Role of methylation in the modification and restriction of chloroplast DNA in *Chlamydomonas*

(genetics/maternal inheritance/5-methylcytosine)

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Contributed by Ruth Sager, December 29, 1978

ABSTRACT The different metabolic paths followed by homologous chloroplast DNAs of maternal and paternal origins in zygotes of *Chlamydomonas* were examined by prelabeling parental cells, before mating them, with [*H*]adenine, [*H*]thymidine, and [*H*]deoxythymidine. Within 6 hr after mating, maternal chloroplast DNA was extensively methylated to 5-methylcytosine and its buoyant density decreased. Paternal chloroplast DNA was largely degraded. Some radioactivity from deoxythymidine of maternal origin reappeared in thymine, and residual paternal DNA contained radioactivity in a base tentatively identified as uracil. These results confirm and extend our previous findings and support our hypothesis that modification (methylation) and restriction enzymes determine maternal inheritance of chloroplast DNA and that the two parental DNAs have different metabolic fates within the zygote.

In 1972, it was postulated (1) that a modification-restriction mechanism analogous to that responsible for host-range restriction of bacteriophage (2) was the molecular basis of maternal inheritance of chloroplast genes in *Chlamydomonas*. This hypothesis was based upon the finding that chloroplast DNAs of male and female origins, indistinguishable in vegetative cells, follow different paths in the zygotes that form after gametic fusion (fertilization). Chloroplast DNA from the female parent persists in the zygote but is shifted to a lighter buoyant density, whereas that from the male is lost soon after zygote formation (1). Thus, the density shift could result from enzymatic modification of chloroplast DNA of female origin; and the loss of chloroplast DNA of male origin could result from attack by a restriction enzyme upon unmodified DNA molecules.

The modification-restriction hypothesis was further supported by studies in which maternal transmission of chloroplast genes was converted to a biparental or paternal pattern by UV-irradiation of female gametes before mating (3), by various drug pretreatments (4), or by a mutant nuclear gene, mat-1 (5). Complementing the genetic studies, in studies of radioisotope prelabeled chloroplast DNAs from zygotes after UV-irradiation and crosses with mat-1 it was found (6, 7) that chloroplast DNA of male origin was preserved in parallel with the genetic markers. These experiments demonstrate that the molecular pattern of inheritance of chloroplast DNA parallels that of chloroplast genes in *Chlamydomonas* (reviewed in refs. 8 and 9).

In this paper, we report that the buoyant density decrease of zygotic chloroplast DNA occurs in concert with the extensive methylation of cytosine residues to form 5-methylcytosine and that a low level of methylation is already present in the gametic chloroplast DNA of female origin and is greatly increased in zygotes in which the genetic pattern of transmission is maternal. These results provide definite support for the modification-restriction mechanism of non-Mendelian inheritance of chloroplast genes in *Chlamydomonas*.

MATERIALS AND METHODS

Cells and Media. The strains used—the wild-type 21gr (maternal), mating type plus (mt*), and the streptomycin-resistant 5177D (paternal), mating type minus (mt—)—have been described (10). They were grown in minimal medium (10) bubbled with 5% CO₂. For radioisotope labeling experiments, cultures were grown for six or seven doublings in the presence of the labeling compound to a density of 5 x 10⁶ cells per ml. Radioisotope was added to 40 ml of culture medium: 2mCi (1 Ci = 3.7 x 10¹² becquerels) of [*H*]deoxythymidine; 500 µCi of [*H*]thymidine; 250 µCi of [¹⁴C]thymidine; 1 mCi of [*⁵⁷C]adenine. For gamete induction and zygote formation, cells were washed once in nitrogen-free minimal medium diluted 1:5 at pH 8 and resuspended in this medium at 5 x 10⁶ cells per ml. Cells doubled twice in 24 hr, producing about 2 x 10⁹ gametes per ml. Equal numbers of gametes of the two mating types were mixed; mating was monitored by phase-contrast microscopy and by plating. Zygote formation reached >90% within 2–3 hr. The yield of viable zygotes in each experiment and the percentage of maternal and exceptional (i.e., biparental and paternal) zygotes was determined by plating at dilutions on agar and replica plating to streptomycin agar as described (10). The frequency of exceptional zygotes averaged 0.5% ± 0.26% in 10 experiments and the yield of viable zygotes was >90%.

