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Demethylation and expression of methylated plasmid DNA stably transfected into HeLa cells

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ABSTRACT

***In vitro* methylation at CG dinucleotides (CpGs) in a transfecting plasmid usually greatly inhibits gene expression in mammalian cells. However, we found that *in vitro* methylation of all CpGs in episomal or non-episomal plasmids containing the SV40 early promoter/enhancer (SV40 Pr/E) driving expression of an antibiotic-resistance gene decreased the formation of antibiotic-resistant colonies by only ~30–45% upon stable transfection of HeLa cells. In contrast, when expression of the antibiotic-resistance gene was driven by the Rous sarcoma virus long terminal repeat or the herpes simplex virus thymidine kinase promoter, this methylation decreased the yield of antibiotic-resistant HeLa transfectant colonies ~100-fold. The low sensitivity of the SV40 Pr/E to silencing by *in vitro* methylation was probably due to demethylation upon stable transfection. This demethylation may be targeted to the promoter and extend into the gene. By genomic sequencing, we showed that four out of six of the transfected SV40 Pr/E's adjacent Sp1 sites were hotspots for demethylation in the HeLa transfectants. High frequency demethylation at Sp1 sites was unexpected for a non-embryonal cell line and suggests that DNA demethylation targeted to certain aberrantly methylated regions may function as a repair system for epigenetic mistakes.**

INTRODUCTION

Although the vast majority of genetically programmed DNA methylation in vertebrates is at CG dinucleotides (CpGs), there are tissue-specific and stage-specific differences in exactly which CpGs in the genome are methylated (1). *In vivo* studies indicate that some of this DNA methylation negatively regulates gene expression and thereby plays critical roles in development, imprinting and carcinogenesis (2–5). Similarly, *in vitro* methylation of expression plasmids at all CpG sites generally greatly decreases reporter gene expression (>90%) upon transient transfection into cultured mammalian cells (6–8). The repressive effects of DNA methylation on transcription can be associated with a high density of m^5C residues and the binding of sequence-nonspecific proteins that have a preference for highly methylated DNA regions (9–11). DNA methylation and linked gene repression may also involve specific sites that are recognized by positive- or negative-acting transcription factors whose DNA binding is altered by methylation (12–15).

Because of the role of chromatin structure and methylation-responsive DNA-binding proteins in mediating the effects of DNA methylation (1,9,16), it is important to examine the expression of methylated plasmids upon stable transfection. Stably transfected DNA should have more chromatin-like interactions with histone and non-histone DNA-binding proteins than transiently transfected DNA (17). In transient transfection experiments, the abnormally open structure of the plasmid DNA may not reveal the long-term, chromatin structure-dependent consequences of DNA methylation (18).

We determined, in stable transfection assays, the effect of *in vitro* DNA methylation on reporter gene expression using plasmids containing various promoters and enhancers. Included in these was the SV40 early promoter/enhancer (SV40 Pr/E), which has six CpG-containing sites recognized by the ubiquitous transcription factor Sp1. Multiple Sp1 sites confer resistance to *de novo* methylation and target localized demethylation during vertebrate embryogenesis or transfection of embryonal cells in culture (19–23) but they were reported not to do so in non-embryonal cells (24). Our study demonstrates that the SV40 promoter is frequently demethylated in a non-embryonal cell line, HeLa cells, upon stable transfection. The demethylation is probably related to our finding that this promoter is unusually resistant to silencing by *in vitro* methylation prior to stable transfection. More variable than demethylation in the SV40 promoter was demethylation in the body of its downstream reporter gene, which might be a consequence of demethylation extending from the promoter.

MATERIALS AND METHODS

Plasmids

The plasmid vector pMC1neo PolyA (abbreviated as pMC1neo; Stratagene), which contains the herpes simplex virus thymidine kinase promoter (HSV TK Pr) driving expression of the G418- and neomycin-resistance (*neo*) gene, was used to construct pPyEhyg by insertion of the enhancer B from polyomavirus DNA upstream of the HSV TK Pr. The episomal pChyg with a hygromycin-resistance (*hyg*) gene (25) contains a promoterless, unexpressed *neo* gene and

