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*CHLOROPLAST DNA IN CHLAMYDOMONAS**

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Extranuclear DNA has not been considered a normal cellular constituent either by cytologists or by geneticists, although exceptional instances have been recognized, such as the cytoplasmic DNA of the amphibian oöcyte.[1] Nonetheless, suggestive evidence has been reported of DNA in chloroplasts,[2, 6, 7] in mitochondria,[3] and in the kinetoplasts.[4]

The present study was prompted by two considerations, one genetical and the other biochemical. We have recently isolated a series of nonchromosomal mutants in the green alga *Chlamydomonas*, which indicate the presence of an extensive nonchromosomal genetic system.[5] Many of these strains cannot grow photosynthetically, suggesting that mutations have occurred in nonchromosomal genes which affect chloroplast traits. Where are these nonchromosomal genes located and are they composed of DNA? A recent report that *Chlamydomonas* contains extranuclear Feulgen-positive particles,[6] and that DNA isolated from *Chlamydomonas* contains a satellite band with a buoyant density lighter than that of the major component,[7] led us to examine the nucleic acid content of chloroplasts isolated from *Chlamydomonas*.

In this paper we describe a procedure for isolating relatively intact chloroplasts free of other cellular debris. DNA extracted from these isolated chloroplasts has been characterized with respect to buoyant density and base composition.

Materials and Methods.—(1) *Isolation of chloroplasts:* Cells (*Chlamydomonas reinhardi*, strain 21 gr) were grown with a six-hr doubling time in 10-liter batches in minimal medium with 5% CO_2,[8] harvested from the logarithmic phase, washed, and resuspended in W medium.[9] W medium contains 0.25 *M* sucrose, 2.5% Ficoll (Pharmacia), 5% dextran-40 (Pharmacia), 0.006 *M* mercaptoethanol, 0.01% bovine serum albumin, 1×10^{-3} *M* $MgCl_2$, 1×10^{-3} *M* $MnCl_2$, and 1.5×10^{-3} *M* $CaCl_2$ in 10^{-2} *M* tris-HCl buffer at pH 7.8. Cells were broken in a cold French pressure cell at 3,000 psi. All subsequent steps in the isolation procedure were carried out at 2°. Five ml of the broken cell mixture was layered onto a discontinuous gradient containing 5 ml each of 2.5 *M*, 2.0 *M*, 1.5 *M*, and 1.0 *M* sucrose in W medium. The gradients were centrifuged in the Spinco Model L at 25,000 rpm in the SW 25.1 rotor for 90 min.

After centrifugation five green bands were present, as shown in Figure 1. Examination in the light microscope revealed that bands 1 and 2 contained broken chloroplasts, band 3 contained largely intact chloroplasts, bands 4 and 5 contained chloroplasts and some unbroken cells. The bands were recovered by piercing the bottom of the tube and collecting drops. Only band 3 was retained for further purification, diluted with W medium not containing sucrose, and banded again in a sucrose density gradient as above. After centrifugation, band 3 was again collected

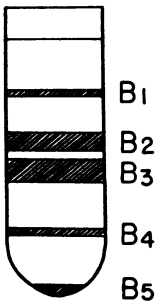

FIG. 1.—Banding of chloroplasts in sucrose gradient. 5 ml of broken cell suspension was layered on a discontinuous gradient, 5 ml each of 1.0, 1.5, 2.0, 2.5 sucrose, top to bottom, all in W medium, and centrifuged at 25,000 rpm in SW 25 rotor, Spinco model L, at 2–3°.

diluted with W medium containing 0.25 *M* sucrose, and washed three times by centrifugation at 2,500 × *g* for 10 min.

(2) *Extraction of DNA:* DNA was extracted by Marmur's method[10] both from cells and from isolated chloroplasts. The chloroplast pellet from the third wash was resuspended in 0.15 *M* NaCl–0.1 *M* EDTA and lysed with 2% sodium lauryl sulphate at 60° for 10 min. After cooling to 25°, sodium perchlorate was added (1 *M*) and the mixture was deproteinized twice with chloroform-amyl alcohol. DNA was precipitated by the addition of 2 volumes of absolute ethanol and resuspended in saline-citrate.

