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Genetics

Identification of 5-methylcytosine in DNA fragments immobilized on nitrocellulose paper

(¹²⁵I-labeled immunoglobulin G/anti-5-methylcytosine antibody/calf thymus satellite I DNA/chloroplast DNA/φX174 virion DNA)

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ABSTRACT **A method to identify 5-methylcytosine (m⁵Cyt) in DNA immobilized on nitrocellulose paper by using antibody against m⁵Cyt raised in rabbits is described. Immobilized restriction fragments of DNA are incubated first with purified antibody against m⁵Cyt and then with goat anti-rabbit IgG labeled with ¹²⁵I. Restriction fragments containing m⁵Cyt are visualized by autoradiography. By using this method, a heavily methylated fragment of about 1700 base pairs was identified in nuclear DNA fom Chinese hamster cells, the methylation pattern of calf thymus satellite I DNA was examined, and chloroplast DNAs that were extracted from various stages of the *Chlamydomonas* life cycle were compared. Little if any methylation was detected in chloroplast DNA from vegetative cells or from male gametes, whereas homologous DNAs from female gametes and from zygotes were heavily methylated. The sensitivity of the method was examined with calf thymus satellite I DNA (which contains approximately 40 m⁵Cyt residues per repeat unit of 1400 base pairs) and with φX174 virion DNA (which contains a single m⁵Cyt per molecule). The presence of m⁵Cyt was detected with as little as 40 ng of φX174 DNA containing 0.02 pmol of m⁵Cyt and with 100 ng of satellite DNA containing about 0.5 pmol of m⁵Cyt. Thus, the method makes possible the identification of individual methylated sites in purified DNAs in the size range of single genes.**

The only known post-replicative modification of eukaryotic DNA is methylation of cytosine residues at the 5-position (1). A function for 5-methylcytosine (m⁵Cyt) in eukaryotic gene regulation, first proposed by Scarano (2), has been suggested in several recent studies (3, 4). In *Chlamydomonas*, we have demonstrated that the maternal inheritance of chloroplast DNA is regulated by a restriction modification system analogous to that in bacteria (5).

Methods have been developed to identify m⁵Cyt, including paper and liquid chromatography (6–8), two-dimensional electrophoresis (9), and mass spectrometery (10); they identify and quantify m⁵Cyt but provide no information on its location. Precise sequence localization can be established by the method of Maxam and Gilbert (11) but only with uncloned DNA, because sequences cloned in bacteria are not methylated at the uncloned methylation sites. By using pairs of isoschizomeric endonucleases such as *Msp* I which cleaves C-^{m}C-G-G and *Hpa* II which cleaves only the unmethylated C-C-G-G, methylated C-C-G-G sequences can be localized to particular restriction sites (12). However, there are additional methylated sites for which pairs of isoschizomeric nucleases are not available—for instance, as revealed by direct sequence determination of 5S rDNA (13). An immunological method used to identify m⁵Cyt in chromosome preparations showed localized regions of heavy DNA methylation at the centromeres (14) but was not applied to the molecular level.

This paper describes a method to identify m⁵Cyt in DNA immobilized on nitrocellulose paper by using antibody raised against m⁵Cyt. With this method, potentially every methylated cytosine in DNA can be detected. The sensitivity of the method, when examined with φX174 virion DNA containing a single m⁵Cyt, is such that single methylated bases can be recognized and as little as 0.02 pmol of m⁵Cyt can be detected. We have applied this method to satellite I of calf thymus DNA to reveal extensive methylation, including at least three C-C-G-G sequences; to nuclear DNA from Chinese hamster cells, identifying a heavily methylated fragment of about 1700 base pairs (bp); and to chloroplast DNA from various stages of the *Chlamydomonas* life cycle, confirming and extending previous evidence of the role of methylation in chloroplast inheritance (5).