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a 9.1 kb fragment from pCEP4 (Invitrogen) with an HSV TK Pr-*hyg* cassette and *oriP* and *EBNA-1* from Epstein–Barr virus (EBV) DNA. From pRC/RSV (Invitrogen), renamed pSVneo, we excised the 0.6 kb *Bgl*II/*Hin*dIII fragment containing the Rous sarcoma virus long terminal repeat (RSV LTR) or the 1.2 kb *Sma*I/*Hin*dIII fragment with the bovine growth hormone (BGH) transcription termination signal, f1 *ori*, and SV40 Pr/E to generate pSneo and pRneo. pRSneo was obtained by deleting the 1.5 kb *Hin*dIII/*Xho*I fragment containing the mouse mammary tumor virus LTR from pMAMneo (Clontech). pCSV was generated from a 2.7 kb *Bam*HI fragment of pMAMneo containing the *neo* gene driven by the SV40 Pr/E that was inserted into the *Bam*HI site of pCEP4's polylinker.

In vitro methylation and transfection of plasmid DNAs

Plasmid DNAs were methylated in standard reactions with bacterial DNA methyltransferases M.*Hpa*II, M.*Hha*I or M.*Sss*I (New England Biolabs) using 2 U of enzyme per µg of DNA for 2 h in a volume of 10 µl. For mock methylation, either AdoMet or M.*Sss*I was omitted. After the reaction, the DNA was extracted with phenol–chloroform and precipitated with ethanol. Each batch of methylated DNA was shown to be undigested upon incubation with *Hpa*II and/or *Hha*I. Calcium chloride-mediated transfection of HeLa cells (ATCC CCL 2) with 10 µg of plasmid was performed as previously (25) using 400 µg/ml of hygromycin B (Hyg, Calbiochem) or 900 µg/ml of G418 (Gibco-BRL) for selection beginning 2 days after the donor DNA was added and changing the medium every 3–5 days. These antibiotics caused massive cell death by 8 or 9 days after transfection and cultures of mock-transfected control cells never recovered from this die-off. Fourteen days after plasmid transfection, either colonies were stained with Giemsa and those containing at least 50 cells were counted or transfectants were further propagated as clones or mixed populations pooled from at least 100 colonies. Unless otherwise stated, transfectants were propagated in the presence of Hyg or G418 for the minimal amount of time necessary to accumulate enough cells for DNA isolation.

Methylation analysis

For Southern blotting, DNA from the transfectants (7 µg) was incubated overnight with *Hpa*II or *Msp*I (20 U/µg of DNA), with internal controls to assure complete digestion. The ^{32}P-labeled probes were as follows: a 2.0 kb *Bam*HI fragment from pSVneo or a 2.7 kb *Bam*HI fragment from pMAMneo containing the *neo* gene or a 0.9 kb *Sac*II–*Bst*BI fragment of pCEP4 containing the *hyg* gene. As copy-number standards, diluted *Bam*HI-digested pSVneo plasmid was added to 7 µg of salmon sperm DNA. Bisulfite-based genomic sequencing on 2 µg of transfectant DNA was as previously described (26) except that the annealing temperature was 55°C and the following primers (20 pmol) were used for the first round of PCR (24 cycles) on 200 ng of bisulfite-treated DNA: 5′-AGTGGATTTTTGTTTTAAATTGGAATAATA-3′ and 5′-AATCATTTCCA(G)AATAACCTCTCCA-3′. In the second round, the former primer plus 5′-AATTTTGTGGAATGTGTGTTAGTTAGG-3′ were used and the second-round PCR products were sequenced.

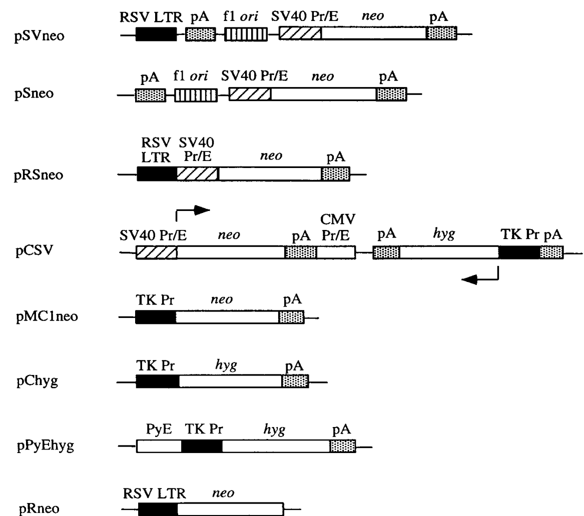

Figure 1. Expression plasmids used for stable transfection after methylation *in vitro*. The structure of the plasmids around the promoters and *neo* or *hyg* selection marker genes under study is diagrammed (not to scale). TK Pr, HSV thymidine kinase promoter; PyE, the polyomavirus B enhancer; SV40 Pr/E, the SV40 virus' early promoter and upstream enhancer; CMV Pr/E, the cytomegalovirus immediate early promoter and upstream enhancer; f1 *ori*, a phage origin of DNA replication; pA, polyadenylation and transcription termination signals from SV40 or, in the case of the pSVneo sequence immediately downstream of the RSV LTR, from the BGH gene.

