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Circular Molecules in the β-Satellite DNA of *Chlamydomonas reinhardii* *

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Summary. The β-satellite DNA (chloroplast DNA) of *Chlamydomonas reinhardii* has been shown to contain a homogeneous population of circular molecules of about 62 μm contour length. Evidence is presented that this molecule is a monomer and that the β-satellite is a homogeneous DNA species. A second class of circular molecules of as yet unknown intracellular origin, having a circumference of about 27 μm, has also been observed. Our data demonstrate that the sizes of circular chloroplast DNA vary considerably even within the *Chlorophyta*.

Introduction

Previous research has revealed that circular chloroplast DNAs (cpDNAs) from widely divergent species apparently share the common property of remarkably uniform contour length. *Euglena* (Manning et al., 1971), about 10 representatives of higher plants including mono- and dicotyledons (Manning et al., 1972; Falk et al., 1974; Herrmann et al., 1975a; Kolodner and Tewari, 1975; Wuttke, 1976), and two fern members (Herrmann et al., 1975b) all contain cyclic forms of cpDNA with an average monomer size of about 45 μm. However, an exception has recently been found for the cpDNA of *Sphaerocarpos donnellii* whose contour length of 37 μm is significantly smaller (Herrmann et al., 1975b).

Our interest in studying the structure of the *Chlamydomonas reinhardii* β-band DNA, which is probably the cpDNA of this green alga, was aroused because its reassociation kinetics consistently revealed a larger genetic complexity than those of higher plant cpDNAs (Bastia et al., 1971a; Wells and Sager, 1971; Howell and Walker, 1976) even when directly compared (Table 2 in Meyer and Herrmann, 1973), and because much genetic, biochemical and cytological work has been carried out on this organism.

We now report experiments which show that the β-satellite DNA of *Chlamydomonas* contains a cyclic form which is larger than that of higher plant cpDNA. Preliminary reports were presented at the 10th meeting of the Federation of European Biochemical Societies (Paris, 1975) and at the Symposium on "Nucleic Acids and Protein Synthesis in Plants", Strasbourg (Hobom et al., 1977).

Materials and Methods

Exponentially grwon *Chlamydomonas reinhardii* cells (axenically) cultured, cell wall deficient nuclear mutant CW 15), synchronized by alternate 12h dark-light cycles, were collected at the end of the light period and washed twice in saline-EDTA (0.15 M NaCl, 0.1 M EDTA, pH 8.0, 0° C). The algae were resuspended in an equal volume of saline-EDTA and diluted with 10–20 volumes of hypotonic Tris-EDTA (50 mM Tris, 20 mM EDTA, pH 8.0, 20° C). Lysis was accomplished by the rapid addition and thorough mixing of 1/10th volume of 10% (w/v) sodium dodecylsarcosinate at 20° C. The viscous lysate was kept at room temperature for 10 minutes and then brought to a refractive index of 1.3660 with a saturated CsCl solution. "Cells", resistant to the detergent treatment, gently lysed at this stage. After 1 h in ice the lysate was centrifuged at 10,000 × g for 15 min, and the DNAs from the central third of this supernatant were collected by an overnight centrifugation into a CsCl cushion as described previously (Herrmann et al., 1975a). The cushions which did not contain much flocculent material were decanted into 5 or 12 ml polyallomer tubes and subjected to CsCl equilibrium or relaxation (Anet and Strayer, 1969) centrifugation. The gradients were subsequently fractionated with an ISCO gradient fractionator at a flow rate of 0.5 ml/min. Aliquots of appropriate fractions were used for electron microscopy and for pycnographic analysis in analytical CsCl gradients (Hermann et al., 1975a). Circular mitochondrial DNA (mtDNA) of *Acanthamoeba castellanii* was a gift from Dr. H.-J. Bohnert of this laboratory.

* There is considerable confusion in the literature with regard to the correct nomenclature for the species *C. reinhardii* (-di, -dii, -dti, -dtii). According to Prof. Dr. J. Gerloff, Botanischer Garten und Museum, Berlin-Dahlem, "*Chlamydomonas reinhardii*" is correct. This term is based on § 73 of the International Code for Botanical Nomenclature and on the fact that Reinhard himself transcribed his name from Russian with "d". Thus, Dangeard's first description "Reinhardtii" is orthographically incorrect

