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Clonal inheritance of the pattern of DNA methylation in mouse cells

(DNA-mediated gene transfer/hemimethylated DNA/maintenance methylation)

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ABSTRACT DNA-mediated gene transfer was used to investigate the mode of inheritance of 5-methylcytosine in mouse L cells. Unmethylated *ϕ*X174 replicative form DNA remains unmethylated after its introduction and integration into these cells. On the other hand, *ϕ*X174 replicative form DNA that was methylated *in vitro* at its C-C-G-G residues retains these methylations as shown by restriction enzyme analysis with *Hpa* II and *Msp* I to detect methylation at this specific site. Although these unselected methylated vectors are prone to lose 30-40% of their methyl moieties upon transfection, this demethylation appears to be random. Once established, the resulting methylation pattern is stable for at least 100 cell generations. In order to examine the specificity of methylation inheritance, fully hemimethylated duplex *ϕ*X174 DNA was synthesized *in vitro* from primed single-strand *ϕ*X174 DNA by using 5-methyl deoxycytidine 5'-triphosphate. This molecule was inserted into mouse L cells by cotransformation and subsequently was analyzed by a series of restriction enzymes. Only methylations located at C-G residues were conserved after many generations of cell growth. The results suggest that the inheritance of the cellular DNA methylation pattern is based on a C-G-specific methylase that operates on newly replicated hemimethylated DNA.

Animal cell DNA contains only one known modified base, 5-methylcytosine (m^5C), and this modification is found almost exclusively at the dinucleotide sequence C-G. Unlike the methylation pattern of prokaryotic DNA, not all C-G sites are methylated in animal cells (1). Through the use of restriction enzymes and specific labeling techniques, it has been estimated that, depending on the specific organism, from 50-70% of these sites are modified in any particular cell (2-4). Furthermore, each methylated site contains two methyl groups symmetrically positioned on both complementary strands ($^{mC\text{-}G}_{G\text{-}Cm}$) (2, 5). Several enzymes, such as *Hpa* II (C-C-G-G) and *Hha* I (G-C-G-C) which are inhibited by methylation at their restriction site C-G residues, can be used to detect site-specific methylations, and together with Southern blotting technology it is possible to assay the methylation state of sites in specific genes (3). From studies on different gene sequences in various tissues of the same organism, it is clear that many genes have a tissue-specific pattern of methylation at C-G sequences (6-8). Thus, at least within one cell type, the pattern of DNA methylation is inherited through many cell divisions (9).

Riggs (10) and Holliday and Pugh (11) constructed a model to explain the mechanism of methylation of DNA. They postulated that symmetrical methylation of both DNA strands coupled with a methylase acting only on half-methylated sites would lead to the maintenance of the methylation pattern on the DNA during replication. Methylated sites would remain methylated; unmethylated sites would remain unmethylated; thus, the total methylation pattern would be clonally inherited.

We have developed a system to test this hypothesis *in vivo* in animal cells growing in culture. By using DNA-mediated gene transfer technology, it is possible to introduce specific unmethylated or methylated DNA sequences into tk^- mouse L cells. The resulting methylation pattern of these gene fragments after many generations in the host cell may then be analyzed by restriction digestion and Southern blot hybridization (9). The results clearly indicate that methylated sites are faithfully inherited but only at C-G-containing sequences.

METHODS

Cell Culture and Transformation. Ltk^- $aprt^-$ mouse cells, a derivative of Ltk^- clone D, was obtained from R. Axel and maintained in Dulbecco's modified Eagle's medium containing 10% (vol/vol) calf serum. These cells were transformed as described (9) with plasmid pBR322 containing the 3.2-kilobase (kb) *tk* DNA fragment of herpes simplex virus type 1, designated pTK DNA. To each Petri dish containing 6×10^5 cells were added 1 ng of pTK DNA, 20 μg of salmon sperm DNA, and 1-2 μg of the unmethylated or *in vitro* methylated cotransforming DNA. In some experiments transfection was performed with 3 ng of pTK DNA that had been methylated *in vitro*. Transformants (tk^+) were selected in Dulbecco's modified Eagle's medium containing hypoxanthine, aminopterin, thymidine, and 10% calf serum. Colonies were picked by using cloning cylinders and grown into mass cultures (9).

