Heterogeneity, Complexity, and Repetition of the Chloroplast DNA of Chlamydomonas reinhardtii

(T=m/density gradient centrifugation/renaturation/hydropatite chromatography)

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ABSTRACT The chloroplast DNA of wild-type Chlamydomonas reinhardtii was isolated in a CsCl density gradient as a single, homogeneous density class with a mean density of 1.695 g/cm³. Irrespective of sheared size, denatured and chloroplast DNA renatured as a single homogeneous species. Compositional heterogeneity, presumably intramolecular, was revealed by the absorbance-temperature profile. The complement of unique nucleotide sequences of the chloroplast DNA, as determined by the rate of renaturation, was 1.94 × 10⁸ daltons. This kinetic complexity is 26-fold less than the DNA content of a single gametic chloroplast, and 52-fold less than the chloroplast of a vegetative cell, which indicates that the chloroplast of C. reinhardtii possesses at least 26 copies of a unique nucleotide sequence.

One of the most extensively studied organelle DNAs is the chloroplast DNA of Chlamydomonas reinhardtii. The chloroplast of this organism was first shown by cytological staining and electron microscopy to contain DNA bodies (1). The chloroplast of C. reinhardtii was later shown to possess DNA molecules distinct from the nuclear DNA (2, 3). The physical continuity of this chloroplast DNA from generation to generation was demonstrated by isotopic labeling studies that confirmed its semiconservative replication (4, 5). Furthermore, the replication of chloroplast DNA was shown to be independent of, and temporally separated from, the chromosomal DNA replication in both meiosis (5, 6) and mitosis (4, 5, 8). Physical conservation of both mating-type chloroplast DNAs through meiosis has also been demonstrated (7). Also, unlike the denatured nuclear DNA of C. reinhardtii, chloroplast DNA renatured readily within a few hours to attain approximately its native density, which indicates that it is much less complex than the nuclear DNA of the same organism (4). Because C. reinhardtii contains only a single chloroplast per cell, the DNA content of the gametic chloroplast has been unequivocally determined to be 8.61 × 10⁻¹⁸ g (4, 5). If this amount of DNA is assumed to be arranged in a single linear molecule, it would be 5.16 × 10⁶ daltons, or about twice the size of Escherichia coli DNA (4). The functional role of this large amount of chloroplast DNA in C. reinhardtii is still unclear. Indirect experimental evidence has suggested that it may serve as a template for the transcription of chloroplast ribosomal RNAs (9). Whether chloroplast DNA consists of a small number of copies of a relatively large genome, or many copies of molecules with a smaller genetic complexity, is obviously a question of considerable interest. A satisfactory estimation of the genomic complexity of the chloroplast DNA by direct physical measurement has not been made because of the experimental difficulty in controlling the purity or the intactness of the DNA molecules isolated from chloroplast preparations.

It has been demonstrated that the kinetic complexity, i.e., the size of the unique nucleotide sequence of a given DNA sample, can be satisfactorily estimated from its rate of renaturation (10, 11). Estimation of the genomic complexity by this technique does not require the isolation of the chloroplast DNA in its unsheared native state. In this paper, we determine the kinetic complexity of the chloroplast DNA of C. reinhardtii to be 1.94 × 10⁸ daltons. Comparison of this value with the DNA content of the chloroplast demonstrates the existence of at least 26 repetitions of the unique nucleotide sequence in a single chloroplast. Furthermore, by denaturation analysis, the chloroplast DNA has been shown to contain considerable compositional heterogeneity as manifested by its bi-or multiphasic melting characteristics. This type of heterogeneity was preserved in the DNA molecule after thermal denaturation and subsequent renaturation, indicating that such heterogeneity is rather specific and of an intramolecular nature.

MATERIAL AND METHODS

Strains and Culture Conditions. The wild-type C. reinhardtii, strain 137C, “plus” mating type (obtained from Dr. R. P. Levine) was used. The composition of the liquid medium HSM and other details of vegetative culture conditions have been described (4, 7, 12).

Preparation of Whole Cell DNA. DNA was isolated by pronase plus RNAse digestion and chloroform–isoamyl alcohol extraction (7).

