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Compositional Heterogeneity of the Chloroplast DNAs from *Euglena gracilis* and *Spinacia oleracea*

Jürgen M. SCHMITT, Hans-Jürgen BOHNERT, Karl H. J. GORDON, Reinhold HERRMANN, Giorgio BERNARDI, and Edwin J. CROUSE

Laboratoire de Génétique Moléculaire, Institut de Recherche en Biologie Moléculaire, Paris, and Botanisches Institut der Universität Düsseldorf

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The chloroplast genomes of *Euglena gracilis* and *Spinacia oleracea* were investigated in their compositional heterogeneity, by using several different experimental approaches.

Euglena chloroplast DNA has a dG+dC content of 28%. Preparations averaging 20×10^6 in molecular weight exhibit a gross heterogeneity in their elution profiles from hydroxyapatite and in their buoyant densities because the rRNA genes have a high rG + rC content. Finer analysis by melting, buoyant density of restriction fragments and micrococcal nuclease degradation have revealed an extended compositional heterogeneity. From micrococcal nuclease digestion data, approximately 30% of the chloroplast genome is as low as 12% in its dG + dC content, whereas 10% is higher than 60% dG + dC. Since the average dG + dC content of large restriction endonuclease fragments varied to a lesser extent, most of dA + dT-rich sequences must occur in short stretches interspersed with dG + dC-rich stretches.

Spinach chloroplast DNA (dG + dC = 36.5%) did not exhibit any gross compositional heterogeneity in its hydroxyapatite elution or in its buoyant density profile. But the higher resolution methods of melting, buoyant densities of restriction fragments and micrococcal nuclease degradation revealed a high degree of heterogeneity which appears to be due to interspersion of short DNA stretches of different base composition. About 30% of genome is as low as 22% in dG + dC, while 10% is higher than 60% in dG + dC.

Studies on the sequence organization of large mitochondrial genomes from a variety of unicellular organisms, including a number of yeasts, *Euglena gracilis* and *Acanthamoeba castellanii* have disclosed a marked compositional heterogeneity [1–4]. In several cases, it has been possible to demonstrate that these genomes share not only a c-value paradox (namely the fact that they contain much more DNA than needed for coding purposes), and a strong compositional heterogeneity, but also the property of containing internally repetitive sequences. In the case of *Saccharomyces cerevisiae*, for example, the 'excess DNA' property is largely due to the presence of 'AT spacers' which contain repeated sequences of dA and dT [1]. At present, it is not known whether the sequence organization of the mitochondrial genomes just mentioned is of more general occurrence.

In view of providing such an answer we have studied here two chloroplast genomes, those of *Euglena* and of spinach. The chloroplast genomes investigated so far (see [5–7] for recent reviews) consist of large circular units varying in contour length from 37 μm to 62 μm. Physical maps are available for a variety of chloroplast DNAs, including those of *Euglena* and spinach [8–14]. Our choice was dictated in one case by the very low dG + dC content of the *Euglena* chloroplast genome, a property allowing easy comparisons with dA + dT-rich mitochondrial genomes. In the other case, our choice was due to the fact that spinach chloroplast DNA is a similar to all other chloroplast DNAs from higher plants and has, in contrast with *Euglena*, a relatively high dG + dC content.

Some of the results reported here were presented in preliminary form at conferences [12, 15].

MATERIALS AND METHODS

Isolation of Chloroplast DNA and Chloroplast rRNA

Chloroplast DNA from *Euglena gracilis* (Z-strain, no. 753, Culture Collection of Algae, Indiana University) was prepared from either autotrophic or mixotrophic cells, and purified by hydroxyapatite chromatography and preparative CsCl density gradient centrifugation, as previously described [16]. Isolation of chloroplast rRNA, labelling *in vitro* with ^{125}I (Radiochemical Center Ltd, Amersham, England) and hybridization to chloroplast DNA were carried out as described earlier [16].

Spinach plants (*Spinacia oleracea*, var. Monopa) were grown in a greenhouse under low-intensity light in order to minimize starch production. Intact chloroplasts were isolated from mature leaves and then incubated for 30 min at 0 °C with 50–100 μg/ml pancreatic DNAase (Boehringer, Mannheim) to degrade contaminating nuclear DNA. After removal of the DNAase, lysis of organelles with 2% sodium dodecyl sarcosinate and treatment with proteinase K and RNAase, the DNA was obtained by several cycles of phenol extraction, dialysis (1 mM Tris/HCl, 0.1 mM EDTA, pH 7.2) and concentration (for details see [7]). The average molecular of the isolated chloroplast DNA was approximately 50×10^6, that is, about half the size of the circular molecule. Hydroxyapatite chromatography and preparative CsCl density gradient centrifugation were performed as described [16]. Chloroplast ribosomal subunits were used for the isolation of the 16-S ribosomal RNA (small subunit) and the 23-S, 5-S and 4.5-S ribosomal RNAs (large subunit) as previously reported [17].

Analytical Ultracentrifugation

Analytical cesium chloride density gradient centrifugation was done as previously described [18].

