Physicochemical Characterization of Mitochondrial DNA from Pea Leaves

(circular conformation/molecular size/renaturation kinetics)

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ABSTRACT

The mitochondrial (mt-) DNA from pea leaves exists in a circular conformation. 25% of the circular molecules exist as supercoils, and 10% of the molecules are dimers. The molecular weight of mitochondrial DNA is about 66 to 70 X 10^6 by electron microscopy, and 74 X 10^6 from its renaturation kinetics. No evidence for inter- and intramolecular heterogeneity is found.

The mitochondrial (mt) DNA from animal tissues exists in the form of closed circular duplex molecules of 10 X 10^6 molecular weight (1). Luck and Reich (2, 3) reported the isolation of linear molecules of molecular weight 13 X 10^6 from Neurospora mitochondria, and calculated that the molecular weight of mt-DNA might be about 66 X 10^6 from its renaturation kinetics. Recent studies (4) have shown that mt-DNA from Neurospora can be obtained in a linear structure 26 μm in length. Similarly, mt-DNA from slime mold Physarium has been isolated in linear form, with molecular weights ranging from 20 to 30 X 10^6 (5). Extensive studies in osmotically shocked preparations of yeast have demonstrated the existence of mt-DNA in circular form (6). However, all attempts to isolate these circular molecules were unsuccessful. The molecular weight of yeast mt-DNA was calculated to be 50 X 10^6 (6). Mt-DNA from protosoz, Tetrahymena, and Paramecium, are linear structures of 17.6 μm in length (7). Mt-DNA from higher plants has always been isolated in linear molecules with mean lengths ranging from 10 to 20 μm (7, 8). We have studied mt-DNA from pea leaves; it has a molecular weight of 74 X 10^6 from its renaturation rate. The DNA released from osmotically shocked mitochondria, as well as that isolated by deproteinization, has a circular form with an average contour length of 30 μm under experimental conditions in which replicative form (RF) II DNA from bacteriophage φ-X174 has a length of 1.45 μm. This is the largest circular mt-DNA isolated to date.

MATERIALS AND METHODS

Isolation of Mitochondrial DNA. 12- to 15-day-old pea leaves were homogenized with 4 volumes of buffer containing 0.3 M mannitol-0.05 M Tris·HCl (pH 8.0)-3 mM EDTA-0.1% bovine-serum albumin-1 mM 2-mercaptoethanol. Homogenates were filtered through cheese cloth and centrifuged at 1020 X g for 15 min; the supernatant was then centrifuged at 12,000 X g for 20 min. The mitochondrial fraction (contaminated with broken nuclei and chloroplasts) was suspended in the buffer (200 ml/kg of leaves) and centrifuged twice at 1020 X g for 15 min; the final mitochondrial pellet was obtained after centrifugation at 12,000 X g for 20 min. This pellet was suspended in the buffer (40 ml/kg of leaves) and treated with 50 μg/ml of DNase for 1 hr at 4°. All subsequent operations to obtain DNA free from RNA and protein were those reported elsewhere (9).

Isolation of Mitochondrial DNA for Electron Microscopy. 100 g of leaves were chopped with razor blades in 250 ml of buffer as reported (10). The mitochondrial pellet obtained as described above was suspended in 10 ml of buffer, treated with DNase, and washed three times by suspension in 3 volumes of washing medium containing 0.35 M sucrose and 0.1 M EDTA (pH 8.0). The final pellet was taken up in 1.0 ml of washing medium. For treatment of osmotic shock 0.02 ml of this suspension was added to 1.0 ml of 4 M ammonium acetate-0.1 M EDTA (pH 8.0), and after 15 min the solution was spread on a hypophase of ice-cold glass-distilled water. After 16 min the monolayer was picked up on carbon-Formvar coated mesh (200) grids and prepared as described (9). For isolations of mt-DNA, 1.0 ml of 0.4% sodium dodecyl sulfate in 20 mM EDTA and 30 NaCl was added to 1.0 ml of the mitochondrial fraction obtained after DNase treatment as described above. The mixture was allowed to stand at 25° for 30 min; then 0.1 ml of Pronase (25 mg/ml) was added, and the mixture was incubated at 37° for 2 hr. Further deproteinization was done with chloroform-isomayl alcohol 95:5. The aqueous phase was dialysed overnight against a solution of 0.15 M NaCl-20 mM EDTA (pH 8.0). The isolated DNA was prepared for electron microscopy as described (9).

