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Molecular Basis of Maternal Inheritance

(cytoplasmic genes/chloroplast DNA/cesium chloride density gradients/^{15}N density label/*Chlamydomonas*)

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ABSTRACT **The mechanism of preferential transmission (i.e., maternal inheritance) of cytoplasmic genes was investigated with chloroplast DNA of *Chlamydomonas* as a model system. The behavior of nuclear and chloroplast DNAs were compared in the sexual cycle; DNAs from male and female parents were distinguished by labeling with ^{14}N- or $^{15}NH_4Cl$ and then by making the crosses: ^{14}N (female) × ^{15}N (male) and the reciprocal. Chloroplast DNAs from the two parents followed different paths in the zygote, but nuclear DNAs showed no differences. Chloroplast DNA from the female parent persists in the zygote, but undergoes a density shift of 0.003–0.005 g/cm^3 to a lighter buoyant density, whereas that from the male disappears soon after zygote formation. The possibility is discussed that a modification-restriction system may be involved.**

Many organisms have been shown to receive certain genetic traits primarily from one parent (1). The best-studied examples have been associated with the DNA and the phenotypes of mitochondria (2, 3) and chloroplasts (1, 4, 5), and have been given the general term cytoplasmic to distinguish them from nuclear genomes. Since all higher organisms contain one or both of these organelles, the importance of this phenomenon of unequal inheritance is evident. This behavior was initially described as maternal inheritance, because all or most genes were transmitted from the female parent, and as non-Mendelian, because the alleles did not segregate with Mendelian ratios among the progeny.

The molecular basis of this loss or preferential exclusion has not been established. In organisms in which one of the parental gametes contributes most of the cytoplasm at fertilization, such as higher plants, animals, and some fungi (e.g., *Neurospora*), it has been widely assumed (with no evidence at the DNA level) that the cytoplasmic genome of the male parent is excluded during formation of the male gametes (sperm, pollen, or conidiospore). Whether or not this *ad hoc* explanation is correct for other organisms, it clearly does not apply to the sexual green alga *Chlamydomonas*.

In *Chlamydomonas*, the pattern of transmission of these cytoplasmic genes in sexual crosses is predominantly maternal, although the female (mt^+) and male (mt^-) gemetes are of equal size, and contribute their entire contents when they fuse to form zygotes (1, 5). Thus, the occurrence of maternal inheritance in *Chlamydomonas* in the face of equal contributions of cytoplasm from both parents raises the question not only of the molecular basis of this phenomenon in this organism, but also of whether similar mechanisms might be operative in other organisms as well.

This paper presents results of studies designed to examine the mechanism of preferential transmission of cytoplasmic genes in *Chlamydomonas*. An extensive investigation led to the discovery of a genetic linkage group (6) or "chromosome" associated with the chloroplast, and probably located in chloroplast DNA (1). Therefore, we compared the behavior of nuclear and chloroplast DNAs from the two parents through the sexual life cycle, distinguishing the DNAs from male and female parents by labeling them with ^{14}N or ^{15}N, and then making crosses: ^{14}N (female) × ^{15}N (male) and the reciprocal. The results show that the chloroplast DNAs from the two parents follow different paths in zygotes, while nuclear DNAs show no such differences. The chloroplast DNA of the female parent persists, while that of the male parent disappears soon after zygote formation. Preliminary evidence implicates a modification-restriction system analogous to that described in bacteria (7).

MATERIAL AND METHODS

Strains used were 5177D, which carries nuclear marker *act-r* (cycloheximide-resistance) and cytoplasmic marker *sm2-r* (streptomycin-resistance) as male (mt^-) parent, and either the wild-type 21 gr (no markers) or strain 6308E with the nuclear marker *ms-r* (methioninesulfoximine-resistance) as female (mt^+) (1). Gametes were prepared by the method of Kates and Jones (8). Parental cultures were grown synchronously in liquid medium, harvested in the middle of the light cycle at population densities of about 4×10^6 cells per ml, collected, and resuspended in a dilute medium free of a nitrogen source (N-free) to permit differentiation of gametes (9). During gametogenesis, each vegetative cell divided to form four gametes. Populations of mt^+ and mt^- gametes, adjusted to equal numbers, were mixed under conditions that permitted over 90% zygote formation within 2 hr, then diluted and shaken gently in the dark to reduce clumping. Zygotes were harvested at appropriate times (see results) by centrifugation, washed to remove the residual unmated cells, centrifuged to a pellet, and stored frozen until use. Control platings were performed to check number and viability of gametes, yield of zygotes, extent of contamination of final zygote pellet by unmated cells, germination of zygotes, and percent of exceptions to the pattern of maternal inheritance (i.e., biparental and paternal zygotes).

