Proc. Natl. Acad. Sci. USA
Vol. 78, No. 5, pp. 3118–3122, May 1981
Genetics

Differential activity of DNA methyltransferase in the life cycle of *Chlamydomonas reinhardi*

[DNA (cytosine-5)-methyltransferase/chloroplast DNA methylation/*mat-1* mutant/symmetrical methylation]

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Contributed by Ruth Sager, January 23, 1981

ABSTRACT **Two molecular weight forms of DNA (cytosine-5-)-methyltransferase [S-adenosyl-L-methionine:DNA (cytosine-5-)-methyltransferase, EC 2.1.1.37], both active in assays *in vitro*, were isolated from the green alga *Chlamydomonas reinhardi* at various stages of the life cycle. The enzyme with M_r 60,000 was found in vegetative cells and gametes of both male (mt^-) and female (mt^+) mating types. The enzyme with M_r 200,000 was specific to gametic cells and zygotes, which are the only stages at which methylation of chloroplast DNA occurs *in vivo*. Chloroplast DNA from gametes was shown to be methylated on both strands at most if not all methylation sites and the M_r 200,000 enzyme was shown to methylate both unmethylated and hemimethylated sites, the latter at an elevated rate. *Micrococcus luteus* DNA showed the same nearest-neighbor frequencies of methylation after methylation by each molecular weight component. The data suggest strongly that the M_r 200,000 enzyme is the active multimeric form of the M_r 60,000 enzyme and that it acts as both initiation and maintenance methylase. It is proposed that methylation of chloroplast DNA in female gametes and zygotes is regulated by assembly of the multimeric M_r 200,000 active enzyme, which in turn determines the maternal inheritance of chloroplast DNA.**

Eukaryotic DNAs contain 5-methylcytosine as a minor component, at concentrations of 2–7% of total cytosine, depending upon the species (1, 2). The introduction of methyl residues into the 5 position of cytosine occurs after replication and is catalyzed by a DNA methyltransferase (EC 2.1.1.37), which transfers the methyl group from S-adenosylmethionine to cytosine in double-stranded DNA (3). This class of enzymes, found in both plant and animal tissues (3), methylates only a small fraction of cytosines, mainly at C-G doublets (4). Very little evidence of site specificity of a eukaryotic methyltransferase has yet been reported, other than in our preliminary study (5).

The physiological roles of methylation in eukaryotic cells are almost entirely unknown (6). Evidence of tissue-specific methylation has been described (7–9), although the significance of the observed methylation patterns is unclear. A function in the regulation of gene expression is suggested especially by the effect of hypomethylation on globin synthesis in Friend cells (10) and the effect of azacytidine on differentiation (11, 12). A role for methylation and restriction in the inactivation or destruction of particular sequences of DNA or even whole chromosomes has been proposed (13). In a series of studies, we have demonstrated that a methylation and restriction system regulates the inheritance of chloroplast DNA in the sexual alga *Chlamydomonas* (14). In this organism, as well as in higher plants, chloroplast DNA from the female parent is preferentially transmitted to progeny, leading to the genetic phenomenon of maternal inheritance.

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In this paper, we describe the isolation and characterization of a DNA methyltransferase from *Chlamydomonas*. Methylating activity has been detected in fractions with M_r 60,000 and M_r 200,000. The M_r 200,000 form is found only in gametic cells and in zygotes, which are the two stages at which active methylation of chloroplast DNA occurs (14).