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Results

Four species of DNA, each characterized in terms of buoyant density, are found in vegetatively growing *Chlamydomonas* (Fig. 1). The predominant DNA species (1.723 g cm^{-3}), comprising some 80% of the total cellular DNA, is almost certainly of nuclear origin. A second DNA species (1.712 g cm^{-3}), corresponding to 0.7–0.9% of the total DNA in the cell is now considered to be nuclear rDNA (Bastia, 1971b; Howell, 1972). A third DNA species (1.706 g cm^{-3}), representing 1–2% of the total cellular DNA, was identified as mtDNA; it contains circular molecules of 4.6 μm contour length (Ryan et al., 1973; Ryan et al., 1974; K.-S. Chiang personal communication). Finally, the major satellite (1.695 g cm^{-3}), amounting to about 15% of the total DNA, is attributed to the chloroplast. Because it is difficult to prepare unbroken and highly purified organelles from this organism, the basis of identification was its enrichment in chloroplast fractions (Sager and Ishida, 1963). While this interpretation is probably correct, it is relevant to point out that the application of the enrichment criterion has led to wrong identification in a number of instances in higher plant cpDNAs (for review see Kirk, 1971). Furthermore, this DNA has not been convincingly demonstrated to consist of only one species.

As shown by electron microscopy *Chlamydomonas* main band DNA consisted of high molecular weight molecules. No circular molecules were seen. However, large circular molecules of uniform size were found in DNA banding within the density range of 1.685–1,715 g cm^{-3}. In order to determine the origin of this population, DNA from the region was separated into 3 fractions, the respective fractions from several gradients were combined and each of these samples was recycled through a relaxation gradient (Anet and Strayer, 1969) to achieve enhanced resolution. The resulting absorbance profiles are shown in Figures 2a–c. In the lightest DNA fraction (Fig. 2c), the β-satellite content was greatly enriched to approximately 50–70% of the total DNA present. This component formed a narrow band in analytical CsCl gradients lacking any detectable satellite DNAs of different buoyant density (Fig. 2e). The large circular molecules were confined to this satellite. The yield of these molecules was usually in the order of 10–20% (on a per molecule basis); but in two other preparation, yields of 40 and 60% were observed.

The predominant circular configuration was the relaxed form; supercoiled molecules beeing seen only rarely (< 5%). A few molecules exhibited rosette patterns lacking visible free ends. These may be preparation artifacts or molecules which have not been completely released from their membrane association analogous to those found with higher plant cpDNAs which have been characterized both cytologically and biochemically (Herrmann et al., 1974).

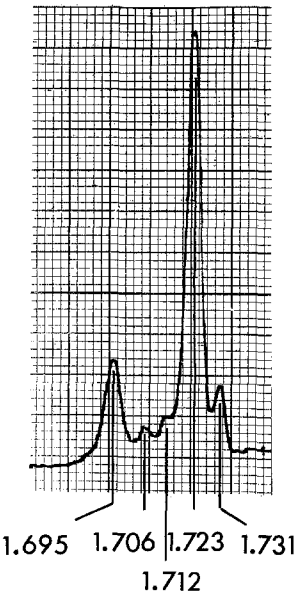

Fig. 1. Analytical CsCl density gradient scan at 262 nm of total cell DNA from *Chlamydomonas reinhardii*. The field is directed to the right. The cpDNA (β-satellite) bands at a density of 1.695 g cm^{-3}, the mtDNA at 1.706 g cm^{-3}. The nuclear rDNA satellite ($\rho = 1.712$ g cm^{-3}) is indicated as shoulder on the light side of the main band nucDNA ($\rho = 1.723$ g cm^{-3}). Centrifugation was performed at 44 Krpm for 21 h at 20° C. *Micrococcus lysodeikticus* DNA ($\rho = 1.731$ g cm^{-3}) served as density reference

The large circular molecules fell into one homogeneous size class. Their average circumference measured 62 μm, corresponding to a molecular weight of 134 megadalton of double stranded DNA based on a mass per unit length of 2.16 megadalton per μm (Bujard, 1969). This contour length is considerably longer than all monomeric molecules of circular cpDNAs so far described. The uniformity, the high percentage of circular molecules in some of our preparations, the occurrence of covalently closed duplex molecules and the method of isolation chosen render the possibility of artificial ring closure during preparation extremely unlikely, and so we conclude that these circular molecules are native components of the β-satellite. The fact that nearly all circular molecules found in this alga were in relaxed form does not imply that this is the predominant conformation in situ. The almost total absence of covalently closed duplex DNA, which is the usual conformation of naturally occurring circular DNA, may be attributed to accidential single strand breaks introduced during lysis or handling the large molecules.