Electron Microscopy. Preparations were examined at a magnification of ×8250, and tracings were made at a final magnification of ×250,000. A Zeiss EM9A with a 30-μm objective aperture was used and calibrated with a diffraction grating (Ladd 28,200 lines/inch).

RESULTS

Purity of mitochondrial DNA

The DNA isolated from crude mitochondrial fractions without DNase treatment showed the OsCl banding pattern presented in Fig. 1A. There are two kinds of DNA, one banding at a density of 1.698 g/cm^3 and the other at a density of 1.706 g/cm^3. DNA banding at the density of 1.698 g/cm^3 could be a mixture of nuclear and chloroplast DNA (10). The DNA obtained from mitochondria treated with DNase showed only one band at a density of 1.706 g/cm^3 (Fig. 1B). The localization of this DNA in the mitochondria of higher plants has been established in a recent review (10). The mt-DNA of pea leaves increased in density by 0.013 g/cm^3
after denaturation (Fig. 2C). Incubation of denatured DNA at 60° for 3 hr resulted in lowering the buoyant density by 0.011 g/cm³. Thus, the mt-DNA obtained here shows the characteristic properties of organelle DNA.

Homogeneity of mitochondrial DNA

Mt-DNA was thermally denatured in 0.15 M NaCl-0.015 M Na citrate. A melting profile of such an experiment is presented in Fig. 3. The mt-DNA melted with a sharp transition midpoint, with a Tm of 88 ± 0.5°, maximal hyperchromicity (hmax) of 0.34, and dispersion (σ 2/3) of 7°. The absorbance temperature profile of Fig. 3 does not show any intramolecular heterogeneity of the kind reported for *Chlamydomonas* chloroplast-DNA (11), which melted over a broad range of temperatures. Since there is more than one molecule of mt-DNA in each mitochondrion (unpublished results), a heterogeneous melting pattern of the isolated DNA would indicate that these molecules have different genetic information or that the base sequences in a given molecule are heterogeneous. The native mt-DNA gave a symmetrical peak in equilibrium centrifugation in CsCl (Fig. 1B), indicating that significant intermolecular heterogeneity does not exist. The melting pattern obtained further demonstrates that it is not possible to detect any inter- and intramolecular heterogeneity within the limits of the sensitivity of the method. We determined the Tm of mt-DNA in 0.01 NaCl-Na citrate in order to further investigate the possibility of heterogeneity. The mt-DNA showed a Tm of 68° but still melted sharply with a σ 2/3 of 7° and hmax of 0.32. On the other hand, nuclear DNA, which showed σ 2/3 of 7° NaCl-Na citrate, a σ 2/3 of 10° in 0.01 NaCl-Na citrate.

The mt-DNA was denatured and brought to 65°. The absorbance was continuously recorded and is presented in

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Birnstiel (12), where the rate-constant plot of lettuce mt-
DNA suggested considerable base-sequence heterogeneity. It
is difficult to explain these differences in results between
mt-DNA of peas and lettuce, since we have found no evidence
for such heterogeneity in mt-DNA isolated from beans,
lettuce, spinach, or tobacco (13).

The renaturation rate-constant of mt-DNA was calculated
from the slope of the curve by the method of Wetmur and
Davidson (14). The values for the renaturation rate constant
($k_2$) for five different preparations of mt-DNA ranged from
16.9 to 17.9 mol$^{-1}$ sec$^{-1}$ (Table 1). Under the same experi-
mental conditions, DNA from bacteriophage T4 gave values of $k_2$
ranging from 12.0 to 12.2 mol$^{-1}$ sec$^{-1}$. The molecular
weight of mt-DNA was calculated from the analyti-
cally determined values for the molecular weight of T4 DNA
of 130 $\times$ 10$^6$ and 106 $\times$ 10$^6$ (15) as a standard. The mean molecular
weight of mt-DNA was calculated to be 90.5 $\times$ 10$^6$ and
73.8 $\times$ 10$^6$, respectively, using the two different values for T4
DNA. A molecular weight of 106 $\times$ 10$^6$ for T4 DNA is more
accurate (15) and is also correct when calculated against pea
chloroplast-DNA as a standard (9). Thus, the molecular
weight of mt-DNA from pea leaves was 73.8 $\times$ 10$^6$ by re-
naturation kinetics.

