemistry 11, 2026–2043.
[7] Rawson, J.R.Y. and Haselkorn, R. (1973) J. Mol. Biol. 77, 125–132.
[8] Edelman, M., Schiff, J.A. and Epstein, H.T. (1965) J. Mol. Biol. 11, 769–774.
[9] Ray, D.S. and Hanawalt, P.C. (1965) J. Mol. Biol. 11, 760–768.
[10] Rawson, J.R.Y. and Stutz, E. (1969) Biochim. Biophys. Acta 190, 368–380.
[11] Brown, R.D. and Haselkorn, R. (1972) Biochim. Biophys. Acta 259, 1–4.
[12] Smith, K.D., Armstrong, G.L. and McCarthy, B.J. (1967) Biochim. Biophys. Acta 142, 323–330.
[13] David, I.B. (1972) J. Mol. Biol. 63, 201–216.
[14] Scott, N.S. and Smillie, R.N. (1967) Biochem. Biophys. Res. Commun. 28, 598–603.
[15] Stutz, E. and Rawson, J.R.Y. (1970) Biochim. Biophys. Acta 209, 16–23.