Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 3025–3029, May 1983
Genetics

Differential methylation of chloroplast DNA regulates maternal inheritance in a methylated mutant of *Chlamydomonas*

(gametogenesis/gel electrophoresis/methylation-sensitive restriction enzymes)

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ABSTRACT **In *Chlamydomonas*, the maternal inheritance of chloroplast genes correlates with the differential methylation of chloroplast DNA (chlDNA) in females (mt^+) but not in males (mt^-). Our previous studies have supported our methylation-restriction model in which the maternal transmission is accounted for by the differential methylation in gametes which protects female but not male chlDNA from degradation during zygote formation. In the mutant *me-1* [Bolen, P. L., Grant, D. M., Swinton, D., Boynton, J. E. & Gillham, N. W. (1982) *Cell* 28, 335–343], chlDNA of vegetative cells of both mating types is heavily methylated even before gametogenesis; nonetheless, maternal inheritance occurs in mutants as in wild type. To investigate the mechanism of maternal inheritance in the *me-1* mutant, we have compared restriction fragment patterns after agarose gel electrophoresis of chlDNAs from mutant vegetative cells and gametes with those from wild type, by using a set of 32 restriction enzymes of which 17 were methylation-sensitive in this system. We find that additional methylation occurs during gametogenesis in the mutant female (mt^+) but not in the corresponding male (mt^-). Thus, gamete-specific, mating-type-specific methylation occurs in the *me-1* mutant as in the wild type, consistent with our methylation-restriction model. In the *me-1* mutant, gametic methylation occurs on a background of vegetative cell methylation not present in wild-type cells and irrelevant to the regulation of chloroplast inheritance. Comparison of the *me-1* mutation with the *mat-1* mutation [Sager, R., Grabowy, C. & Sano, H. (1981) *Cell* 24, 41–47] provides evidence for the existence of two different chlDNA methylation control systems: *mat-1*, linked to the mating type locus and regulating the mating-type-specific methylation that correlates with maternal inheritance, and *me-1*, unlinked to the mating type locus and unrelated to the regulation of maternal inheritance.**

Methylation-restriction systems in bacteria function to degrade foreign DNA while leaving intact the homologous or heterologous DNA of the host. The molecular mechanism underlying this differential degradation is site-specific methylation of the host DNA at sites which are recognized in the best-studied examples by a corresponding type I endonuclease, leading to cleavage and degradation of foreign DNAs unprotected by prior methylation (reviewed in refs. 1–3).

About 10 years ago (4, 5) we proposed that the maternal inheritance of chloroplast DNA (chlDNA) in *Chlamydomonas* and in some higher plants (6) was governed by a methylation-restriction system analogous to that in bacteria. The initial evidence was: (*i*) loss of chlDNA from male (mt^-) parents in zygotes in which the homologous chlDNA of female origin was preserved, shown by means of differential isotopic prelabeling of parental DNAs in reciprocal crosses, and (*ii*) a density shift in chlDNA isolated from zygotes after disappearance of male chlDNA, consistent with extensive methylation. Over the subsequent years, increasingly definitive evidence in support of this hypothesis has been accruing.

This evidence has been recently reviewed (7) and will only be cited briefly here. The first identification of 5-methylcytosine (mC) in chlDNA was achieved by prelabeling with [G-^{3}H]deoxycytidine in reciprocal crosses of labeled × unlabeled parental cells. The presence of mC was detected by HPLC analysis of bases from chlDNA of zygotes only when the female parent had been prelabeled. Methylation was essentially complete within 6 hr after zygote formation, and no bases other than cytosine were methylated (8). Subsequently, the absence of C-C-G-G methylation in vegetative cells, and its presence in female gametes and zygotes but not in male gametes, was demonstrated by differences in *Msp* I and *Hpa* II restriction fragment patterns (9).

Because C-C-G-G sites represent only a fraction of total methylatable sites, we have developed other methods to provide a more complete analysis of the extent and location of methylation. One such method utilizes antibodies directed against mC. Highly specific rabbit anti-mC antibodies are prepared and used then for quantitation and localization of mC bases in DNA restriction fragments transferred to nitrocellulose paper. The fragments are reacted with the anti-mC antibody and then reacted with ^{125}I-labeled goat anti-rabbit IgG (10). With this highly sensitive method, no mC was detected in chlDNA from male gametes, whereas in chlDNA from female gametes, we found extensive methylation, as well as evidence of its nonrandom distribution: extent of methylation was not proportional to fragment size (11).

