RIBOSOMAL DNA SATELLITE OF *EUGLENA GRACILIS* CHLOROPLAST DNA

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1. Introduction

A chloroplast of *Euglena gracilis* contains between 1.2×10^{-15} [1] and 1×10^{-14} g [2] of double stranded DNA. Electron microscopic studies have shown this DNA to be in the form of circular molecules of about 40 μm in contour length *in situ* with an estimated molecular weight of 8.3×10^7 daltons [3]. From a study of chloroplast DNA renaturation rates [4] we concluded that the kinetic complexity was in the range of 1.8×10^8 daltons which, if corrected according to Wetmur and Davidson [5] for the low GC content, is equivalent to a molecular weight of 9×10^7 daltons. The results from the electron microscopic study and the renaturation data coincide within acceptable limits, suggesting that the circular molecule carries all the genetic information located in the chloroplasts. However, from DNA/RNA hybridization experiments it was known [6, 7] that chloroplast ribosomal RNA (rRNA) anneals with approximately 1 to 1.2% (wt/wt) of the chloroplast DNA. Relating this value to the size of the circular molecule one obtains a nucleotide sequence equivalent to 0.53 and 0.64 rRNA cistrons, respectively*. Obviously, this means that either the circular DNA molecule does not contain all the genetic information or the reported hybridization data are too small.

In this report we shall investigate the second possibility and show that the DNA/rRNA hybridization value is considerably larger, namely, in the range of 6%. This higher value was obtained with *Euglena* chloroplast DNA which was isolated in a somewhat modified way. The DNA preparation showed a novel DNA component with a buoyant density in the range of 1.701 g/ml in addition to the usual main component (ρ = 1.685 g/cm^3). If we accept the circular DNA molecule to be the only type of chromosome in the chloroplast then there would be 3 cistrons per circle.

* The following numerical values were used: 1.28×10^5 base pairs per chloroplast DNA circle; 4700 nucleotides per 23 S + 16 S chloroplast *r*RNA.

2. Materials and methods

Euglena gracilis Klebs (z-strain) cells were grown under autotrophic conditions, harvested, washed and stored at $-20°$ as reported earlier [8]. Chloroplasts were isolated from trypsinized cells [8] and the chloroplast pellet was further purified in a density gradient using LUDOX HS, a gift from the E.I. DuPont de Nemours Sales Office, Chicago. The chloroplast DNA and ribosomal RNA were isolated as described earlier [7, 8] with the modification for DNA given below. The buoyant density of the DNA samples were determined in a Spinco Model E, An-D rotor, 12 mm center piece, room temperature, 44,770 rpm. The RNA/DNA hybridization experiments were done according to the filter method of Gillespie and Spiegelman [9]. For the DNA melting curves we used a Gilford Spectrophotometer model 2400 with temperature controlled cuvette chamber and automatic recording device. Ribosomal RNA was labeled with ^{32}P-phosphate as reported [8]. The radioactivity was monitored in a Packard Tri-Carb Liquid Scintillation Spectrometer.

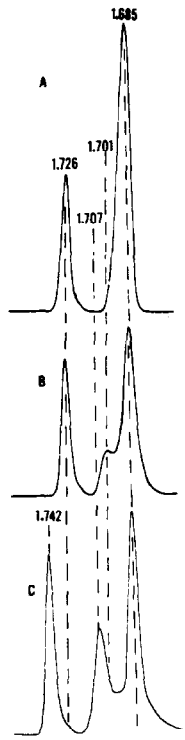

Fig. 1. Buoyant density centrifugation of *Euglena* DNA. (A) 2 μg native chloroplant DNA isolated from the peak fraction of a CsCl preparative density gradient. (B) Approximately 2 μg of chloroplast DNA isolated and purified as described in the text. The photographs were taken after 18–20 hr and traced with a Joyce Loebl & Co. microdensitometer. *Pseudomonas aeruginosa* DNA (density 1.726 g/ml) and ^{15}N-DNA from the same organism (density 1.742 g/ml) were used as reference DNA. Calculation of densities according to Sueoka [15].