DNA Extraction and Fractionation. Isotopically labeled gametes and zygotes were harvested by centrifugation and washed once in NET buffer (0.15 M NaCl/0.1 M Na₂EDTA/50 mM Tris-HCl, pH 8) and resuspended in NET at 5 x 10⁶ cells per ml. The cells were then mixed with 10⁹ cells of unlabeled 21gr vegetative cells at the same cell density and 90% broken in a French pressure cell [1200 psi (8.27 mPa) for gametes and T₀ zygotes, 3000 psi for 6-hr zygotes, and 4000 psi for 24-hr zygotes]. Each sample was lysed with 2% sodium dodecyl sulfate plus 2% Sarkosyl at 50°C for 10 min and then frozen at −20°C. Lysates were thawed, diluted 1:1 with NET, and digested with Pronase (200 µg/ml) added three times at 1-hr intervals at 37°C and further incubated for 18 hr at 50°C.

Lysates were then extracted twice with equal volumes of distilled phenol saturated with 0.1 M Tris-HCl (pH 8) and once with an equal volume of chloroform/isoamyl alcohol, 24:1 (vol/vol). Remaining CIA was removed by ether extraction. Nucleic acids were precipitated with 2 vol of cold absolute ethanol at −20°C for 30 min. The precipitate was pelleted, dissolved in 15 ml of TE buffer (10 mM Tris/0.1 mM EDTA, pH 8), and dialyzed for 2 hr against TE. Samples were dialyzed

Abbreviation: HPLC, high-pressure liquid chromatography.

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against 0.15 M NaCl/0.015 M Na citrate, pH 7, at 37°C and treated with RNase T1 (20 units/ml) and pancreatic RNase (200 μg/ml) for 18 hr. Samples were extracted with phenol as before until the interface was clear. The amount of DNA was then determined spectrophotometrically. One milligram of DNA in TE buffer was mixed with CsCl to give a density of 1.706 g/ml. Gradients were run in a vertical Ti 65 rotor at 40,000 rpm for 18 hr at 20°C and fractionated to separate chloroplast and nuclear DNAs; appropriate fractions were pooled and rerun. Chloroplast DNA fractions from the second CsCl gradient were pooled on the basis of absorbance profiles and isotope incorporation and dialyzed against TE buffer to remove CsCl.

Hydrolysis and Chromatographic Separation of Bases. DNA samples from the second CsCl gradient were extracted with chloroform/isomyl alcohol, ethanol precipitated, redissolved in 200 μl of 98% anhydrous formic acid, and heated to 180°C in sealed pasteur pipettes for 25 min. The hydrolysates were cooled to room temperature, dried, redissolved in 100 μl of elution buffer (20 mM ammonium carbonate, pH 9.95), and heated to 68°C for 5 min. The bases were identified by the method of Singhal (11, 12). Each total hydrolysate was applied to a high-pressure liquid chromatography (HPLC) Aminex A-6 column operated at a flow rate of 0.25 ml/min. The eluate was monitored by an UV optical unit at 254 nm. Fractions (0.25 ml) were collected, 10 ml of Aquasol (New England Nuclear) was added to each vial, and the radioactivity was determined in a LS-9000 Beckman scintillation counter. The elution positions of the bases were determined with a set of standards: cytosine and 5-methylcytosine from Sigma; adenine, guanine, thymine, and uracil from Calbiochem. Recovery of bases was monitored with DNAs of known base composition: λ DNA, maize nuclear DNA containing 5-methylcytosine (gift of L. Bogorad), and Chlamydomonas nuclear and chloroplast DNAs. [\(^{14}C\)]Adenine, [\(^{3}H\)]adenine, [\(^{3}H\)]thymidine, [5-\(^{3}H\)]deoxycytidine, and [G-\(^{3}H\)]deoxycytidine were from New England Nuclear.

RESULTS

Bouyant Density Shift. In 1972 it was reported (1) that chloroplast DNA extracted from zygotes as early as 6 hr after zygote formation has undergone a bouyant density shift to a value approximately 5 mg/cm³ lighter than that of homologous DNAs from vegetative cells and from gametes of both mating types. We confirmed this observation in the experiments shown in Fig. 1, in which the chloroplast DNA of each parent was prelabeled by growth in medium containing [\(^{3}H\)]thymidine. Each parent was then mated to unlabeled cells of the opposite mating type in a pair of reciprocal crosses, and chloroplast DNA was extracted from zygotes immediately after mating (T0) and after 6 and 24 hr. The data are from a second preparative CsCl gradient containing chloroplast DNA pooled from the first gradient in which a small amount of nuclear DNA was present. By utilizing the absorbance peaks of nuclear and chloroplast DNAs as reference points and assuming a linear gradient, we have estimated a bouyant density decrease of about 6–10 mg/cm³ in the triturated chloroplast DNA of maternal origin. The homologous DNA from the paternal parent was largely degraded within 6 hr after zygote formation.