Furthermore, with this method we were able to determine the mechanism underlying the biparental inheritance of chloroplast genes that occurs in crosses with the *mat-1* mutant (11). The *mat-1* mutation was discovered in a male (mt^-) population (12) and is closely linked to the mating type locus: thus, all *mat-1* segregants from crosses are mt^-, and chloroplast genetic markers are transmitted from both wild-type mt^+ and *mat-1* mt^- parents to all progeny. We found that the chlDNA of *mat-1* mt^- gametes is partially methylated, intermediate in extent between wild-type males and females, as shown both by restriction fragment patterns and by the antibody binding method (10). Methyl transferase with M_r 200,000, presumed to be the active form of the enzyme because it is found only in wild-type female gametes and in zygotes in which chlDNA is being methylated, is also present in *mat-1* male gametes (13).

These results further confirmed the role of methylation in chloroplast heredity, by correlating the extent of methylation occurring during gametogenesis directly with the genetic transmission of chloroplast genes, as well as with the enzymatic activity of a methyl transferase, in wild-type and *mat-1* mutant cells.

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Abbreviations: chlDNA, chloroplast DNA; mC, 5-methylcytosine.

Recently, a new mutant of *Chlamydomonas*, *me-1*, has been described (14) in which maternal inheritance occurs as in the wild type, but in which vegetative cells of both mating types contain methylated chlDNA, in contrast to the nonmethylated state of the chlDNA in vegetative cells of wild type. The *me-1* mutation is unlinked to *mat-1* or to mating type and represents a second nuclear gene shown to influence the methylation of chlDNA. The *me-1* mutation has provided us with an opportunity to test the methylation-restriction hypothesis in a mutant showing a high level of background methylation. In this paper, we compare the methylation patterns of chlDNAs from vegetative and gametic cells of both strains and both mating types to find out whether additional gamete-specific and mating-type-specific methylation occurs during gametogenesis.

We find in the *me-1* mutant, that superimposed upon the background level of methylation in vegetative cells, further methylation occurs during gametogenesis in the female gametes but not in the males. Thus, the same differential pattern of methylation is present in the *me-1* mutant as in the wild-type cells.

MATERIALS AND METHODS

Cells and Culture Conditions. *Chlamydomonas reinhardi* strain 21gr (female, mt^+) is our standard wild-type stock (9); strains carrying the nuclear mutation *me-1* mt^+ (CC-1154) and mt^- (CC-1155) were provided by N. W. Gillham (14). Cells were grown in liquid minimal medium at pH 7.2 (bubbled with 5% CO_2 in air) in continuous light and were harvested at a density of 2–4 $\times 10^6$ cells per ml for vegetative preparations or for induction of gametes. Gametogenesis was induced by pelleting vegetative cells at 5,000 $\times$ *g* for 5 min at 15°C and washing one time in gamete induction medium (1/5 dilution of minimal medium lacking nitrogen and buffered at pH 8); cells then were resuspended in this medium at the original density in continuous light for 24 hr at which time gametes were harvested. Mating efficiency was assayed by mixing 1–2 ml with gametes of opposite mating type and zygote formation observed after 30 min. At this time at least 90% of cells are zygotes.

Gels and Restriction Enzyme Digests. chlDNA was isolated as described (9). Digestions with endonucleases were performed under conditions suggested by distributors. The following restriction enzymes were obtained from Bethesda Research Laboratories: *Hin*fI, *Hpa* II, *Hha* I, *Cfo* I, *Bam*HI, *Tha* I, and *Sal* I. All other restriction enzymes were obtained from New England BioLabs. Restriction fragments were separated on horizontal 0.8% agarose 30-cm gels; the bands were visualized by ethidium bromide fluorescence and photographed as described (9). The intensity of smaller molecular weight fragments was enhanced by longer exposure times, and the corresponding films were matched and joined to make the final prints.