RESULTS

Effects of *in vitro* CpG methylation on gene expression driven by various promoters

To study the effects of DNA methylation on gene expression, we stably transfected HeLa cells with various methylated or mock-methylated expression plasmids containing *neo* or *hyg* reporter genes (Fig. 1). Following transfection, G418- or hygromycin-resistant (G418^R or Hyg^R) transfectants were selected. After 2 weeks, the number of colonies from HeLa cells stably transfected with methylated plasmid was compared to the colony yield from the parallel transfection with mock-methylated DNA. *In vitro* methylation at all CpGs of non-episomal plasmids containing the SV40 early promoter and enhancer driving expression of the *neo* gene caused a decrease of only ~30–40% in the formation of antibiotic-resistant transfectant colonies (Table 1, pSVneo, pSneo and pRSneo). No difference was seen in the size distribution of G418^R colonies from the cells transfected with these M.*Sss*I-methylated or mock-methylated plasmids at 11 days after transfection, when colonies averaged ~10 cells. This was also the case at 14 days after transfection, when almost all colonies had >50 cells.

In contrast to the small decrease in the number of antibiotic-resistant colonies upon *in vitro* methylation of SV40 Pr/E-*neo* plasmids, CpG methylation reduced by ~100-fold the number of antibiotic-resistant colonies obtained from plasmids in which *neo* or *hyg* gene expression was driven by the HSV TK Pr (Table 1). Similar results were obtained from the non-episomal pMC1neo

and the episomally maintained pChyg. When the polyomavirus enhancer B was present upstream of the HSV TK Pr in the transfecting DNA, CpG methylation still repressed the formation of most of the antibiotic-resistant colonies although not to as large an extent as in the absence of this enhancer (Table 1, pPyEhyg). CpG methylation of a transfecting plasmid containing the RSV LTR with its enhancer and promoter sequences driving *neo* gene expression caused a >100-fold decrease in the number of G418^R transfectants (Table 1, pRneo). The presence of the RSV LTR's enhancer did not significantly alter the frequency of G418^R transfectants when it was present upstream of an SV40 Pr/E-*neo* cassette.

Table 1. Effects of CpG methylation on expression from reporter plasmids stably transfecting HeLa cells

Plasmid	(Enhancer)(promoter)reporter gene[a]	Number of Hyg^R or G418^R colonies[b]		Relative colony formation (B/A)
		A	B	
		Unmethylated plasmid	M.Sss I-meth. plasmid	
pSVneo	(RSV LTR)(SV40 Pr/E)*neo*	346 ± 119	247 ± 11	0.71
pSneo	(SV40 Pr/E)*neo*	320 ± 22	225 ± 18	0.70
pRSneo	(RSV LTR)(SV40 Pr/E)*neo*	288 ± 23	165 ± 6	0.57
pCSV	(SV40 Pr/E)*neo*	470 ± 14	255 ± 7	0.54
pCSV	(HSV TK Pr)*hyg*	274 ± 18	0 ± 0	<0.01
pMC1neo	(HSV TK Pr)*neo*	74 ± 3	1 ± 1	0.01
pChyg	(HSV TK Pr)*hyg*	50 ± 3	0 ± 0	<0.02
pPyEhyg	(PyE)(HSV TK Pr)*hyg*	133 ± 9	15 ± 1	0.11
pRneo	(RSV LTR)*neo*	796 ± 113	3 ± 4	<0.01

[a]The promoters and enhancers upstream of the *neo* and *hyg* reporter genes (Fig. 1) were the RSV LTR promoter-enhancer, the SV40 early promoter-enhancer (SV40 Pr/E), the HSV TK promoter (HSV TK Pr) and the polyomavirus enhancer B (PyE). The plasmids in this table which are episomally maintained in HeLa cells are pCSV and pChyg.
[b]5×10^5 HeLa cells were stably transfected with 10 µg of M.*Sss*I-methylated or mock-methylated plasmid and the resulting G418^R or Hyg^R colonies were scored, depending on whether the *neo* or *hyg* gene was present in the plasmid. For pCSV, which contained both genes, selection was for G418^R in the fourth row of the table and Hyg^R in the fifth row. The average results from duplicate 100 mm plates are shown.