Thawed zygotes were resuspended at 2×10^8/ml in 4% sodium dodecyl sulfate–0.15 M NaCl–0.10 M Na_3citrate–1 mM EDTA, pH 7.9, then frozen and thawed again. Sarkosyl, sodium deoxycholate, and Triton X-100 were added at final concentrations of 2%, 1%, and 4%, respectively, and the mixture was incubated at 60° for 2 hr. NaCl was added to 1 M, and the mixture was deproteinized by the Sevag procedure

(10). The supernate from this first extraction was kept separate (denoted *a*), while the interface (*b*) was again extracted in 1 M NaCl–0.015 M Na_3citrate by repeated enzyme treatments with ribonucleases (pancreatic, 100 μg/ml and T1, 50 U/ml) and α-amylase (100 μg/ml) at 37° for 30 min, followed by Pronase (1 mg/ml) at 37° for 4 hr (or 60° for 16 hr), and deproteinized. (Stock 50-times concentrated ribonuclease and Pronase solutions were heated at 80° for 10 min to destroy any contaminating DNase activity.) The supernates of both fractions were precipitated with ethanol, dissolved in 0.15 M NaCl–0.015 M Na_3citrate, treated with enzymes as above, and deproteinized at least twice. Yields of DNA from zygotes were 50–70% of the starting material, with losses primarily in initial penetration of zygote wall, and with about 25% recovered in the (*a*) fraction and 75% in the (*b*) fraction. Yields from gametes extracted by the same procedure were over 90%, with most of the DNA in the (*a*) fraction.

CsCl equilibrium gradients were made by the technique of Meselson, Stahl, and Vinograd (11); a 4-place rotor (An-F) was used at 44,000 rpm and 25°. Density and quantitation markers used were *Bacillus subtilis* phage 15 DNA, ρ = 1.761–2 g/cm³ (gift of Dr. Julius Marmur); crab poly(dAT), ρ = 1.680 g/cm³ (gift of Dr. Noboru Sueoka); and *Escherichia coli* DNA, ρ = 1.710 g/cm³ used to calibrate densities of the other markers. Films were traced with an Analytrol microdensitometer, and densities of nuclear and chloroplast DNAs were estimated from the positions of markers by the method of Schildkraut, Marmur, and Doty (12).

RESULTS

Our first clue to understanding the behavior of chloroplast DNA in the zygote came from a study of control crosses: $^{14}N \times {}^{14}N$. An example is shown in Fig. 1, in which the DNA extracted from 24-hr zygotes (Fig. 1*a*) is compared with DNA from one of the parental strains (Fig. 1*b*). The tracing in Fig. 1*b* shows a typical profile: nuclear DNA banding at 1.724 g/cm³ and chloroplast DNA at 1.694 g/cm³ (13, 14). Similar results have been obtained with DNAs from both mating types, extracted either from vegetative cells or from gametes, with the mean value for chloroplast DNA from all preparations being 1.695 ± 0.0005 g/cm³. Chloroplast DNA of *Chlamydomonas* was originally identified by extraction from isolated chloroplasts (13). Mitochondrial DNA has not been identified with certainty in this organism. The M band, described in zygote DNA by Sueoka *et al.* (14), was not seen in any of our preparations.

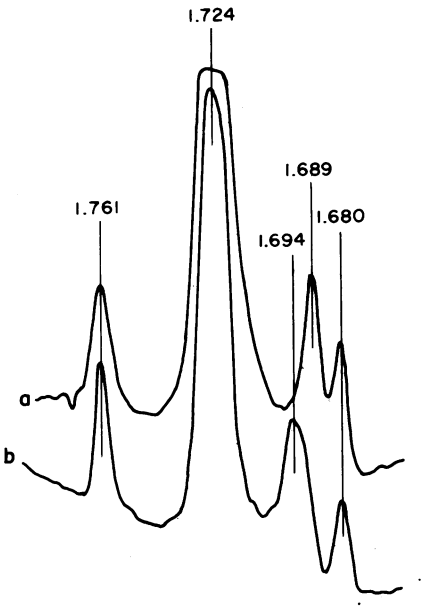

FIG. 1. Microdensitometer tracings of UV-absorption bands of DNA after CsCl density equilibrium centrifugation. (*a*) DNA from 24-hr zygotes, from $^{14}N \times {}^{14}N$ cross, fraction *a* (see *Methods*). (*b*) gamete DNA from one of the parents. *Outside peaks* are markers; SP-15 DNA at 1.761 g/cm³ and poly(dAT) at 1.680 g/cm³. Nuclear DNA (overloaded) at 1.724 g/cm³, chloroplast DNA at 1.694 g/cm³ in (*b*), and at 1.689 g/cm³ (shifted density) in (*a*). (44,000 rpm, 20 hr, 25°).