Preparative CsCl Gradients. 200–500 μg of DNA in 1.5 ml of 0.15 M NaCl–0.015 M Na₂citrate was mixed with 4.5 ml of 65% (w/w) CsCl solution in 10 mM Tris–1 mM EDTA (pH 8.0). The density of this mixture was adjusted to 1.700 g/cm³. Centrifugation was in a Beckman L2-65 with a no. 65 fixed-angle rotor at 50,000 rpm (159,000 × g) for 40 hr at 25°C. Ten-drop fractions were collected. In general, a total of 350–420 drops was obtained from each gradient.

Analytical CsCl Equilibrium Centrifugation and Calculation of Buoyant Density of DNA. The detailed method has been described elsewhere (7, 13).

Determination of Melting Curves. The procedure described by Mandei and Marmur (14) was followed. The hyperchromicity was recorded automatically on a Gilford 2000 spectro-
photometer. The rate of temperature elevation was approximately 0.1°C per minute.

Shearing of DNA. The chloroplast DNA, as well as other reference DNA samples from _E. coli_ and T4 DNA (gifts from Dr. E. P. Geiduschek), were sonicated in a Branson model 8-125 sonicator for 30–120 sec. The size of the sheared DNA samples was determined by analytical sedimentation in 0.1 M NaOH–0.9 M NaCl (pH 13.0) as described by Studier (15).

Determination of Renaturation Rate Constant, _k_₂, and the Kinetic Complexity, _N_ₐ. The following two equations, derived by Wetmur and Davidson (11), were used for the calculation of _k_₂ and _N_ₐ.

\[
\frac{1}{(A_i - A_\infty)} = 2.04 \times 10^{-4} k_2 t + 1/0.36 A_\infty
\]

\[
N_D = 5.5 \times 10^6 \left(\frac{\epsilon_{260\text{cm}^{-1}\text{ml}^{-1}\text{mg}^{-1}}}{k_2}\right)^{1/3}
\]

where _A_∞ is the absorbance of native DNA, and _A_i_ is the absorbance of renaturing DNA at a time _t_. A Gilford 2000 automatic recording spectrophotometer was used for the renaturation studies. Other experimental conditions for the DNA renaturation studies were as described by Wetmur and Davidson (11).

**RESULTS**

**Purity and homogeneity of the chloroplast DNA sample**

The whole-cell DNA of _C. reinhardtii_ was first fractionated on an hydroxyapatite column into a major peak and a minor peak, with maxima eluting at 0.20 and 0.275 M phosphate buffer, respectively. The experimental details of this chromatography of _C. reinhardtii_ DNA will be reported elsewhere. DNA recovered from the minor peak consisted of about equal amounts of chloroplast and nuclear DNA (see Fig. 1). This recovered DNA sample was further purified by centrifugation in a preparative gradient of CsCl. After this final fractionation, the chloroplast DNA was then isolated (shaded area in Fig. 1). The purity and the homogeneity of this DNA were attested to by its profile in an analytical CsCl gradient (Fig. 2). All of the subsequent physical experiments were performed with this chloroplast DNA sample, which had a density of 1.695 g/cm³.

**Intramolecular heterogeneity of the chloroplast DNA**

Heterogeneous melting patterns of the chloroplast DNA were observed in the course of the determination of the hyperchromicity factor of chloroplast DNA in 1 M salt (11) (0.7 M NaCl–0.1 M NaOH–0.2 M NaH₂PO₄, pH 7.8) for the renaturation kinetics studies. For this reason, an extensive study on the thermal denaturation of the chloroplast DNA was performed in 0.15 M NaCl–0.015 M NaH₂PO₄ since the thermal expansion correction factor of this solvent is known (14). T4 DNA, which is similar to the chloroplast DNA in both G + C content and _T_m_, was used as a control.

The melting characteristics of the chloroplast DNA (Fig. 3a) can be summarized as follows: (a) the progressive melting of the chloroplast DNA occurred over a considerably broader temperature range than that of T4, and (b) the cooperative transition of the double helix of the chloroplast DNA appeared to be nonhomogeneous within the melting temperature range. When the original melting data of the chloroplast DNA were transformed into quadratic derivatives, the heterogeneity with respect to the cooperative hyperchromic transition became quite obvious (Fig. 4a). The progressive melting appeared to be unevenly spread over the melting temperature range and consisted of at least two main parts (Fig. 4a). The native chloroplast DNA, on the other hand, gave a symmetrical peak characteristic of a single homogeneous species by equilibrium centrifugation in CsCl (see Fig. 2). Therefore, the heterogeneity revealed here by the denaturation studies probably results from a considerable intramolecular heterogeneity in the G·C base-pair distribution in segments of the chloroplast DNA molecule. The two hetero-
heterogeneous segments of the molecule, as revealed by a differential plot of the melting curve, were separated by a difference of 3.5°C in the melting temperature, which corresponds to a difference in G + C content of about 8.5%.