MATERIALS AND METHODS

Enzymes. *DNA methyltransferase.* Cells of *Chlamydomonas reinhardi*, strain 21gr as the female (mt^+) and strain 5177D as the male (mt^-), were grown in 4-liter batches in M medium (5). Vegetative cells were harvested in the midlogarithmic phase of growth and gametes and zygotes were produced as described (15). DNA methyltransferase was isolated according to the method described in ref. 5. Cells were disrupted by a French press at 180 kg/cm^2 (18 MPa) and centrifuged at 40,000 rpm in a Beckman 60 Ti rotor for 2 hr, and the resulting clear supernate was treated with Sepharose-bound DNase I. The crude extract was dialyzed against buffer containing 20 mM potassium phosphate at pH 7.1, 1 mM 2-mercaptoethanol, 1 mM EDTA, and 5% (vol/vol) glycerol and then chromatographed on a DEAE-cellulose column equilibrated with the buffer. The protein was eluted with a linear gradient of NaCl (0.02–0.5 M) and enzyme activity was assayed (Fig. 1). Aliquots from appropriate fractions were applied to a 10–30% glycerol gradient and centrifuged at 40,000 rpm for 17 hr in a Beckman 60 VTi rotor. The enzyme activity was assayed and peak fractions were pooled (Fig. 2).

Other enzymes. Escherichia coli alkaline phosphatase, and pancreatic DNase I were purchased from Worthington. Restriction endonucleases *Eco*RI and *Hpa* II were from Bethesda Research Laboratories (Rockville, MD). Polynucleotide kinase was from New England BioLabs. Units for these enzymes are those defined by the suppliers.

DNA Methyltransferase Assay. One unit of enzyme activity is defined as 1 pmol of methyl group incorporated into DNA per 60 min at 37°C in the presence of *Micrococcus luteus* DNA at 70 μg/ml. The standard assay was performed under the conditions described (5). A 50-μl reaction mixture containing 25 mM Tris·HCl at pH 8.0, 6 mM dithiothreitol, 20 mM EDTA, 5 μM S-adenosyl[*methyl*-^{3}H]methionine (specific activity 6 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels), (2–5 μg of DNA, and 1–2 units of the enzyme was incubated at 37°C for 1 hr. After further incubation in the presence of 7 μg of protease K and 0.4% sodium dodecyl sulfate, the solution was made 0.4 M NaOH and incubated at 60°C for 30 min to destroy any RNAs that might possibly be methylated with contaminating RNA methyltransferase (5). The whole reaction mixture was then neutralized with HCl and extracted with phenol. The aqueous phase was washed with ether and spotted on a Whatman no. 3 paper disc that had

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been soaked in 0.2 M EDTA and dried prior to use. The disc was dried, washed three times with 7% trichloroacetic acid, and counted for radioactivity.

Product Analysis. The product was analyzed as described (5). Briefly, *in vitro* methylated DNA from *M. luteus* was eluted from filter paper discs after intensive washing with 6% trichloroacetic acid. The eluate was hydrolyzed with 98% (wt/wt) formic acid and resulting bases were analyzed by paper chromatography.

Nearest-Neighbor Frequency Analysis. The nearest-neighbor analysis of methylation was performed as described (5). *M. luteus* DNA was selected because in unpublished experiments we found no detectable m^5C by using the very sensitive antibody detection method (16). Various amounts of *M. luteus* DNA were methylated *in vitro* with *S*-adenosyl[^{3}H]methionine by each enzyme and digested with DNase I in the presence of 10 mM $MgCl_2$ and 2 mM $CaCl_2$. Dinucleotides, which were more than 50% of total products, were collected by DEAE-cellulose column chromatography and separated by high-voltage paper electrophoresis.

Agarose Gel Electrophoresis of Reassociated Chloroplast DNA. Two micrograms of gamete DNA was incubated with 12.5 units of *Eco*RI in 25-μl reaction mixtures for 4 hr at 37°C, then 50 units of bacterial alkaline phosphatase was added and the mixture was incubated further for 2 hr. The reaction mixture was extracted with phenol, and the DNA was precipitated from the aqueous phase with 75% (vol/vol) ethanol and labeled at the 5′ ends with [^{32}P]phosphate. Then 0.2 μg of radioactive DNA was mixed with 2 μg of vegetative DNA that had been digested with *Eco*RI, heated at 100°C for 5 min, and incubated at 60°C for 2 hr. Reassociated DNA was recovered by ethanol precipitation. DNA samples were digested with *Hpa* II (7.5 units) in a 25-μl reaction mixture for 4 hr at 37°C and electrophoresed in a 0.8% agarose gel containing ethidium bromide at 0.5 μg/ml, for 40 hr at 12 mA. After being photographed under ultraviolet light, the gel was dried on a glass plate at 37°C for 16 hr and autoradiographed.

