Methylation of foreign DNA sequences in eukaryotic cells

(DNA-mediated gene transfer/thymidine kinase/5-methylcytosine/Southern blotting)

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ABSTRACT The herpesvirus thymidine kinase gene has been used to introduce foreign DNA sequences into mouse L cells by DNA-mediated gene transfer. These inserted genes were then assayed for methylation at the specific sequence C-C-G-G by using the restriction enzyme isoschizomers *Hpa* II and *Msp* I. Despite the fact that 70% of the cellular C-C-G-G sites are methylated, herpesvirus sequences, plasmid DNA, and growth hormone gene DNA were found to remain unmethylated in 90% of the clones that contain these genes. DNA that had been methylated *in vitro* with *Hpa* II methylase was also inserted into L cells. The presence of this modification in the vector DNA did not, however, guarantee that these sequences remained methylated in the recipient clones. Only 10% of all transformed clones were found to contain methylated C-C-G-G sequences in the vector DNA, and these modifications were stable for 25-50 generations. *Hha* I and *Mbo* I were used to probe for methyl groups at these restriction sites, but none of the inserted sequences acquired these modifications. These results are discussed in relation to various models put forth to explain the process of methylation in eukaryotic cells.

The role of DNA methylation in eukaryotes is not well understood. 5-Methylcytosine is the only methylated base in eukaryotic DNA, and it is present at a level of about 1% of the total nucleotides (1). These methyl groups are not randomly distributed in the genome; 50-90% of the 5-methylcytosine residues are concentrated in highly repeated satellite sequences (2-4). Methylcytosine moieties also seem to be preferentially localized on regions of the chromosome that are protected from micrococcal nuclease digestion (1).

Over 90% of these methyl groups are present in the dinucleotide CpG (5), and restriction enzymes that recognize 5-methylcytosine and CpG can be used to assay and locate methyl groups in eukaryotic DNA. One of these enzymes, *Hpa* II, recognizes the tetranucleotide sequence C-C-G-G but will not cut at this site if the internal C is methylated (6). In contrast, *Msp* I cuts at this same sequence even when the internal C is methylated. Together, these enzymes can be used to identify methyl moieties in specific gene sequences.

In this study we address our attention to the mechanism of methylation in cultured L cells. There are numerous questions about the mode of methylation that have remained unanswered due to the lack of a suitable system for studying this process. Is there *de novo* methylation in eukaryotic cells? Are methyl groups passed on to succeeding generations by a semiconservative mechanism? Is there active demethylation of DNA? By using DNA-mediated gene transfer of the thymidine kinase (*tk*) gene into *tk*⁻ cells (7), we have succeeded in inserting foreign DNA into mammalian cells and analyzing its methylation pattern. The results show that this foreign DNA is rarely methylated in its host chromosome and suggest that methylation is controlled by *cis*- rather than *trans*-acting factors.

METHODS

Cell Culture and Transformation. Ltk⁻aprt⁻, a derivative of Ltk⁻ clone D (8), 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 (7) with plasmid pBR322 containing the 3.2-kilobase *tk* DNA fragment of herpes simplex virus type 1 (HSV-1) (pTK). To each petri dish containing 6×10^5 cells were added 1 ng of pTK DNA or pTK DNA that had been methylated *in vitro* and 20 μ g of salmon sperm DNA. Cotransformation (9) was carried out in the presence of 1 μ g of pBR322 or methylated pBR322 DNA. Transformants (*tk*⁺) were selected in modified Eagle's medium containing hypoxanthine, aminopterin, and thymidine and 10% calf serum. Colonies were picked by using cloning cylinders and grown into mass cultures.