Base Composition of DNA and RNA

This was determined by nucleoside analysis on columns of Aminex A6 (Bio-Rad, Richmond, CA) after enzymatic degradation [19].

Absorbance/Temperature Profiles

High-resolution absorbance/temperature profiles were determined as described elsewhere [4]. DNA solutions in standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) having an A_{270} value between 0.5 and 1.0, were heated at a rate of 0.1°/min while monitoring the change in absorbance at 270 nm.

Micrococcal Nuclease Digestions

DNA solutions were dialyzed against 0.1 M sodium borate, pH 8.8, and adjusted to an absorbance at 260 nm of 1.0 and to 2.5 mM $CaCl_2$ by additions of 0.5 M $CaCl_2$. Digestions were performed at 6 °C. DNA digests were filtered on Sephadex G-25 columns (Pharmacia, Uppsala, Sweden). 1-ml DNA solutions were loaded on columns (0.67 × 31 cm) equilibrated with 0.05 M ammonium acetate, pH 5.5. Flow rate was kept at 6 ml/h using a Technicon (Chauncey, NJ) peristaltic pump. All other conditions were described in detail elsewhere [20].

Restriction Endonuclease Cleavage Analysis of Chloroplast DNA

Restriction endonucleases *Xho*I (from New England Biolabs), *Sma*I, *Hin*dIII and *Eco*RI (from Boehringer, Mannheim) were used as recommended by the suppliers. *Sal*I and *Xma*I (isoschizomer of *Sma*I) were prepared from frozen bacteria using the methods described [21, 22]. Degradation of DNA (0.5 – 2 µg) was carried out under conditions detailed by the suppliers. *Sal*I assays contained 10 mM Tris/HCl, 100 mM NaCl, 5 mM mercaptoethanol, pH 7.4. Those of *Xma*I 10 mM Tris/HCl, 40 mM NaCl and 5 mM mercaptoethanol, pH 7.2. The reaction mixtures were incubated for 30 – 60 min at 37 °C except those of *Sma*I which were kept 15 min at room temperature. Tracking dye (0.1% bromophenol blue in aqueous 50% glycerol) and 0.2 vol. aqueous 0.7% Seaplaque agarose (Marine Colloids) were added before the samples were layered into the gel slots. Addition of agarose prevents band tailing.

Electrophoretic separation of DNA fragments was performed in horizontal 0.5 – 2.0% Seakem agarose (Marine Colloids, Rockland, ME) slab gels (20 × 20 × 0.4 cm) in 40 mM Tris/acetate, 20 mM sodium acetate, 1 mM EDTA (pH 7.4) containing 0.5 µg ethidium bromide per ml. Gels were run at 50 mA, for about 14 h, at room temperature. DNA fragments were visualized with ethidium bromide under ultraviolet light and photographed on Polaroid 665 film.

Separation of DNA Fragments from Agarose by Electroelution

DNA restriction fragments for buoyant density analyses were obtained by preparative electrophoresis. Individual bands were eluted by electrophoresis [23]. In case of cross-contamination, the bands were re-purified by the same method.

Separation of DNA Fragments from Agarose by Hydroxyapatite Chromatography

Preparative Seaplaque (Marine Colloids, Rockland, ME) agarose gels (0.5 – 1.0%), containing 36 mM Tris/HCl, 30 mM NaH_2PO_4, pH 7.5 and 0.5 µg/ml of ethidium bromide, were used to separate the DNA fragments. No EDTA was used in the electrophoresis buffer in order to avoid interference with the subsequent hydroxyapatite chromatography.

DNA fragments were cut from ethidium-bromide-stained agarose gels and the agarose was liquefied at 72 °C for 50 – 70 s after increasing the sodium concentration to more than 0.1 M. The sol was cooled to 40 – 60 °C and diluted to a final concentration of 0.12 M sodium phosphate pH 6.8 (equimolar amounts of NaH_2PO_4 and Na_2HPO_4) and about 0.05% agarose. Preparation of hydroxyapatite and chromatography of DNA on 1-ml hydroxyapatite columns at 40 – 60 °C were performed as described by Bernardi [24]. After loading the sample and washing the column with 2 vol. of 0.12 M sodium phosphate buffer, the DNA which was essentially free of agarose (as determined by electron microscopy) and ethidium bromide, was eluted with 0.5 – 1 ml of 0.4 M sodium phosphate buffer.

Radioactive DNA was used to estimate the yields from the columns. It was prepared by growing *Escherichia coli* in [^{3}H]thymidine (Radiochemical Center Ltd, Amersham, England) purified by hydroxyapatite chromatography and preparative CsCl gradients, dialysed and concentrated; the specific activity was 23000 counts min^{-1} μg^{-1}. The recovery was usually over 95%.

Unlabelled *Clostridium perfringens* DNA, salmon sperm DNA, and *E. coli* DNA were obtained commercially (Worthington and Serva) and *Chlamydomonas* nuclear DNA isolated as described [25]. These DNAs were used as standard controls for the buoyant density determination of DNA recovered by liquid agarose-hydroxyapatite chromatography.

