Molecular Basis of Maternal Inheritance

(cytoplasmic genes/chloroplast DNA/cesium chloride density gradients/14N 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 14N- or 15NH4Cl and then by making the crosses: 14N (female) × 15N (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/cm³ 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−) gametes 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 14N or 15N, and then making crosses: 14N (female) × 15N (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− (cycloheximide-resistance) and cytoplasmic marker sm2− (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 × 10⁶ 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 × 10⁶/ml in 4% sodium dodecyl sulfate-0.15 M NaCl-0.10 M Na3citrate-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.

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(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 citrate by repeated enzyme treatments with ribonucleases (pancreatic, 100 μg/ml and T1, 50 U/ml) and α-amylose (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 citrate, 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: 14N x 14N. An example is shown in Fig. 1, in which the DNA extracted from 24-hr zygotes (Fig. 1a) is compared with DNA from one of the parental strains (Fig. 1b). The tracing in Fig. 1b 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.

The zygote DNA shown in Fig. 1a resembles that of the gamete preparation in Fig. 1b, 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 14N- and with 15NCl 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 14N x 15N and as 15N x 14N, 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. 1.** Microdensitometer tracings of UV-absorption bands of DNA after CsCl density equilibrium centrifugation. (a) DNA from 24-hr zygotes, from 14N x 14N 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 14N- and the other with 15NCl. Outside markers are SP-15 DNA at 1.761 g/cm³ and poly(dAT) at 1.681 g/cm³. 14N- and 15N-labeled nuclear DNAs are at 1.738 and 1.723 g/cm³, respectively, and chloroplastDNAs are at 1.694 g/cm³ (14N) and at 1.706 g/cm³ (15N), 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.)
of gametes, one strain grown with 14N- and the other strain with 15NH4Cl as sole nitrogen source. The major components seen at buoyant densities of 1.723 and 1.738 g/cm3 correspond to the 14N and 15N nuclear DNAs, respectively, while the satellite components banding at 1.694 g/cm3 and at about 1.706 g/cm3 (here a shoulder on the 15N-nuclear peak) correspond to the 14N and 15N chloroplast DNAs (13, 14). In gradients with DNA from 14N-grown gametes, the density of the chloroplast DNA peak is 1.708 g/cm3. The peak at 1.712 g/cm3, usually seen as a shoulder in 14N-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. 1a 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. 1a, 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/cm3 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. 3a and 4a, the peak is at 1.692 g/cm3, lighter than that of the 14N female parent, and no peak is seen in the region corresponding to that of the density-shifted 14N chloroplast DNA, which is at 1.698 g/cm3 in the reciprocal crosses (Figs. 3b and 4b). Similarly, in Figs. 3b and 4b, no peak is seen in the position expected from Figs. 1a, 3a, and 4a for a density-shifted 14N 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 14N x 14N control (Fig. 1a) 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 14N x 14N, and 1.698 ± 0.0013 for the reciprocal cross. The mean value for chloroplast DNA from 24-hr 14N x 14N zygotes was 1.690 ± 0.001 g/cm3. 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/cm3) is seen, as well as very dense material in the region around 1.770 g/cm3. 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 14N x 14N and 14N x 15N 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 14N x 14N control crosses, which showed only one peak, and the position of gamete DNA, but shifted about 0.005 g/cm3 to the lighter density of 1.690 g/cm3. In the 14N x 15N crosses, the single peak seen at 1.692 g/cm3 is inferred to be the density-shifted 14N DNA from the female parent. (Whether the mean values of 1.692 and 1.690 g/cm3 are the same or different cannot be concluded from these data.) If chloroplast DNA from the male parent
were present at its unshifted density, it might be hidden under
the nuclear peak, but the control cross $^{14}$N x $^{14}$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 x $^{14}$N zygotes, it should be located at a density-
shifted position that would readily be seen. In the reciprocal
cross, $^{14}$N x $^{14}$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, $\alpha$-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). Al-
though 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 $[^3]$H-) and 0.008–0.010 g/cm$^3$ for $[^4]$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 com-
ponent 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
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 re-
semble 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 pre-

tented 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 hy-
pothesis that the location of the cytoplasmic linkage group of
*Chlamydomonas* (6) is in chloroplast DNA, a hypothesis al-
ready supported by a great deal of indirect evidence (1).

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