Presence of 5-Methylcytosine in Zygote Chloroplast DNA. To account for the density shift, experiments were designed to look for altered bases in zygote chloroplast DNAs. Purified DNA preparations were hydrolyzed with formic acid and the bases were separated by HPLC. A typical profile based on the absorbances of bases recovered after formic acid hydrolysis of chloroplast DNA is shown in Fig. 2, together with an absorbance profile of a mixture of authentic bases run under the same conditions. The molar ratios of bases computed from the absorbance profiles of chloroplast DNAs corresponded well with published values. The identities of the bases were further confirmed in experiments in which cells were prelabeled with [\(^{3}H\)]adenine, [\(^{3}H\)]thymidine, or [\(^{3}H\)]deoxycytidine.

Results are given in Fig. 3 and Table 1 of two experiments in which parental cells were prelabeled with [\(^{3}H\)]deoxycytidine and reciprocal crosses were made between labeled and unlabeled cells. Chloroplast DNAs were prepared from vegetative cells, from gametes, and from zygotes recovered 6 and 24 hr after cell fusion. The DNAs were hydrolyzed with formic acid and the resulting bases were separated by HPLC on Aminex A-6. When the female parent (21gr) was labeled, a definite peak in the position of 5-methylcytosine was seen in both the 6- and 24-hr zygote preparations (Fig. 3 upper), amounting to 22 and 27%, respectively, of the total radioactivity (Table 1). In contrast, when the male parent (5177D) was prelabeled (Fig. 3 lower), only a low level of incorporation was seen in the 5-methylcytosine position. This dramatic difference in labeling pattern represents definitive evidence that chloroplast DNA of maternal origin is selectively methylated during the first 6 hr of zygote formation. No labeling was detected in the 5-
methylcytosine peaks of chloroplast DNA from vegetative cells of either parent, but a small peak of radioactivity was found in the maternal gamete DNA. This 5-methylcytosine peak was also seen in large-scale preparations of unlabeled gamete DNA.

Experiments similar to those described above in Fig. 3 and Table 1 were carried out with unlabeled cells and with cells prelabeled either with [3H]thymidine (reciprocal crosses of labeled × unlabeled cells) or with adenine (one parent labeled with [3H]adenine, the other with [14C]adenine). In the experiments with unlabeled cells, performed without carrier DNA, the amount of 5-methylcytosine present in 6- and 24-hr zygotes was estimated as representing 4–8% of the total absorbance (estimated from absorbance tracings). In the experiments with prelabeled cells, label appeared only in thymidine in the thymidine labeling experiment (Fig. 1) and only in adenine and guanine in the adenine labeling experiment (data not shown).

Nuclear DNAs were examined in the experiments using unlabeled cells and those using cells labeled with [3H]adenine and [14C]adenine. No peaks were seen in the position of 5-methylcytosine or uracil. Nuclear DNAs were unlabeled in the experiments with [3H]thymidine because the thymidine kinase responsible for phosphorylation is a chloroplast enzyme (13).

Different Metabolic Paths of Maternal and Paternal Chloroplast DNAs. In addition to these anticipated findings, additional peaks of radioactivity were observed in other positions. When the maternal parent was prelabeled (Fig. 3 upper) the percentage of total label in cytosine decreased substantially in the interval between 6 and 24 hr after mating (Table 1),

Table 1. Percentage distribution of radioactivity from [G-3H]deoxyctydine–prelabeled cells present in chloroplast DNAs