Isolation of DNA. Cells were harvested by trypsinization and centrifugation at $1000 \times g$ for 10 min. The pellet was resuspended into 100 vol of 10 mM Tris·HCl, pH 8.0/400 mM NaCl/10 mM EDTA, and $NaDodSO_4$ and proteinase K were added to 0.2% and 100 μg/ml, respectively. The lysate was incubated at 37°C for 3 hr, extracted sequentially with buffer-saturated phenol and chloroform, and the high molecular weight DNA was isolated by ethanol precipitation. This DNA was dissolved in 10 mM Tris·HCl, pH 7.9/0.1 M NaCl/5 mM EDTA and treated for 1 hr at 37°C with RNase (25 μg/ml). After extraction with chloroform, the DNA was precipitated with ethanol and redissolved at a high concentration (5-10 mg/ml). Plasmid pBR322 and its derivatives were propagated in *Escherichia coli* K-12 and purified by the method of Clewell (12). The human growth hormone-containing plasmid, designated pGH, was obtained from H. Goodman. Bacteriophage *ϕ*X174 and *ϕ*X174 replicative form DNAs were prepared as described (13).

Filter Hybridization. DNA (20-50 μg) from transformed cells was digested with restriction endonucleases as recommended by the supplier (New England BioLabs, Bethesda Research Laboratories, or Boehringer Mannheim). Digestions

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Abbreviations: kb, kilobase; m^5C, 5-methylcytosine; m^5dCTP, 5-methyldeoxycytidine 5'-triphosphate.

were carried out at an enzyme-to-DNA ratio of 1.5 units/μg for 2 hr at 37°C. Under these conditions, digestion was found to be complete as determined by restriction analysis of the same cellular DNA together with an internal DNA marker. Reactions were terminated by the addition of EDTA, and the products were electrophoresed on agarose slab gels. DNA fragments were transferred to nitrocellulose sheets, hybridized, and washed as described (9). pBR322, pTK, and ϕX174 replicative form DNAs were nick translated with [α-^{32}P]dATP and [α-^{32}P]dCTP (New England Nuclear) to a level of 2–3 × 10^8 cpm/μg (9).

DNA Methylation *in Vitro*. *Hpa* II DNA methylase was purified from frozen *Hemophilus parainfluenza* through the Sephadex G-50 column chromatography step as described (14). This preparation lacked endonucleolytic activity and *Hpa* I methylase activity (15). Plasmid DNA was incubated at 50 μg/ml in 50 mM Tris, pH 7.9/5 μM S-adenosylmethionine/5 mM dithiothreitol at 37°C for 1 hr with a saturating amount of enzyme (as determined experimentally). The reaction was brought to 0.4 M NaCl/1 mM EDTA/0.2% $NaDodSO_4$ and extracted once with phenol and once with chloroform/isoamyl alcohol, 24:1 (vol/vol), and concentrated by ethanol precipitation. DNA methylated *in vitro* was tested for methylation at the *Hpa* II sites by restriction digestion analysis. Furthermore, the degree of methylation was measured directly by labeling the internal cytosine of the sequence C-C-G-G after digestion with the enzyme *Msp* I (2). In every case methylation was over 98% complete. Every preparation of methylated DNA was treated with a large excess of *Hpa* II to ensure the complete digestion of all unmethylated or partially methylated molecules.

Hemimethylated DNA was synthesized *in vitro* by using primed repair synthesis of bacteriophage ϕX174 single-strand DNA as template. The reaction mixture (100 μl) contained 50 μM each of dATP, dTTP, dGTP, and 5-methyldeoxycytidine 5′-triphosphate (m^5dCTP), 10 mM dithiothreitol, 66 mM Tris·HCl (pH 7.4), 6.6 mM $MgCl_2$, 0.1 mM ATP, 1.5 μg of single-strand ϕX174 DNA, 0.5 μg of 17-mer oligodeoxynucleotide as primer, 15 units of *E. coli* DNA polymerase I, and 1 unit of T4 DNA ligase (16). After incubation at 30°C for 1 hr, the DNA was extracted with phenol and precipitated with ethanol. The ϕX174 DNA was essentially completely converted to the circular duplex form as judged by analytical gel electrophoresis.