(3) *CsCl density gradient centrifugation.*[11] The DNA-CsCl mixtures were adjusted to a density (g/ml) of 1.710 and centrifuged in the SW 39 rotor of the Spinco Model L at 33,000 rpm for 66–70 hours at 18–20°C. The tubes were harvested dropwise from the bottom after centrifugation, two drops per tube; tris-HCl buffer was added to each tube and the OD_{260} determined.

(4) *Chemical analysis of cell fractions:* Cell fractions were extracted with cold 10% trichloracetic acid and centrifuged; nucleic acids were extracted from the pellets with 1 *N* hot perchloric acid. DNA was determined by the reaction with diphenylamine[12] and RNA by the orcinol reaction.[13] For protein determination, samples were dissolved in 1*N* NaOH or concentrated formic acid, and protein was measured by the Folin reaction using bovine serum albumin as a standard.[14] Chlorophyll concentration was determined by measuring the OD_{665} after extraction of cell fractions with 85% acetone. The number of chloroplasts in each preparation was determined by counting with a hemocytometer.

(5) *Base composition of DNA fractions from CsCl gradients:* Drops collected from each peak region after centrifugation were pooled and dialyzed against water for 48 hr at 2°C. to remove CsCl. DNA was hydrolyzed with 7.5 *N* perchloric acid at 100°C for one hr, and the hydrolysates were spotted on Whatman No. 1 filter paper strips. Descending chromatograms were run in isopropanol-HCl-water at room temperature for 22 hr.[15] After drying, spots containing bases were cut out and eluted with 0.1 *N* HCl for 48 hr at room temperature. OD_{260} of eluates was determined.

Results.—(1) *Chemical composition during purification:* The chlorophyll, protein, and DNA contents of the isolated chloroplasts from one experiment are given in Table 1. Similar results have been obtained in two subsequent experiments. Since there is but one chloroplast per cell in this species, the data reported per

TABLE 1

DNA, PROTEIN, AND CHLOROPHYLL CONTENT OF CHLOROPLASTS DURING ISOLATION PROCEDURE

Steps of isolation	Per cent intact chloroplasts	Amounts (per cell or intact chloroplasts) Chlorophyll $\times 10^9$ molecules	Protein ($\times 10^{-6}$ mg)	DNA ($\times 10^{-10}$ mg)
Intact cell	100.0	1.22	5.0	3.5
After 1st S.G.C.*	48.7	1.57	2.90	2.30
After 2nd S.G.C.*	28.3	1.54	2.29	0.62
After 1st wash	20.4	1.30	0.96	0.21
After 2nd wash	12.4	1.19	0.76	0.11
After 3rd wash	10.6	1.28	0.77	0.12

* Sucrose gradient centrifugation.

chloroplast can be directly compared with the initial values for intact cells. The data could also be reported on a chlorophyll basis, since the amount of chlorophyll per plastid remained constant during the purification procedure. This constancy of chlorophyll content is not surprising, since the pigment is membrane-bound. The protein content fell, during washing, from about 40 per cent of the cellular protein after the second sucrose gradient centrifugation to about 15 per cent after the second wash. These data suggest that the chloroplasts retained most of the soluble proteins until the washing procedure began. Since the chloroplast represents 50 per cent of the volume of the cell, a protein content of 40 per cent seems reasonable.

After the second sucrose gradient, band 3 contained about 18 per cent of the cellular DNA, but upon washing this fraction decreased to 3.3 per cent and remained constant in the second and third washes.

(2) *Microscopy:* Examination of fractions with the light microscope revealed a very low level of contamination by other cell particulates. Very few intact or broken nuclei were seen in the chloroplast bands recovered from sucrose gradient centrifugation. The isolated chloroplasts remained cup-shaped and scarcely swollen even after the third wash, with pyrenoid and eyespot still *in situ*.[16]

(3) *Characterization of DNA in CsCl density gradients:* The existence of a satellite DNA in *Chlamydomonas* with a lower buoyant density in CsCl than the major component is shown in Figure 2*c*. In this preparation, DNA was isolated from whole cells and centrifuged to equilibrium in a CsCl density gradient in the Spinco Model L. The major peak contains about 94 per cent of the DNA, while the satellite peak which is much lighter contains the remaining 6 per cent. Figures 2*a* and 2*b* show the pattern of banding obtained with DNA extracted from chloroplasts after the third wash (cf. Table 1). In these preparations, the satellite DNA is about 40 per cent of the total, representing a sevenfold enrichment in concentration over that present in whole cell DNA.