<table>
<thead>
<tr>
<th>Distribution, %</th>
<th>Total incorporation, °</th>
<th>Maternal parent prelabeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Vegetative cells (21gr)</td>
<td>38 8 54 —</td>
<td>9,861</td>
</tr>
<tr>
<td>Gametes</td>
<td>22 13 63 2</td>
<td>17,049</td>
</tr>
<tr>
<td>Zygotes at 6 hr</td>
<td>4 21 53 22</td>
<td>6,120</td>
</tr>
<tr>
<td>Zygotes at 24 hr</td>
<td>13 42 18 27</td>
<td>5,254</td>
</tr>
<tr>
<td>Paternal parent prelabeled†</td>
<td>12 9 80 —</td>
<td>7,764</td>
</tr>
<tr>
<td>Vegetative cells (5177D)</td>
<td>6 10 83 —</td>
<td>11,755</td>
</tr>
<tr>
<td>Gametes</td>
<td>33 10 53 4</td>
<td>3,015</td>
</tr>
<tr>
<td>Zygotes at 24 hr</td>
<td>43 6 45 5</td>
<td>3,421</td>
</tr>
</tbody>
</table>

Vegetative cells were grown with 1 mCi of [3H]deoxyctydine per 40-ml culture; other cells were grown with 2 mCi/40-ml culture. U, uracil; T, thymine; C, cytosine; 5MeC, 5-methylcytosine.

† Excluding void volume.

In this experiment, a peak between U and T contained 4% of the counts in the 6- and 24-hr preparations.
much of it reappearing in thymine. When the paternal cells were prelabeled (Fig. 3 lower), the relative incorporation into thymine did not change but the radioactivity lost from cytosine reappeared in the position of uracil, mostly within the first 6 hr after mating.

As noted in Table 1, losses of total radioactivity occurred in all zygote preparations compared with the gametic aliquots, in part owing to the difficulty in cracking the zygote wall to release DNA. In addition, a differentially greater loss was seen of paternal than of maternal label. This loss was less than that seen with [3H]thymidine label (Fig. 1), suggesting that some reincorporation from paternal into maternal DNA occurs with deoxycytidine but not with thymidine.

This loss and redistribution of label provides further evidence of the breakdown of the chloroplast DNA of male origin and suggests that some turnover is occurring in the chloroplast DNA of female origin. Thus, the increase in radioactivity in the positions of thymine and uracil must reflect previously undetected processes of degradation and resynthesis in which the precursor pools available within chloroplasts of paternal and maternal origins are different. If confirmed, the presence of uracil is an interesting clue that merits further investigation.

**DISCUSSION**

This paper describes the different metabolic paths that chloroplast DNAs of maternal and paternal origin follow in zygotes of *Chlamydomonas* during the first 6 hr after mating and during the subsequent 18 hr. As previously described, pairs of gametes of opposite mating type fuse to form zygotes in which the chloroplasts remain unfused in separate compartments within a common cytoplasm for some 5–6 hr (14). We now report the different fates of homologousDNAs facilitated by this temporary compartmentalization. During the first 6 hr, the chloroplast DNA of maternal origin is methylated to the extent of 4–8% (molar basis) of 5-methylcytosine, and a concomitant decrease is seen in buoyant density of this DNA. In the same time interval, the homologous DNA of paternal origin is degraded as described (1, 3, 9) and as further documented here, especially with thymidine labeling. In addition, the maternal chloroplast DNA undergoes further changes, mainly between 6 and 24 hr, suggesting some new synthesis or repair.

These new findings provide strong evidence in support of the hypothesis formulated earlier (1, 3, 15) that the maternal inheritance of chloroplast DNA in *Chlamydomonas* is determined at the molecular level by the action of DNA modification (methylation) and restriction enzymes, leading to modification (i.e., methylation) of the maternal DNA and destruction of the homologous paternal DNA. Additional support for this hypothesis comes from the isolation of a site-specific endonuclease (16) and of a methyl transferase (17) from *Chlamydomonas*.

A general role for modification-restriction systems in the regulation of gene expression in eukaryotes has been proposed (15), in part because of similarities between the chloroplast genetics of *Chlamydomonas* and of *Pelargonium* (18, 19) and in part because of parallels between these organelle systems and the differential chromosomal loss and inactivation seen in insects and in mammals (15). A role for methylation of DNA in eukaryotic gene regulation has also been proposed by Scarano (20), by Holliday and Pugh (21), by Comings (22), and by Riggs (23).