MATERIALS AND METHODS

Antibody Preparation. A 100-mg sample of 5-methylcytidine was conjugated to 280 mg of bovine serum albumin (15) and 1 mg with complete Freund's adjuvant was injected into each rabbit at 2-week intervals (16). When the precipitin test became positive, 50-ml blood samples were taken, and antibody against m⁵Cyt was prepared from serum as described (16) using m⁵Cyt-bovine serum albumin-conjugated Sepharose 4B. Antiserum to rabbit IgG produced in goats were purified by using rabbit immunoglobulin-conjugated Sepharose 4B. Antibody preparations were iodinated as described (17). The iodinated protein was fractionated by Sephadex G-50 column chromatography and purified further by three cycles of precipitation with 40% ammonium sulfate. The final pellet was dissolved in buffer I (10 mM K phosphate, pH 7.1/0.15 M NaCl) and stored at −20°C. The specific activity was 10^6 cpm/μg when prepared.

Filter Binding Assay. Sonicated and denatured DNAs from calf thymus and *Escherichia coli* were labeled at the 5′ end with [γ-³²P]ATP by polynucleotide kinase (11). DNA was incubated with antibody against m⁵Cyt, and the DNA–protein complex was collected on nitrocellulose paper (18). The paper was washed and dried, and radioactivity was measured.

DNA Transfer and Antibody Binding. After restriction endonuclease digestion, the DNAs were loaded onto horizontal agarose gels containing 0.5 μg of ethidium bromide per ml in buffer II (40 mM Tris·HCl, pH 7.8, 5 mM sodium acetate, 1 mM EDTA). Gels (14.5 × 21 × 0.4 cm) were electrophoresed (15 mA for 16 hr), and DNA was detected by UV transillumination. After photography, DNAs were denatured and transferred to nitrocellulose sheets (Schleicher & Schuell, Ba-85, 13 × 16 cm, 0.45 μm pore size) (19), which were baked at 80°C for 4 hr and used for antibody binding. The immunoreaction was performed at 37°C with gentle shaking. The nitrocellulose

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Abbreviations: m⁵Cyt, 5-methylcytosine; bp, base pairs; DBM, diazobenzyloxymethyl.

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sheet was washed in buffer I for 1–2 hr, sealed in a polyethylene bag with 40 ml of buffer I containing 1% gamma globulin and 0.01% sodium azide, and incubated 4 hr. Then, the solution was removed and buffer I (40 ml) containing 100 μg of antibody against m^5Cyt, 0.1% gamma globulin, and 0.01% sodium azide was added, and the mixture was incubated 16 hr. The sheet was washed three times with 300 ml of buffer I, sealed again, and preequilibrated with 40 ml of buffer I containing 0.1% gamma globulin for at least 1 hr. The solution was removed and 40 ml of buffer I containing 20 μg of ^{125}I-labeled anti-rabbit IgG, 0.1% gamma globulin, and 0.01% sodium azide was added, and the mixture was incubated 16 hr. The sheet was washed several times for 2–3 hr with 300 ml of buffer I containing 1% Triton X-100 and 1% deoxycholate until the ^{125}I radioactivity in the washing solution was less than 100 cpm/ml. Finally, the sheet was rinsed well in distilled water, dried at 60°C, and exposed for several hours at −70°C to Kodak XR-5 x-ray film with an intensifying screen.

Other Materials. Restriction endonucleases (*Eco*RI, *Bam*HI, *Hae* III, *Msp* I), φX174 virion DNAs, and polynucleotide kinase were purchased from New England BioLabs. *Hpa* II was from Bethesda Research Laboratories (Rockville, MD). Alkaline phosphatase was from Worthington. 5-Methylcytidine, human gamma globulin, and DNAs from calf thymus and *E. coli* (strain B) were from Sigma. *Chlamydomonas reinhardi* chloroplast DNA (20), and Chinese hamster DNA (21) were prepared as described. Bolton–Hunter reagent (4000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from New England Nuclear. Antiserum to rabbit IgG was purchased from Miles.