Chloroplast purification with LUDOX HS [10]: Chloroplasts which were obtained from 50 g cells [8] were resuspended in 120 ml of buffered LUDOX (40 ml LUDOX in buffer I). The gently homogenized mixture was transferred to a 250 ml centrifuge bottle and overlayered with 80 ml of buffered LUDOX (25 ml LUDOX in buffer I) and finally with 20 ml of buffer I. The sample was spun in the Sorvall GSA rotor at 11,000 rpm, 30 min, 4°. Two green banding zones were usually generated by this flotation procedure. We harvested only the top green band at the interface between the 25% LUDOX layer and buffer I. The chloroplasts were washed two times in buffer I and the final pellet was used for DNA extraction.

Modified DNA extraction procedure: Purified chloroplasts from 50 g cells were resuspended in buffer I (10 ml) and heated to 60° for 10 min. The suspension was adjusted to 1 M $NaClO_4$ and shaken for 1 hr at room temperature, with 2 volumes of chloroform–isoamylalcohol (24: 1, v/v). The phases were separated by centrifugation and the extraction was repeated with the aqueous layer. The final aqueous layer was dialyzed in the cold overnight against buffer III. The dialyzate was loaded on a 0.8 × 10 cm methylated albumin kieselguhr column (MAK) [11] and the nucleic acids were eluted with a linear salt gradient from 0.4 to 1.2 M Na^+, in 0.05 M Na phosphate, pH 6.8. The DNA fractions were pooled, dialyzed against 0.01 × SSC (standard saline citrate buffer, pH 7.5) 0.1 mM EDTA, 4° and concentrated *in vacua*.

Buffer I: 0.7 M sucrose, 0.01 M Tris-HCl, pH 7.5, 0.04 M KCl, 5 mM 2-mercaptoethanol, 0.01 M EDTA; Buffer II: 0.1 M Tris-HCl, pH 7.5, 2.5% sodium dodecylsulfate, 0.01 M EDTA; Buffer III: 0.1 M NaCl, 0.05 M Na phosphate buffer, pH 6.8, 0.1 mM EDTA.

3. Results and discussion

The large difference in buoyant density between the chloroplast DNA (ρ = 1.685 g/ml) and the nuclear DNA (ρ = 1.708 g/ml) [1, 2] from *Euglena* made it relatively simple to purify chloroplast DNA on a preparative CsCl density gradient by pooling the fractions in the proper density regions. In fig. 1 we show the density profile of such a chloroplast DNA (pattern A) and compare it with the density profile of a chloroplast DNA isolated as described above (pattern B). Both preparations show the same main band with a mean density of 1.685 g/ml, but pattern B shows in addition a pronounced shoulder at a density of 1.701 g/ml. Since both preparations have approximately the same average molecular weight (approx. 5 × 10^6) the shoulder cannot be the result of different degrees in shearing. Rather, we argue that the heavier component was selectively lost in case A by pooling from the CsCl gradient only the peak fractions. We can exclude the shoulder to be nuclear DNA which bands distinctly different at 1.708 g/ml (pattern C). We can also exclude the novel band to be mitochondrial DNA which has a