There was also a large difference in the extent of silencing by *in vitro* methylation of an HSV TK Pr-*hyg* cassette and an SV40 Pr/E-*neo* cassette when both were present on a single plasmid, namely, the episomal pCSV (Fig. 1; Table 1). Furthermore, when methylated pCSV was used to generate a mixed population of G418^R HeLa transfectants and 10^7 of these cells were selected immediately with Hyg or after maintenance in G418-containing medium for 2 months, no Hyg^R colonies were obtained. When, as a control, 10^6 cells from the mixed population of G418^R HeLa transfectants containing mock-methylated pCSV DNA were incubated with Hyg instead of G418, a confluent monolayer was obtained 10 days after Hyg addition. Therefore, sufficient DNA methylation was maintained in the transfected CpG-methylated plasmid over the course of >80 cell population doublings to silence the expression of the *hyg* gene while the *neo* gene in the same plasmid was expressed.

Also, we tested the effect of methylating pCSV only at its 49 *Hha*I sites (5′-GCGC-3′), 64 *Hpa*II sites (5′-CCGG-3′), or both types of sites. In the HSV TK Pr there are 25 CpGs, including one

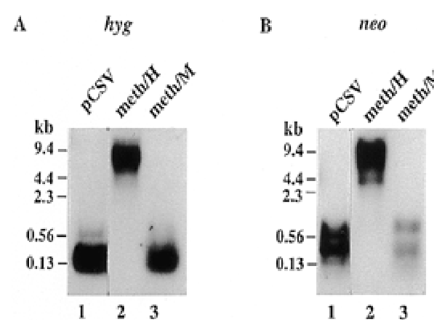

Figure 2. Extensive methylation in the body of the *neo* and *hyg* reporter genes of the transfecting pCSV in mixed populations of HeLa transfectants. DNA (7 µg) was analyzed from a mixed population of G418^R transfectants obtained with M.*Sss*I-methylated episomal pCSV, which contains the HSV TK Pr-*hyg* and SV40 Pr/E-*neo* gene cassettes. The DNA was digested with *Hpa*II (meth/H) or *Msp*I (meth/M) and blot-hybridized to *hyg* (**A**) or *neo* (**B**) gene probes. The probes spanned from just downstream of the HSV TK Pr past the transcription termination sequence of the *hyg* gene or from the SV40 Pr/E almost to the end of the *neo* gene. Purified diluted, unmethylated pCSV plasmid DNA digested with *Hpa*II was present in the first lane of each panel. The sizes of molecular-weight markers are indicated.

*Hha*I site overlapping a low-affinity Sp1 site (27) and one *Hpa*II site further upstream. The HSV TK Pr contains one additional, moderate-affinity Sp1 site (27). Methylation at only the *Hha*I or *Hpa*II sites or at both types of sites led to decreases of 79, 19 and 100%, respectively, in the yield of Hyg^R colonies. Therefore, HSV TK Pr-*hyg* expression was much more sensitive to repression by methylation at a small subset of CpGs than was SV40 Pr/E-*neo* expression to *in vitro* methylation at all CpG sites.

Variable extents of spontaneous demethylation in the body of the *neo* reporter gene in G418^R transfectants containing an SV40 Pr/E-driven *neo* gene

Southern blot analysis on digests obtained with *Hpa*II, which is inhibited by CpG methylation at its cognate sites, was used to assess DNA methylation in the region of the *neo* gene in transfectants. Although there are no CpG methylation-sensitive restriction sites in the SV40 Pr/E region, retention of methylation in the gene as well as in the transcription regulatory upstream region should be examined because even DNA methylation confined to the body of a reporter gene can greatly decrease reporter gene expression upon transfection (28,29). Methylation was assessed on DNA from a mixed population of stable G418^R transfectants obtained with methylated pCSV. Because this plasmid also contains the *hyg* gene, methylation in this region too was analyzed. If the examined gene regions were completely demethylated, the hybridizing DNA fragments from *Hpa*II-digested DNA should be of <1 kb (236, 233, 207, 187 and 117 bp with the *hyg* probe and 863, 434, 275, 181, 171, 131, 102 and 90 bp with the *neo* probe). In digested DNA from pooled transfectants that were G418^R and Hyg-sensitive, no hybridizing bands of <4 kb were observed with the *hyg* probe, indicating a very high degree of retention of CpG methylation at the tested *Hpa*II sites (Fig. 2A, lane 2). Similar results were obtained with the *neo* probe (Fig. 2B, lane 2) despite sufficient expression of the *neo* gene to confer resistance to G418 on the transfectants.