RESULTS

Specificity of Antibody Against m^5Cyt. Antibody against m^5Cyt conjugated to bovine serum albumin was produced in rabbits and purified as described above. DNAs from calf thymus, which contain approximately 1.4% m^5Cyt (22), and from *E. coli* were sonicated and denatured. The average chain length obtained was estimated as 100 bases by sucrose density gradient centrifugation. Each DNA fragment was labeled at the 5′ end with ^{32}P and incubated with antibody against m^5Cyt. The DNA–protein complex was collected on a nitrocellulose filter and the amount of DNA was determined. The antibody precipitated only calf thymus DNA (Fig. 1). Nonimmune rabbit gamma globulin reacted with neither DNA. These results show that the antibody reacts exclusively with m^5Cyt in DNA and that nonspecific binding of DNA by protein does not occur under the conditions used. Antibody specificity was checked also by competitive inhibition of calf thymus DNA precipitation. Under the experimental conditions shown in Fig. 1, 50 μM deoxy-5-methylcytidine 5′-monophosphate inhibited the reaction completely, whereas the same amount of deoxycytidine 5′-monophosphate caused less than 10% inhibition.

Identification of m^5Cyt in DNAs Immobilized on Nitrocellulose Paper. Various DNAs digested with restriction endonucleases were electrophoresed on agarose gels and transferred to nitrocellulose sheets. The DNAs were incubated with purified rabbit antibody against m^5Cyt, and then with the second antibody, ^{125}I-labeled goat anti-rabbit IgG. Restriction fragments containing m^5Cyt were identified by autoradiography. Fig. 2 shows the distribution pattern of m^5Cyt in calf thymus DNA digested with *Eco*RI. Satellite I DNA with a repeat length of 1400 bp contains 1.4 mol% of m^5Cyt (22). The satellite DNA was purified and digested with restriction endonucleases, and its m^5Cyt distribution was examined. *Hin*cII cleaved it into three fragments, which seemed to be methylated equally. *Msp* I produced at least four fragments, but *Hpa* II did not cleave this DNA. Thus, the 1400-bp DNA contains at least three C-C-G-G sequences in which the second C is methylated (12). In Fig. 2 (lane g), φX174 RF DNA digested with *Hae* II is shown as a control that does not contain m^5Cyt.