Hydroxyapatite Chromatography and Ultracentrifugation Experiments

Hydroxyapatite chromatography and analytical CsCl density gradient centrifugation of the column fractions were performed as previously described [20]. Sedimentation velocity experiments were made using the band sedimentation method described by Prunell and Bernardi [26].

RESULTS

Hydroxyapatite Chromatography and Buoyant Density

The hydroxyapatite elution profile and the bimodal buoyant density profile of *Euglena* chloroplast DNA was found to depend upon the molecular weight of the sample. High-molecular-weight preparations ($M_r \approx 20 \times 10^6$) are eluted at a high sodium phosphate concentration (0.29 M) but the elution profile is skewed on the low-concentration side (Fig. 1); their CsCl profile is characterized by a main band, $\varrho = 1.685$ g/cm^3, and a heavy shoulder extending to ϱ values as high as 1.703 g/cm^3 (Fig. 2A). Sheared DNA preparations ($M_r \approx 4 \times 10^6$) present a broader elution peak centered at 0.29 M phosphate but extended to lower concentrations (not shown); their CsCl profiles are characterized by a broad main peak ($\varrho = 1.686$ g/cm^3), with a heavy shoulder and a heavy minor peak (Fig. 2B). When these sheared DNA preparations are chromatographed on hydroxyapatite, the early eluting

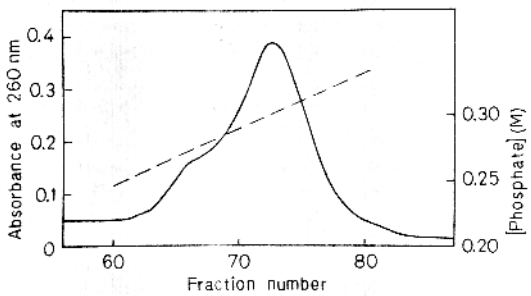

Fig. 1. *Chromatography of* Euglena *chloroplast DNA (35* A_{260} *units;* $M_r \approx 20 \times 10^6$*) on an hydroxyapatide column (1 × 25 cm).* A linear gradient of phosphate (0.1 – 0.5 M); pH 6.8) was used for elution

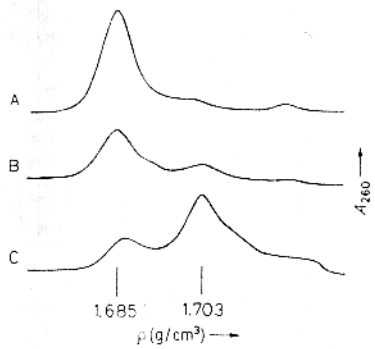

Fig. 2. *CsCl analytical profiles of* Euglena *chloroplast DNA.* Sample A had a molecular weight of about 20×10^6; sample B had been sheared to a molecular weight of about 4×10^6; sample C was the low-eluting fraction from hydroxyapatite of sample B

Table 1. *Hybridization of chloroplast rRNA to total chloroplast DNA and fractions eluted from hydroxyapatite*

Chloroplast DNA	rRNA (M_r)	Hybridization	Number of copies per circular molecule
		%	
Euglena	16-S + 23-S (1.6×10^6)	5.4	3.2
Fraction I	16-S + 23-S	8.9	–
Fraction II	16-S + 23-S	2.8	–
Spinach	16-S + 23-S (1.6×10^6)	3.4	2.0
	precursor (2.7×10^6)	5.6	2.0

material is highly enriched in this minor component (Fig. 2C). This minor fraction contains the ribosomal DNA sequences as can be shown by hybridization experiments. Under saturation conditions, chloroplast ribosomal RNA hybridizes to an extent of 5.4%, a value corresponding to three sets of ribosomal genes per chloroplast genome unit of M_r 9.2×10^7. In contrast, the early-eluting fraction hybridized up to 8.9% and the late-eluting fraction only up to 2.8% (Table 1). The more mechanically degraded the molecule, the progressively earlier those regions richer in dG + dC composition eluted from hydroxyapatite. During the course of this degradation,

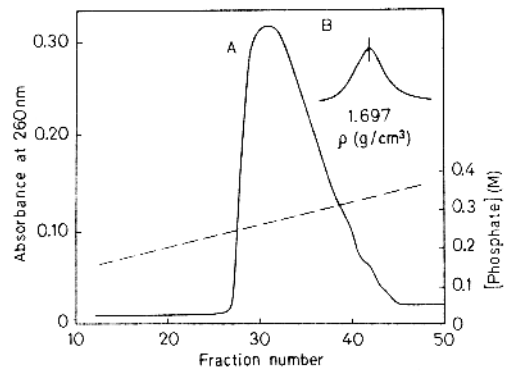

Fig. 3. *Chromatography (A) and CsCl analytical profile (B) of spinach chloroplast DNA.* 18 A_{260} units of spinach chloroplast DNA ($M_r \approx 50 \times 10^6$) were used for chromatography on hydroxyapatite. Conditions as in Fig. 1