The second-order rate-constants calculated for mt-DNA
were independent of the initial reactant concentration (Table
2). The renaturation rate-constants were dependent on the
molecular weight of DNA and exhibited the square root
relationship between the second-order rate-constant and the
molecular weight (Table 3).

Electron microscopy of mitochondrial DNA

Osmotically shocked mitochondria were prepared as described
in Methods and examined in the electron microscope. The

**TABLE 1. Molecular weight of mitochondrial DNA**

<table>
<thead>
<tr>
<th>Preparation no.</th>
<th>Renaturation rate-constant (mol$^{-1}$ sec$^{-1}$)</th>
<th>Mol. wt. of mt-DNA (10$^6$) calculated from T4 DNA assuming mol. wt. of</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T4 DNA</td>
<td>mt-DNA</td>
<td>130 $\times$ 10$^6$</td>
</tr>
<tr>
<td>1</td>
<td>12.2</td>
<td>17.8</td>
<td>89.0</td>
</tr>
<tr>
<td>2</td>
<td>12.1</td>
<td>17.4</td>
<td>90.4</td>
</tr>
<tr>
<td>3</td>
<td>12.1</td>
<td>17.1</td>
<td>92.0</td>
</tr>
<tr>
<td>4</td>
<td>12.2</td>
<td>16.9</td>
<td>93.8</td>
</tr>
<tr>
<td>5</td>
<td>12.0</td>
<td>17.9</td>
<td>97.2</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td></td>
<td>90.48</td>
</tr>
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</table>

**TABLE 2. Renaturation rates at different concentrations of mitochondrial DNA**

<table>
<thead>
<tr>
<th>Concentration of mt-DNA (µg/ml)</th>
<th>Rate-constant $k_2$</th>
<th>Mol. wt. of mt-DNA (10$^6$) calculated from T4 DNA assuming mol. wt. of</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>130 $\times$ 10$^6$</td>
<td>106 $\times$ 10$^6$</td>
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<tr>
<td>10</td>
<td>18.1</td>
<td>86.0</td>
<td>70.3</td>
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<tr>
<td>15</td>
<td>17.2</td>
<td>90.6</td>
<td>74.0</td>
</tr>
<tr>
<td>20</td>
<td>16.6</td>
<td>93.4</td>
<td>76.6</td>
</tr>
<tr>
<td>25</td>
<td>16.3</td>
<td>95.7</td>
<td>78.0</td>
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<tr>
<td>30</td>
<td>17.7</td>
<td>88.1</td>
<td>71.8</td>
</tr>
<tr>
<td>40</td>
<td>18.6</td>
<td>83.8</td>
<td>68.4</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>89.6</td>
<td>73.2</td>
</tr>
</tbody>
</table>

**TABLE 3. Renaturation rate-constants of mitochondrial DNA at different molecular weights**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Molecular weight* $\times 10^6$</th>
<th>Rate-constant $k_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>1.14</td>
</tr>
<tr>
<td></td>
<td>(obtained by sonication of mt-DNA)</td>
<td>12.7</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>3.37</td>
</tr>
<tr>
<td></td>
<td>(obtained by passage of DNA through No. 27 gauge needle)</td>
<td>17.4</td>
</tr>
<tr>
<td>3</td>
<td>26</td>
<td>13.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30.0</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>24.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39.9</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>35.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45.8</td>
</tr>
</tbody>
</table>

* Calculated by Studier’s method (16).

preparation contained circular forms of DNA. The frequency
distribution of the lengths of DNA molecules is presented in
Fig. 5. 56% of the DNA released was in the circular confor-
mation of monomer length 30.3 µm (SD ± 1.0 µm). 12% of
the DNA was in the form of circles, ranging in contour lengths
from 3 to 5 µm. 10% of the circular molecules were present in
dimer-length circles. The remaining DNA was linear, and the
molecules ranged in length from 2 to 27 µm. The circular
molecules found in these preparations are of mitochondrial
origin because no such molecules were seen in the nuclear or
chloroplast fractions. Furthermore, chloroplast-DNA exists
in circular molecules of 39 µm in length (9). The deproteinin-
ed mt-DNA was prepared as described in Methods. 25% of the
isolated mt-DNA was in the form of circles of contour length
29.9 µm (SD ± 1.0 µm). The supercoiled molecules constituted
30% of the circular molecules. A typical picture of an open
circle is presented in Fig. 6.