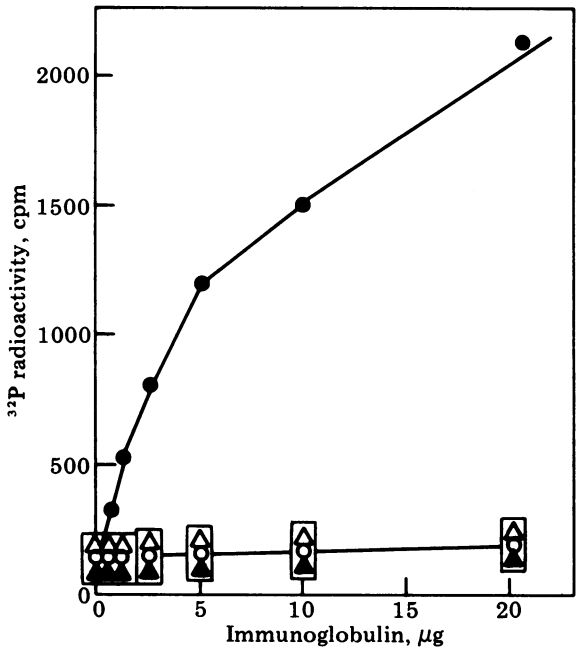

FIG. 1. Precipitation of DNA by antibody against m^5Cyt. DNA in 50 mM Tris·HCl/1 mM EDTA was sonicated three times (15 sec each) at 0°C, precipitated with 2.5 vol of ethanol, dissolved in 50 mM Tris·HCl, pH 9.5 (0.5 mg/ml), heated at 100°C for 10 min, and cooled quickly to 0°C. The solution was incubated (60°C, 1 hr) with 3 units of alkaline phosphatase, and was extracted with phenol. DNA was precipitated with ethanol and was end-labeled with ^{32}P by using polynucleotide kinase. The specific activities of calf thymus DNA and *E. coli* were 7.5×10^4 and 6×10^4 cpm/ng, respectively. A 200-μl reaction mixture for antibody binding containing 10 mM K-phosphate (pH 7.1), 0.15 M NaCl, 1 μg of ^{32}P-labeled DNA, and 10 μg of antibody against m^5Cyt was incubated at 37°C for 30 min; this mixture was made up to 500 μl with buffer I containing 20 μg of tRNA and applied to a membrane filter (HA, 0.45 μm) that had been pretreated with 0.5 M KOH for 30 min and kept in buffer I prior to use. The filter was washed three times with 5 ml of buffer I, dried, and measured for radioactivity. ●, Calf thymus DNA with antibody against m^5Cyt; ○, calf thymus DNA with nonimmune rabbit immunoglobulin; ▲, *E. coli* DNA with antibody against m^5Cyt; △, *E. coli* DNA with nonimmune rabbit immunoglobulin.

The DNA-antibody binding technique was applied to identify m^5Cyt in Chinese hamster cell lines 204-Bu50 (tumorigenic) and 204-Bu50-TuK1 (tumor derived). The latter cell line contains a homogeneous staining region on chromosome 3 (23). It was of interest to see whether we could detect a difference in the m^5Cyt distribution in DNAs from these two cell lines. Nuclear DNA was digested with two restriction endonucleases *Eco*RI and *Bam*HI simultaneously, and positions of m^5Cyt were visualized as shown in Fig. 3. A heavily methylated band corresponding to about 1700 bp was detected by the antibody-binding method in the DNAs from both strains, although it was not visible after ethidium bromide staining. Thus, we have detected a methylated fragment in Chinese hamster DNA present in both cell lines that presumably does not correspond to the homogeneous staining region of 204-Bu50-TuK1.

Differences in methylation of chloroplast DNAs from vegetative cells, gametes, and zygotes of *Chlamydomonas* were shown previously by using the restriction enzymes *Msp* I and *Hpa* II to reveal the methylation of C-C-G-G sites in female

