In Vitro Methylation of Bovine Papillomavirus Alters Its Ability To Transform Mouse Cells

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Bovine papillomavirus (BPV) was methylated in vitro at either the 29 *HpaII* sites, the 27 *HhaI* sites, or both. Methylation of the *HpaII* sites reduced transformation by the virus two- to sixfold, while methylation at *HhaI* sites increased transformation two- to fourfold. DNA methylated at both *HpaII* and *HhaI* sites did not differ detectably from unmethylated DNA in its efficiency of transformation. These results indicate that specific methylation sites, rather than the absolute level of methylated cytosine residues, are important in determining the effects on transformation and that the negative effects of methylation at some sites can be compensated for by methylation at other sites. BPV molecules in cells transformed by methylated BPV DNA contained little or no methylation, indicating that the pattern of methylation was not faithfully retained in these extrachromosomally replicating molecules. Methylation at the *HpaII* sites (but not the *HhaI* sites) in the cloned BPV plasmid or in pBR322 also inhibited transformation of the plasmids into *Escherichia coli* HB101 cells.

Bovine papillomavirus type 1 (BPV-1) is a DNA virus which causes benign fibropapillomas in cattle but which can cause fibrosarcomas in other hosts (20). In culture, the virus transforms some mouse fibroblast lines, including NIH 3T3 and C127 cells (7). NIH 3T3 or C127 cells that are transformed by the viral DNA are anchorage independent and tumorigenic in nude mice (7). In vivo and in vitro, the viral DNA persists in the transformed cells as an extrachromosomal element (23). In culture, a 69% subgenomic fragment is sufficient to induce transformation (24), and the remainder of the viral genome, which encodes structural proteins, is not expressed at a detectable level in mouse cells (14).

BPV has been used successfully as a vector for the extrachromosomal maintenance of several different genes in mouse fibroblast cells, including the rat preproinsulin gene (37), the phosphotransferase neomycin resistance gene of Tn5 (22), the human metallothionein gene (16), the human β -globin gene (5), the human beta interferon gene (31, 51), and other genes (32, 48). Its use as a vector system has numerous advantages, including the fact that the inserted gene of interest is maintained extrachromosomally, thereby eliminating position effects due to the position of integration of a transferred sequence. Another advantage to the extrachromosomal location is that BPV DNA or chromatin or both can be partially purified from the bulk of the genome (32, 34).

A large number of studies have implicated methylation of cytosine residues in the control of eucaryotic gene expression and other cellular processes. An inverse correlation between the extent of methylation and the amount of expression has been established for a large number of genes (6), while a positive correlation has been noted in a few cases (1, 44). For still other genes, no correlation can be detected by available techniques (30, 50). Studies involving the use of in vitro methylation with purified bacterial or mammalian methylase enzymes have demonstrated that methylation is sufficient to reduce or eliminate the expression of some genes (6). Although a strong correlation has been established, the mechanism(s) by which methylation affects gene expression is still largely unknown. Several mechanisms

have been proposed, including direct inhibition of the polymerase as a result of protrusion of the methyl groups into the major groove of the DNA helix, effects on binding of regulatory proteins, and changes in chromatin conformation brought about by methylation of regions of the DNA (6, 8).

Several types of viral DNA often contain methylated cytosine residues when they are integrated into the host chromosome. In several cases, the presence of this methylation has been correlated with the lack of expression of certain viral genes (11, 43, 46). In vitro methylation of some (29, 38), but not all (9, 41), viral DNAs appears to inhibit their ability to transform or infect cells in culture.

In this study, we examined the effects of in vitro methylation on transformation by cloned BPV DNA. The frequency of transformation of C127 cells was decreased two- to sixfold by methylation at *HpaII* sites and increased two- to fourfold by methylation at *HhaI* sites. Since there are similar numbers of sites for the two enzymes in the virus and plasmid sequences, the results suggest that the sites or arrangement of sites, rather than the overall level of methylation, are important for determining the effects of methylation. Cloned viral DNA which was methylated at both *HpaII* and *HhaI* sites was not reduced in its ability to transform C127 cells, suggesting that the effects of methylation at *HhaI* and *HpaII* sites were additive and able to compensate for each other. These results are consistent with the hypothesis that methylation affects transformation by altering the conformation of the supercoiled viral DNA. Alternatively, the two sets of methylation sites could affect different regulatory proteins, one which exerts a positive effect on transformation and one which exerts a negative effect on transformation.