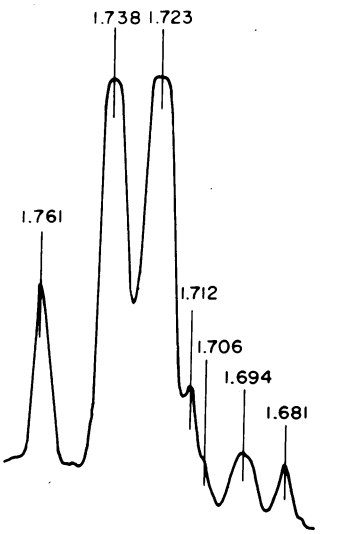

FIG. 2. CsCl density equilibrium centrifugation, as in Fig. 1. DNA extracted from a 1:1 mixture of gametes, one strain grown with ^{14}N- and the other with $^{15}NH_4Cl$. *Outside markers* are SP-15 DNA at 1.761 g/cm³ and poly(dAT) at 1.681 g/cm³. ^{15}N- and ^{14}N-labeled nuclear DNAs are at 1.738 and 1.723 g/cm³, respectively, and chloroplast DNAs are at 1.694 g/cm³ (^{14}N) and at 1.706 g/cm³ (^{15}N), seen as a shoulder on the peak at 1.712 g/cm³. (44,000 rpm, 20 hr, 25°). A different poly(dAT) preparation was used here).

The zygote DNA shown in Fig. 1*a* resembles that of the gamete preparation in Fig. 1*b*, except that the banding position of the chloroplast DNA is shifted from 1.694 to 1.689 g/cm³. In other preparations a similar shift was seen, the mean density being 1.690 ± 0.001 g/cm³. In a study of later times in zygote development (1, 15, and manuscript in preparation), it was found that the density shifted position was maintained until the first round of replication, which occurred several days later under our conditions.

Do the chloroplast DNAs from both male and female parents undergo this density shift? To answer this question, and to examine the behavior of chloroplast DNAs from the two parents individually, parental strains were grown with ^{14}N- and with $^{15}NH_4Cl$ to provide a density label in the DNA. DNAs were then prepared from zygotes produced in reciprocal crosses. (In the following discussion, zygotes from reciprocal crosses will be referred to as $^{14}N \times {}^{15}N$ and as $^{15}N \times {}^{14}N$, respectively, with the female parent cited first.)

The ease of identifying nuclear and chloroplast DNAs in these experiments is exemplified by the peaks seen in Fig. 2. The tracing shows the separation by centrifugation in a cesium chloride density gradient of DNA isolated from a 1:1 mixture

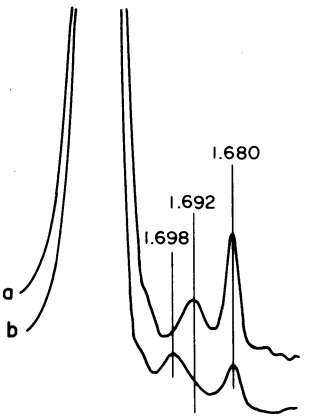

FIG. 3. CsCl density equilibrium centrifugation. (*a*) DNA extracted from ^{14}N x ^{15}N zygotes, 6 hr after mating. (*b*) DNA from ^{15}N x ^{14}N zygotes, extracted at 6 hr, as in (*a*). Nuclear peaks are overloaded; positions of single chloroplast peaks at 1.692 g/cm^3 in (*a*) and at 1.698 g/cm^3 in (*b*) were estimated from poly(dAT) at 1.680 g/cm^3. DNAs from extraction fraction *b*: dense DNAs in region of 1.770 g/cm^3 obscure heavy marker (see text). (44,000 rpm, 20 hr, 25°).

of gametes, one strain grown with ^{14}N- and the other strain with $^{15}NH_4Cl$ as sole nitrogen source. The major components seen at buoyant densities of 1.723 and 1.738 g/cm^3 correspond to the ^{14}N and ^{15}N nuclear DNAs, respectively, while the satellite components banding at 1.694 g/cm^3 and at about 1.706 g/cm^3 (here a shoulder on the ^{14}N-nuclear peak) correspond to the ^{14}N and ^{15}N chloroplast DNAs (13, 14). In gradients with DNA from ^{15}N-grown gametes, the density of the chloroplast DNA peak is 1.708 g/cm^3. The peak at 1.712 g/cm^3, usually seen as a shoulder in ^{14}N-DNA, has not been identified.