By Southern blotting, we also confirmed that plasmids isolated from the mixed G418^R population obtained with the M.*Sss*I-

methylated episomal pCSV were mostly maintained as episomes, as seen by their linearization by *Hin*dIII (expected single cleavage of pCSV) and resistance to cleavage by *Afl*II (expected no cleavage of pCSV). By Southern blotting using diluted untransfected plasmid as a copy-number standard, we also determined that transfectants obtained with methylated DNA had an average plasmid copy number of about 10 while those obtained with mock-methylated plasmid had a copy number of about one. However, examination of individual transfectant clones generated with the non-episomal pSVneo indicated that transfectants with low-copy-number plasmid DNA could retain most of their *in vitro*-introduced *neo* methylation and, nonetheless, express the plasmid's selection marker (pSVmeth clones 3 and 6; Table 2 and Fig. 3).

Table 2. Retention of DNA methylation at *Hpa*II sites in the *neo* gene of HeLa transfectants

pSVmeth transfectants	Copy no[a]	Rearrangements in the vicinity of the *neo* gene[b]	Extent of demethylation at Hpa II sites of *neo*[c]
Mix 1	10	-	none
Mix 2	20	-	partial
Clone 1	50	-	none
Clone 2	1	+	high
Clone 3	1	in ~25% of copies	very low
Clone 4	1	+	ND
Clone 6	3	in~50% of copies	low

[a]Clones or mixed populations (mix 1 or 2) of transfected HeLa cells were obtained after introduction of M.*Sss*I-methylated pSVneo. The approximate average copy numbers of the plasmid DNA in these stably transfected populations were determined by Southern blot analysis with a *neo* gene probe on *Bam*HI-digested DNA from transfectants in comparison to copy-number standards.
[b]Rearrangements in the transfectants in the ~2 kb region, including the 0.7 kb *neo* gene, were assessed by the appearance of unexpected sizes of hybridizing bands in Southern blot analysis of *Bam*HI digests (data not shown) or *Msp*I digests, as seen in lanes 11 and 12 of Figure 3.
[c]The relative extents of methylation at *Hpa*II sites in the *neo* gene were approximated from the fraction of low-molecular-weight hybridizing DNA fragments after *Hpa*II digestion as illustrated in Figure 3. For mix 1, no bands of unmethylated hybridizing DNA fragments were visible (Fig. 3, lanes 3 and 5). ND, not determined.

The extent of retention of methylation at *Hpa*II sites within the *neo* gene varied greatly among SVmeth transfectant clones (Table 2 and Fig. 3). In two mixed populations of transfectants obtained with CpG-methylated pSVneo, one showed no demethylation within the *neo* gene (pSVmeth mix 1; Fig. 3, lane 19) and another from a transfection conducted identically, except that the cells were cultured for 8 weeks after transfection instead of 3, displayed much *neo* demethylation although a fraction of the sequences retained methylation (pSVmeth mix 2; Fig. 3, lane 7). The difference in the extent of *neo* demethylation in the pSVmeth mix 1 and mix 2 transfectant populations cannot be due to differences in the plasmid's copy number because the average copy number for both of these populations of mixed transfectants was high (Table 2). It might be the result of different numbers of cell division cycles before harvesting transfectants, which may have allowed more time for spreading of demethylation, as seen for an *in vitro*-methylated non-functional, synthetic promoter transfected into a murine embryonal carcinoma cell line (22).