MATERIALS AND METHODS

Cell culture and gene transfer. Mouse C127 cells were obtained from the American Type Culture Collection, Rockville, Md., and maintained in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% fetal calf serum. Transfections were done by the calcium phosphate precipitation method (10). Cells were seeded at 0.5×10^6 to 1.0×10^6 cells per 60-mm dish. The following day, 0.5 to 1.0 μ g of plasmid DNA and 10 μ g

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of salmon sperm carrier DNA were added directly to the medium. DNA was removed after 12 to 24 h of incubation with the cells. In most experiments, cells were split 1:3 24 h after removal of the DNA. Foci were scored at 2 to 3 weeks.

Plasmid DNA. Plasmid pdBPV-1 was a gift of Peter Howley, National Cancer Institute, Bethesda, Md. The construct contains the entire BPV-1 viral genome inserted into pML2d, a deletion derivative of pBR322 which lacks the poison sequences (26, 36). Plasmid DNAs were grown up in *Escherichia coli* HB101 (*dam*⁺) cells and purified by the method of Guerry et al. (12).

In vitro methylation. *Hpa*II and *Hha*I methylase enzymes were obtained from New England BioLabs, Inc., Beverly, Mass. DNA was methylated in vitro; the conditions suggested by the manufacturer were used with a few modifications. Briefly, DNA was incubated with 1 to 2 U of methylase enzyme per μ g of DNA at 37°C in a reaction mixture containing 50 mM Tris (pH 7.5), 10 mM EDTA, 5 mM 2-mercaptoethanol, and 100 μ g of *S*-adenosylmethionine per ml. Incubations were carried out for several hours. Mock-methylated DNA was prepared in a similar manner, except that *S*-adenosylmethionine was omitted from the reaction mixture. Following methylation, the DNA was extracted once with phenol, once with phenol-chloroform (1:1, vol/vol), and once with chloroform. Extracted DNA was ethanol precipitated prior to use. The extent of methylation was estimated by digestion with the appropriate enzyme followed by gel electrophoresis and ethidium bromide staining or Southern blotting and hybridization, or both.

RESULTS

In vitro methylation of cloned BPV-1 DNA. Plasmid pdBPV-1 (142-6) (Fig. 1) contains 29 *Hpa*II sites, 12 within the viral sequences (3) and 17 within the plasmid vector sequences (42). Many of the *Hpa*II sites in the virus are clustered near the viral origin of replication and postulated regulatory sequences (Fig. 1). There are no *Hpa*II sites within the 39% nontransforming region of the virus. The plasmid also contains 27 *Hha*I sites (Fig. 1), 10 within the viral sequences (3) and 17 within the plasmid vector sequences (42).

Plasmid pdBPV-1 was methylated in vitro with either *Hpa*II or *Hha*I methylase. After the methylation reaction, the DNA was digested with the appropriate endonuclease and electrophoresed through 1% agarose to monitor the extent of methylation. The fragments generated were visualized by ethidium bromide staining (Fig. 2a) and by Southern blotting and hybridization to labeled pdBPV-1 plasmid DNA (Fig. 2b). The methylation typically was estimated to be more than 90% complete, although some variability was observed from reaction to reaction and some lots of methylase enzyme possessed more activity than others. Interestingly, certain *Hpa*II sites on the plasmid appeared to be more refractory to methylation than others (data not shown).