Except for the slight broadening of the progressive melting temperature range, the chloroplast DNA after denaturation and subsequent renaturation still exhibited an unaltered Tm and heterogeneous melting characteristics similar to those of the native chloroplast DNA (Fig. 3b and 4b). This result indicates that during the renaturation process, the intramolecular heterogeneous segments must re-form without significant deviation from their respective native states. This result is to be expected if the heterogeneous segments of the chloroplast DNA molecules are indeed intramolecular and have their own unique and noncross-hybridizable sequences.

Renaturation kinetics and complexities of chloroplast DNA

T4 and E. coli DNA whose analytical complexities were previously determined by other physical means, were also analyzed under our conditions. Chloroplast and T4 DNA samples were sheared to generate three size classes, and the s_{av}.w^{0.14} of each size class was determined by analytical band sedimentation. The molecular homogeneity of each size class was apparent from the symmetry and sharpness of the bands, as seen in the microdensitometer traces of UV photographs taken during the sedimentation analysis. The second-order renaturation rate constant of chloroplast and T4 DNA was determined for the different size classes. It is important to point out that under our experimental conditions, the time required for the absorbance measuring system (DNA solution, blanks, cuvettes, cuvette holder, etc.) to equilibrate to the desired temperature was about 7 min. Even with prior warming, temperature equilibration, as measured directly by a thermometer immersed in the cuvette, required about 7 min for the Gilford 2000 spectrophotometer. Other investigators, using the same apparatus, also report a temperature equilibration time of 7 min (16). Therefore, any fast-renaturing species, which completed its major hypochromic shift before 7 min, would not be detected under our experimental conditions.

The chloroplast DNA renatured as a single kinetic class, up to 35% of its hypochromic shift (Fig. 5). The k_2 values for each of three different size classes, and the corresponding kinetic complexity values, are presented in Table 1. These results show that while the complexity of the chloroplast DNA is independent of the sedimentation coefficients of the differentially sheared and denatured DNA samples (s_{av}.w^{0.14}), a linear relationship exists between the sedimentation coefficient and the second-order renaturation rate constant (k_2). This linearity between s_{av}.w^{0.14} and k_2 is shown in Fig. 6 (a log-log plot). The slope of the straight line in Fig. 6 for the chloroplast DNA, 0.80, is close to that of 0.78, demonstrated...
**DISCUSSION**

The G + C content of DNA is known to be one of the factors that influence the rate constant ($k_2$) of its renaturation reaction and, consequently, the kinetic complexity ($N_k$) of a given DNA species. The magnitude and the direction of the effect, however, remain unclear since contradictory results have been reported (11, 16). Therefore, no attempt was made to correct the kinetic complexity value of chloroplast DNA calculated from the experimentally observed value of $k_2$ (see Table 1).

No special temperature-jump apparatus was used in the present study. Any small quantity of an exceedingly fast-renaturing species that completed most of its hypochromic shift before 7 min would have escaped detection.

From the chemical determination of DNA by the diphenylamine method, it has been shown that the gamete of *C. reinhardtii* contains $1.23 \pm 0.064 \times 10^{-11}$ g of DNA, of which 6.9% or $8.61 \times 10^{-11}$ g is chloroplast DNA (4, 5). Since gametes possess only one chloroplast per cell, the entire $8.61 \times 10^{-12}$ g, or $5.16 \times 10^9$ daltons, of DNA is present in a single chloroplast (4, 5). From our renaturation analysis, the kinetic complexity of the unique nucleotide sequence of the chloroplast DNA is only $1.94 \times 10^9$ daltons. The gametic chloroplast must, therefore, have about 26.6 repetitions of the unique nucleotide sequence. About 52 repetitions must exist in the chloroplast of the vegetative cell since it, too, contains only one chloroplast per cell and $1.722 \times 10^{-14}$ g of chloroplast DNA (4, 5).