Frequent spontaneous demethylation in the SV40 promoter region upstream of the *neo* gene in G418^R transfectants

Because CpG-rich regions (CpG islands) overlapping the 5′ ends of expressed genes are often hypomethylated in contrast to the sequences in the body of the genes (1,30), demethylation of the promoter and the 5′ end of the gene in the SV40 Pr/E-*neo* cassette may be more important for its expression than demethylation of the central portion of the gene. In order to assay for demethylation of the M.*Sss*I-methylated SV40 promoter region upstream of the *neo* gene in G418^R transfectants, we did genomic sequencing using bisulfite-modified DNA from HeLa transfectant clones obtained with methylated pSVneo (pSVmeth clones). After bisulfite modification, subsequent deamination, PCR amplification and molecular cloning, the methylated C residues of individual strands of DNA are evidenced by persisting C-specific (or complementary G-specific) bands in DNA sequencing gels while unmethylated C residues are converted to T residues. We used PCR primers to amplify ~240 bases that encompassed 13 CpGs from the transcribed strand of the SV40 promoter and the immediate cap-downstream region, but not the adjacent CpG-deficient SV40 enhancer. From the amplified DNA of five transfectant clones, a total of 17 PCR-derived molecular clones were sequenced. Only ~1% of the C residues at non-CpG sites were unconverted after the bisulfite modification and PCR and these were in random locations. Therefore, the efficiency of modification and deamination at unmethylated Cs was ~99% and no significant level of atypical, non-CpG methylation was observed. As a control, five molecular clones from the M.*Sss*I-methylated pSVneo used for transfection were subject to genomic sequencing. They were fully methylated at the examined CpGs.

All but two of the 17 sequenced molecular clones from G418^R HeLa transfectants obtained with M.*Sss*I-methylated pSVneo were demethylated in ~30–90% of the 13 CpGs in the SV40 Pr and 5′ region of the *neo* gene (Table 3). Those two molecular clones were from one transfectant colony, pSVmeth 6, and were unmethylated at one CpG in this region while the third molecular clone from this transfectant was unmethylated at 11 of the 13 examined CpGs. In all the molecular clones except the two above-mentioned hypermethylated ones from transfectant pSVmeth 6, the CpGs in four Sp1 sites were unmethylated, namely, those at positions –106, –94, –85 and –73, relative to the major transcription start site of the gene (31). At most of the other examined CpGs, there was considerable variation in the exact patterns of methylation from DNA molecule to DNA molecule, which is seen sometimes (26), but not always (32), at m^{5}CpG-rich regions in human tissue DNAs. Even the SV40 promoter's CpGs at positions –63 and –52, which are also located in Sp1 sites, albeit moderate- or low-affinity ones (27), showed heterogeneity in their methylation state among the molecular clones.

DISCUSSION

Upon stably transfecting HeLa cells with methylated reporter plasmids, we demonstrated, for the first time, that a non-embryonal cell type could frequently demethylate Sp1 sites in a promoter, namely the SV40 early promoter, and that a large extent