Isolation of DNA. Cells were harvested by trypsinization and centrifugation at $1000 \times g$ for 10 min. The pellet was resuspended in 100 vol of 10 mM Tris-HCl, pH 8.0/400 mM NaCl/10 mM EDTA, and NaDodSO₄ and proteinase K were added to 0.2% and 100 μ g/ml, respectively. The lysate was incubated at 37°C for 3 hr and then extracted sequentially with buffer-saturated phenol and chloroform. High molecular weight DNA was isolated by mixing the aqueous phase with 2 vol of cold ethanol and immediately removing the precipitate that formed. 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 25 μ g of RNase per ml. After extraction with chloroform, the DNA was precipitated with ethanol and redissolved at a high concentration (5-10 mg/ml). Plasmid pBR322 was propagated in *Escherichia coli* K-12 and purified by the method of Clewell (10).

Filter Hybridization. DNA from transformed cells was digested with restriction endonucleases under the conditions given by the supplier (New England BioLabs or Boehringer Mannheim). Digestions were performed at an enzyme-to-DNA ratio of 1.5 units/ μ g for 2 hr at 37°C. Reactions were terminated by addition of EDTA and the products were electrophoresed on agarose slab gels. DNA fragments were transferred to nitrocellulose sheets, hybridized, and washed as described (11). pTK DNA was nick translated with [α -³²P]dATP and dCTP (New England Nuclear) to a level of $2-3 \times 10^8$ cpm/ μ g (11).

In Vitro DNA Methylation. *Hpa* II DNA methylase was purified from frozen *Hemophilus parainfluenza* through the Sephadex G-50 column chromatography step by the method of Mann and Smith (12). This preparation lacked endonucleolytic activity as well as *Hpa* I methylase activity. Plasmid DNA was incubated in 50 mM Tris-HCl, pH 7.9/5 μ M S-adenosylmethionine/5 mM dithiothreitol at 37°C for 1 hr at a DNA concentration of 50 μ g/ml with a saturating amount of enzyme (as determined experimentally). The mixture was brought to

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Abbreviations: HSV, herpes simplex virus; *tk*, thymidine kinase gene; BRL, Buffalo rat liver.

0.4 M NaCl/1 mM EDTA/0.2% NaDodSO₄, extracted once with phenol and once with chloroform/isoamyl alcohol, 24:1 (vol/vol), and concentrated by ethanol precipitation.

RESULTS

Mouse L cells lacking the thymidine kinase (*Ltk*⁻) gene can be transformed by a cloned *tk* gene originally isolated from herpes simplex virus (HSV) DNA (7). Resulting transformants naturally contain copies of the vector DNA (13). Additional DNA sequences for which there is no selection procedure may be inserted into the same mouse cells by taking advantage of co-transformation (9). In this way, ϕ X174, pBR322, and rabbit β -globin gene sequences have been introduced into mouse cells (9).

We have isolated and examined 14 clones that have been transformed by the recombinant plasmid pTK which carries the HSV 3.2-kilobase *Bam*HI fragment containing the *tk* gene. These clones have been studied for the presence of methylation at the site C-C-G-G by Southern blotting after digestion of the DNA with the isoschizomers *Hpa* II and *Msp* I. pBR322, the parental plasmid, has 26 sites for these enzymes (14), and the HSV fragment probably contains a large, although unknown, number of sites. The pattern of digestion of pTK DNA with *Hpa* II is shown in Fig. 1. By comparison of the bands derived from pBR322 alone with those obtained from pTK, one can determine which fragments are derived from the HSV *Bam*HI fragment containing the *tk* gene. It is not known whether any of these bands are part of the *tk* gene itself. Most of the fragments produced by *Hpa* II digestion are too small to be detected by this gel electrophoresis/blotting system.

The digestion pattern of several *tk*⁺ transformed clones with *Hpa* II and *Msp* I is shown in Fig. 1. Because both digestion patterns are identical, we can conclude that the pTK DNA sequences in all of these clones are unmethylated at the C-C-G-G sites. We have estimated by restriction mapping that most of these clones contain between one and three copies of the pTK plasmid. One clone, PLH4, prepared by cotransformation with a large excess of pBR322 DNA, carries multiple copies (10–20) of the pBR322 sequences; all of these seem to be unmethylated.