In vitro methylation at *Hpa*II or *Hha*I sites affected the frequency of focus formation in mouse fibroblast cells. The efficiency of transformation of *Hpa*II-methylated pdBPV-1 DNA was tested by calcium phosphate precipitation into mouse C127 cells (Table 1). The number of foci induced by *Hpa*II-methylated DNA reproducibly was reduced two- to sixfold relative to those for mock-methylated DNA. To eliminate the possibility that some or all of the foci generated by the methylated DNA were due to the presence of unmethylated molecules within the methylated population,

FIG. 1. Partial restriction map of plasmid pdBPV-1(142-6). Plasmid pdBPV-1(142-6) consists of the entire BPV-1 genome (7.95 kilobases) inserted into the single *Bam*HI site of plasmid pML2d (2.65 kilobases), a deletion derivative of pML2, which lacks the poison sequences (26, 36). The stippled area corresponds to the 69% transforming region of the virus, which is necessary and sufficient for transformation in cultured mouse cells (24). The open area represents the 31% nontransforming region of the virus, which is not detectably expressed in cultured mouse cells (14). The two *Bam*HI sites and the single *Hind*III site are denoted by B and H, respectively. The 29 *Hpa*II sites in the plasmid and viral sequences are represented by open circles. The 27 *Hha*I sites in the plasmid and viral sequences are represented by closed squares. The regions of the viral genome which are transcribed into early (E) or late (L) mRNAs are noted. The actual locations of the various transcripts are not represented. The plasmid maintenance sequences, regions of the virus which are important for its maintenance as an extrachromosomal element (27), are denoted PMS1 and PMS2. Two separate putative enhancer-activator elements have been described (25, 39). One is termed the distal enhancer and is located between bases 4390 and 4451, approximately 200 base pairs 3' to the polyadenylation site (25). However, recent evidence has suggested that this putative enhancer element is not essential for transformation or plasmid maintenance, but does appear to suppress the effects of a *cis*-inhibitory element in pML2 sequences (15). A second enhancer element has recently been described in the noncoding region of the virus (39), which has previously been shown to contain other regulatory elements, including the origin of viral replication and a sequence necessary for extrachromosomal maintenance of the plasmid. Locations of the TATAAA and AATAAA elements are marked. The region of the virus which contains the putative origin of replication is located within the region of the virus which is not transcribed, near the *Hind*III site within the 69% transforming fragment (3).

DNA was digested with *Hpa*II endonuclease prior to transfection. In these experiments, *Hpa*II cleavage did not significantly decrease the frequency of focus formation of methylated DNA, although the ability of the mock-methylated DNA to transform the cells was completely abolished by *Hpa*II digestion (not shown). These data indicate that the foci generated by the methylated DNA preparations were induced by methylated *Hpa*II-resistant molecules.

In contrast to the results obtained with methylation at *Hpa*II sites, DNA methylated at *Hha*I sites had a higher efficiency of transformation than mock-methylated DNA (Table 2). In both experiments, methylation of the pdBPV-1 plasmid at *Hha*I sites before transfection increased the

FIG. 2. Analysis of DNA methylated in vitro with *HpaII* methylase and *HhaI* methylase. Plasmid pdBPV-1(142-6) (0.5 μ g per lane) was methylated in vitro with *HpaII* methylase alone (lanes 1 and 5), *HhaI* methylase alone (lanes 2 and 6), or both methylases (lanes 3 and 7), or mock-methylated (lanes 4 and 8). (a) Methylated DNAs were digested with either *HpaII* (lanes 1 through 4) or *HhaI* (lanes 5 through 8), electrophoresed through 1% agarose, and stained with 25 μ g of ethidium bromide per ml. (b) The same preparation of DNA, 50 pg per lane, electrophoresed through 1% agarose, transferred to nitrocellulose, and hybridized to labeled pdBPV-1(142-6) DNA. The three high-molecular-weight bands visible in lanes 1, 3, 6, and 7 correspond to open circular, linear, and supercoiled forms of the plasmid DNA. As estimated from these data, the *HpaII* methylation reaction was approximately 90% complete, while the *HhaI* methylation reaction was approximately 95% complete.

frequency of transformation by about two- to fourfold relative to the frequency obtained with mock-methylated DNA.

To eliminate the possibility that the effects of methylation were mediated through sites on the plasmid portion of the molecule, the BPV DNA was removed from pBR322 by digestion with *Bam*HI after methylation at the *HpaII* sites. In three experiments, *HpaII* methylation reduced the number of foci induced by linearized BPV alone by a factor of 2 to 4, while in the same experiments, the frequency of focus formation by intact plasmid was reduced by a factor of approximately 3 (data not shown). These data demonstrate that most or all of the reduction in transformation frequency after *HpaII* methylation was a result of methylation of sites within the BPV genome.