Table 2. *Base composition of chloroplast DNA and ribosomal RNAs*

DNA	dT	dG	dA	dC	dG + dC
	mol/100 mol				
Euglena	35.5	14.2	36.3	14.0	28.2
Spinach	31.4	18.0	32.1	18.5	36.5
rRNA	rU	rG	rA	rC	rG + rC
	mol/100 mol				
Euglena					
23-S + 16-S	26.0	28.2	26.9	18.6	46.8
Spinach					
23-S + 16-S	26.3	29.4	22.6	21.7	51.1
23-S	26.2	29.0	22.9	21.9	50.9
16-S	26.9	29.7	21.6	21.8	51.5
pre-rRNA	30.1	27.5	21.2	21.2	48.7

satellite DNAs having buoyant densities shifting from 1.692 to 1.696 and to 1.701 g/cm³ can be observed. The fact that the dG + dC-rich fragments coding for the rRNA sequences are eluted at a lower sodium phosphate concentration compared to the bulk chloroplast DNA was used for separating these components. The total DNA and the two subfractions were used for the measurements reported below.

Spinach chloroplast DNA was eluted from hydroxyapatite (Fig. 3A) in a single peak centered at 0.26 M sodium phosphate and showed a broad, unimodal profile ($\varrho = 1.697$/cm³) when centrifuged in CsCl (Fig. 3B). When sheared DNA preparations were studied, both the chromatogram on hydroxyapatite and the CsCl profile showed only a small change, consisting in a skewness towards lower elucion concentrations and higher buoyant densities, respectively (not shown). Hybridization of chloroplast ribosomal RNAs to chromatographic fractions showed a slightly higher level on the low-eluting side. Hybridization experiments, using either the mature 16-S and 23-S ribosomal RNAs or the common precursor, indicate the presence of two sets of ribosomal genes per chloroplast genome unit of M_r 9.5×10^7 (Table 1).

Base Composition

Table 2 presents the base composition of the chloroplast DNAs from *Euglena* and spinach as well as those of the

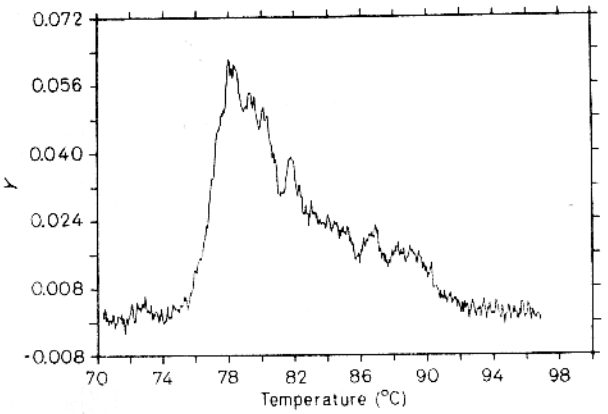

Fig. 4. *Differential melting curve of* Euglena *chloroplast DNA.* Y is the increment in relative absorbance per degree: $(A_{t_2}-A_{t_1})/A_{25}(t_2-t_1)$, where A_{t_1}, A_{t_2}, A_{25} are absorbance at 270 nm, at temperatures t_1, t_2 and 25 °C, respectively. Absorbance data were smoothed over 19 points

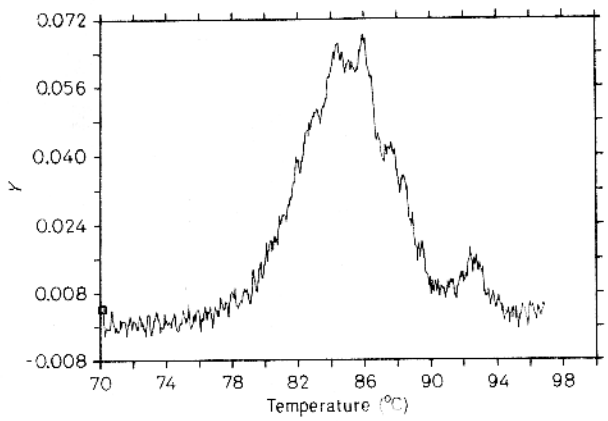

Fig. 5. *Differential melting curve of spinach chloroplast DNA.* Details as in Fig. 4

corresponding ribosomal RNAs. It should be noted that the analytical dG + dC content of *Euglena* chloroplast DNA is higher (28.2%) than estimated from its buoyant density (25%), a discrepancy in the opposite direction of that found in dA + dT-rich mitochondrial DNAs [4]. In contrast, the chloroplast DNA of spinach has a dG + dC content which is slightly lower (36.5%) than expected from its buoyant density (38%). In both cases, the ribosomal RNAs are much higher in rG + rC than the corresponding dG + dC of total chloroplast DNAs. This difference is much stronger in the case of *Euglena* (47% vs 28%) than in the case of spinach (49% for the common precursor RNA vs 36.5%).

It should be remarked that neither *Euglena* nor spinach chloroplast DNA appear to be glucosylated, since digestion with spleen exonuclease is not hindered, and that no evidence for 5-methylcytidine was observed in the nucleoside chromatograms.