RESULTS

Identification of Methylation-Sensitive Enzymes. When this work began, only a few methylation-sensitive restriction enzymes were known (15). In order to compare multiple DNA sequences, it was necessary to identify additional methylation-sensitive restriction enzymes. We did so by comparing restriction fragment patterns of chlDNA from wild-type vegetative cells in which this DNA is unmethylated with the homologous DNA from female gametes in which considerable methylation has occurred. We found that the majority of enzymes tested in this way were methylation-sensitive, including 17 of the 20 enzymes listed in Table 1. The other three enzymes—*Mbo* I, *Taq* I, and *Eco*RV—cleaved several sites uniformly in all chlDNAs tested and either the enzymes were methylation-resistant, or the sites were totally unmethylated. In addition, the following enzymes, tested in the same way but not listed in Table 1, were methylation-sensitive: *Tha* I and *Fnu*dII, each with several sites, and *Sma* I, *Sal* I, and *Sst* I, each having very few sites in chlDNA. The following enzymes cleaved no sites: *Pvu* I, *Xor* I, *Xho* I, *Nae* I, *Nar* I, *Nru* I, and *Pae*R71.

Table 1. Comparative methylation of chlDNAs from female (mt^+) vegetative cells and gametes

Enzyme	Site	Methylation 1*	2†	3‡
Msp I	C-C-G-G	+ (5′ C)	++	a
Hpa II	C-C-G-G	+++ (Inner C)	+++	b
Cfo I	G-C-G-C	++	+++	+
Hha I	G-C-G-C	++	+++	++
*Sau*3A	G-A-T-C	++	++	a
Mbo I	G-A-T-C	−	ND	−
Hae III	G-G-C-C	−	++	+
Ava II	G-G-$\frac{A}{T}$-C-C	+++	+++	a
*Sau*96I	G-G-N-C-C	++	+++	a
*Hin*fI	G-A-N-T-C	+ (Few extra bands)	+++	a
*Scr*FI	C-C-N-G-G	+	++	a
*Fnu*4HI	G-C-N-G-C	++	+++	+
*Bam*HI	G-G-A-T-C-C	+ (Few extra bands)	+++	b
Hpa I	G-T-T-A-A-C	−	+++	+
*Eco*RV	G-A-T-A-T-C	−	−	−
Hae II	R G-C-G-C Y	++	+++	a
*Hgi*AI	C-$\frac{T}{A}$-G-C-$\frac{T}{A}$-C	−	+++	+
Kpn I	G-G-T-A-C-C	+ (Few extra bands)	+++	a
Acc I	G-T-$\frac{A\ G}{C\ T}$-A-C	++	+++	+
Taq I	T-C-G-A	−	−	−

+, ++, and +++, Increased extent of methylation; −, no change. ND, not determined. R, purine; Y, pyrimidine.

* Methylation change in wild-type gametogenesis: vegetative to gamete.

† Methylation change in gametogenesis of *me-1* mutant: vegetative to gamete.

‡ Methylation change, comparing wild-type gamete to *me-1* mutant, vegetative. a, Similar extent but differences in band positions; b, less methylation in *me-1* vegetative than wild-type gamete.

Comparison of Methylation in chlDNAs of Female (mt^+) Gametes from Wild-Type and *me-1* Mutant. The results summarized in Table 1 are based upon electrophoretic separation on agarose gels of restriction enzyme digests of chlDNAs. These gels, consisting of two-lane (female vegetative and gametic either wild-type or *me-1* mutant) and four-lane (female vegetative and gametic wild-type and *me-1* mutant) comparisons of which Fig. 1 is an example, are all of similar quality.

In Fig. 1*A*, *Hae* II digestion produced similar but not identical band patterns in the wild-type gametes and mutant vegetative cells. Thus, the overall extent of methylation appeared similar in the two preparations, both showing a series of *Hae* II fragments not blocked by methylation. In contrast, the mutant gamete DNA consisted entirely of high molecular weight fragments. In Fig. 2*B*, *Scr*FI digestion also produced a similar pattern of methylated sites, as shown by comparison with the unmethylated wild-type vegetative chlDNA. However, many bands were also present in the mutant gamete lane, demonstrating that many unmethylated *Scr*FI sites were still present. These results represent a sample of the patterns seen in the other restriction digests compared in Table 1. Two different DNA preparations from each cell line were used during the study of 32 enzyme digests; no differences in patterns attributable to variations from one preparation to the other were noted in control digests.