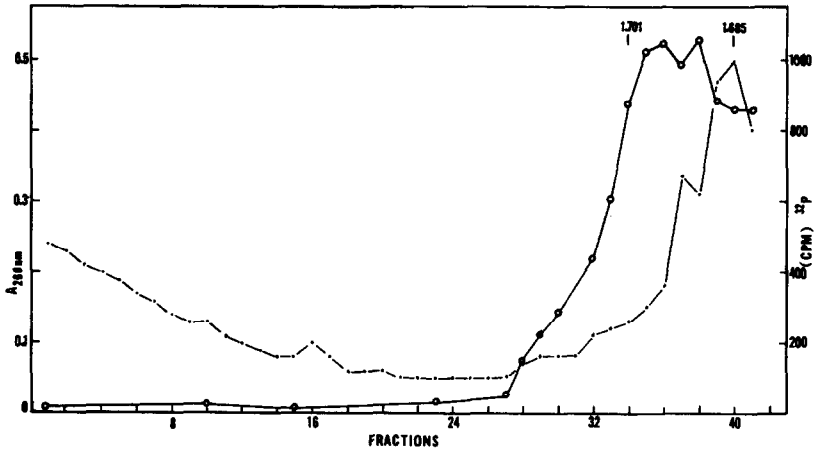

Fig. 2. Preparative CsCl density gradient of type B chloroplast DNA. Approximately 100 μg of DNA were equilibrated in a 4.5 ml preparative CsCl density gradient. Centrifugation: Spinco 40 rotor, 72 hr, 25°, 33,000 rpm. The fractions were diluted with 0.1 × SSC and the absorbance at 260 nm was measured. The samples were alkaline denatured at room temperature and after quenching in ice neutralized with NaH_2PO_4. The samples were diluted with 2 × SSC (total volume of 5 ml) and passed through nitrocellulose filters (S & S B6, 25 mm). Ten charged filters along with blanks were incubated together in plastic vials containing 5 ml of 2 × SCC with 20 μg ^{32}P-rRNA. Noise level on blank filters was between 25 and 41 cpm. The filters were processed as described earlier [7] (–·–) absorbance at 260 nm; (–○–) cpm per filter corrected for noise.

Table 1
Hybridization of 23 S/16 S chloroplast rRNA with chloroplast RNA.

Chloroplast DNA Type	DNA/filter (μg)	^{32}P-RNA (cpm)	Hybrid (%)	rRNA cistron number*
A	10.5	371	1.05	0.53
		401	1.10	0.58
B	9.0	2169	6.8	3.6
		2041	6.5	3.5

^{32}P-RNA; 3480 cpm/1 μg RNA, corrected for noise; incubation at 64°, 12 hr, 2 × SSC.
* Assumed molecular weight per circle; 8.3×10^7 [3].

buoyant density of 1.691 g/ml [3]. However, the novel component could be chloroplast DNA enriched in rRNA cistrons which have to be richer in GC content than the bulk DNA according to the base composition of the respective chloroplast rRNA [8].

To test this hypothesis we equilibrated type B *Euglena* chloroplast DNA on a preparative CsCl density gradient and challenged the various fractions against ^{32}P-labeled chloroplast rRNA. The results of this DNA/rRNA hybridization experiment are plotted in fig. 2. The main absorbance peak centers around the expected buoyant density (1.685 g/ml) while the ^{32}P-label is strongly displaced towards the heavier buoyant densities in the range of 1.700 g/ml. The absorbance units (260 nm) and radioactivity when totaled up between the fraction numbers 28 to 41 yield a corrected hybridization value of 4.8%.

From a parallel preparative CsCl density gradient we pooled the respective fractions and hybridized the total DNA (type B) with radioactive chloroplast rRNA along with a DNA preparation of type A. The results are given in table 1. Type B DNA with the shoulder on the heavy side hybridized to better than 6% compared to the 1% of the type A DNA. This strongly supports the possibility that the shoulder around a density of 1.701 g/ml is chloroplast DNA rich in ribosomal RNA cistrons.

Provided that there is only one type of chromosome within the chloroplast, this DNA molecule must be heterogeneous in its base sequences. Such considerable heterogeneity is reflected in the differential melting profiles of two chloroplast DNA pre-