Melting Transitions

The differential melting curves of the two chloroplast DNAs were broad, asymmetric and multimodal (Fig. 4 and 5). T_m values corresponded to dG + dC contents of 30.4% in the case of *Euglena* DNA and 38.8% in the case of spinach DNA; both values were higher than the value determined by base analysis.

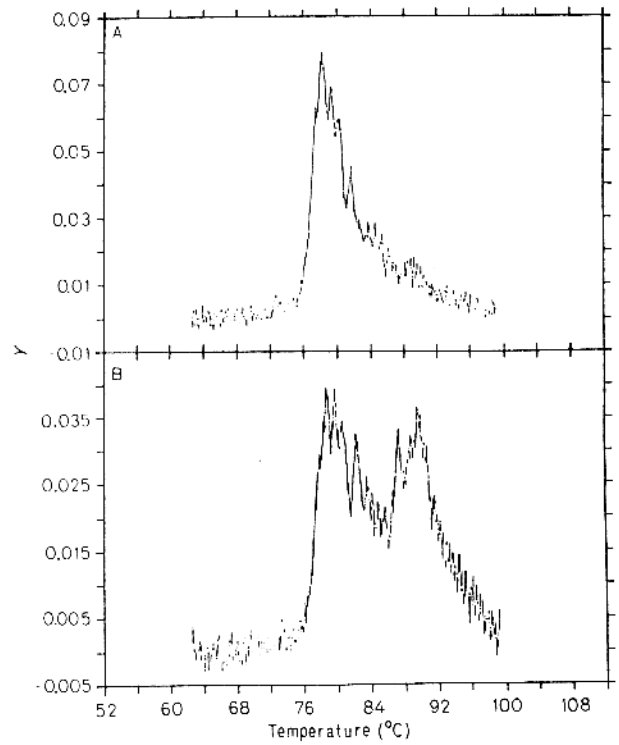

Fig. 6. *Differential melting curve of (A) early-eluting and (B) late-eluting fractions of* Euglena *chloroplast DNA.* Details as in Fig. 4

Table 3. *Melting spectrum of chloroplasts DNAs*

DNA	t_1-t_2	dG + dC	Amount
	°C	%	
Euglena	71.0–74.0	7.8	1.1
	75.5–77.8	18.8	13.0
	77.7–78.8	21.8	14.7
	78.8–79.8	24.4	11.5
	79.8–81.2	27.3	13.4
	81.2–82.8	31.0	11.4
	82.8–85.6	36.3	14.2
	85.6–87.6	42.2	7.8
	87.6–92.5	50.6	11.6
Spinach	76.0–82.1	23.8	16.4
	82.1–83.3	32.7	11.3
	83.3–84.8	36.0	18.0
	84.8–85.5	38.7	9.1
	85.5–87.1	41.5	18.2
	87.1–88.1	44.6	8.1
	88.1–89.1	47.1	5.8
	89.1–89.9	49.3	2.7
	89.9–91.5	52.5	3.1
	91.5–94.4	57.7	6.3

The melting of the two hydroxyapatite fractions of *Euglena* DNA (Fig. 1) indicated, as expected, an enrichment in high-melting components for the low-eluting fraction (Fig. 6A) and a depletion in the same components for the high-eluting fraction (Fig. 6B).

Table 3 presents the relative amounts of both DNAs melting within certain temperature values, and the corresponding dG + dC contents.

Table 4. *Buoyant density and average dG + dC content of fragments produced by* Eco*RI cleavage of* Euglena *chloroplast DNA*
The + sign indicates DNA fragments from repeated region of the molecule. The number given in brackets refers to multiple copies

Fragment M_r ($\times 10^{-6}$)	ϱ	dG + dC
	g/cm^3	%
15	1.686	27
13	1.686	27
7.5	1.690	31
6.8	1.685	26
5.2	1.687	28
5.2	1.693	34
4.7	1.688	29
3.6	1.689	30
3.1	1.690	31
2.4 (2×)	1.693	34
22.2	1.693	34
2.1 (3×) +	1.702	43
1.9	1.690	31
1.7	1.694	35
1.6 (3×) +	1.707	48
1.1	1.693	34
1.0	–	–
0.81	1.691	32
0.65 (2×)	1.691	32
0.57 (2×)	1.691	32
0.48	–	–
91.4 total M_r		

Buoyant Densities of Restriction Fragments

Tables 4 and 5 presents the buoyant densities obtained for the *Eco*RI fragments of *Euglena* and for the *Sal*I, *Xma*I, *Xho*I and *Hin*dIII fragments of spinach DNA. The range of buoyant densities extends from 1.685 to 1.707 g/cm^3 (namely from about 26% to about 48% dG + dC) in the first case, and from 1.693 to 1.717 g/cm^3 (namely from about 34% to about 58% dG + dC) in the second case.

Micrococcal Nuclease Degradation

Degradation by micrococcal nuclease (Fig. 7) showed that about 30% of the DNAs had dG + dC levels as low as 12% in the case of *Euglena*, and 22% in the case of spinach. In both cases about 10% of the DNAs had dG + dC levels higher than 60%.