Analysis of DNA Demethylation. In many of the cotransformation experiments described here, fully methylated DNA inserted into mouse L cells was found to undergo a certain amount of demethylation. The degree of demethylation could be determined by Southern blot hybridization analysis with the enzyme *Hpa* II. If P is the probability that any particular *Hpa* II restriction site would undergo demethylation, then P^2 is the probability that any particular restriction fragment would appear on the gel because the formation of any band requires two cleavage events. By comparing *Msp* I and *Hpa* II digestion patterns, it is possible to estimate what percentage of the *Msp* I bands remain after *Hpa* II digestion and, thus, to calculate the value of P. For example, if 25% as much DNA appears in a particular *Hpa* II band as compared to the same band produced by *Msp* I, then $P^2 = 0.25$ and $P = 0.5$ suggesting 50% demethylation of any particular site. This analysis can be carried out only for defined ϕX174 DNA restriction fragments in order to avoid the effects of methylations in flanking sequences. In one experiment we estimated the degree of demethylation by monitoring the stability of a ϕX174 DNA *Taq* I fragment that contains three potentially methylated *Hpa* II sites. In this case, the percentage of the *Taq* I sites remaining after *Hpa* II digestion is equal to $(1 - P)^3$ because this fragment will remain intact only if all three *Hpa* II sites remain methylated.

RESULTS

Inheritance of Methylation at C-C-G-G Sequences in Unselected Genes. Although 70% of the C-G dinucleotides in mouse cell DNA are in a methylated state (4), unmethylated foreign DNA sequences introduced into L cells by DNA-mediated gene transfer appear to remain completely unmodified after many generations in the host cells (9). This demonstrates the relative lack of *de novo* methylation in these cells and supports the proposal that unmethylated sites remain unmethylated through cell propagation. In order to test the hypothesis that methylation at specific sites can be clonally inherited, it was necessary to introduce into the cell a DNA molecule methylated at specific C-G residues. To this end, duplex DNA of the bacteriophage ϕX174 (replicative form) was methylated *in vitro* by using the *Hpa* II bacterial methylase, which recognizes and methylates the sequence C-C-G-G. This DNA was proven to be methylated at these sites by the fact that the resulting DNA molecules could not be digested by the restriction enzyme *Hpa* II (15). The methylated ϕX174 molecules were inserted into mouse tk$^-$ L cells by cotransfection with the Herpes simplex virus thymidine kinase (*tk*) gene as a selective marker, and the resulting ϕX174 DNA-containing mouse L cell clones were then analyzed by Southern blot hybridization for the presence of methyl moieties at the *Hpa* II restriction sites in ϕX174 DNA (Fig. 1). The presence of methylation was validated by comparing the digestion pattern obtained with *Hpa* II to that obtained with *Msp* I, an isoschizomer that does not recognize the internal cytosine methylation and, therefore, cleaves normally at methylated sites. Out of 10 clones isolated by cotransfection with methylated ϕX174 DNA, all were found to be either totally or partially methylated at the C-C-G-G sites. In contrast to this, five clones prepared by cotransfection with unmodified ϕX174 DNA remained unmethylated at the *Hpa* II restriction sites.

The inheritance of methyl groups at the C-C-G-G sites was not unique to ϕX174 DNA. When pBR322 (Fig. 1) or pGH (data