RESULTS

Elution Patterns of the Enzyme After DEAE-Cellulose Column Chromatography. The crude extract was treated with Sepharose-bound DNase and the supernatant after high speed centrifugation was loaded onto a DEAE-cellulose column. The elution profiles of the enzyme recovered from cells at various stages in the life cycle are depicted in Fig. 1. The female vegetative cells contained two active fractions eluting at 0.08 M (peak I) and 0.15 M (peak II) NaCl, respectively (Fig. 1*A*). Extracts of female gametes showed an elution pattern similar to that of vegetative cells except that the second peak (peak II) increased in activity (Fig. 1*C*). The male vegetative and gamete cells contained a single active fraction eluting at 0.08 M NaCl (Fig. 1*B*). The zygotes showed a single peak eluting at 0.11 M NaCl (Fig. 1*D*).

Judging from these elution patterns, the peak I activity was found in all vegetative cells and gametes, both male and female. The peak II activity was specific to female cells. The relationship between the zygote enzyme activity and that of peak II was clarified by further experiments, described below. Essentially, we found no differences in nearest neighbor frequencies of methylation or in molecular weight of these two preparations, although their elution positions from DEAE-cellulose columns were slightly different. We therefore consider the zygote activity to be the same as the peak II activity.

Molecular Weight Estimation. Molecular weights of the enzymes obtained after DEAE-cellulose column chromatography were estimated by glycerol density gradient centrifugation (Fig. 2).

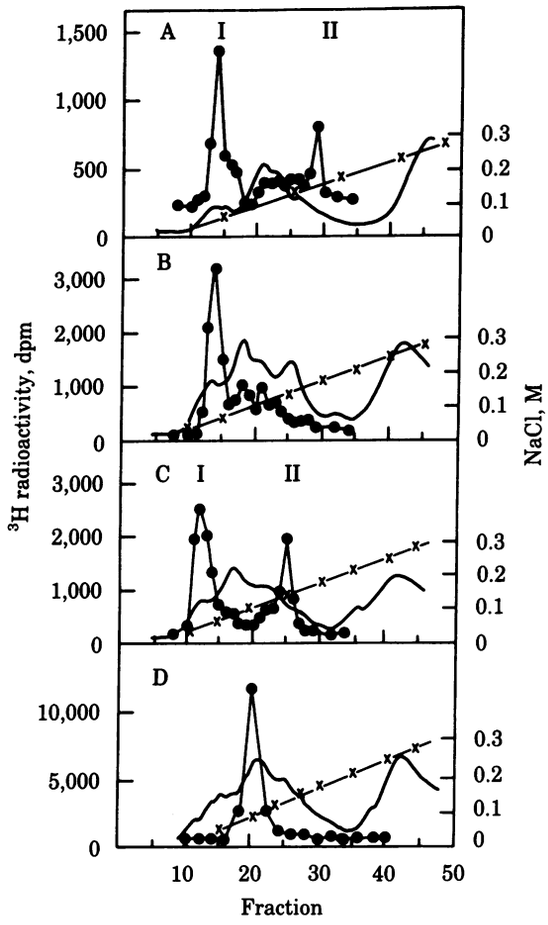

FIG. 1. Elution patterns of DNA methyltransferases from DEAE-cellulose. Extracts were from: (*A*) 21gr vegetative cells (female); (*B*) 5177D gamete cells (male); (*C*) 21gr gametes cells (female); and (*D*) zygotes. ●, Enzyme activity; ——, relative absorbance at 278 nm; ×, NaCl concentration measured by conductivity. Peak I is enzyme activity eluted at 0.08 M NaCl; peak II is enzyme activity eluted at 0.15 M NaCl.