Plasmid pdBPV-1 DNA methylated in vitro with both *HpaII* and *HhaI* methylases was tested for the ability to transform C127 cells. The efficiency of focus formation of

TABLE 1. Efficiency of focus formation with *HpaII*-methylated or mock-methylated DNA

Expt	No. of foci/ μ g of DNA for			% of control (Mock-methylated)
	Untreated DNA	Mock-methylated DNA	Methylated DNA	
1	54.4	ND	17.2	32 ^a
2	12.4	11.6	5.4	47
3	18.0	12.5	2.0	16
4	10.0	10.0	3.6	36
5	28.3	13.7	3.7	27
6	ND	122.5	39.0	32
7	60.0	86.4	4.0	5

^a Untreated DNA used as control for experiment 1.

TABLE 2. Efficiency of focus formation with *HhaI*-methylated or mock-methylated BPV DNA

Expt	No. of foci/ μ g of DNA for:			% of control
	Untreated DNA	Mock-methylated DNA	Methylated DNA	
1	103.5	73.5	283.2	385
2	122.5	102	287.1	281

the doubly methylated DNA was approximately the same as for mock-methylated DNA, suggesting that the effects of *HpaII* and *HhaI* methylation were additive and counteracted each other (Table 3).

Lack of methylation of BPV sequences in individual foci generated from methylated DNA. To analyze the state of methylation of the BPV sequences in the transformed cells, individual transformed foci were picked and grown into mass culture. DNA was isolated and analyzed by restriction endonuclease digestion, electrophoresis, Southern blotting, and hybridization to labeled pdBPV-1 plasmid DNA. BPV sequences isolated from cells transformed with either methylated or mock-methylated BPV DNA were unmethylated at all detectable sites (Fig. 3 shows the *HpaII* analysis; *HhaI* analysis is not shown). In general, BPV molecules were extrachromosomal, whether the DNA used as donor was methylated or unmethylated, although evidence of occasional occurrence of integrated molecules was noted (not shown).

Inhibition of ability of plasmid to transform *E. coli* HB101 by methylation of pdBPV-1 DNA with *HpaII* methylase but not *HhaI* methylase. Molecules methylated at *HpaII* or *HhaI* sites were used to transform *E. coli* HB101 cells to ampicillin resistance (Table 4). Each methylated molecule also contained methylated adenine residues, since the plasmids used for in vitro methylation were grown in strain HB101. Methylation of the *HpaII* sites in the pdBPV-1 plasmid reduced its ability to transform strain HB101 to ampicillin resistance 28- to 39-fold relative to that of mock-methylated or untreated DNA. A similar (14-fold) decrease was seen when pBR322 alone was used for the methylation reaction. Similar to the results obtained with mouse cells, no significant reduction in the frequency of transformation was obtained following *HhaI* methylation. These data suggest that cytosine methylation at *HpaII* sites, but not at *HhaI* sites, was inconsistent with plasmid uptake or maintenance in *E. coli* cells and therefore that the sites of methylation are important in conferring the ability of the plasmids to transform bacteria as well as mammalian cells.

DISCUSSION

The data presented here indicate that in vitro methylation of cloned BPV-1 viral DNA at *HpaII* sites reduces its ability to induce morphological transformation of C127 mouse

TABLE 3. Efficiency of focus-formation with *HpaII*-methylated, *HhaI*-methylated, doubly methylated, or mock-methylated BPV DNA

Expt	No. of foci/ μ g of DNA (% of control) for methylation conditions:				
	None	<i>HpaII</i>	<i>HhaI</i>	<i>HpaII</i> + <i>HhaI</i>	Mock
1	37.5	12.0 (20%)	49.2 (80%)	54.0 (88%)	61.5
2	15.0	8.0 (27%)	72.0 (246%)	24.0 (83%)	29.3
3	20.6	3.9 (16%)	49.5 (200%)	22.9 (92%)	24.8
4	7.9	1.7 (19%)	30.7 (353%)	7.9 (91%)	8.7