The necessity of including an internal standard when
making molecular weight determinations from contour-length
measurements of DNA molecules has been discussed (9).
Therefore, φX174 RF II DNA was spread along with isolated

**Fig. 5. Frequency distribution of the DNA released from
osmotically shocked mitochondria. The values expressed are the
percentage of the total DNA lengths measured, which was 800
µm. Three of the molecules shown are of dimer lengths. Only one
of these could be distinguished as a circular dimer. The others are
either catenated or tangled circles.**
Mitochondrial DNA in Pea Leaves

DNA (Fig. 7). In 20 molecules measured, contour lengths of φX174 RF II DNA ranged from 1.30 to 1.45 μm (SD ± 0.08 μm). Sixteen molecules of mt-DNA ranged from 28.4 to 31.8 μm, with an average contour length of 29.9 μm (SD ± 1.0 μm). The ratio of lengths between mt-DNA and φX174 RF II DNA is 20.6. From this ratio, and assuming a molecular weight of 3.2 × 10^6 for φX174 RF II DNA (Strider and Warner, personal communication), the molecular weight of mt-DNA is 66 × 10^6.

DISCUSSION

The isolation of mt-DNA from sources other than animal tissues has always resulted in linear molecules (1-8). In *Euglena gracilis*, the longest linear molecule found was 19 μm, even though circular molecules of 40 μm could be isolated from chloroplasts (17). Recent studies in pea mitochondria by Mikulska et al. (18) showed that only linear molecules of DNA were present in the lysed mitochondria, and the size of mt-DNA in the isolated DNA was only 10 μm. Our success in isolating intact mt-DNA in circular forms of contour lengths of 30 μm depends on gentle conditions of lysis. These circular molecules could not be due to non-DNA links, since such molecules amounted to 25% of the DNA isolated after extraction with detergent, Pronase, and chloroform–isoamyl alcohol. Wong and Wildman (19) have reported the presence of circular molecules with contour lengths of 2 μm in the

![Electron micrograph of a typical DNA molecule isolated from mitochondria. The contour length of the molecule is 28.6 μm. Bar length equals 1 μm.](image)
mitochondrial fraction of tobacco leaves. We have observed such molecules in our preparations (Fig. 5), but these constituted an insignificant amount of the total DNA. Small molecules are also present in yeast mitochondria (6), but their significance has yet to be understood.

The molecular weight of mt-DNA from pea leaves has been calculated to be $70 \times 10^6$ and $66 \times 10^6$, assuming corresponding values of $3.4 \times 10^6$ and $3.2 \times 10^6$ for φX174 RF II DNA (9). The calculation of the molecular weight of mt-DNA from renaturation rates yields a value of $73.8 \times 10^6$. Thus, the mean value for the molecular weight of pea mt-DNA is $70 \times 10^6$.

The banding in CsCl density gradients and the melting characteristics of mt-DNA have failed to show any intramolecular heterogeneity in the base compositions of different molecules. Also, there is no evidence of repeating sequences in the mt-DNA. The excellent agreement observed between the molecular weight of $70 \times 10^6$ obtained by electron microscopy and $73.8 \times 10^6$ obtained by kinetics analysis precludes the presence of mt-DNA molecules containing different base sequences.

Mt-DNA of pea leaves of molecular weight $70 \times 10^6$ is the largest DNA molecule reported in mitochondria. The question arises whether mt-DNA from sources other than animal mitochondria are of similar complexity or differ in their molecular sizes. Our experiments with spinach, lettuce, and beans have shown that the molecular weight of mt-DNA in these plants is also about $70 \times 10^6$ (13).

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