The peak I enzyme from the female migrated at the position of M_r 60,000 (Fig. 2*A*). The peak II enzyme, however, showed two active peaks at M_r 60,000 and 200,000 (Fig. 2*B*), with the M_r 200,000 enzyme accounting for more than 72% of the total activity. The peak I enzyme from the male was M_r 60,000 (Fig. 2*C*), and the zygote enzyme was M_r 200,000 (Fig. 2*D*). We infer that the peak I enzyme, present in vegetative and gamete cells, is the M_r 60,000 species, and that the peak II and zygote enzyme preparations contain the M_r 200,000 enzyme species.

Nearest-Neighbor Frequency Analysis. The product of *in vitro* methylation was analyzed by hydrolyzing *M. luteus* DNA with formic acid and separating the products by paper chromatography (5). All methylase activities obtained by DEAE-cellulose chromatography yielded solely 5-methylcytosine (data not shown). The two enzymatic activities corresponding to the 60,000 and 200,000 M_r species were compared with respect to the site specificity of their activities, using *M. luteus* DNA as the substrate. The DNA was methylated with *S*-adenosyl-[^{3}H]methionine and digested with DNase I. Dinucleotides containing ^{3}H were identified after high-voltage electrophoresis and their frequencies were determined. 5-[^{3}H]Methyldeoxycytidine was found in all four of the cytosine-containing dinucleotides (data not shown). The frequency of each dinucleotide was estimated and the results are shown in

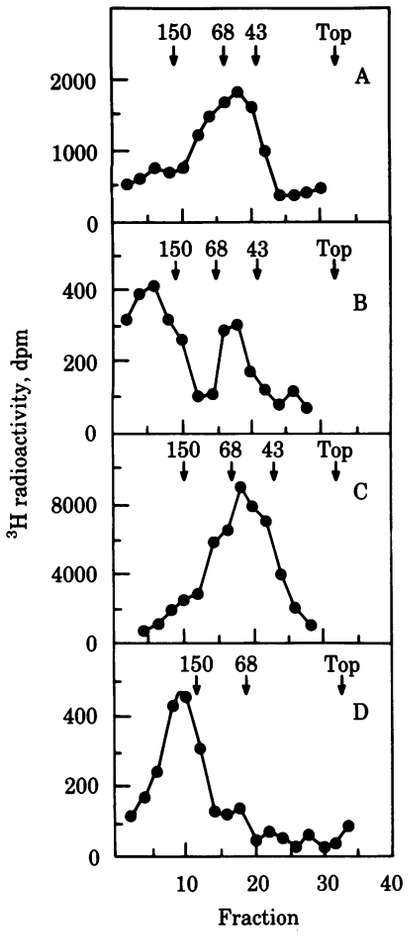

FIG. 2. Glycerol density gradient centrifugation of DNA methyltransferase. Appropriate fractions from DEAE-cellulose eluates were applied onto a 10–30% glycerol gradient equilibrated with 20 mM potassium phosphate, pH 7.1/20 mM NaCl/1 mM 2-mercaptoethanol/1 mM Na_2EDTA and centrifuged at 40,000 rpm at 4°C for 17 hr in a Beckman 60 VTi rotor. Fractions were collected and refractive index and enzyme activities were measured. Samples are from: (*A*) peak I fraction of 21gr (female) gamete cells (Fig. 1*C*); (*B*) peak II fraction of 21gr (female) gamete cells (Fig. 1*C*); (*C*) 5177D (male) gamete cells (Fig. 1*B*); and (*D*) zygote cells (Fig. 1*D*). The molecular weight markers (given $\times 10^{-3}$) were ovalbumin (M_r 43,000), bovine serum albumin (M_r 68,000), and immunoglobulin G (M_r 150,000). The densities at their migrating positions were determined from refractive index measurements.

Table 1. Each of the enzyme preparations showed a similar pattern of dinucleotide frequencies—i.e., the d(m^5C,G) doublet contained about 72% of the total radioactivity and the other three doublets, d(m^5C,C), d(m^5C,A), and d(m^5C,T), each contained about 9%. The frequencies of dinucleotides containing m^5C do not differ in the products methylated by M_r 60,000 and M_r 200,000 enzymes. This result is consistent with the hypothesis that the catalytic site is the same in the two enzymes and therefore that they are closely related polypeptides, the M_r 60,000 protein probably being a subunit of the M_r 200,000 enzyme.