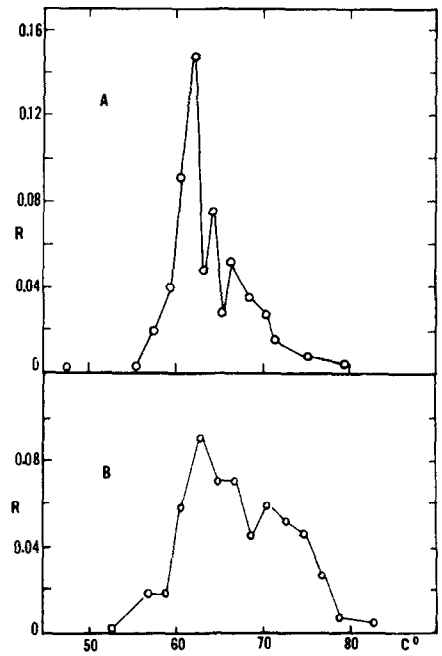

Fig. 3. Differential melting curve of chloroplast DNA. The chloroplast DNA samples were dialyzed over night, 4° against 0.1 × SSC (CRC Multicavity Dialysis Cells) and transferred into stoppered quartz cuvettes. The increase in absorbance (260 nm) was continuously measured as a function of the temperature increase (0.25°/min). (A) Chloroplast DNA with 2% hybridization capacity (A_{260} initial 0.62). (B) Chloroplast DNA with 6% hybridization capacity (A_{260} initial, 0.46).

$$R = \frac{A_{t2} - A_{t1}/A_{100} - A_{20°}}{t_2 - t_1}$$

parations, shown in fig. 3. Pattern A was obtained from a DNA sample which had hybridized with rRNA to approximately 2%, pattern B was obtained from a DNA sample, which had hybridized to approximately 6%. Both profiles show a main maximum at 62° which is equivalent, in 0.1 × SSC, to a GC content of 21% [12]. Profile A shows relative to the main peak two minor maxima at 65° and 67° respectively and a trailing edge in the 70° region. In profile B the maxima at 65° and 70°, corresponding to a GC content of 31 and 41%, respectively, are quite pronounced relative to the main peak. Again, the base sequence heterogenity correlates with the hybridization capacity. Under identical experimental conditions a T-7 phage DNA gave a symmetrical differential melting profile with a maximum at 74° (not shown) indicating that the heterogeneity found with chloroplast DNA was not an experimental artifact. Multiphasic differential melting profiles were also reported for chloroplast DNA from chlamydomonas [13, 14].

Combining the various data we postulate that the novel DNA component comes from the chloroplast and probably is part of the circular DNA molecule. If this assumption is correct a circular molecule would accomodate three copies of the chloroplast rRNA (23 S + 16 S RNA) cistron. This amounts to approximately 12% of the contour length, possible rRNA precursors not included.

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References

[1] M. Edelman, C.A. Cowan, H.T. Epstein and J.A. Schiff, Proc. Natl. Acad. Sci. U.S. 52 (1964) 1214.
[2] G. Brawerman and J.M. Eisenstadt, Biochim. Biophys. Acta 91 (1964) 477.
[3] J.E. Manning, D.R. Wolstenholme, R.S. Ryan, J.A. Hunter and O.C. Richards, Proc. Natl. Acad. Sci. U.S. 68 (1971) 1169.
[4] E. Stutz, FEBS Letters 8 (1970) 25.
[5] J.G. Wetmur and N. Davidson, J. Mol. Biol. 31 (1968) 349.
[6] N.S. Scott and R.N. Smillie, Biochem. Biophys. Res. Commun. 28 (1967) 598.
[7] E. Stutz and J.R. Rawson, Biochim. Biophys. Acta 209 (1970) 16.
[8] J.R. Rawson and E. Stutz, Biochim. Biophys. Acta 190 (1969) 368.
[9] D. Gillespie and S. Spiegelman, J. Mol. Biol. 12 (1965) 829.
[10] J.W. Lyttleton, Anal. Biochem. 38 (1970) 277.
[11] J.D. Mandell and A.D. Hershey, Anal. Biochem. 1 (1960) 66.
[12] M. Mandel and J. Marmur, in: Methods in Enzymology, Vol. XIIB, eds. L. Grossman and K. Moldave (Academic Press, New York, 1968) p. 195.
[13] R. Wells and R. Sager, J. Mol. Biol. 58 (1971) 611.
[14] D. Bastia, K.-S. Chiang, H. Swift and P. Siersman, Proc. Natl. Acad. Sci. U.S. 68 (1971) 1157.
[15] N. Sueoka, J. Mol. Biol. 3 (1961) 31.