The fact that the DNA methylation pattern of a eukaryotic DNA is stable suggests the existence of a mechanism for transmitting this pattern from generation to generation, probably during DNA replication. Because most, if not all, of these methyl groups are symmetrically placed complementary to each other (15), it is tempting to postulate that these methyl

groups are replicated in a semiconservative manner. Immediately after DNA replication, the newly synthesized DNA strands would be methylated at a site complementary to the methyl moieties on the parental strand. This mechanism preserves the state of methylation of the genome during cell division and predicts that foreign unmethylated genes would not undergo methylation. One way to test this hypothesis is to insert *methylated* gene sequences into L cells by means of DNA-mediated gene transfer. To this end pTK and pBR322 DNAs were methylated *in vitro* by using the C-C-G-G-specific methylase isolated from *H. parainfluenza*. DNA methylated in this manner was completely resistant to digestion by *Hpa* II while giving a normal digestion pattern with *Msp* I (results not shown).

Despite the heavy level of methylation, pTK DNA was competent to transform *tk*⁻ cells. As shown in Fig. 2, this DNA was 50% as efficient as unmethylated pTK DNA in a biological assay for gene activity. In numerous experiments of this sort we consistently observed a 2- to 5-fold inhibition of transformation with a methylated *tk* gene.

The transformation activity of methylated DNA is not due to the presence of unmethylated pTK DNA molecules in the plasmid population. In order to ensure maximal methylation, all *in vitro* preparations were treated with a 4-fold excess of *Hpa* II to digest any plasmid DNA molecules that may have escaped the methylase reaction. The percentage of DNA molecules that are not completely methylated *in vitro* has been quantitated by Southern blot analysis of the methylated plasmid DNA digested with *Hpa* II. This highly sensitive assay demonstrated that less than 0.001% of the plasmid molecules are digestible by *Hpa* II.

When clones obtained from transformation with methylated pTK DNA were analyzed by blotting, it was found that both the HSV *tk* and pBR322 sequences were present in the host in an unmethylated state (Table 1). These results suggest that the presence of a modification in the vector DNA, even in a potentially methylatable sequence (C-C-G-G), is not sufficient to ensure its propagation in eukaryotic cells.

Although most clones were found to be unmethylated, in both the pBR322 plasmid sequences and the fragment derived from HSV three clones were observed in which the integrated foreign sequences were indeed methylated (Fig. 3). Each of these clones gave the usual pattern when digested with *Msp* I but an altered pattern upon digestion with *Hpa* II. LH10 has several copies

FIG. 1. Digestion of transformed cell DNA with *Hpa* II and *Msp* I. High molecular weight DNA from *tk*⁺ clones was digested with *Msp* I or *Hpa* II, electrophoresed on 2.4% agarose, and analyzed by Southern blotting and hybridization with pTK DNA. Fifty micrograms of DNA from clones LH6, LH9, and LH8 and 20 μ g of DNA from clone PLH4 were digested with *Msp* I (lanes 3, 5, 8, and 11) or *Hpa* II (lanes 4, 6, 9, and 12). One hundred picograms of pBR322 DNA (lane 1) or pTK DNA (lanes 2, 7, and 10) were digested with *Msp* I to provide markers for the gel.

FIG. 2. DNA-mediated gene transfer with pTK DNA that had been methylated *in vitro*. Methylated (Δ) and unmethylated ($\circ$) pTK DNAs were used in a typical transfection reaction. The actual amounts of vector DNA (ng/plate) were quantitated by blotting analysis. Each data point represents the average number of colonies from three plates.