Enzyme Activity at Various Stages in the Life Cycle. The quantitative estimation of each enzyme at various stages in the life cycle is summarized in Table 2. The level of M_r 60,000 enzyme activity is similar in male (mt^-) and female (mt^+) gametes, but the activity of the M_r 200,000 fraction is increased 2-fold in female gametes and 30-fold in zygotes when compared with levels in vegetative cells. The increase of female-specific enzyme in gametes and zygotes coincides with the methylation that occurs specifically in female chloroplast DNA in gametes and zygotes (14, 15).

Table 1. Frequencies of ^{3}H-labeled dinucleotides

Enzyme source	^{3}H in products, dpm (frequency, %) d(m^5C,C)	d(m^5C,A)	d(m^5C,G)	d(m^5C,T)
mt^+ gamete				
M_r 60,000	360 (10)	450 (12)	2,470 (69)	310 (9)
M_r 200,000	170 (12)	140 (10)	990 (70)	110 (8)
mt^- gamete				
M_r 60,000	190 (8)	160 (7)	1,910 (78)	170 (7)
Zygote				
M_r 200,000	13,000 (10)	6000 (6)	90,000 (72)	16,000 (12)

A 100-μl reaction mixture contained 25 mM Tris/acetate at pH 8.0, 6 mM dithiothreitol, 20 mM EDTA, 5 μM *S*-adenosyl[^{3}H]methionine (specific activity 5 Ci/mmol), and appropriate amounts of enzyme solution and was incubated at 37°C for 6 hr. The reaction mixture was subjected to phenol extraction and the DNA precipitated by 75% ethanol was digested with DNase I as described (5). Dinucleotides containing 5-[^{3}H]methylcytosine were obtained by DEAE-cellulose column chromatography in the presence of 7 M urea mixed with nonradioactive marker dinucleotides and were separated by high-voltage paper electrophoresis (5). The paper was cut into 1-cm strips and radioactivity was measured in a toluol-based scintillator. mt^+ is strain 21gr (female) and mt^- is strain 5177D (male). Reactions were run two to five times with each enzyme preparation.

Presence of M_r 200,000 Enzyme in *mat-1* Mutant. Further evidence that the M_r 200,000 enzyme methylates gametic chloroplast DNA comes from a study of the *mat-1* mutation, in which biparental inheritance of chloroplast DNA occurs. The *mat-1* mutation carried by the male and linked to the mt^- nuclear gene gives 50% biparental transmission upon crossing with wild-type females (17). We have recently found that chloroplast DNA of *mat-1* gametes is methylated, in contrast to that of the wild-type male, which is unmethylated (14). If this methylation occurs by the action of the M_r 200,000 methyltransferase, then *mat-1* gametes should contain this enzyme which is missing in the male wild-type (Fig. 1*B*). As shown in Fig. 3, we have found that the M_r 200,000 enzyme is present in extracts from *mat-1* gametes, to the extent of 24% of the total *in vitro* methylating activity.

Symmetrical Methylation Sites in Gamete Chloroplast DNA. Gamete chloroplast DNA is resistant to *Hpa* II cleavage (15), indicating that the internal cytosine in the C-C-G-G sequence is methylated (18). When gamete DNA is digested successively by *Eco*RI and *Hpa* II, the pattern of fragments separated on agarose gels differs considerably from that of vegetative chloroplast DNA treated similarly with the two enzymes (Fig. 4, lanes a and b). The result shows that C-C-G-G

Table 2. DNA methyltransferase activity at various stages in life cycle of *Chlamydomonas*

Stage in life cycle	No. of exps.	Enzyme, units per 10^9 cells (% of total) Total	M_r 60,000	M_r 200,000
mt^+ vegetative	6	3.4	2.8 (86)	0.6 (14)
mt^+ gamete	2	3.1	1.7 (55)	1.4 (45)
mt^- gamete	2	1.8	1.8 (100)	— —
mt^- *mat-1* gamete	2	6.7	5.1 (76)	1.6 (24)
Zygote	3	22.8	— —	22.8 (100)

Total activity was estimated from elution profiles of enzymes from DEAE-cellulose column chromatography in replicate experiments.