Our identification of 5-methylcytosine is based on three lines of evidence. (i) The peak position in the elution profiles (fraction 47 in Figs. 2 and 3) coincides with that of the authentic base. (ii) This position is labeled when [C-3H]deoxycytidine but not when [3H]thymidine or [3H]adenine is incorporated into DNA. (iii) This position is not labeled when [5-3H]deoxycytidine is used as prelabel. (Both the 4 and 5 positions of the pyrimidine ring are tritiated in [G-3H]deoxycytidine and consequently the base remains radioactive after the H in the 5 position has been replaced by an unlabeled methyl group.)

The decrease in buoyant density in zygotic chloroplast DNA of maternal origin (Fig. 1) of approximately 6–10 mg/cm² can be accounted for (24) by the extent of methylation we have estimated from the absorbance profiles of large-scale nonradioisotope-labeled preparations: 4–5% (molar basis) of the total chloroplast DNA.

The results of reciprocal crosses between [3H]thymidine-labeled and unlabeled gametes are important also in unambiguously demonstrating the degradation of chloroplast DNA of paternal origin. When adenine is used to prelabel chloroplast DNA, a significant precursor pool from the breakdown of ribosomal RNAs in gametes (25) becomes available for incorporation into newly synthesized DNA and confuses the identification of paternal DNA. This pool problem led to Chiang's erroneous conclusion that both parental chloroplast DNAs are physically conserved in zygotes (26). Previous experiments using either [15N]adenine (1) or [3H]adenine (7) as prelabel established the loss of paternal DNA in 6- and 24-hr zygotes, but the results were less definitive than those shown here with [3H]thymidine.

In addition to methylation and degradation, other differences were found in the metabolic fates of chloroplast DNAs of maternal and paternal origins. When [3H]deoxycytidine was used as a prelabel of the maternal DNA, a large increase was seen in the thymine peak, especially at 24 hr. This result suggests that, in the young zygotes, some synthesis or repair of chloroplast DNA of maternal origin is occurring, in which free deoxycytidine is converted to thymidine triphosphate and incorporated into DNA.

No comparable increase in the thymine peak is seen in Fig. 3 lower, in which the paternal parent had been prelabeled. On the contrary, the poor recovery of total radioactivity indicated loss of paternal chloroplast DNA, but not as fully as with thymidine. Thus, some recycling of deoxycytidine may occur, leading to some incorporation of this paternal label into maternal DNA. A similar explanation probably accounts also for the small 5-methylcytosine peak in Fig. 3 lower.

In the residual paternal chloroplast DNA (Fig. 3 lower), there was a large incorporation into a peak tentatively identified as uracil from its position in the elution profile (Fig. 2). If no new synthesis of paternal DNA is occurring, as shown by the thymidine incorporation experiment (Fig. 1), then the only source of uracil in this residual DNA must be deamination of cytosine residues on the macromolecule. To our knowledge, no enzyme with this activity has been described. We may speculate that the accumulation of uracil is a signal to DNA degradation. Enzymes that remove uracil and repair DNA have been described (27). Perhaps *Chlamydomonas* contains a restriction enzyme that degrades the DNA from which uracil has been removed.

The findings reported in this paper have a direct bearing on genetic data proposed to estimate the number of copies of maternal and paternal chloroplast DNAs present in zygotes (28, 29). In particular, Wurtz et al. (29) reported that pretreatment of the maternal parent with FdUrd decreases the amount of chloroplast DNA and leads to the retention of paternal chloroplast genes in zygotes. They proposed that these results rule out our modification-restriction mechanism because "the normal, maternal pattern of inheritance of chloroplast genes should not be altered by FdUrd treatment" (29).

It has been found (5) that low-dose UV-irradiation of ma-
ternal gametes just before mating, followed by photoreactivation to repair damage to the DNA, prevented loss of the paternal chloroplast genes. Two effects of UV irradiation were postulated (5): the blocking of induction of DNA "processing" enzymes in the zygote at low doses comparable to phage induction; and direct inactivating effects on the irradiated chloroplast DNA at high doses. It is probable that, like UV radiation (30), FdUrd has additional effects on enzymes of DNA synthesis, processing, and degradation and thus that FdUrd interferes with some or all of the processes described in this paper.

The methylation, destruction, and complex enzymatic processing of zygotic chloroplast DNAs described in this paper are predicted by our hypothesis but not by others (26, 28, 29). We show here unequivocally that the DNAs of maternal and paternal origins undergo very different processing reactions in the zygote. The effects of FdUrd and UV pretreatments on these reactions can now be dissected directly at the molecular level.

This work was supported by National Institutes of Health Grant GM-22874.