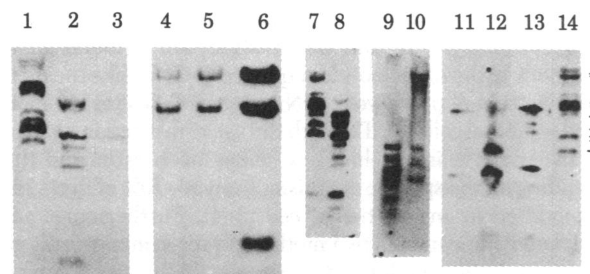

FIG. 1. Detection of methylated DNA in tk$^+$ clones. DNA methylated *in vitro* by the *Hpa* II methylase was inserted into Ltk$^-$ mouse cells by DNA-mediated gene transfer. Methylated ϕX174 replicative form (clones RMϕLH2, RMϕLH7, and MϕLH1) and unmethylated ϕX174 replicative form (clone RϕLH1) DNAs were introduced into cells by cotransformation using pTK DNA as the selectable gene vector. Clone MLH13 was obtained after transformation of L cells with pTK DNA methylated *in vitro*. High molecular weight DNA (50 μg) from each of these clones was restriction digested and analyzed by Southern blot hybridization with nick-translated ϕX174 replicative form probe (lanes 1–8, 11–14) or pBR322 probes (lanes 9 and 10). The presence of methylated C-C-G-G residues was detected by digestion with *Hpa* II or *Msp* I. Lanes: 1 and 2, clone RMϕLH7; 4 and 5, clone RϕLH1; 7 and 8, clone RMϕLH2; 9 and 10, clone MLH13; 3 and 6, marker ϕX174 replicative form DNA digested with *Hpa* II (the two largest fragments are 2.7 and 1.7 kb); 12–14, clone MϕLH1 digested with *Taq* I (lane 13), *Hpa* II (lane 14), or both *Taq* I/*Hpa* II (lane 12). All possible *Hpa* II partial digestion products of ϕX174 DNA are indicated in the space adjacent to lane 14. The heavy lines correspond to the 2.7- and 1.7-kb fragments. Lane 11 contains the marker ϕX174 replicative form DNA digested with *Taq* I (the two bands seen in this blot are 2.9 and 1.2 kb).

not shown) were methylated *in vitro* in the same manner, these DNAs also retained their *Hpa* II methylations after transfer to mouse L cells. In both cases, unmodified DNA remained unmethylated in recipient clones.

Cotransfection experiments using methylated DNA were all performed with DNA in which greater than 98% of the *Hpa* II restriction sites were methylated in both strands, as judged by the direct detection of m^5C at these sites (15). In contrast to the almost complete methylation of the vector molecule, the integrated sequences were found to be only partially methylated. This can be readily seen from the blot hybridization shown in Figs. 1 and 2 because in clones containing methylated DNA, several of the bands appearing in the *Msp* I lane also appear more faintly in the *Hpa* II digests. Furthermore, in several cases the additional bands produced by *Hpa* II correspond to the partial digestion products of the original DNA sequences. This is especially obvious for clone MϕLH 1 shown in Fig. 1. All of the clones that we examined probably contain multiple copies of the ϕX174 genome as estimated from the band intensities as compared to single copy gene standards. The digestion patterns of these clones suggest that demethylation occurs randomly at almost all possible sites in the ϕX174 molecule.

The restriction digestion pattern of integrated ϕX174 DNA cleaved with *Hpa* II can be used to quantitatively estimate the degree of demethylation of these molecules. Comparing the intensities of specific identified fragments from restriction analysis of >10 methylated clones (including those shown in Figs. 1 and 2), we estimate that about 10–20% of the DNA seen in the *Msp* I bands remained after digestion with *Hpa* II. Because the formation of each band required two separate cleavage events, we calculate that approximately 30–40% of the C-C-G-G sites underwent demethylation. An alternative technique for measuring the amount of demethylation is to look at the sensitivity of a defined methylated DNA fragment to the enzyme *Hpa* II. Digestion of ϕX174 DNA with *Taq* I produced 10 fragments including one of 2.9 kb that contains three clustered sites for the enzyme *Hpa* II (17). When ϕX174-containing L cell DNA was digested with both *Taq* I and *Hpa* II, approximately 20–30% of the 2.9-kb *Taq* I fragment remained intact. This was examined in three separate clones, one of which is shown in Fig. 1. Because this fragment will remain undigested only in the case that all three C-C-G-G sites are methylated, it can be calculated that 30–40% of these specific sites underwent demethylation. It should be kept in mind that these calculations represent rough estimates of the actual degree of demethylation and that there are large variations between clones.