Table 1. Analysis of the clones obtained by transformation with methylated and unmethylated DNA

Vector DNA	Total no. of clones	No. of methylated clones
pTK and pBR322	14	1
pTK and pBR322 (methylated)	8	2
pGH	8	0

tk⁻ mouse L cells were transformed with plasmid pTK and cotransformed with pBR322, and both the HSV and plasmid sequences were analyzed for methylation at the site C-C-G-G by restriction enzyme analysis. One methylated clone (LH10) was obtained from these experiments. Two types of experiments were performed with methylated *tk* DNA. Out of four clones transformed with pTK DNA that had been methylated *in vitro*, none of the incorporated sequences showed methylation. Clones produced by cotransformation with unmethylated pTK DNA with a large excess of pBR322 DNA that had been methylated *in vitro* were also tested for methylation. Two methylated clones, MPLH4 and MPLH5, were identified from this series of experiments. It is possible that the other two clones may not have picked up methylated pBR322 sequences in the cotransformation. BRL clones containing the human variant growth hormone gene plasmid (pGH) were isolated after DNA-mediated gene transfer of *tk*⁻ BRL cells by using pTK and pGH as vectors. These BRL clones were isolated by R. Axel.

of the pTK genome, at least one of which seems to be completely unmethylated. The methylated DNA appears as a high molecular weight, broad band. Due to the high-percentage agarose gel used in this experiment, transfer of DNA was not efficient in this size range, resulting in a loss of material. Clones MPLH4 and MPLH5 are particularly interesting because they were prepared by cotransformation of unmethylated pTK DNA with a large excess of pBR322 DNA that had been methylated *in vitro*. By restriction mapping and comparison with other clones, we estimate that MPLH5 contains 20–30 copies of the pBR322 DNA. As seen in Fig. 3, almost all of these copies are highly methylated at the C-C-G-G sites. The DNA used in Fig. 3 was obtained from dividing cells after 25–30 divisions. When grown an additional 25 generations, clones MPLH4 and MPLH5 retained their methyl groups, whereas clone LH10 lost many of its methyl groups.

Because in most of our clones the foreign DNA was unmethylated, we questioned whether this was unique to the HSV and plasmid DNAs used in the study. These vectors are not naturally methylated at the C-C-G-G sites and may lack some

intrinsic signals for L-cell methylation present only in eukaryotic DNA. In order to test this possibility, we took advantage of clones prepared by the transfer of pTK DNA into *tk*⁻ Buffalo rat liver (BRL) cells and cotransformation with excess quantities of a cloned human variant growth hormone plasmid. These BRL clones contain multiple copies of the growth hormone genome. When assayed by restriction enzymes, it was found that in all clones the growth hormone gene remained unmethylated at C-C-G-G sites (Fig. 4). The pBR322 sequences present in these same cells were also completely unmethylated (data not shown). In summary, only 10% of the clones examined in this study showed methylation of transferred DNA (Table 1). One of these clones (LH10) appeared to result from *de novo* methylation, whereas two clones (MPLH4 and MPLH5) probably inherited the methyl moieties that were inserted *in vitro*.

Methylation at C-C-G-G sites represents only a fraction of the cellular methylation sites. In order to gain a more complete picture of the metabolism of methylation, we analyzed possible methylations at other sites. Because eukaryotic methylation occurs almost exclusively at sites containing the sequence CpG, we analyzed transformed clones with enzymes that recognize these sites. The enzyme *Hha* I (recognition sequence G-C-G-C) is unable to digest when the internal C is methylated (6). By using this enzyme it has been demonstrated that mouse DNA is about 70–80% methylated at this site (6, 16). Ten clones were examined with *Hha* I and the inserted sequences were found to be unmethylated in every case. Even those clones that have methyl groups at C-C-G-G sites within the inserted DNA were not methylated at the *Hha* I recognition sites (Fig. 5). Two clones were digested by *Taq* I (recognition sequence T-C-G-A) and found to be unmodified at this site. There is no evidence, however, that this site is at all methylated in eukaryotic DNA.