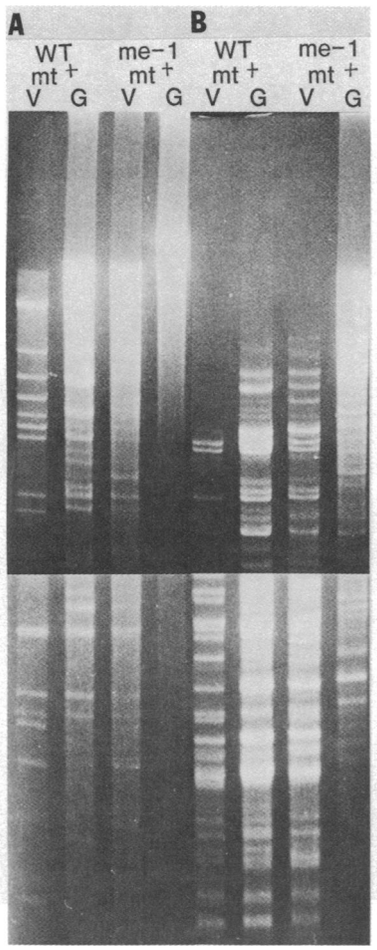

FIG. 1. Restriction fragment patterns of chlDNA digested with (*A*) *Hae* II or (*B*) *Scr*FI. V, vegetative; G, gamete; mt$^+$, female; WT, wild type; me-1, methylated mutant.

Three sets of comparisons of chlDNAs from female cells are listed in Table 1: column 1 compares wild-type vegetative and gametic chlDNAs; column 2 compares *me-1* mutant vegetative and gametic chlDNAs; and column 3 compares wild-type gametic with mutant vegetative chlDNAs. Column 1 shows the variable extent of gametic methylation of chlDNAs seen at different restriction sites. Because the total methylation occurring in wild-type gametes is about 20–25% of the cytosines (H. Sano, personal communication), it is evident that the 20 enzymes listed in Table 1 identify only a sample of the sites at which methylation occurs. Of particular interest are the three enzymes *Hae* III, *Hpa* I, and *Hgi*AI, with no apparent sequence resemblance, in which no methylation detectable by this method occurs in wild-type gametes, but in the *me-1* mutant methylation occurs at a few sites in vegetative cells and extensively in gametes.

Methylation is extensive in the gametes of the *me-1* mutant, representing, for most sites, a dramatic increase over the methylation present at those sites in the mutant vegetative cells. This increase is evident in the patterns shown in Figs. 1 and 2, as well as in Table 1. Nonetheless, with about 60% of the enzymes, bands of moderate and low molecular weight DNAs were still present in the *me-1 mt*$^+$ gamete lane, indicating that not all methylatable sites had been methylated.

Methylation patterns in chlDNA from wild-type gametes are compared with those from mutant vegetative cells in column 3. The comparison is particularly interesting because the majority of restriction sites showed very similar methylation patterns in the two chlDNAs being compared (noted in Table 1 as a and b). Only *Hha* I sites showed extensively increased methylation, and *Cfo* I, *Hae* III, *Fnu*4HI, *Hpa* I, *Hgi*AI, and *Acc* I sites showed some increase (compare *Hae* III in Fig. 2). The column 2 comparisons show that many methylatable cytosines were still available in the vegetative mutant, for further methylation during gametogenesis.

Absence of Gametic Methylation in *me-1* Males (*mt*$^-$). Six-lane comparisons of chlDNAs restricted with five enzymes—*Msp* I, *Hpa* II, *Hin*fI, *Hae* III, and *Kpn* I—are shown in Fig. 2. Here it is evident that no further methylation has occurred in the mutant males during gametogenesis. Their restriction fragment patterns are identical in DNAs from vegetative cells and from gametes and also do not differ from those of the female vegetative cells. In contrast, the female gametes are more methylated than the vegetative cells from which they developed. Thus, these patterns demonstrate unambiguously that differential methylation of male and female gametes occurs during gametogenesis in the *me-1* mutant just as in the wild type. The difference between the mutant and the wild type lies in the level of methylation already present in the mutant vegetative cells upon which the gametic methylation is superimposed.

DISCUSSION

This paper compares the chlDNA methylation that occurs during gametogenesis in wild-type cells and in the *me-1* mutant which is extensively methylated in the vegetative state before gametogenesis (14). The results establish that: (*i*) further methylation occurs during gametogenesis in female (*mt*$^+$) *me-1* mutant cells as it does in the wild type; (*ii*) no further methylation occurs during gametogenesis in *me-1* males (*mt*$^-$); and (*iii*) the overall level of methylation in *me-1* vegetative cells is similar to that in wild-type female gametes for most restriction sites, but the detailed banding patterns are different.