To exclude the possibility that the observed size disparity between higher plant and *Chlamydomonas*

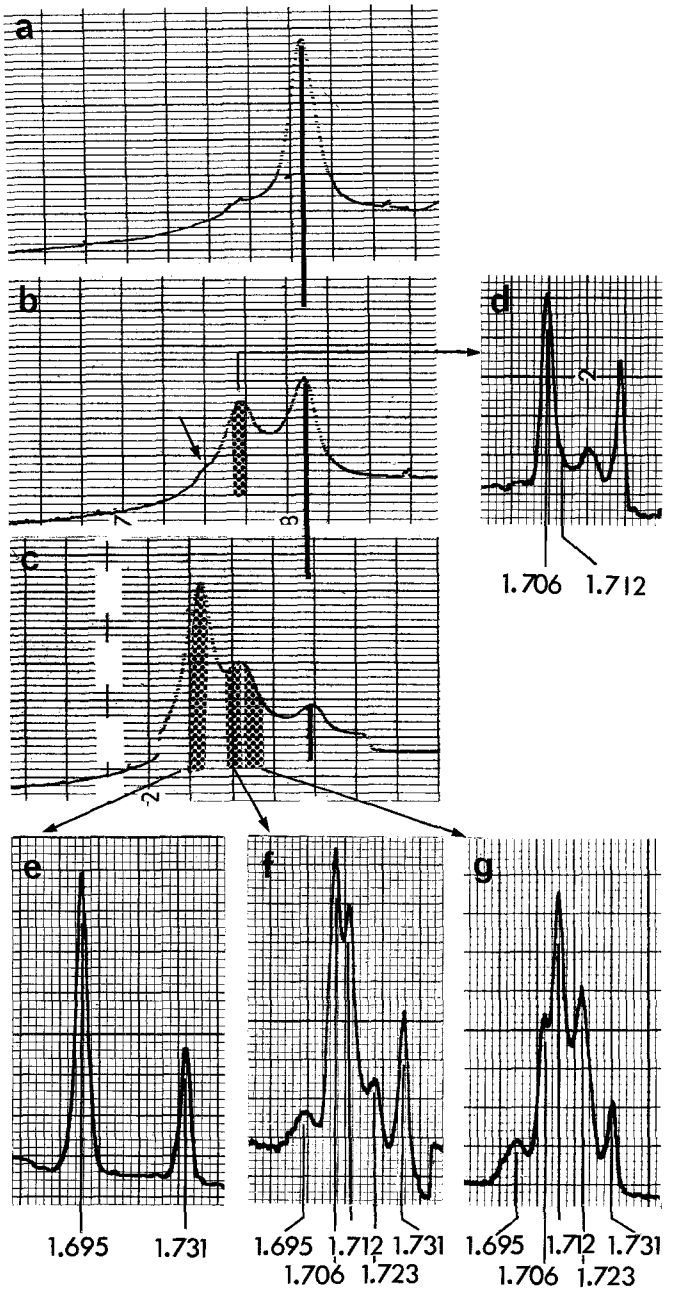

Fig. 2a–g. Fractionation of the *Chlamydomonas* DNA satellites in CsCl relaxation gradients. Whole-cell DNA was first centrifuged in a preparative CsCl gradient and the satellite-rich region cut into three fractions. These fractions were centrifuged to equilibrium for 52 h at 39 Krpm at 20° C in a 6 × 5 ml SW 40 swinging bucket rotor (WKF-Hitachi P65 preparative ultracentrifuge) and the gradients relaxed for 40 h at 27 Krpm. **a–c** show the resulting absorbance profiles (scale extention $A_{260}=0.5$) of these three fractions in order of decreasing density. The DNA species were isolated by drop collection and aliquots of fractions were examined in the electron microscope and by analytical ultracentrifugation. The banding pattern in analytical CsCl gradients of selected DNA fractions (hatched areas) are shown in the **d–g**

cpDNAs was due to a preparation artefact and to determine more accurately their relative contour lengths, two kinds of experiments were devised. Purified circular cpDNA of *Spinacia oleracea* ($c_{av}=45$ μm; Manning et al., 1972; Herrmann et al., 1975a), *Sphaerocarpos donnellii* ($c_{av}=37$ μm; Herrmann et al., 1975b) and *Chlamydomonas* β-band DNA were mixed in pairs in various combinations. In addition, as in our previous work, circular mtDNA of *Acanthamoeba castellanii* ($c_{av}=12.7$ μm; $\rho=1.691$ g cm^{-3}; Herrmann et al., 1975a) was chosen as an internal size standard, because its molecular weight is approximately one quarter of that of higher plant plastid DNA, thus minimizing measuring errors by allowing size comparisons at the same microscope magnification. Moreover, significant variations in molecule length could arise from the existence of cellular contaminants, which may resist removal during the DNA preparation and behave like some intercalating dyes considerably increasing DNA length (Freifelder, 1971). To exclude this possibility a second experiment was designed in which *Chlamydomonas* cells were added to *Spinacia* tissue prior to blending. The resulting homogenate was filtered before plastids