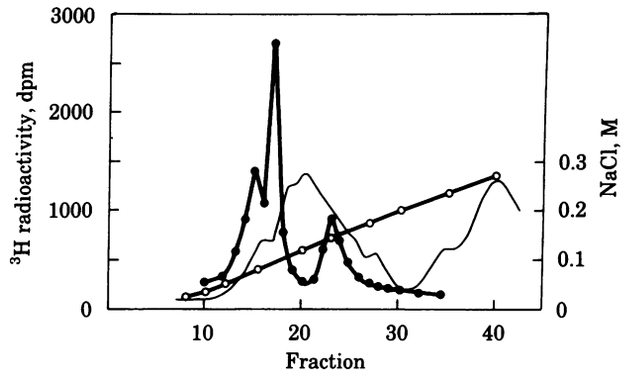

FIG. 3. DEAE-cellulose chromatography of DNA methyltransferase from *mat-1* gametes. ●, Enzyme activity; ○, salt concentration; ——, relative absorbance at 278 nm.

sequences in *Eco*RI fragments of gamete chloroplast DNA are methylated, as previously described (15).

In order to determine whether gamete DNA is methylated on one or both strands, we dissociated it and then annealed it with vegetative DNA (19). Chloroplast DNA from female gametes (which is methylated) was digested with *Eco*RI and labeled at the 5′ ends with [^{32}P]phosphate. A 10-fold excess of vegetative DNA, which is unmethylated, was also digested with *Eco*RI and annealed with ^{32}P-labeled gamete DNA fragments. After reassociation, the DNA was digested with *Hpa* II, which does not cleave the C-C-G-G site in hemimethylated double-stranded DNA (19). If the gametic DNA were biologically hemimethylated, then after dissociation and annealing with vegetative unmethylated DNA, there should be sites available to cleavage by *Hpa* II, such that the final patterns of bands seen by autoradiography would resemble the pattern of vegetative DNA. However, if the gametic DNA had been fully methylated on both strands, then the reassociated material should not be cleaved by *Hpa* II, and the autoradiograms should resemble those of gametic DNA.

In Fig. 4, lanes c and d, it is seen that both vegetative and reassociated DNAs have the same banding pattern as seen by ethidium bromide staining, after digestion with *Eco*RI and *Hpa* II, because the annealed sample contains a 10-fold excess of vegetative DNA. In the autoradiograms, however, the reassociated DNA resembles the gametic pattern, not the vegetative pattern, because the reassociated DNA is resistant to *Hpa* II digestion. This result demonstrates that female gametic DNA is methylated symmetrically on both strands.

Mode of Methylation by M_r 200,000 Enzyme. The methyl group acceptor capacity of chloroplast DNAs from cells at various stages of the life cycle was examined. Because zygote DNA is heavily methylated *in vivo* (15, 16), it should accept fewer methyl groups than vegetative DNA in the *in vitro* methylating reaction catalyzed by the M_r 200,000 enzyme. As shown in Fig. 5*A*, chloroplast DNA isolated from vegetative cells was a better substrate for methylation than that from female gametes. DNA from zygotes accepted less than one-third as many methyl groups as vegetative cell DNA. To determine whether the zygote enzyme can methylate hemimethylated sites, equal amounts of gamete and vegetative DNAs were mixed and digested with *Eco*RI. After denaturation and annealing, DNA was examined for its methylation by the M_r 200,000 enzyme. As shown in Fig. 5*B*, hemimethylated DNA was a better methyl group acceptor than was nonmethylated DNA, indicating that M_r 200,000 enzyme methylates half-methylated sites in DNA more rapidly than it does the nonmethylated sites.