Thus, the differential methylation of chlDNA from male and female gametes, which provides the basis for differential destruction of chlDNA of male origin in zygotes, occurs in the *me-1* mutant as it does in the wild type. The hypothesis that chloroplast heredity is regulated by a methylation-restriction system operating in a manner analogous to that in bacteria is not contradicted by the existence of *Chlamydomonas* mutants with a high background of methylation in the vegetative state.

Methylation-restriction systems in bacteria are site-specific. Methylation or cleavage of a few sites is sufficient for operation of the system. Methylation blocks restriction activity, and cleavage of a few sites that are unmethylated provides the substrates for subsequent rapid nonspecific degradation.

Since the inception of our studies of chlDNA methylation, we have wondered about the extensive methylation occurring during gametogenesis in wild-type cells. We showed that this methylation is not limited to a single recognition site by demonstrating the extensive methylation within chlDNA fragments that had been cleaved by *Msp* I, *Bam*HI, or *Eco*RI (11) using the anti-mC antibody method (10). One way to reconcile extensive methylation with the bacterial model is to postulate that most of the methylation is nonspecific and irrelevant to the mechanism of maternal inheritance and that site-specific methylation is present but masked by the high nonspecific background.

Methylation in the *me-1* mutant described here supports this hypothesis, by providing an example of high background methylation that does not alter the pattern of maternal transmission of chlDNA. The results reconfirm the methylation-restriction hypothesis by demonstrating the occurrence of gamete-spe-