Stability of the DNA Methylation Pattern. The mouse DNAs used for the hybridization analyses were obtained from transformed clones after 25–30 generations of growth in selective medium because this is generally the time necessary to obtain enough DNA for restriction studies. In order to examine the genetic stability of this modification, cells were grown for additional periods of time. Even after 100 generations of growth, the methylation pattern of any particular clone remained constant, indicating that the methyl modification, once established, is stably inherited for many generations (Fig. 2).

The C-G residues of the specific sequence C-C-G-G represent only a small fraction (about 6%) of the total C-Gs of any animal DNA. Of course, the *in vitro* methylated DNA is unmodified at all C-G sites other than at the specific sequence C-C-G-G. It was of interest to determine whether these other C-G-containing sites undergo methylation together with the *Hpa* II sites after the introduction of this DNA into L cells by cotransfection. To this end, we digested the DNA from several of the methylated ϕX174-containing clones with *Hha* I, an enzyme that recognizes the tetranucleotide sequence G-C-G-C and is inhibited by methylation of the internal C. These sites are also heavily methylated in cellular DNA (18). The *Hha* I sites in ϕX174 replicative form remained unmethylated (Fig. 3), further supporting the concept that methylated sites are inherited, but even adjacent unmethylated sites remain unmodified. The ϕX174 replicative form DNA used in these studies was isolated from ϕX174-infected *E. coli* and, therefore, is methylated at the internal cytosine residue of the sequence C-C-$\frac{A}{T}$-G-G (19). Although the enzyme *Eco*RII will not cut these methylated sites in ϕX174 DNA, an isoschizomer, *Bst*NI cleaves this DNA normally (20). ϕX174 DNA, which integrated into the cellular genome after transfection, lost these methyl moieties, as shown by the fact that both *Eco*RII and *Bst*nI cleaved this DNA identically (Fig. 3). This suggests that maintenance methylation does not function at every cytosine-containing sequence and is probably specific to the symmetrical dinucleotide C-G.

Sequence Specificity of Maintenance Methylation. The experiments with DNA methylated *in vitro* at the *Hpa* II sites showed that methylation at this specific sequence (C-C-G-G)

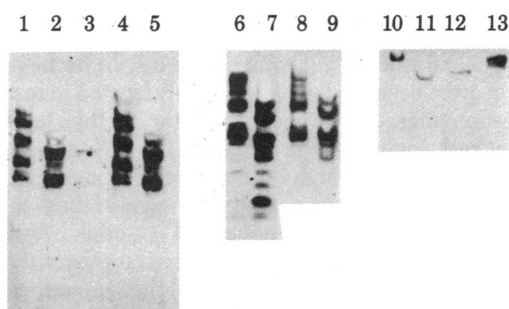

FIG. 2. Stability of DNA methylation in tk$^+$ transformants. Mouse Ltk$^-$ cells were transformed with pTK DNA and cotransformed with ϕX174 replicative form DNA methylated *in vitro* by *Hpa* II methylase. Three ϕX174 DNA-containing clones were isolated and grown into mass culture. DNA was isolated from these clones after growth for 30 and 100 generations and analyzed by Southern blot hybridization with a nick-translated ϕX174 replicative form DNA probe. All DNA samples were digested with *Hpa* II or *Msp* I: clone MϕLH1 at 30 generations (lanes 1 and 2) and 100 generations (lanes 4 and 5), clone MϕLH3 at 30 generations (lanes 6 and 7) and 100 generations (lanes 8 and 9), and clone MϕLH4 at 30 generations (lanes 10 and 11) and 100 generations (lanes 13 and 12). Marker ϕX174 replicative form DNA digested with *Msp* I is shown in lane 3.