FIG. 3. *HpaII* and *MspI* digests of DNA isolated from foci produced by transfection with *HpaII*-methylated pdBPV-1 DNA. DNA was isolated from foci produced by transfection with methylated pdBPV-1 DNA in three different gene transfer experiments. The DNAs were digested with either *HpaII* (lanes h) or *MspI* (lanes m), electrophoresed through 1% agarose, transferred to nitrocellulose, and probed with labeled pdBPV-1. DNA from five separate transformed foci are shown (lanes 1 through 5). Lanes 6 contain C127 DNA only, while lanes 7 contain 25 pg of pdBPV-1(142-6) plasmid DNA mixed with C127 DNA. From a comparison of the *HpaII* and *MspI* digests, it is apparent that most of the BPV sequences present in the transformed foci are unmethylated at *HpaII* sites.

fibroblast cells. These results are in contrast to those obtained with the other papovaviruses polyoma and simian virus 40, for which in vitro methylation at *HpaII* sites or at all CpG sites did not reduce the ability to transform cells and did not inhibit the production of viral early proteins, including T antigen (9, 41). The reduction in the focus-inducing ability of cloned BPV molecules when methylated at *HpaII* sites could be due to one or more of several factors. First, methylation at the *HpaII* sites could inhibit the expression of viral genes that are necessary for morphologic transformation. Methylation of several genes of viral and nonviral origin previously has been shown to inhibit their expression (21, 40, 45). For BPV, it may be that *HpaII* methylation reduces, rather than abolishes, the level of gene expression, since the frequency of focus induction is decreased only by a factor of about 3. A second possibility is that methylation at *HpaII* sites inhibits the expression of genes required for viral replication. Third, methylation could directly inhibit

replication, even in the presence of all the required proteins. Experiments are now in progress to distinguish among these possibilities.

In contrast to the results obtained with *HpaII* methylase, in vitro methylation of the *HhaI* sites in the cloned BPV molecule increased its ability to transform C127 cells. The number of sites for the two enzymes is similar (29 *HpaII* sites versus 27 *HhaI* sites), but the distribution of the two types of sites is different. Many of the *HpaII* sites are clustered in the noncoding region of the virus, which is postulated to contain the origin of replication and other important regulatory elements (Fig. 1). Most of the *HhaI* sites are located in other regions of the virus, although there are some *HhaI* sites in the noncoding region. The fact that methylation at *HhaI* sites affects transformation differently from methylation at *HpaII* sites indicates that the effect is not due simply to the overall level of cytosine methylation, but that the sites of methylation are important in determining whether, and how, a particular gene is affected by methylation.

Methylation at specific sites or regions has been implicated in the negative regulation of several genes, including the adenovirus E1a and E2a genes (19, 21), the human gamma-globin gene (2), and the hamster adenine phosphoribosyltransferase gene (40). In contrast, expression of the herpesvirus thymidine kinase (tk) gene was inhibited strongly by methylation in either the 5' or 3' region (17, 47). These results indicate that the locations and clustering of methylation sites are important in determining whether, and how, a particular gene is affected by methylation.

The observation that methylation of the cloned BPV molecule at both *HpaII* and *HhaI* sites did not decrease its focus-inducing ability suggests that the methylation at *HpaII* and *HhaI* sites have opposite or compensatory effects. This observation could be explained by postulating that *HpaII* methylation interfered with expression or binding of a *trans*-acting positive regulatory protein, while *HhaI* methylation interfered with expression or binding of a negative regulatory protein. A precedent for this hypothesis exists, since methylation at guanine residues within the *O_R1* region of lambda has been shown to affect binding of lambda repressor (13). Reduction of the effects of both factors might fortuitously bring the virus back to wild-type levels of transformation. This hypothesis is interesting in light of the analysis of BPV deletion mutants carried out by Lusky and Botchan (28). They demonstrated that deletion mutants that inactivated the E7 open reading frame (ORF) reduced transformation by approximately 10-fold, while a deletion mutant that inactivated the E1 ORF increased focus formation by ap-