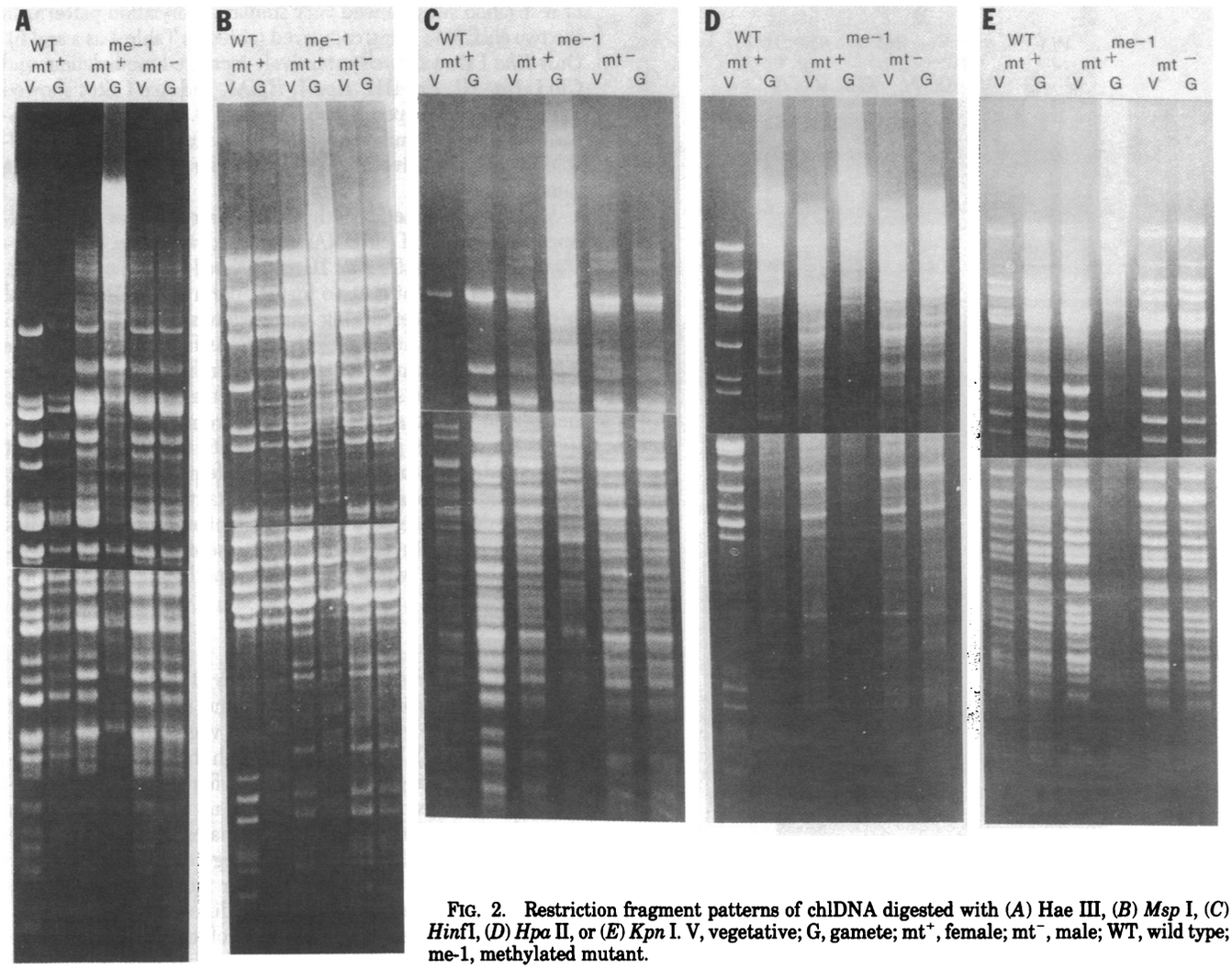

FIG. 2. Restriction fragment patterns of chlDNA digested with (*A*) Hae III, (*B*) *Msp* I, (*C*) *Hin*fI, (*D*) *Hpa* II, or (*E*) *Kpn* I. V, vegetative; G, gamete; mt^+, female; mt^-, male; WT, wild type; me-1, methylated mutant.

cific, mating-type-specific additional methylation in the *me-1* mutant female cells.

Our findings with the *me-1* mutation reported here provide additional information concerning the methylation events that occur during gametogenesis. We have incorporated the available evidence from chlDNA methylation studies with the wild type, the *mat-1* mutation, and the *me-1* mutation into a two-enzyme hypothesis, which may be oversimplified but provides a working model for further research.

According to this hypothesis, two methyltransferases with different specificities are active in gametogenesis. Enzyme I is a site-specific methyltransferase that methylates only a few sites, and its activity is regulated by the *mat-1* gene and the mating type locus. The same sites are also recognized by a restriction endonuclease present in zygotes and blocked by methylation at these sites. Enzyme II is a different methyltransferase with broad site specificity, responsible for the extensive methylation seen in vegetative cells of both mating types that carry the *me-1* mutation. Thus, the *me-1* gene regulates expression of enzyme II in vegetative cells.

In the simplest version of this hypothesis, enzyme II is responsible not only for the methylation of *me-1* vegetative cells but also for the further methylation that occurs in mt^+ gametes and subsequently in zygotes. These stages of the life cycle show increasing extents of methylation, which may result from increased levels of enzyme activity. This rheostat-like control of enzyme II activity may be regulated by new signals that are turned on in gametogenesis and after zygote formation. Alternatively, the methylation seen in gametogenesis and in young zygotes may result from the activation of additional methyltransferases. The two-enzyme hypothesis is attractive for its simplicity and because the extent and pattern of methylation seen in vegetative *me-1* cells resembles that seen in wild-type mt^+ gametes. Furthermore, the extent of methylation in *me-1* mt^+ gametes resembles that seen in wild-type zygotes. At both stages about 70% of the cytosines are methylated (unpublished data). Studies must be undertaken to test the two-enzyme working hypothesis by characterizing the methyltransferases present at various stages of the life cycle.

A different question concerns the function of extensive chlDNA methylation seen in gametogenesis and in young zygotes. In wild-type mt^+ gametes we find that 20–25% of cytosines are methylated, and in 6-hr zygotes, that fraction has increased to about 70%. If maternal inheritance is regulated by site-specific methylation of a few sites, what is the function of the massive methylation? In the nuclei of mammalian cells, DNA methylation appears to play a role, perhaps in concert with chromatin conformation, in the control of transcription (15). It seems unlikely that methylation of chlDNA plays an important role in the control of transcription in *Chlamydomonas*, because the chlDNA of the *me-1* mutant vegetative cells is heavily methylated without interfering with growth (14). chlDNA is not organized into nucleosomes and its transcriptional controls may be characteristically prokaryotic. In bacteria, adenine meth-

ylation is important in mismatch repair (16), as well as in protection against foreign DNA (1–3), but no adenine methylation has been detected in chlDNA of *Chlamydomonas* (8). The extent of cytosine methylation in bacteria is low and has been associated with the methylation-restriction systems (17); no other functions have been described.

Thus, the extensive chlDNA methylation occurring in gametogenesis and in young zygotes appears to be a process as yet unreported in other organisms. Definition of its function awaits further research.

We thank Dr. N. W. Gillham for providing *me-1 mt*$^+$ and *me-1 mt*$^-$ strains. We thank S. James for preparing the manuscript. This research was supported by National Institutes of Health Grant GM22874 to R.S.

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