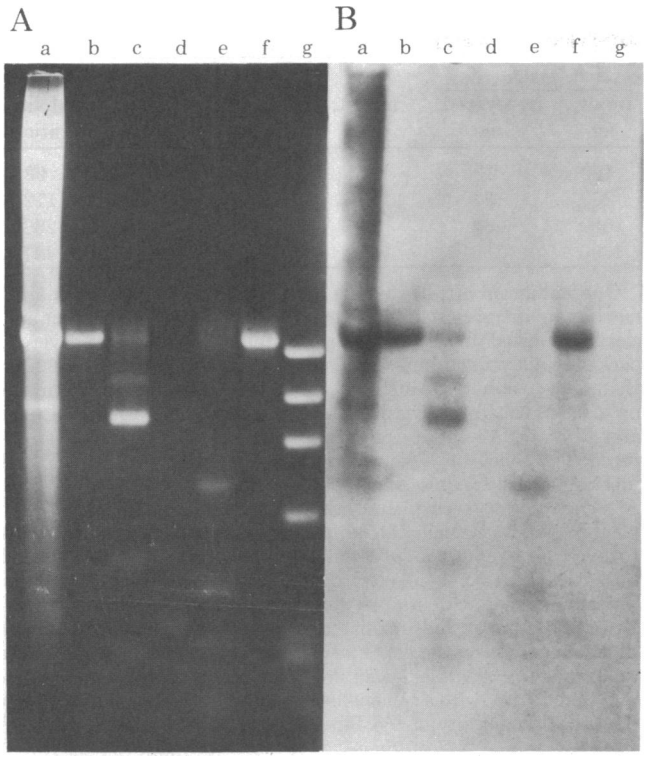

FIG. 2. Identification of m^{5}Cyt in calf thymus DNA. Satellite I DNA (1400 bp) was prepared as described in Table 1; 20-μl reaction mixtures containing 10 mM Tris·HCl (pH 7.6), 6 mM NaCl, 6 mM $MgCl_2$, 6 mM dithiothreitol, 0.5 μg of DNA, and 5 units of restriction endonuclease were incubated at 37°C for 3 hr. Samples were electrophoresed in 1.4% agarose gel at 100 V for 16 hr and photographed after ethidium bromide staining (*A*). After transfer to nitrocellulose paper, DNAs were incubated with antibody and positions of m^{5}Cyt were visualized by autoradiography (*B*). Lanes: a, total calf thymus DNA (10 μg) digested with *Eco*RI; b, purified satellite I DNA; c, satellite I DNA digested with *Hin*cII; d, satellite I DNA digested with *Hae* III; e, satellite I DNA digested with *Msp* I; f, satellite I DNA digested with *Hpa* II; and g, coliphage ϕX174 replicative form DNA (2 μg) digested with *Hae* III.

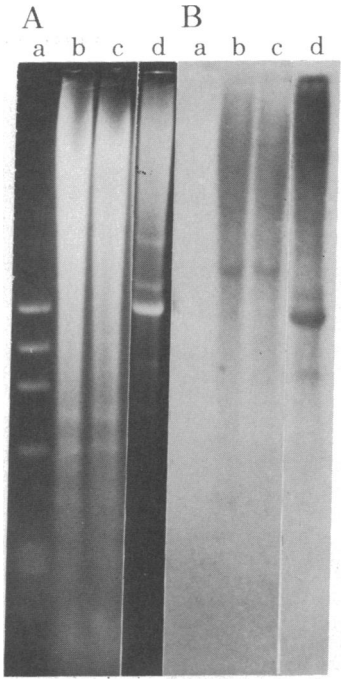

FIG. 3. Identification of m^{5}Cyt in nuclear DNA of Chinese hamster cells. Reaction mixtures (20 μl) containing 0.1 M Tris·HCl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$, 10 μg DNA, and 5 units each of *Eco*RI and *Bam*HI were incubated at 37°C for 3 hr. Samples were treated as described in Fig. 2. (*A*) Ethidium bromide staining. (*B*) Autoradiography. Lanes: a, coliphage ϕX174 replicative form DNA digested with *Hae* III (2 μg); b, Chinese hamster strain 204-Bu50 DNA; c, Chinese hamster strain 204-Bu50-TuK1 DNA; and d, calf thymus total DNA (10 μg) digested with *Eco*RI.

(mt^+) but not in male (mt^-) gametes and in zygotes (20). Fig. 4 shows the *Eco*RI restriction patterns of these DNAs revealed by ethidium bromide staining (Fig. 4*A*) and by antibody against m^{5}Cyt (Fig. 4*B*). No methylation was detected in vegetative DNAs, and just a trace was in the male gamete DNA, whereas both the female gamete and zygote DNAs were heavily methylated.

Quantitative Measurement of m^{5}Cyt. To quantitate the amount of m^{5}Cyt in DNA that can be detected by this method, the following experiments were performed. Calf thymus DNA was digested with *Eco*RI, and satellite I DNA (1400 bp) was purified. The 5′ end of this fragment was labeled with ^{32}P, and different amounts of DNA were electrophoresed, transferred to nitrocellulose paper, and incubated with antibody against m^{5}Cyt and with ^{125}I-labeled goat anti-rabbit IgG as described. After identifying the positions of DNA, the bands were cut out of the nitrocellulose paper and radioactivities of ^{125}I and ^{32}P were measured. Results are shown in Table 1. The amount of DNA recovered was only about 14% of the input amount. This low yield may be due to prolonged incubation at 37°C (72 hr). Antibody binding was not linear with increasing DNA concentration. The amount of m^{5}Cyt present was calculated from the amount of DNA of known m^{5}Cyt content retained on the filter (22). With an input of 100 ng of DNA, for example, approximately 1 m^{5}Cyt was detected for every 100 m^{5}Cyt residues present; this ratio of m^{5}Cyt present to antibody bound decreased as the DNA input concentration was increased (Fig. 5). The reciprocal plot shown in Fig. 5*B* has been used as a standard curve for quantitation.