DISCUSSION

Euglena *Chloroplast DNA*

This DNA presents a gross compositional heterogeneity in that its three pairs of ribosomal RNA genes have an rG + rC content almost 20% higher than the average value of 28% (Table 2). As a consequence, the routine DNA preparations are grossly heterogeneous in both elution from hydroxyapatite (Fig. 1) and in buoyant density (Fig. 2A). This heterogeneity becomes apparent as the molecular weight

Table 5. *Buoyant density and average dG + dC content of some DNA fragments produced by restriction endonuclease cleavage of the spinach chloroplast DNA*
The + sign indicates DNA fragments from repeated region of the molecule. The number given in backets refers to multiple copies

*Sal*I cleavage			*Xma*I cleavage			*Xho*I cleavage			*Hin*dIII cleavage		
M_r ($\times 10^{-6}$)	ϱ	dG + dC	M_r ($\times 10^{-6}$)	ϱ	dG + dC	M_r ($\times 10^{-6}$)	ϱ	dG + dC	M_r ($\times 10^{-6}$)	ϱ	dG + dC
	g/cm^3	%		g/cm^3	%		g/cm^3	%		g/cm^3	%
29.7	1.701	42	22.3	1.696	37	28.0	1.697	38	6.6 (2×)	1.696	37
13.8	1.698	39	12.2	1.699	40	10.2 (2×)	1.703	45	6.2	1.697	38
12.7	1.698	39	6.1	1.701	42	8.6	1.699	40	5.6 (3×) +	1.708	49
8.6	1.699	40	5.6	1.697	38	7.8	1.699	40	5.4	1.697	38
6.6 (2×) +	1.702	43	5.2	1.697	38	7.5	1.698	39	4.5 (3×) +	1.698	39
5.6	1.694	35	5.0 (3×) +	1.700	41	6.3	1.698	39	3.7	1.696	37
3.7	1.699	40	3.8	1.699	40	3.6	1.701	42	3.5	1.699	40
3.2	1.697	38	3.5	1.696	37	2.0 (2×) +	1.715	56	3.3	1.695	36
2.5			3.4 (2×) +	1.707	48	1.7			2.85 (2×)	1.700	41
1.5			2.8	1.698	39	1.55			2.55	1.700	41
0.45			2.4 (2×) +	1.717	58	1.1			2.35 (2×) +	1.698	39
			1.7	1.704	45	1.0			1.68	1.693	34
			1.2 (2×) +	1.704	45	0.95			1.65	1.695	36
			1.15 (2×) +	1.713	54	0.60 (2×) +			1.56	1.695	36
						0.40			1.42	1.703	44
						0.35			1.25		
						0.15			1.05		
									0.93 (2×) +		
									0.77 (2×) +		
									0.73 (2×) +		
									0.66		
									0.56		
95.0 total M_r			94.5 total M_r			95.2 total M_r			93.2 total M_r		

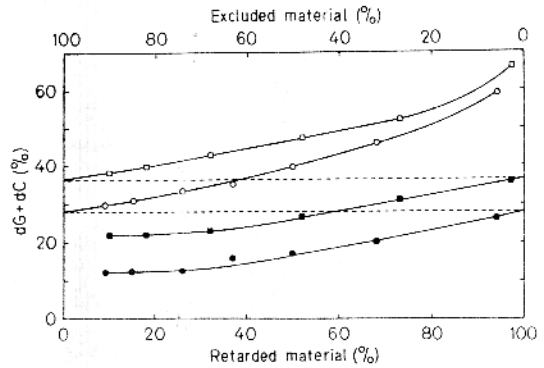

Fig. 7. *Micrococcal nuclease digestions.* Plot of the dG + dC contents of the excluded (○) and retarded (●) fractions of *Euglena* chloroplast DNA and excluded (□) and retarded (■) fractions of spinach chloroplast DNA against the relative amount of excluded or retarded material after Sephadex G-25 chromatography of the micrococcal nuclease digests (6 °C)

of the DNA is decreased by shearing. During the shearing process the dG + dC-rich rDNA regions are separated from the bulk DNA and appear as satellites having buoyant densities of 1.692, 1.696 or 1.702 g/cm^3 depending on the degree of fragmentation. The fragments enriched in the ribosomal genes band at a higher density than the other ones, and are eluted at a lower phosphate concentration from hydroxyapatite. This heterogeneity, which is reflected in the high density of two *Eco*RI fragments (see Table 4), is far from being the only one in the genome, as shown by other experimental approaches.

The melting transition indicates that about 30% of the chloroplast DNA is lower than 22% dG + dC (Fig. 4 and Table 3) and that the remaining DNA is strongly heterogeneous in base composition (see also [27, 28]. The minor component melting at 88 – 89 °C can tentatively be assigned to ribosomal cistrons, since it corresponds to the expected dG + dC level (47%) and amount (10%). This heterogeneity was confirmed by the wide range of buoyant densities of restriction fragments (Table 4 and [29]).