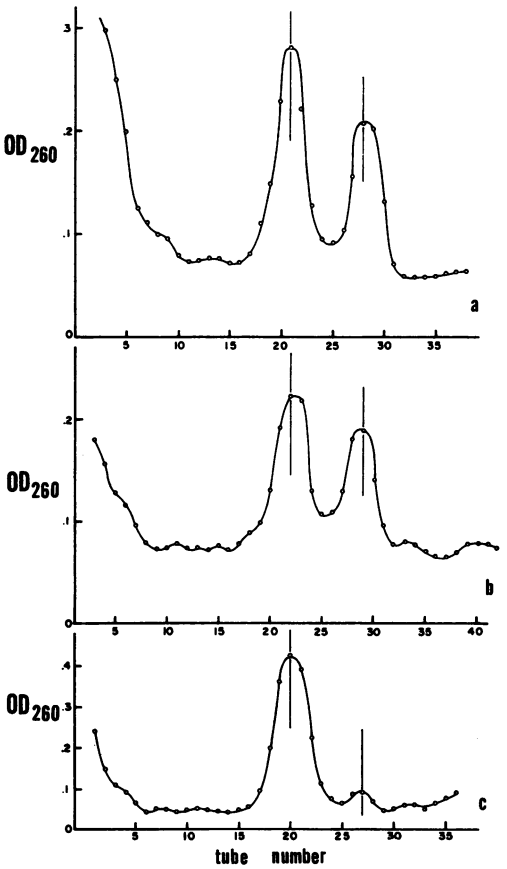

FIG. 2.—Banding of DNA from whole cells and chloroplasts in CsCl gradient, SW 39 rotor, Spinco Model L, 33,000 rpm, 70 hr, 18–20°. (*a*) and (*b*) Chloroplast DNA from two experiments. The satellite band is 39% of the total DNA in each. (*c*) Whole cell DNA with major band (94%) and satellite band (6%).

TABLE 2

BASE COMPOSITION OF DNA FROM *Chlamydomonas*

	Total	Major band	Satellite band
Adenine	18.0 ± 0.42	18.0 17.9 17.5	29.8 30.0
Guanine	29.3 ± 0.39	29.7 30.2 30.4	20.9 20.3
Cytosine	32.1 ± 1.0	32.2 32.2 31.7	19.0 18.3
Thymine	20.5 ± 1.1	20.1 19.7 20.4	30.3 31.3
Pu/Py	0.90	0.92	1.02
6-keto/6-amino	0.99	1.01	1.06
CG content (%)	61.4	62.1	39.3

(4) *Base composition of DNA fractions:* The considerable difference in bouyant density of the two DNA components was sufficient to permit their separation in the Spinco Model L in a CsCl gradient and their recovery for analytical purposes. DNA samples recovered in three different experiments, each beginning with 20 liters of culture medium, were pooled to provide enough material for base analyses. The results are shown in Table 2. The DNAs of both fractions may be double-stranded, since they contain equal amounts of the complementary base pairs. No unusual bases were detected in either fraction. The buoyant density of the major component, determined in the analytical ultracentrifuge as 1.728[17] compares well with the value of 1.726 calculated from the G-C content. By calculation the satellite DNA has a buoyant density of 1.702.

Discussion.—In this paper we report the isolation of DNA from chloroplasts of *Chlamydomonas.* The success of the method depends in the first instance upon the separation of intact chloroplasts from other cell particulates and debris by two rounds of banding in a discontinuous sucrose gradient. The integrity of the isolated chloroplasts is maintained by a complex medium developed by Honda and Wildman for higher plant chloroplasts[9] and adapted by us to *Chlamydomonas.*

The critical evidence that *Chlamydomonas* chloroplasts contain DNA rests upon its distinctive base composition. Total cell DNA contains about 6 per cent of a satellite band, seen in a CsCl density gradient as a light component. DNA extracted from isolated, washed chloroplasts also contains both the major and the satellite bands, but here the satellite is 25–40 per cent of the total (in three different preparations). On a weight basis the isolated, washed chloroplasts contain about 25 per cent of the total cellular satellite DNA and only about 2% of the major component. This enrichment of satellite DNA during purification indicates clearly the association *in vivo* of satellite DNA with the chloroplast. Whether the other component is also native to the chloroplast or is a contaminant from the nucleus cannot be ascertained from these data.