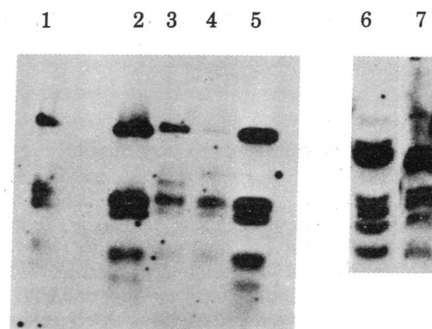

FIG. 3. Analysis of methylation at *Hha* I and *Eco*RII restriction sites. High molecular weight DNA was obtained from clones cotransformed with ϕX174 DNA methylated *in vitro* by *Hpa* II methylase. This DNA was digested by restriction enzymes and analyzed by Southern blot hybridization with a nick-translated ϕX174 replicative form DNA probe. Clones MϕLH1 (lane 1), MϕLH3 (lane 3), and MϕLH4 (lane 4) were digested with *Hha* I. Marker ϕX174 replicative form DNA (lanes 2 and 5) was digested with *Hha* I (the bands correspond to 1.6, 0.6, 0.5, and 0.3 kb). Clone MϕLH1 was also digested with *Eco*RII (lane 6) and *Bst*NI (lane 7).

can be inherited and, thus, passed on to succeeding generations. This is presumably carried out by a maintenance enzyme that, during DNA replication, uses the methyl group on the template strand to direct the accurate methylation of the newly synthesized DNA. It was of great interest to study the specificity of this process and, in particular, to determine which m^5C residues are inheritable. This experiment could be done by introducing into L cells a DNA molecule in which all cytosine residues were methylated. For this purpose, we synthesized ϕX174 replicative form DNA *in vitro* using primed repair synthesis in the presence of m^5dCTP in place of dCTP. In the resulting ϕX174 replicative form DNA, the complementary strand (−) is totally methylated at every cytosine (except the five cytosine residues of the primer) whereas the viral strand (+) is unmethylated. This hemimethylated molecule is resistant to digestion by every restriction enzyme that is responsive to m^5C in its restriction site, including the enzymes *Hpa* II (C-C-G-G), *Msp* I (C-C-G-G), *Hae* III (G-G-C-C), *Alu* I (A-G-C-T), *Hha* I (G-C-G-C), *Eco*RII (C-C$\frac{A}{T}$-G-G), and *Sac* II (C-C-G-C-G-G) (20). This DNA was introduced into L cells by cotransfection, and the resulting ϕX174 DNA-containing clones were analyzed by restriction digestion and hybridization. Each clone contains multiple copies of the ϕX174 genome. As expected, all of the integrated ϕX174 molecules were highly resistant to *Hpa* II, indicating that these sites remained methylated at the internal cytosine of the sequence C-C-G-G (Fig. 4).

In striking contrast, *Msp* I cleaved the ϕX174 DNA at all of its C-C-G-G sites. Because this enzyme is known to be inhibited by methylation of the external cytosine (21), we may conclude that this methyl moiety was not preserved in these cells. Thus, despite the fact that both cytosine residues were methylated in the original molecule, only the methylated C-G was clonally inherited by the daughter cells. Two other restriction sites that contain C-G in their recognition sequence were found to remain highly methylated as shown by the fact that *Hha* I (Fig. 4) and *Sac* II (data not shown) were unable to cleave the integrated ϕX174 DNA. On the other hand, the enzymes *Alu* I (A-G-C-T) (data not shown), *Hae* III (G-G-C-C) (Fig. 4), and *Eco*RII (C-C$\frac{A}{T}$-G-G) (Fig. 3; unpublished results) cleaved the ϕX174 genome normally. The data suggest that only methylated C-G sequences can be genetically transmitted.