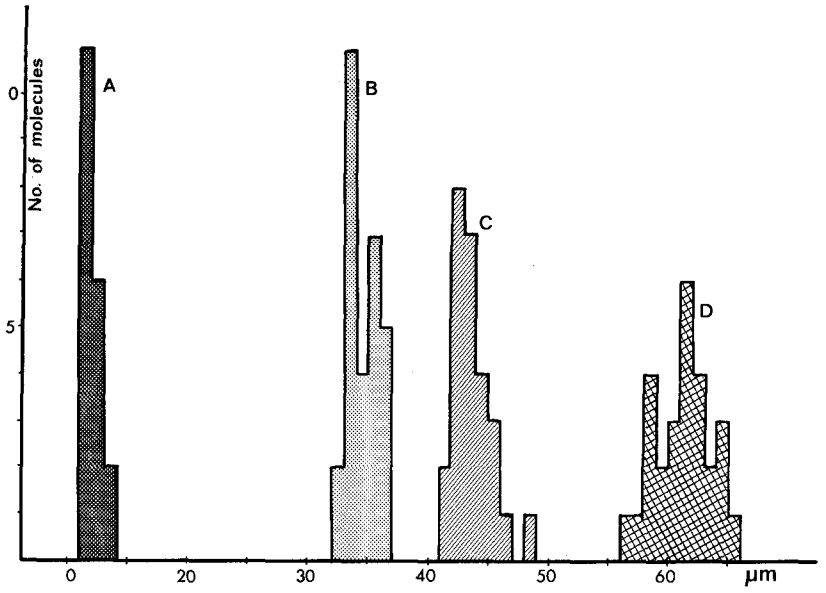

Fig. 3. Combined frequency distribution of sizes of open circular DNAs from mitochondria of *Acanthamoeba castellanii* (A; 12.5, S.D. 0.5µm), plastid fractions of *Sphaerocarpos donnellii* (B; 36.9, S.D. 1.1 µm) and of *Spinacia oleracea* (C; 43.9, S.D. 1.8 µm), and from the *Chlamydomonas* β-satellite (D; 61.8, S.D. 2.4 µm). The histogram was constructed from data obtained by mixing DNA species in pairs. The same preparation for each DNA species was used in this experiment. Results from 7 other preparations were quantitatively similar (S.D. of means 0.15%)

and algal cells were collected by centrifugation. The pellet material was incubated with DNAase to remove *Spinacia* nuclear DNA, washed twice in saline-EDTA and lysed to recover the DNA (for procedural detail see Herrmann et al., 1975a).

The frequency distribution of circular cpDNAs of the three plant species as well as that of *Acanthamoeba* mtDNA is shown in Figure 3. Regardless of the method of preparation used, the characteristic lengths were always obtained. This implies that the observed size differences are real and also that the contour lengths of different circular cpDNAs are not necessarily equal. Small variations in size have recently been postulated for higher plant plastid DNAs (Kolodner and Tewari, 1975); however, since no control experiments such as the type described above were performed, it remains to be shown that these variations reflect real differences in molecular weight.

In two preparations a few relaxed circular molecules were observed in the density region adjacent to that of the β-satellite (Fig. 2c). About 20 molecules that could be traced unambiguously had contour lengths between 25 and 28 µm. These represent a third class of circular DNA molecules from *Chlamydomonas*. Their significance and subcellular localization has not yet been established because in both of these DNA preparations more than one density mode in CsCl gradients was observed (Fig. 2f, g). Because this DNA is similar in size to ring-shaped mtDNA from *Pisum* (Kolodner and Tewari, 1972) it is tempting to speculate that it originated in the mitochondrion, however, Chiang's observation of 4.6 µm covalently closed circular molecules in *Chlamydomonas* mitochondrial DNA makes this rather unlikely (Ryan et al., 1974; K.-S. Chiang personal communication).