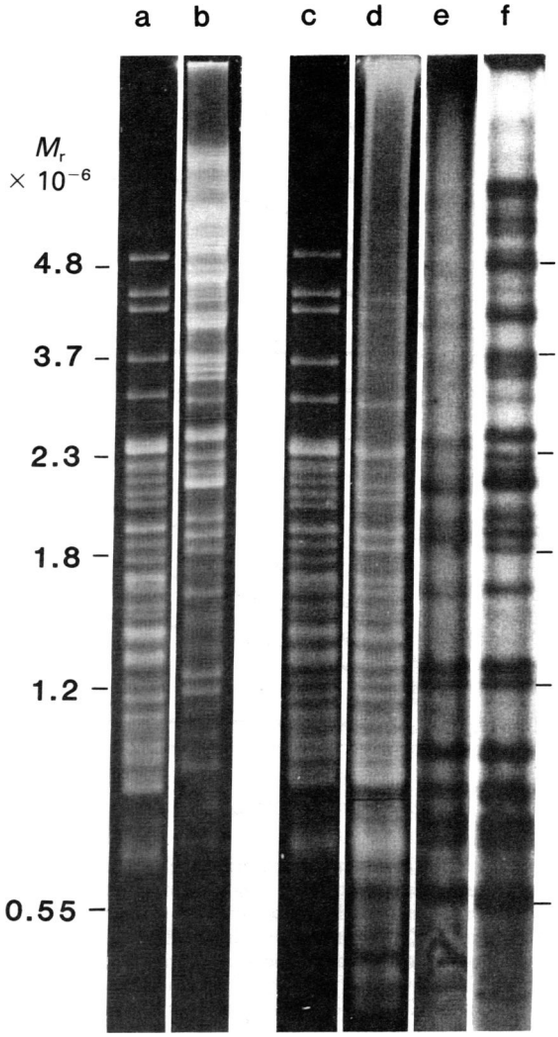

FIG. 4. Effects of *Hpa* II on *Eco*RI fragments of chloroplast DNA. Two micrograms of female vegetative chloroplast DNA (lane a) and gametic chloroplast DNAs (lane b) were digested successively with *Eco*RI and *Hpa* II and electrophoresed on a 0.8% agarose gel. *Eco*RI fragments of 2 μg of female vegetative chloroplast DNA (lane c) and reassociated DNA formed by 0.4 μg of ^{32}P-labeled gamete chloroplast DNA and 5 μg of nonlabeled female vegetative chloroplast DNA (lane d) were digested with *Hpa* II, electrophoresed, and visualized by ethidium bromide staining. The autoradiography of lane d and ^{32}P-labeled gamete chloroplast DNA digested with *Eco*RI and *Hpa* II are shown in lanes e and f, respectively.

DISCUSSION

We have isolated two forms of a DNA methyltransferase from *Chlamydomonas* by DEAE-cellulose chromatography. The enzyme with M_r 60,000 eluted at 0.08 M NaCl and that with M_r 200,000 eluted at 0.15 M NaCl. Attempts to purify these activities further with various ion-exchange and affinity chromatography procedures were not successful because of enzyme lability. We have previously reported a DNA methyltransferase in a different stock of *Chlamydomonas* that eluted from DEAE-cellulose at 0.12 M NaCl (5). Its M_r was estimated as 58,000–60,000, but it differs from the enzyme(s) reported here: (*i*) it adsorbs to phosphocellulose, whereas the present enzymes do not; (*ii*) it methylates primarily T-C-R, whereas the enzymes discussed here methylate primarily the d(C,G) doublet.

The enzyme with M_r 60,000 was found in vegetative and gametic cells of both male and female, with a level of activity of