Identification of m^{5}Cyt in ϕX174 Virion DNA. Whether similar quantitative measurements can be applied to DNA containing just a single methylated base per molecule was examined by using ϕX174 virion DNA, which contains one m^{5}Cyt in its 5386-base single-stranded DNA (9) (but none in the double-stranded replicative form). Experiments were performed as described above, except that viral DNA was not prelabeled with ^{32}P. The amount of bound IgG was determined from ^{125}I radioactivity and the corresponding m^{5}Cyt content was calculated in two ways: (*i*) from the known input of DNA, with 8% recovery (data not shown); and (*ii*) from extrapolation of the standard curve (Fig. 5*B*) based on the satellite I DNA experiment. As shown in Table 2, there is a good coincidence between the observed and calculated amounts of m^{5}Cyt. With an input of 40 ng of ϕX174 DNA, 1 m^{5}Cyt was detected for every 30 m^{5}Cyt residues present and, as with satellite I DNA, this ratio decreased with increasing DNA concentration.

Detection of m^{5}Cyt in DNA Covalently Bound to Diazobenzyloxymethyl (DBM)-Paper. We investigated whether the m^{5}Cyt residues in DNA linked to diazotized (DBM-paper) paper are available for binding to antibody. DBM-paper was synthesized as described (24), and *Eco*RI digests of calf thymus DNA were electrophoresed in agarose gels and transferred to it. After transfer, the remaining reactive diazonium groups were inactivated by incubating the paper in 100 mM Tris, pH 9/0.25% gelatin/1% glycine at 37°C overnight. The paper was then processed for antibody binding and autoradiographed as above.

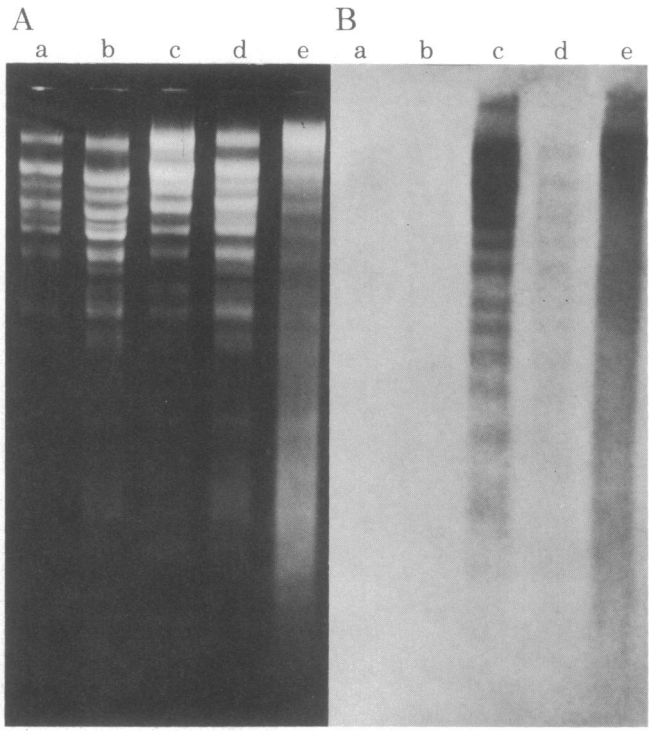

FIG. 4. Identification of m^{5}Cyt in chloroplast DNA of *Chlamydomonas*. Reaction mixtures (50 μl) containing 0.1 M Tris·HCl (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$, 2 μg of DNA, and 25 units of *Eco*RI were incubated at 37°C for 6 hr. Samples were electrophoresed at 100 V for 17 hr. DNAs were visualized by ethidium bromide staining (*A*) and m^{5}Cyt was identified by antibody binding (*B*). Lanes; a, DNA from female vegetative cells; b, DNA from male vegetative cells; c, DNA from female gamete cells; d, DNA from male gamete cells; and e, DNA from zygote cells.