Finally, degradation by micrococcal nuclease showed that a least 30% of chloroplast DNA has a dG + dC content of 12% or less. In fact, the amount of DNA quoted may be underestimated, whereas the dG + dC content may be overestimated because of 'leakage' of dG + dC-rich material [20]. The different sets of data on the compositional heterogeneity do not contradict each other, each experimental approach involving stretches of different lengths.

Spinach Chloroplast DNA

In contrast to the preceding case, chloroplast DNA from spinach did not show an anomalously high elution concentration nor, when sheared, a fractionation according to base composition. This was confirmed by the CsCl profile of sheared DNA (not shown). The results indicate an absence of the gross heterogeneity exhibited by *Euglena* chloroplast DNA. It should be recalled that spinach chloroplast DNA only contains two sets of ribosomal cistrons instead of three as in *Euglena*. The dG + dC content of the DNA fragments carrying the rRNA genes was found to be between 48% and 58% (Table 4). This is in agreement with the high rG + rC level determined for the rRNAs as well as for a precursor to the rRNAs (Table 2). The fact that the dG + dC content of the spinach rDNA cistrons is only 12% higher than the average value, compared to 22% in *Euglena*, prevents the rDNA regions from separating on hydroxyapatite or in density gradient from the bulk DNA even with sheared DNA.

The thermal transition of spinach chloroplast DNA revealed, however, a multimodal curve covering a wide range of dG + dC contents. As in *Euglena*, a transition with a melting point of 92 °C could be found, which can be related to the rDNA in mass (9.4%) and dG + dC content (56%). The buoyant densities of restriction fragments also showed a very wide spectrum, which is in overall agreement with the melting curve.

The finer probing of compositional heterogeneity allowed by micrococcal nuclease digestion, showed that in fact the dG + dC range was even wider than indicated by the other two approaches just mentioned. These sequences would have to be very short and widely distributed since DNA fragments produced by the restriction enzymes used did not have corresponding low buoyant densities.

Intramolecular Compositional Heterogeneity of Chloroplast DNA

As indicated in a preceding paper [4], the mitochondrial DNAs of protists investigated so far appear to share a great compositional heterogeneity and the presence of DNA stretches very rich in dA and dT. In the cases where the primary structure is known, these appear to be internally repetitive in sequence, as originally found in the case of the mitochondrial genome of *Saccharomyces cerevisiae*.

In the present work, two chloroplast genomes were investigated to see whether the sequence organization found in the mitochondrial genomes of protists is also present in the chloroplast genomes of the protist *Euglena gracilis* and the higher plant *Spinacia oleracea*. The chloroplast DNAs of *Euglena* and spinach have about the same contour length of 45 μm [30, 31]. Early studies had indicated a gross heterogeneity for the *Euglena* chloroplast genome, but not for the chloroplast genome of higher plants (for a review see [32]). Refinement of the methods leads to a revision on this concept. High-resolution melting, buoyant density determination of restriction fragments and micrococcal nuclease digestion reveal base compositional heterogeneity in the spinach chloroplast DNA molecule. The heterogeneity in the case of spinach is less pronounced than in the case of *Euglena*. These results, along with electron microscopic denaturation studies [33, 34], and fine thermal denaturation studies in other plant genera [35] and DNA sequencing studies [36, 37] indicate that intramolecular heterogeneity is a basic feature of the chloroplast genome.

APPENDIX

Restriction Endonuclease Site Map of *Spinacia oleracea* Chloroplast DNA

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Many restriction endonucleases were screened with regard to the number and size of fragments generated from spinach chloroplast DNA. The following four enzymes were selected in this work for site mapping: *Sal*I, *Xma*I, *Xho*I and *Hin*dIII (see Table 5). DNA fragments smaller than those listed in Table 5 have been disregarded in this study. Double and triple