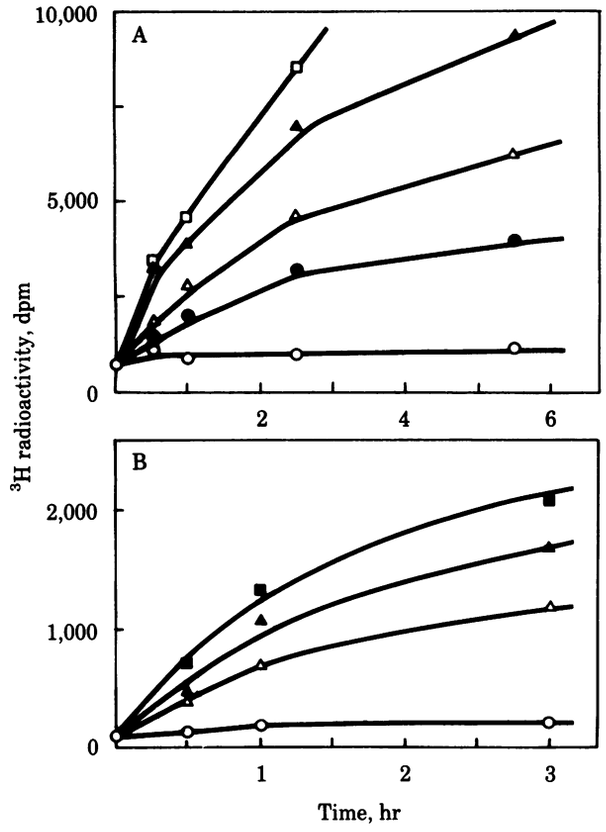

FIG. 5. Kinetics of *in vitro* methylation of chloroplast DNA. (*A*) A 100-μl reaction mixture containing 2 μg of chloroplast DNA and standard assay components was incubated with 3.7 units of zygote enzyme (M_r 200,000) at 37°C. At various time intervals, 20-μl aliquots were withdrawn, treated with phenol, and spotted on a Whatman 3MM paper disc, which was washed with 6% trichloroacetic acid and counted for radioactivity as described. DNA samples are from: ▲, female vegetative cells; △, female gamete cells; ●, zygote cells; and □, *M. luteus*. As the control, the reaction mixture without DNA added was analyzed (○). (*B*) The reaction conditions were the same as for *A* except that DNAs were cleaved by *Eco*RI and reassociated prior to methylation. DNA samples were from: ▲, female vegetative cells; △, female gamete cells; and ■, the reassociation product of equal amounts of female vegetative and female gamete DNAs. As the control, reaction in the absence of DNA is shown (○).

approximately 2 units per 10^9 cells. The enzyme with M_r 200,000 was found specifically in the female gamete, in zygotes, and in gametes of the *mat-1* mutant. The activity of this high molecular weight component was 2-fold higher in the female gametes and 30-fold higher in zygotes than in vegetative cells (Table 2). This increase in activity agrees well with the appearance of methylation in chloroplast DNA as revealed by the antibody (against 5-methylcytosine) binding assay (16).

It is generally accepted that, in eukaryotic DNA, methylation occurs symmetrically and primarily at C-G (20) but the mechanism of methyltransferase activity has remained unclear. We have found that the *Chlamydomonas* methyltransferase described here introduces methyl groups preferentially into d(C,G) and that the reaction occurs with both unmethylated and hemimethylated DNAs, but much more rapidly with the latter. Rapidity of the second-step methylation is similar to that catalyzed by the endo R·*Eco*B enzyme of *E. coli*, which can function as either a restriction nuclease or a modification methylase (21–23).

We propose that the M_r 200,000 enzyme is a multimer of the M_r 60,000 form, and is the active form *in vivo* for the following reasons: (*i*) The two forms show the same nearest-neighbor frequencies of methylation. (*ii*) The peak II activity recovered from DEAE-cellulose, repurified on glycerol gradients, gave both peak I (M_r 60,000) and peak II (M_r 200,000) components, indicating that peak I was being generated from peak II. (*iii*) The M_r 200,000 form was found in gametes of the male mutant *mat-1*, in which gametic chloroplast DNA is methylated (14) and in which inheritance of chloroplast genes is biparental, not maternal. Further support for this proposal will require purification of the enzyme. The possibility that the M_r 200,000 moiety contains a different subunit in addition to the M_r 60,000 form also needs to be examined with purified preparations.

We thank S. James for preparing the manuscript. This work was supported by National Science Foundation Grant PCM78-07643 and National Institutes of Health Grant GM22874 to R.S.

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