TABLE 4. Effects of *HpaII* or *HhaI* methylation on bacterial transformation

Methylation agent	Plasmid	Expt	No. of Ampicillin-resistant colonies/ μ g of DNA for:			% of control
			Untreated DNA	Mock-methylated DNA	Methylated DNA	
<i>HpaII</i>	142-6	1	4.4×10^4	7.7×10^4	2.1×10^3	2.7
		2	3.3×10^4	3.95×10^4	1.4×10^3	3.5
		3	4.1×10^4	1.1×10^5	2.8×10^3	2.6
	pBR322	1	1.0×10^5	1.1×10^5	7.6×10^3	6.9
<i>HhaI</i>	142-6	1	3.2×10^4	3.8×10^4	3.7×10^4	97
		2	4.1×10^4	1.0×10^5	1.0×10^5	100
	pBR322	1	1.0×10^5	1.4×10^5	9.7×10^4	69

proximately 30-fold. There is one *HpaII* site, but no *HhaI* sites, within the E7 ORF and two *HpaII* and three *HhaI* sites within the E1 ORF. It is possible that methylation of sites within these ORFs had effects similar to those seen with the similar deletions. Since the changes in frequency of focus formation were not as great after methylation as they were with the deletion mutants, it might be that methylation induced a partial reduction in the expression or binding of regulatory factors.

An alternative hypothesis consistent with these data is that methylation operates by altering the conformation of the DNA in such a way that it affects the binding of a factor(s) necessary for expression, replication, or both. Since the BPV plasmid is a supercoiled circular molecule, changes in the supercoiling in one area of the molecule would affect the supercoiling in other areas of the molecule. The *HpaII* sites might be situated so as to alter the conformation of the molecule in a way which reduces expression or replication or both, while methylation at the *HhaI* sites could alter the conformation differently. The two different alterations in conformation might have additive effects and thus result in only a small net change in behavior.

When C127 cells were transformed by BPV molecules methylated at either the *HpaII* or *HhaI* site and individual foci were picked and grown up for DNA analysis, the viral sequences contained within the nuclei of the cells were unmethylated at all detectable *HpaII* or *HhaI* sites (Fig. 3). These data could indicate that the methylation pattern is not maintained in BPV molecules that are maintained in an extrachromosomal state, either because the methylation machinery cannot work efficiently on extrachromosomal molecules or because the virus contains a mechanism for avoiding methylation by the host machinery. Similarly, Subramanian (41) found that methylation at the *HpaII* sites in polyomavirus DNA (which did not affect the infectivity of the virus) was not maintained in the transformed cells. It was suggested that the maintenance methylase enzyme may be unable to keep up with the faster rate of replication of the polyomavirus DNA (41). This also is a possibility for BPV. Although BPV replicates once per cell cycle in productively transformed cells (unpublished observations), there is a burst of replication at the initial stages of infection (Peter Howley, personal communication), and it may be that the cellular methylation machinery cannot keep up with this burst. Since molecules isolated from transformed cells are not methylated, methylation must affect early events in the initiation of transformation, so that methylation affects the establishment rather than the maintenance of transformation.

Methylation is not incompatible with extrachromosomal maintenance per se, since there are some viral DNAs which contain methylated sites when maintained in an extrachromosomal state (4, 18, 33, 35, 49). For example, certain sites were methylated in Shope papillomavirus (49), Epstein-Barr virus (18, 33, 35), and human papillomavirus type 1a (4). Our unpublished data suggest that BPV DNA isolated from bovine papillomas or from virions also contains methylated sites. Therefore, the absence of methylation seen in viral DNA in these studies probably reflects species- or tissue-specific factors or both. The finding of methylation at sites in virion and papilloma DNA or suggests that methylation is important to the biology of the virus and that specific sites of methylation may have been selected for.

The fact that methylation at *HpaII* sites inhibits the ability of the pdBPV-1 plasmid, as well as pBR322, to transform *E. coli* HB101 to ampicillin resistance indicates that methyla-

tion of these cytosine residues affected the ability of the plasmid genes to be expressed in *E. coli*, the uptake of the DNA into the bacteria, or the ability of the molecules to replicate. The fact that methylation at the *HhaI* sites did not inhibit transformation in *E. coli* indicates that cytosine methylation in general does not preclude the uptake and expression of plasmid DNA in *E. coli*, but that the locations of the sites are important. Similar effects in mammalian and bacterial cells may indicate that methylation affects a fundamental property of expression or replication.

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