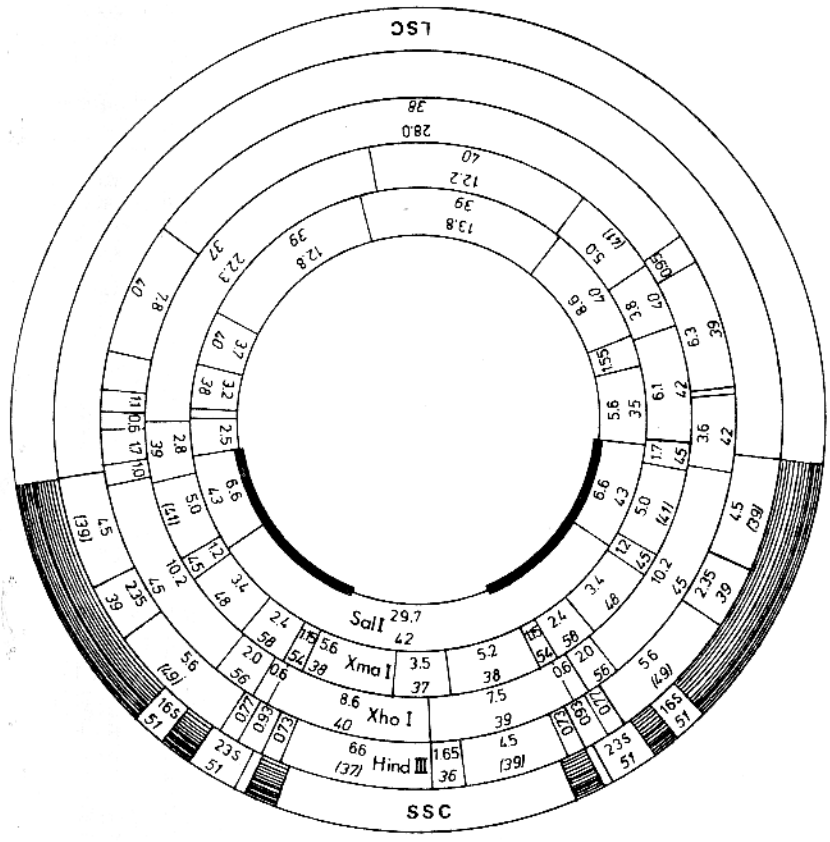

Fig. A1. Restriction endonuclease cleavage site map of the spinach chloroplast DNA molecule and the average G + C content of some DNA fragments. Inner circle to outer circle: *Sal*I, *Xma*I, *Xho*I and *Hin*dIII cleavage site maps (cf. references [6, 11–13]) and the location of the four major regions of the molecule (outermost circle), i.e. small single copy region (SSC, $M_r = 12 \times 10^6$), large single copy region (LSC, $M_r = 52 \times 10^6$) and inverted repeat regions ($M_r = 15 \times 10^6$, each). Within each inverted repeat region, the position (and G + C content, see Table 2) of the 16-S and 23-S rRNA genes are shown. DNA fragments are identified by their relative molecular mass (upper numbers = $10^{-6} \times M_r$) and their average G + C content (lower numbers = % G + C), see Table 5. The numbers enclosed in brackets indicate the average G + C value of different fragments having the same size (see below).

DNA fragment order (counter-clockwise from 6 o'clock): for *Sal*I, – 29.7 – 6.6a – 5.6 – 1.55 – 8.6 – 13.8 – 12.8 – 3.7 – 3.2 – 0.45 – 2.45 – 6.6a – ; for *Xma*I, – 3.5 – 5.2 – 1.15a – 2.4a – 3.4a – 1.2a – 5.0a – 1.7 – 6.1 – 3.8 – 5.0b – 12.2 – 22.3 – 2.8 – 5.0a – 1.2a – 3.4a – 2.4a – 1.15a – 5.6 – ; for *Xho*I, – 8.6 – 7.5 – 0.6a – 2.0a – 10.2a – 3.6 – 0.4 – 6.3 – 0.95 – 28.0 – 7.8 – (1.55 – 0.35 – 0.15 (relative order has not been determined) – 1.1 – 0.6b – 1.7 – 1.0 – 10.2a – 2.0a – 0.6a – ; for *Hin*dIII, – 6.6a – 1.65 – 4.5b – 0.73a – 0.93a – 0.77a – 5.6a – 2.35a – 4.5a – (6.6b – 6.2 – 5.6b – 5.4 – 3.7 – 3.5 – 3.3 – 2.85a – 2.85b – 2.55 – 1.68 – 1.56 – 1.42 – 1.25 – 1.05 – 0.66 – 0.56 (relative order has not been determined) – 4.5a – 2.35a – 5.6a – 0.77a – 0.93a – 0.73a – ; where 'a' = fragments of identical size and nucleotide sequence and 'b' = fragments of identical size but different nucleotide sequence.

All the cleavage sites in the small single copy region might have the reverse orientation relative to the orientation shown in the large single copy region

digests were used to construct the map [6, 13]. The relative order of some cleavage sites (for example many *Hin*dIII sites) still remain to be determined. The outstanding characteristic of the spinach chloroplast DNA map (Fig. A1) is the duplication and inverted orientation of a region of about 15 megadaltons. These regions are separated along the circular molecule by two regions, one of about 53 megadaltons and the other of about 13 megadaltons. For the location of the ribosomal RNA genes, the individual 16-S and 28-S ribosomal RNAs were isolated from subunits of chloroplast ribosomes and labelled *in vitro* with ^{125}I.

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J. M. Schmitt, Botanisches Institut der Julius-Maximilians-Universität Würzburg, Dallenbergweg 38, D-8700 Würzburg, Federal Republic of Germany

H. J. Bohnert, Europäisches Laboratorium für Molekularbiologie, Postfach 102209, D-6900 Heidelberg, Federal Republic of Germany

K. H. J. Gordon, Department of Biochemistry, University of Adelaide, Adelaide, South Australia, Australia 5001

R. Herrmann, Botanisches Institut der Universität Düsseldorf, Universitätsstraße 1, D-4000 Düsseldorf, Federal Republic of Germany

G. Bernardi, Laboratoire de Génétique Moléculaire, Institut de Recherche en Biologie Moléculaire du Centre National de la Recherche Scientifique, Université de Paris VII, Tour 43, 2 Place Jussieu, F-75221 Paris-Cedex-05, France

E. J. Crouse, Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique, 15 Rue René-Descartes, Esplanade, F-67084 Strasbourg-Cedex, France