With 4 μg of DNA we obtained intense labeling of the satellite bands and good labeling of other m^{5}Cyt containing fragments comparable to the results in Fig. 2. The nonspecific background was low. Thus, m^{5}Cyt in DNA covalently linked to DBM-paper is accessible to the antibody. DBM-paper is useful because hybridized DNA or RNA can be removed after autoradiography without affecting the underlying DNA that previously had been covalently linked. With our method, one can look for m^{5}Cyt residues in DNA fragments that were previously localized in the paper by DNA or RNA hybridization.

DISCUSSION

A simple method was developed to identify m^{5}Cyt in DNA fragments transferred to nitrocellulose paper after agarose gel electrophoresis. The m^{5}Cyt is visualized by autoradiography after the DNA is allowed to react first with purified antibody raised in rabbits against m^{5}Cyt and then with ^{125}I-labeled goat anti-rabbit IgG. With this method, we identified methylated regions in calf thymus satellite I DNA, in Chinese hamster nuclear DNA digested with *Eco*RI plus *Bam*HI, and in the chloroplast DNA of *Chlamydomonas* isolated from zygotes and from female gametes but not in the chloroplast DNA from male gametes or from vegetative cells. The results with *Chlamydomonas* confirm and extend previous evidence for the role of methylation in the maternal inheritance of chloroplast DNA (5, 8, 20).

We attempted to quantitate the sensitivity of our method in experiments with purified calf thymus satellite I DNA which contains approximately 40 m^{5}Cyt residues in 1400 bp and with ϕX174 virion DNA which contains only 1 m^{5}Cyt per 5386 bases. The recovery of DNA was about 10% after the long incubation at 37°C with antibody preparations and the required extensive washing. The ratio of m^{5}Cyt present in the DNA to antibody bound (measured by γ-emission of ^{125}I) was about 30:1 for ϕX174 and about 100:1 for satellite I DNA at the lowest DNA concentrations tested. With both DNAs, the ratios in-

Table 1. Quantitative measurement of m^{5}Cyt in calf thymus satellite I DNA

DNA Input, ng	DNA Recovered, ng	m^{5}Cyt, pmol	Antibody* cpm	Antibody* pmol	Molar ratio†
100	13	0.55	660	0.0059	93
200	34	1.48	970	0.0086	172
500	67	2.9	1170	0.0104	288
1000	135	5.8	1530	0.0136	427

One milligram of calf thymus total DNA was incubated in 1-ml reaction mixtures containing 25 units of *Eco*RI at 37°C for 16 hr and electrophoresed in 0.8% agarose gel. Satellite I DNA was recovered, packed in dialyzing tubing with 2 ml of buffer II, submerged in buffer II, and electrophoresed at 15 mA for 16 hr. Elution buffer was made up to 0.3 M Na acetate, washed three times with both isoamyl alcohol and ether. DNA was precipitated with 2.5 vol of ethanol and centrifuged. The pellet was dissolved in 100 μl 50 mM Tris·HCl (pH 9) and incubated with 1.5 units of alkaline phosphatase at 40°C for 30 min. After extraction with phenol, the DNA was ^{32}P-labeled at the 5′ position with polynucleotide kinase and mixed with unlabeled DNA to give 7×10^4 cpm/μg. Different amounts of this DNA were loaded on a 1.4% agarose gel and electrophoresed at 75 V (15 mA) for 18 hr. After photography of stained gels, the DNA was transferred to nitrocellulose paper and incubated with antibody preparations. Radioactive bands on the paper were cut into 3-mm strips which were analyzed for ^{125}I with a γ-emission counter (Packard Auto-Gamma) and then for ^{32}P with a toluene-based scintillator (Beckman LS9000). The specific activity of ^{125}I-labeled anti-rabbit IgG was 750 cpm/ng.

* ^{125}I-Labeled anti-rabbit IgG.

† Shown as m^{5}Cyt present/antibody detected.