Zygote formation involves the complete fusion of two cells, (i.e., gametes), of opposite mating type, including fusion of the two chloroplasts. Since the gamete DNAs shown in Fig. 2 are incorporated into the zygote in the mating process, one might expect to see a similar pattern in the DNAs extracted from zygotes just after fusion. Since the density shift seen in Fig. 1*a* was found in 24-hr zygotes, we thought that it might not have occurred in 6 hr, the earliest time after mating at which zygotes can be conveniently harvested. The DNAs shown in Figs. 3 and 4 are from 6-hr zygotes, and it is apparent that the density shift has already occurred. As noted below, similar results were seen in the 24-hr samples.

The DNAs shown in Figs. 3 and 4 are very similar in the chloroplast region, although the preparations differ in at least two important ways. First, the DNAs of Fig. 3 are from the (*b*) fraction, while those of Fig. 4 are, like Fig. 1*a*, from the (*a*) fraction (see *Methods*). Secondly, the DNAs in Fig. 4 come from an experiment in which gametogenesis occurred in the presence of unlabeled adenine. In these preparations (to be reported in detail elsewhere), a new light peak at about 1.684 g/cm^3 appeared in the gametes and in the young zygotes, but was seen in zygote DNA only after Pronase extraction of the interface (i.e., in fraction *b*).

In all four tracings, only one peak is seen in the chloroplast region. In Figs. 3*a* and 4*a*, the peak is at 1.692 g/cm^3, lighter than that of the ^{14}N female parent, and no peak is seen in the region corresponding to that of the density-shifted ^{15}N chloroplast DNA, which is at 1.698 g/cm^3 in the reciprocal crosses (Figs. 3*b* and 4*b*). Similarly, in Figs. 3*b* and 4*b*, no peak is seen in the position expected from Figs. 1*a*, 3*a*, and 4*a* for a density-shifted ^{14}N chloroplast DNA from the male parent.

The results shown in Figs. 3 and 4 were confirmed in studies of zygotes sampled after 24 hr of maturation in the same experiments, in agreement with the ^{14}N x ^{14}N control (Fig. 1*a*) sampled at 24 hr. The mean values of the chloroplast DNA from eight samples (*a* and *b* fractions; 6 and 24 hr; two experiments) were 1.692 ± 0.001 for ^{14}N x ^{15}N, and 1.698 ± 0.0013 for the reciprocal cross. The mean value for chloroplast DNA from 24-hr ^{14}N x ^{14}N zygotes was 1.690 ± 0.001 g/cm^3. These results will be discussed below.

No changes were seen in the position of the nuclear peaks until late in zygote maturation, after at least one round of replication of chloroplast DNA. However, a nuclear peak of intermediate density (between 1.723 and 1.738 g/cm^3) is seen, as well as very dense material in the region around 1.770 g/cm^3. These results, to be presented elsewhere, are based on gradients run at low DNA concentration, whereas the gradients shown here were overloaded in order to visualize the chloroplast fraction.

DISCUSSION

The results presented in this paper demonstrate that chloroplast DNAs from male and female gametes of *Chlamydomonas* undergo different fates during the initial stages of zygote development. Two principal observations support this conclusion. (*i*) Only one DNA peak is regularly seen in the chloroplast region in DNA preparations from 6- and 24-hr zygotes produced in reciprocal ^{14}N x ^{15}N and ^{15}N x ^{14}N crosses. (*ii*) The density of this DNA resembles the density-shifted position of chloroplast DNA from the female parent, whereas the chloroplast DNA of the male parent is no longer seen.

The identification of the zygote DNAs that banded in the chloroplast region was clarified by ^{14}N x ^{14}N control crosses, which showed only one peak, not at the position of gamete DNA, but shifted about 0.005 g/cm^3 to the lighter density of 1.690 g/cm^3. In the ^{14}N x ^{15}N crosses, the single peak seen at 1.692 g/cm^3 is inferred to be the density-shifted ^{14}N DNA from the female parent. (Whether the mean values of 1.692 and 1.690 g/cm^3 are the same or different cannot be concluded from these data.) If chloroplast DNA from the male parent