We have taken the value of 1.2×10^{-11} mg to represent the amount of plastid DNA, having recovered this quantity after washing the chloroplasts in three separate experiments. However, it is not evident whether the higher values found in

plastids taken directly from sucrose gradient bands are the result of contamination or whether the low values obtained after washing are the result of losses.

It seems likely that the Feulgen-positive particles in the cytoplasm of *Chlamydomonas*, first reported by Ris and Plaut,[6] represents all or part of the DNA we have isolated, but we have no direct evidence on this point. However, it is highly probable that the satellite DNA which we have characterized is the same fraction detected in total DNA extracts from *Chlamydomonas* by Chun, Vaughan, and Rich[7] (to whom we are indebted for bringing its existence to our attention). However, their value of $\rho = 1.695$ (from a CsCl gradient) differs somewhat from our value of $\rho = 1.702$ computed from the GC content of the purified DNA.

What is the functional role of chloroplast DNA? The genetical literature contains many reports of nonchromosomal hereditary factors which influence chloroplast development. With *Chlamydomonas*, we have recently obtained a number of different nonchromosomal gene mutations leading to loss of photosynthetic activity.[5] These mutants contain normal amounts of photosynthetic pigments but require acetate for growth in the light. Another mutant, y_1, is blocked in the dark conversion of protochlorophyll to chlorophyll, shows unit factor segregation, but may be nonchromosomal because it does not map and is mutated by streptomycin.[18]

The existence of nonchromosomal genetic systems poses urgent questions of their chemical identity, location, and mode of action. Does satellite DNA code for plastid proteins? Are the nonchromosomal genes of *Chlamydomonas* composed of satellite DNA? Do nonchromosomal genetic systems act in other ways than by coding for the primary structure of proteins?

A structural subunit of the chloroplast lamella, the "quantasome" in spinach, has been described. It is about 200 Å in diameter[19, 20] and contains 100–200 chlorophyll molecules. If we assume a similar molecular organization in the chloroplast lamellae of *Chlamydomonas*, there would be some 5×10^6 quantasomes per plastid. Our finding of 1×10^{-11} mg of DNA per chloroplast would provide only about 20 nucleotide pairs per quantasome. Thus, it is evident that genetic DNA cannot be a constituent of each quantasome. If the organization of molecules into the quantasome is template-controlled (i.e., genetic), then the template is either not DNA or it is used many times over.

The *Chlamydomonas* chloroplast also contains RNA, most of it in the form of ribosomes,[21] and isolated chloroplasts incorporate amino acids into polypeptide.[22] Thus, it should be possible to examine the role of DNA in the synthesis of chloroplast RNA and protein fractions, and thereby to investigate the capabilities of chloroplast DNA in cell-free systems. It is hoped that the distinctive base composition of satellite DNA will aid us in elucidating its role, and that the nonchromosomal chloroplast mutants will provide material for a more penetrating analysis of genetic control mechanisms.

Summary.—A method has been developed for the isolation of relatively intact chloroplasts from the alga *Chlamydomonas*. These chloroplasts contain about 3 per cent of the cellular DNA, of which 25–40 per cent (in three separate preparations) consists of a satellite band with a buoyant density of 1.702 gm/cm^3 and GC content of 39.3 per cent. The enrichment of satellite DNA in the chloroplast extract sevenfold over its concentration in total cell extracts provides strong evidence

for its association with the chloroplast *in vivo*. Light microscope examination of the isolated chloroplast preparations indicates very low contamination with other cell particulates.

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THE CYCLIC HELIX AND CYCLIC COIL FORMS OF POLYOMA VIRAL DNA

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The DNA extracted from polyoma virus exhibits certain properties which have not been reported for other viral base-paired DNA's.[1] The DNA renatures monomolecularly. The loss of helical configuration does not impair biological activity. Heating at 100° for 10–20 min followed by rapid cooling does not reduce the infective titer. Dulbecco has given evidence suggesting that a fraction of the polyoma DNA molecules are cyclic.[2] This form would in part account for the above properties.