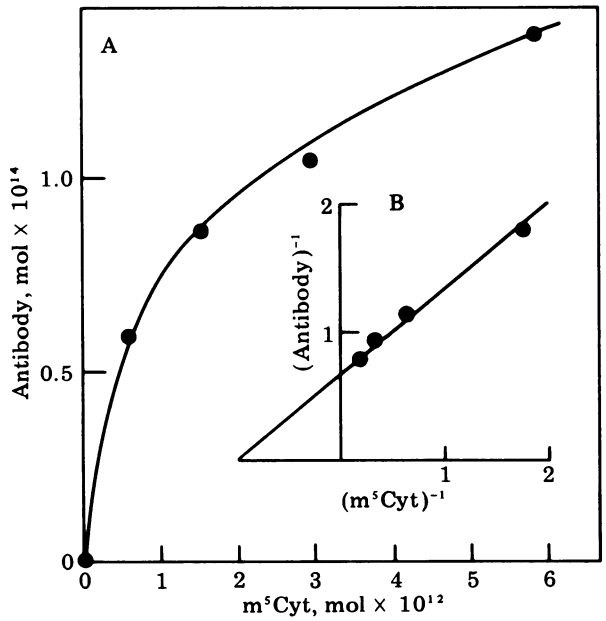

FIG. 5. Quantitation of m^{5}Cyt by antibody reaction. Data of Table 1 plotted to show relationship between antibody bound and m^{5}Cyt content of the satellite I DNA used in the reaction. (*A*) Hyperbolic curve. (*B*) Reciprocal plot used to calculate constants and to serve as the standard curve to estimate m^{5}Cyt concentration from observed antibody concentration. Ordinate is 1/antibody in mol^{-1} $\times 10^{-14}$. Abscissa is 1/m^{5}Cyt in mol^{-1} $\times 10^{-12}$.

Table 2. Identification of m^5Cyt in $\phi X174$ virion DNA

$\phi X174$ virion DNA Recovered, μg	m^5Cyt, pmol	Antibody, pmol $\times 10^2$	Estimated m^5Cyt content, pmol
0.008	0.012	ND*	—
0.04	0.024	0.08	0.05
0.08	0.047	0.15	0.10
0.2	0.12	0.32	0.26
0.4	0.24	0.39	0.33
0.8	0.47	0.48	0.45
1.6	0.94	0.57	0.61

Various amounts of $\phi X174$ virion DNA were electrophoresed in 1% agarose gel at 200 V for 4 hr and treated as described in legend for Table 1. The recovery efficiency of DNA was estimated to be 8% from ^{32}P-labeled calf thymus satellite I DNA electrophoresed in the same gel with samples.
* ND, nondetectable.

creased at higher DNA concentrations (Fig. 5), showing that antibody is bound less efficiently as the DNA concentration is increased. A reciprocal plot gave a straight line which we used as a standard curve to calculate m^5Cyt content from ^{125}I radioactivity. With $\phi X174$ virion DNA, the lowest concentration in which m^5Cyt could be detected by the antibody method was 40 ng, which contains 0.02 pmol of m^5Cyt. With satellite I DNA, the lowest concentration was 100 ng of DNA, which contains 0.5 pmol of m^5Cyt. The greater sensitivity of the method with virion DNA than with satellite I DNA may result from differences in spacing of m^5Cyt along the DNA. The spacing may be too close for optimal antibody binding in satellite I DNA. Also, partial renaturation of double-stranded DNA may occur on the nitrocellulose paper. The decreasing efficiency of detection of m^5Cyt with increasing DNA concentration of both DNAs may result from steric hindrance by DNA (25).

In summary, a single methylated site can be detected in a 5000-bp molecule or fragment, starting with 500 ng of DNA banded in an agarose gel. Under the conditions described, about 50 ng of DNA will be retained after transfer to nitrocellulose paper and incubation with antibody preparations. This level of sensitivity makes possible the identification of single methylated sites in uncloned purified DNAs in the size range of single genes.

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