All of the DNAs used in this study for DNA-mediated gene transfer were obtained from plasmids grown in *E. coli* K-12. Because this bacterium contains its own methylases, all plasmid-associated DNA is modified by the methylation of adenine at the site G-A-T-C. As a result, these DNAs cannot be digested by the enzyme *Mbo* I (14) but they are sensitive to its isoschizomer *Sau*3A (Fig. 5). It was of interest to examine whether this premethylated site remained methylated after transformation. As shown in Fig. 5, the vector's G-A-T-C sites were all sensitive to *Mbo* I digestion, even in clones that contained methyl moieties at C-C-G-G sites. This result, of course, is not surprising in lieu of the fact that 6-methyladenine has not so far been described in vertebrate cells.

FIG. 3. Detection of methylated pTK and pBR322 sequences in *tk*⁺ clones. DNA from clones MPLH5 (20 μ g), MPLH4 (40 μ g), and LH10 (50 μ g) was digested with either *Msp* I (lanes 3, 5, and 6) or *Hpa* II (lanes 2, 4, and 7) and subjected to Southern blotting analysis with nick-translated pTK DNA as probe. Lanes 1 and 8 contain 100 pg of pTK DNA cut with *Msp* I.

FIG. 4. Analysis of human variant growth hormone genes in BRL cells. *tk*⁻ cells transformed with the HSV *tk* gene and cotransformed with a pBR322 plasmid containing the human growth hormone gene were analyzed by Southern blot techniques. Ten micrograms of four separate clones were digested with *Hpa* II (lanes 2, 4, 6, and 8) and *Msp* I (lanes 3, 5, 7, and 9). One hundred picograms of the growth hormone gene-containing plasmid was digested with *Msp* I and used as a marker (lane 1). Growth hormone-specific DNA was probed by using the *Eco*RI fragment of the pBR plasmid.

FIG. 5. Analysis of *tk*⁺ transformed clones with other restriction enzymes sensitive to methylation. Twenty micrograms of PLH4 DNA was cleaved with *Hha* I (lane 6) or *Mbo* I (lane 4). Seven micrograms of MPLH5 DNA was digested with *Hha* I (lane 7), *Mbo* I (lane 5), or *Taq* I (lane 2). Nick-translated pTK DNA was used as a probe. The schematic patterns of pTK DNA digested with *Mbo* I (lane 3) and *Taq* I (lane 1) or pBR322 DNA digested with *Hha* I (lane 8) are provided for reference. These digestion profiles were determined experimentally by using *Taq* I, *Hha* I, and *Sau*3A (isoschizomer of *Mbo* I).

DISCUSSION

Although the methylation pattern of mammalian DNA is not well established, it is clear that specific sequences are highly methylated in these cells. In particular, the sequences C-C-G-G (*Hpa* II recognition site) and G-C-G-C (*Hha* I recognition site) are 70–80% methylated. Exogenous DNA sequences integrated into this cellular DNA, on the other hand, are, in most cases, completely unmethylated at these same sites. These observations point out the specificity and perhaps the importance of the methylation reaction. Unlike prokaryotes, in which particular sites in the DNA are consistently methylated, eukaryotic cells clearly have a mechanism for the differential placement of methyl moieties.

In attempting to understand this phenomenon, we investigated the possibility that methylation is a semiconservative process whereby the pattern of methyl groups is transferred to the next generation during replication by insertion of complementary methyl groups at sites that are already methylated. If this were the case, unmethylated DNA vectors would always remain unmethylated after integration whereas methylated DNA would retain its methyl moieties because it provides a proper template for methylation. Our data suggest that this is indeed the case; in almost every instance, unmethylated gene sequences remained unmethylated in the transformed clone. On the other hand, in two clones prepared by cotransformation of unmethylated pTK DNA with pBR322 DNA that had been methylated *in vitro* the methyl moieties appeared to be inherited.

pTK vector DNA that had been methylated *in vitro*, although transforming *tk*[−] L cells, did not, in general, retain its methyl groups. Several explanations could account for this observation. Although a demethylase could have removed the methyl moieties either before or after integration, it is more likely that these methylation sites were diluted out by replication in the absence of concomitant methylation. We cannot rule out the possibility that the methyl groups were, in fact, inherited for several generations but slowly diluted out by the time the cells were harvested and analyzed (25–30 generations). Whatever the explanation, these results suggest that the presence of a methyl group is not sufficient to guarantee its inheritance in eukaryotic cells.