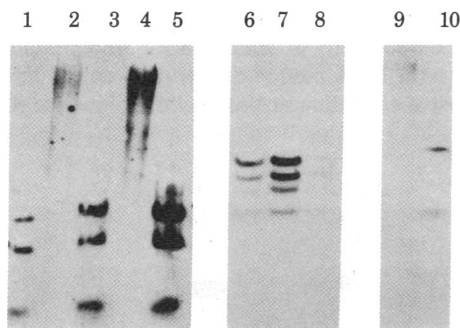

FIG. 4. Detection of methylation in tk$^+$ clones cotransformed with hemimethylated ϕX174 DNA. Ltk$^-$ mouse cells were transformed with pTK DNA and cotransformed with hemimethylated ϕX174 DNA. DNA prepared from ϕX174-containing clones was digested with various restriction enzymes and analyzed by Southern blot hybridization with a nick-translated ϕX174 replicative form DNA probe. Clone HMϕLH1 was digested with *Msp* I (lane 5), *Hpa* II (lane 4), *Hae* III (lane 7), and *Hha* I (lane 9). Clone HMϕLH2 was digested with *Msp* I (lane 3), *Hpa* II (lane 2), and *Hae* III (lane 6). Marker ϕX174 replicative form DNA was digested with *Msp* I (lane 1), *Hae* III (lane 8) (the larger fragments correspond to 1.35, 1.1, 0.9, and 0.6 kb), and *Hha* I (lane 10). Several other digestions of these same clones are not shown in this figure. Both *Alu* I and *Eco*RII digested the integrated ϕX174 DNA as expected. To test the methylation of the one ϕX174 DNA *Sac* II restriction site, DNA was double digested with *Hpa* I/*Sac* II. In all cases the 3.7-kb *Hpa* I fragment containing the *Sac* II site remained intact after digestion with *Sac* II.

DISCUSSION

DNA-mediated gene transfer has been used to investigate the metabolism and inheritance of methyl groups on DNA. Gene sequences introduced into Ltk$^-$ mouse cells by cotransfection together with the selective marker thymidine kinase appear to be integrated into the host genome and stably inherited over many generations (22). Our results conclusively show that the methylation pattern of these foreign DNA sequences is clonally inherited if these methyl moieties are present in C-G residues. These conclusions are based on two experimental observations. DNA that is selectively methylated at the specific restriction site for *Hpa* II (C-C-G-G) remains methylated at these and *only these* sites after many generations of growth within animal cells. Furthermore, ϕX174 replicative form DNA methylated at every cytosine of the complementary strand synthesized *in vitro* retained its m^5C at C-G sites, while totally losing methylations of cytosine residues at other sequences.

These observations essentially confirm the model of DNA methylation suggested by Riggs (10) and by Holliday and Pugh (11). Because DNA methylation is a postreplication modification, during synthesis the DNA is methylated only in the parental strand. A maintenance methylase that is specific for hemimethylated sites will then methylate the newly synthesized strand. Thus, the parental methyl moieties serve as a form of template for the methylation of the opposite strand. By this process, methylated sites will continually remain methylated, whereas unmethylated sites will stay unmethylated in daughter cells.

The key element in this model is the symmetry of the methylated sites. Without some form of strand symmetry it would be impossible to faithfully transfer the methylation pattern from generation to generation. In animal cells this symmetry is provided by the unique dinucleotide sequence C-G. If we assume that methylation is based on dinucleotide symmetry, no other cytosine-containing dinucleotide can be inheritably methylated for lack of a cytosine in the opposite strand. Although the sequence G-C has symmetrical cytosines in both strands, the methylating enzyme does not recognize this as a methylatable site. The symmetrical placement of methylated moieties seems to be a general rule for all living organisms. In bacteria methylation of cytosine and adenine are mainly located in restriction modification sites, and in most cases the methylated base is found on both complementary strands (19). DNAs from higher plants have a relatively high content of m^5C, sometimes reaching 29% of the total cytosine. Although many of these m^5C residues are in the dinucleotide sequence C-G, other cytosine residues are also methylated. Recent studies show that these m^5Cs are always found on both DNA strands at the symmetrical trinucleotide sequence C-X-G, where X can be A, T, or C (29).

Whereas methylation occurs at C-X-G sequences in plant DNA, these methylations are not inheritable in mouse L cells. This can be deduced from the observation that methylation at *Eco*RII (C-C$\frac{A}{T}$-G-G) sites is not preserved in these animal cells, although this sequence is highly methylated in plant DNA. Furthermore, the animal cell methylase was unable to transmit methylations at the external cytosine residue of the *Msp* I (C-C-G-G) sites. This cytosine is partially methylated in higher plants. The results of this experiment are especially puzzling because it has been reported that this cytosine is methylated in a tissue-specific manner at *Msp* I sites (23), including those at the 5′ ends of the human γ^A and γ^G genes (21). Since meth-

ylation of the external cytosine of the sequence C-C-G-G is very rare in animal cells, these gene-specific methylations may represent a special type of DNA modification, which may occur only in specific cell types or certain chromosomal settings.