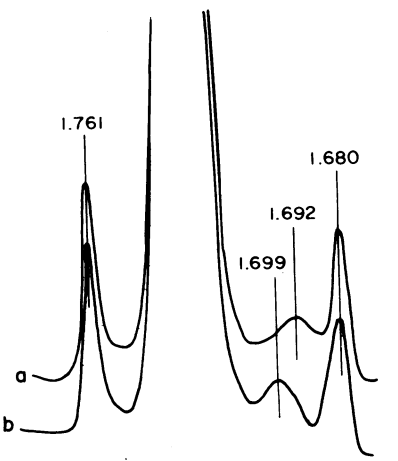

FIG. 4. CsCl density equilibrium centrifugation. (*a*) DNA extracted from ^{14}N x ^{15}N zygotes, 6 hr after mating. (*b*) DNA from ^{15}N x ^{14}N zygotes, extracted at 6 hr, as in (*a*). Nuclear peaks are overloaded; positions of single chloroplast peaks at 1.692 g/cm^3 in (*a*) and at 1.699 g/cm^3 in (*b*) are estimated from poly(dAT) at 1.680 g/cm^3. DNAs from extraction fraction *a*. (44,000 rpm, 20 hr, 25°).

were present at its unshifted density, it might be hidden under the nuclear peak, but the control cross $^{14}N \times {}^{14}N$ has shown that if DNA from the male were present, it too would be shifted (since only one peak was seen in the control crosses). Therefore, if chloroplast DNA from the male parent persisted in the $^{14}N \times {}^{15}N$ zygotes, it should be located at a density-shifted position that would readily be seen. In the reciprocal cross, $^{15}N \times {}^{14}N$, the absence of chloroplast DNA from the male parent, expected on the basis of the control and reciprocal crosses to band at 1.690–1.692 g/cm^3, is evident.

The density shift seen in chloroplast DNA from zygotes is resistant to extensive digestion with Pronase, α-amylase, and pancreatic and T1 ribonucleases. Thus, addition of covalently bound components such as methyl groups or sugar residues seems a more likely explanation than noncovalent binding of protein. Methyl groups produce a density shift in cesium chloride of about 0.001 g/cm^3 per 1% methylation (16). Although difficult to obtain in sufficient quantity, purified chloroplast DNA from zygotes should be examined for methylation, and such experiments are in progress. However, the differential density shift seen in the reciprocal crosses (0.003–0.005 g/cm^3 for [^{14}N]- and 0.008–0.010 g/cm^3 for [^{15}N]-DNA) requires an additional explanation. Estimation of the amount and density of an added component, on the simplest assumptions, indicates addition of a large amount of a component with a density in the range of DNA, suggesting that some new synthesis of DNA may be involved in the density shift. However, other interpretations are possible.

Chiang studied the behavior of chloroplast DNAs in zygotes of *Chlamydomonas*, initially using radioisotope labelling with [^{3}H]- and [^{14}C]adenine (17). He examined chloroplast DNA late in zygote maturation, and in progeny recovered after zygote germination. At these late times, he found that the ratio of ^{3}H–^{14}C in the chloroplast region was about the same as in the nuclear region. By examining late times only, Chiang missed seeing the density shifts and clear differences in the results of reciprocal crosses that we have reported here. More recently, Chiang reported the results of a pair of reciprocal crosses involving both radioisotope and density labeling (18). There, differences in reciprocal crosses could be seen, but the peaks were not as well resolved as those presented here, and the intermediate positions of the peaks were interpreted as the result of recombination. Our extensive genetic evidence shows that recombination of chloroplast markers occurs very rarely in the zygote. Consequently, it is unlikely that recombination explains the intermediate densities of chloroplast DNAs.

The mechanism of maternal inheritance may in part resemble that of modification-restriction systems of bacteria and viruses (7). If so, the density shift seen in chloroplast DNA could be the work of a modification enzyme, and the loss of chloroplast DNA from the male could result from attack by a restriction enzyme. Evidence that at least two components are involved, one from each parent, comes from genetic studies showing that the male cytoplasmic genome can be preserved by UV irradiation of the female parent before mating (19), or by treatment of the female gametes with spectinomycin (20), or by treatment of the male parent during gametogenesis with cycloheximide (20).

Whatever the mechanism of the density shift, the results presented here are consistent with the genetic evidence that chloroplast genes from the female, and not from the male, are regularly transmitted to progeny. Thus, the findings presented here support the hypothesis that the loss of genetic markers from the male parent is the direct consequence of the loss of the corresponding chloroplast DNA. This correlation between genetic and physical behavior further strengthens the hypothesis that the location of the cytoplasmic linkage group of *Chlamydomonas* (6) is in chloroplast DNA, a hypothesis already supported by a great deal of indirect evidence (1).

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