Discussion

A circle size of 62 µm for the cpDNA of *Chlamydomonas* is in excellent agreement with recent experiments by Rochaix (1977) and Howell et al. (1977) who determined the physical size of the β-satellite DNA by an independent approach. Using digestion with various restriction enzymes, they obtained values between 120 and 130 megadalton by summing the molecular weights of the fragments. The coincidence of both results proves that the β-satellite of *Chlamydomonas reinhardii* is a homogeneous DNA species and suggests that its basic complement is the 62 µm circle.

A molecular weight of 134 megadalton, calculated for the 62 µm circular molecules, is significantly lower than the genetic complexity of 190 to 200 megadalton found by reassociation kinetics (Bastia et al., 1971a; Wells and Sager 1971; Howell and Walker, 1976), but agrees better with that of 140 megadalton found in our laboratory (Table 2 in Meyer and Herrmann, 1973). This discrepancy can be attributed to uncertainities inherent in the estimation of genetic complex-

ities of cpDNA by reassociation kinetics. These uncertainties are illustrated by the fact that both biphasic (Wells and Sager, 1971) and homophasic (Bastia et al., 1971; Howell and Walker, 1976) kinetics have been reported for the β-satellite. Meaningful kinetics have to account for differences in G+C content compared to standard DNA, for the presence of inverted repeats which contribute to "zero-time" DNA annealing, for base misparing at low temperatures (Tr < Tm-20; Bohnert and Herrmann, 1974,) and for intramolecular compositional heterogeneity at more stringent reassociation temperatures (Tr < Tm-15). *Chlamydomonas* cpDNA exhibits considerable compositional heterogeneity (Wells and Sager, 1971; Bastia et al., 1971 a; Bastia et al., 1971 b) and may contain a repeated region(s) (cf. fragment stoichiometry in the restriction endonuclease digests reported by Rochaix, 1977 and Howell et al., 1977) similar to the inverted repeats of leucoplast DNA in the closely related organism *Polytoma obtusum* (Siu et al., 1975) and to cpDNAs of some higher plants (Herrmann et al., 1976; Bedbrook, 1977). Depending on the choice of experimental conditions these properties can influence reassociation kinetics yielding, at best, only approximations for the potential coding capacity of cpDNAs.

It is not yet possible to relate the disparities in size between cpDNAs to genetic functions and evolutionary processes since knowledge of the functions of cpDNAs is still very limited and the comparative study of their physico-chemical properties has not progressed very far. An important question is whether the extra DNA in the *Chlamydomonas* chromosome implies increased coding capacity, sequence duplications or only an accumulation of non-functional sequences. An increase in the A+T-rich sequences may be inferred from the slightly lower density in CsCl as compared to that of higher plant cpDNAs (1.695 vs. 1.697 g cm^{-3}). Of interest in this respect is a comparison with the leucoplast of *Polytoma* which obviously still possesses a functional transcription and translation system but its DNA is much higher in A+T content than the *Chlamydomonas* cpDNA (Siu et al., 1975). Variation in the length of A+T-rich sequences in closely related extrakaryotic DNAs has recently been reported in the mtDNAs of different species of *Drosophila melanogaster* (Fauron and Wolstenholme, 1976), and different strains of yeast (Prunell et al., 1977).

Three possibilities can be envisaged to explain the evolutionary implications of the increased size of *Chlamydomonas* cpDNA. The excess sequences may have been lost during evolution in higher plant cpDNAs, could have been taken over by the nucleus, or may reflect merely fortuitous differences. The uniformity in size of higher plant cpDNA compared to the larger size of this algal cpDNA has an interesting analogy to the size differences found between animal and protist mtDNAs. Animal mtDNA consist of cirles with an uniform contour length of about 5 μm (Borst, 1972) whereas protists contain considerably larger ring-shaped mtDNAs (Borst, 1970; Bohnert, 1973; Sanders et al., 1974). The evolutionary conservation of size and shape of animal mtDNAs has been interpreted in terms of a reductive evolution that apparently reached its minimum size with the 5 μm circle (Borst, 1970). It is not easy, however, to reconcile the length fluctuations in cpDNAs with the simple hypothesis of a reductive evolution of the plastome in view of the fact that circular molecules of cpDNA which are about 15% smaller than those of higher plant cpDNAs have been found in *Sphaerocarpos donnellii* (Herrmann et al., 1975b) and in *Vaucheria sessilis* (*Xanthophyceae*) cpDNA (Hennig and Kowallik, unpublished observation).

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