The original models of DNA methylation predicted that modification is carried out by a methylating enzyme that is specific for hemimethylated DNA. Studies *in vitro* with partially purified DNA methylases from animal cells clearly show that both double-strand and single-strand DNA are relatively poor substrates for methylation, whereas presumed hemimethylated DNA is more active (1). We recently used hemimethylated ϕX174 replicative form DNA as a substrate for a partially purified methylase from mouse ascites cells and obtained methylation of the unmethylated strand at every C-G residue. Furthermore, this substrate was methylated at a rate 200-fold greater than that of ϕX174 replicative form double-strand DNA (unpublished results). This type of selectivity could explain how the cell methylase can transfer existing methylations to succeeding generations without causing extensive methylation at previously unmethylated sites. *De novo* methylation (24, 25) may occur through a second enzyme or by means of the maintenance enzyme acting under special conditions. In all of our experiments we have detected little, if any, *de novo* methylation at the *Hpa* II or *Hha* I sites of DNA sequences inserted into mouse L cells by DNA-mediated gene transfer (9). It should be noted, however, that if this process does take place at a low rate, it would go undetected in our clones unless it occurred early in the transfection experiment.

Although methylation is usually coupled to DNA replication, the methylase enzyme may also be active during other periods of the cell cycle. Hemimethylated ϕX174 DNA, which was introduced into mouse L cells, was found to be fully methylated in ϕX174-containing clones. These molecules must have undergone modification of the complementary strand preceding replication because otherwise only 50% of the DNA sequences would retain the methyl groups. This type of replication-independent methylation might be involved in other cell functions, such as DNA repair synthesis.

DNA methylated *in vitro* at C-C-G-G sites underwent demethylation upon transfer and integration into mouse L cells. This demethylation was of the order of 30–40% for unselected markers such as ϕX174 and pBR322 DNAs. The mechanism of this apparent demethylation is not understood, but one could suggest several possible mechanisms. (*i*) Demethylation may occur prior to replication by an enzyme that selectively removes methyl groups from either one or both strands of the DNA. (*ii*) Demethylation may occur prior to replication by DNA repair synthesis without subsequent methylation of the appropriate cytosines. (*iii*) Demethylation may occur during cell replication if the normal DNA maintenance methylase is inhibited. In all of these possibilities, of course, any demethylation would be transmitted to future generations of cells. Our results indicate that demethylation of unselected markers is a one-time event that probably occurs early in the transfection process because, in all of the clones we examined, the DNA methylation pattern remained fixed for more than at least 100 cell generations. Because the processing and integration of transferred DNA is not well understood, it would be difficult to speculate on the correctness of any of these models. The high degree of demethylation may be a function of the type or extent of *in vitro* methylation of the vector molecule. Hemimethylated ϕX174 DNA, for example, underwent almost no demethylation at *Hpa* II, *Hha* I, or *Sac* II restriction sites.

Studies involving restriction analysis of various genes have clearly demonstrated a close correlation between specific gene activity and undermethylation of certain restriction sites (26). Furthermore, it has been shown that on the average, active genes are about 50% undermethylated at all C-G residues (27). The results of *tk* transfection experiments support this correlation because the efficiency of DNA-mediated gene transfer with *tk* gene that was methylated *in vitro* was 2–5 times lower than with the unmethylated plasmid (9, 28). Vector DNA from clones obtained from cells transfected with *in vitro* methylated *tk* gene (and selected for the tk^+ phenotype) were found to be highly unmethylated. Additionally, we observed that this undermethylation is greatest in the region of the *tk* gene itself, as judged by the analysis of a specific C-G site in the coding region of the gene (unpublished results). The results support the model that demethylation may afford an advantage to the thymidine kinase gene when these cells are grown in selective medium.

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