In one case, integrated foreign DNA underwent *de novo* methylation at sites that were previously unmethylated. This modification was found to be only partially stable, however, and was considerably decreased after 75–100 generations of growth. *De novo* methylation has been predicted by several theoretical models (17, 18) and has been detected in integrated viral sequences from transformed cells (19, 20). It is clear that further work is necessary to elucidate the frequency and mechanism of *de novo* methylation in eukaryotes.

These observations, when taken together, give us a good indication of the potential of the cell to manipulate DNA methylation. Although the methylation pattern of the cell is relatively fixed and passed on to future generations, the cell has the mechanisms necessary to add new methyl groups and remove previously existing modifications. Presumably this capability also applies to the normal genetic material of the cell.

One possible function of methyl groups in DNA is protection of the DNA from site-specific endonucleases. Such systems have not been reported in eukaryotes. Furthermore, the partial methylation of particular DNA sequences in eukaryotic DNA argues against such a role for methylation. In support of this idea we have shown that the methylated *tk* gene is almost as efficient as unmethylated DNA in the transfer of this marker to *tk*[−] cells. This strongly suggests that the methylations at the C-C-G-G sites are not part of a restriction modification system.

Several observations suggest that there is a relationship between DNA methylation and gene expression. In particular, certain genes seem to be relatively undermethylated in cells in which that gene is expressed. This correlation has been observed in the globin gene (21, 22), the ovalbumin gene (23, 24), and several viral genes (19, 20). In the present study it is difficult to make any association between gene activity and methylation. It should be noted, however, that methylation of the *tk* gene partly inhibits its ability to transform Ltk[−] cells. In addition, all the clones transformed by methylated pTK DNA contained only unmethylated pTK DNA sequences. In other clones found to contain methylated pTK DNA sequences (Fig. 3), it is difficult to determine whether the expressed *tk* gene itself was methylated. Because we have no specific map of the HSV 3.2-kilobase fragment, we cannot identify the fragments originating from the *tk* gene. It is likely that these fragments are very small and not detectable in our gel system. The fact that several clones contain multiple copies of pTK DNA also complicates the picture because there is no way to know which copies are actively transcribed.

We propose the following model to explain the pattern of methylation in eukaryotes. Several lines of evidence point to the idea that there are domains of methyl groups separated by areas that are undermethylated. That this is indeed the case in sea urchins has been elegantly demonstrated by Bird *et al.* (25). It is also suggested both by the fact that highly methylated satellite sequences are tandemly distributed and by experiments using DNA fiber autoradiography (26). Because methylation would occur primarily within these domains, foreign DNA might undergo methylation only when integrated within these regions. This could explain why some clones were highly methylated while others remained completely unmethylated. The observation that most of the clones remained unmethylated may suggest selective pressure against integration of the gene within a methylated domain, an idea that is consistent with evidence that active genes are relatively undermethylated. This selection process, inherent in the DNA-mediated gene transfer technique, may also explain why we were unable to detect any methylation at sites for the enzyme *Hha* I. Perhaps undermethylation at these sites is a prerequisite for gene expression.

Note Added in Proof. In recent experiments we have inserted *in vitro* methylated ϕ X DNA into mouse L cells. We find that this DNA remains partially methylated after many cell generations, further suggesting that 5-methylcytosine is inherited in a semiconservative manner.

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