The strikingly high frequency of multiple copies of chloroplast DNA in *C. reinhardtii* is of considerable interest, though the exact nature of the physical repetitions is not known. The following alternatives are possible: (a) Micro-intermolecular heterogeneity exists between these $26$ repetitive sequences in the gametic chloroplast DNA; the micro-heterogeneity in nucleotide sequence, although undetectable by the renaturation kinetics studies, is sufficient to cause heterogeneity in the informational content of these DNA molecules. (b) All the chloroplast DNA molecules are homogeneous in their nucleotide sequence and the $26$ repetitions of chloroplast DNA reflect true duplications or redundancies of the chloroplast genome. Whichever of these possibilities may prove correct, repetition of cellular organelle DNA in a given organism may have evolutionary advantages for the survival of the organelle via selection and recombination of its DNA.

It was pointed out by Granick (23) that algal species with single large chloroplasts may be considered as "polyploid", with multiple genome copies. Our recent hybridization studies with chloroplast ribosomal RNA in *C. reinhardtii* indicate the presence of only three rDNA cistrons per copy, i.e., $2 \times 10^8$ daltons (manuscript in preparation). Consequently, multiple copies of the chloroplast genome could conceivably be required for periods of rapid synthesis of chloroplast rRNA.

**Table 1. Kinetic and analytical complexities of DNAs**

<table>
<thead>
<tr>
<th>DNA</th>
<th>$s_{20,w}p_{H2}^{13}$</th>
<th>$k_2$</th>
<th>Kinetic</th>
<th>Average kinetic</th>
<th>Analytical</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4</td>
<td>31.4</td>
<td>217.45</td>
<td>$1.88 \times 10^9$</td>
<td>$2.075 \times 10^9$</td>
<td>$1.3 \pm 0.1 \times 10^9$</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>7.3</td>
<td>29.0</td>
<td>$2.27 \times 10^9$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. reinhardtii</em> chloroplast</td>
<td>4.0</td>
<td>16.05</td>
<td>$1.93 \times 10^9$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.48</td>
<td>55.3</td>
<td>$1.92 \times 10^9$</td>
<td>$1.94 \times 10^9$</td>
<td>$5.16 \times 10^9$</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>17.1</td>
<td>95.7</td>
<td>$1.99 \times 10^9$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>14.15</td>
<td>6.166</td>
<td>$2.44 \times 10^9$</td>
<td>$2.45 \times 10^9$</td>
<td>$2.5 \pm 0.5 \times 10^9$</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td>14.15</td>
<td>6.16</td>
<td>$2.45 \times 10^9$</td>
<td></td>
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</tr>
</tbody>
</table>
It is not known at present how the 26 copies of DNA sequences are organized in the gametic chloroplast, nor how the 52 copies are organized in the vegetative chloroplast. For the simplest case, in the gametic chloroplast it is possible that these sequences either exist as 26 single units or are linked in tandem in some fashion to one or several groups. In this connection, the finding (24) that preparations of *Euglena gracilis* chloroplast DNA exist as separate 40-μm circles is of interest.

The kinetic complexity of the chloroplast DNA of *C. reinhardtii* is similar to the kinetic complexity of the chloroplast DNA in higher plants, i.e., 1.2 x 10^8 daltons for lettuce (17) and for tobacco (18). The kinetic complexity of *Euglena* chloroplast DNA is around 1 x 10^8 daltons (19). The similarities in kinetic complexity of chloroplast DNA among these organisms may be entirely coincidental, or it could signify that the information content necessary for chloroplast development and function is basically similar for *C. reinhardtii*, *Euglena*, and higher plants. This informational content of chloroplast DNA, 1–2 x 10^8, is close to that of bacteriophage T4, which is large enough to contain a few hundred genes.

The first derivatives of the melting curves of both the native and renatured chloroplast DNA deviate substantially from a Gaussian distribution (Fig. 4a and b). At least two main components were revealed. These features are strikingly different from those shown by T4 DNA. It appears, therefore, that chloroplast DNA is formed by segments of quite different average base composition. That this compositional heterogeneity is intramolecular, but not intermolecular, even in sheared molecules, is suggested by the single and essentially symmetrical bands formed by our purified chloroplast DNA sample in a CsCl density gradient, despite the 8.5% G + C difference between the two main melting components estimated from Fig. 4a. A similar situation appears to exist in a respiratory-deficient, cytoplasmic "petite", mutant strain of yeast (20).

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