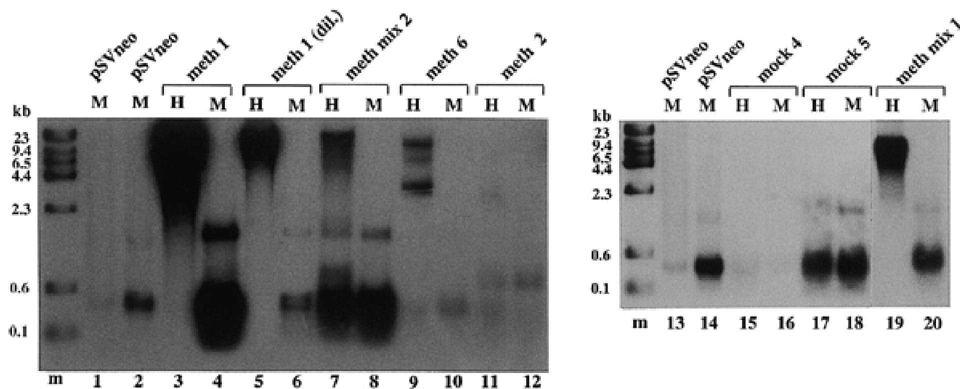

Figure 3. Methylation of the body of the *neo* reporter gene in the transfecting pSVneo DNA in different HeLa clones. DNA from mixed populations (meth mix 1 or 2) or clones (meth 1, 2 or 6) of G418^R transfectants obtained with the non-episomal M.*Sss*I-methylated pSVneo plasmid or transfectant clones of the analogous mock-methylated plasmid (mock 4 or 5) was digested with *Hpa*II (H) or *Msp*I (M). Southern blotting was performed as in Figure 2 with a *neo* gene probe and 3 µg of transfectant cell DNA for lanes 3, 4, 7, 8, 11 and 12; 1.5 µg for lanes 9, 10 and 15–20; and 0.3 µg from SVmeth clone 1 (meth 1 dil.) for lanes 5 and 6. In lanes 1 and 13, there was *Msp*I-digested DNA from 10^6 molecules and lanes 2 and 14 had 10 times as much of the same DNA. The *neo* probe for this analysis contained additional sequences from the F1 *ori* upstream of the SV40 Pr/E (Fig. 1) so that an extra, weak 1.5-kb fragment hybridizing with ~130 bp of the probe is seen from vector sequences upstream of the SV40Pr/E, which was not seen with the 0.7-kb shorter *neo* probe used in Figure 2B. Molecular weight markers are shown in lane m.

of resistance to methylation-induced gene silencing was associated with this demethylation. *In vitro* methylation of transfected episomal or non-episomal plasmids containing the SV40 promoter and enhancer (SV40 Pr/E) driving *neo* expression gave no noticeable decrease in transfectant colony size and a decrease of only ~30–45% in the yield of G418^R transfectants compared to an ~100-fold reduction in transfectant yield when *neo* expression from the plasmid was driven by the HSV TK promoter or the RSV LTR. Therefore, the high frequencies of SV40 Pr/E-*neo* expression and of SV40 promoter demethylation are probably a reflection of their frequency *in vivo* and not the result of selection for G418^R cells.

The effect of *in vitro* DNA methylation on gene expression has generally been analyzed in transient rather than stable transfection experiments. In some transient transfection studies on HeLa, L929 or NIH 3T3 cells, gene expression driven by the SV40 Pr/E was highly susceptible to CpG methylation-induced silencing (29,33,34), while in others, it showed only a low sensitivity (6,35,36). *In vitro* methylation of stably transfected plasmids containing a variety of promoter-reporter gene cassettes greatly inhibits gene expression (28,37–39). However, preferential demethylation of several restriction sites in the 5′ region of a transfected dihydrofolate reductase minigene was seen in stable transfectants of Chinese hamster ovary cells even without selection for minigene expression (39). Also, in a transfected M.*Sss*I-methylated episomal plasmid, EBV *oriP* sequences undergo very frequent and targeted demethylation at sites recognized by a viral DNA-binding protein, EBNA-1, starting several days after transfection into a human embryonic kidney carcinoma cell line (40).

In our study, four of the six tightly clustered Sp1 sites in the SV40 Pr were hotspots for demethylation upon stable transfection of HeLa cells. There was much more variability in the extent of demethylation in DNA sequences surrounding these Sp1 sites and in the central portion of the reporter gene. The variation in methylation patterns could be partly due to differences in plasmid copy number. Demethylation can be inhibited by a high copy number as well as a high m^{5}CpG density (41). The transfectant clone with the highest copy number (pSVmeth clone 1) had the most methylation of the *neo* gene and a lack of detectable unmethylated 5′-CCGG-3′ sites (Fig. 3). Given the G418^R phenotype of pSVmeth clones 3 and 6, which had average plasmid copy numbers of only one or three and yet were also highly methylated in their *neo* gene (Table 3 and Fig. 3), retention of most of the CpG methylation within the *neo* gene itself does not appear to preclude *neo* expression.

Targeting of demethylation to multiple Sp1 sites and the spreading of demethylation to the 5′ end of the *neo* gene could have led to the relative resistance of the SV40 Pr/E to *in vitro* methylation-induced inactivation. This targeted demethylation could be relevant to the establishment and maintenance of hypomethylation of CpG islands spanning the 5′ ends of genes, which often have multiple potential Sp1 sites (42). Clustered Sp1 sites confer local protection against *de novo* methylation in murine embryonal stem cells, embryonal carcinoma cell lines and early embryos (20,21,23). Also, in murine embryonal cells transfected with *in vitro*-methylated plasmids containing Sp1 sites in the adenine phosphoribosyl transferase (APRT) promoter or an inactive synthetic promoter, the wild-type Sp1 sites direct local demethylation of CpGs (20,22). However, it was reported that Sp1 site-associated demethylation, detected by Southern blot analysis, occurred in embryonal cells but not in non-embryonal L-cell fibroblasts and L8 myoblasts transfected with a methylated plasmid containing the hamster APRT promoter (24). In our study, it appears that demethylation in HeLa cells, monitored precisely by genomic sequencing, was specifically targeted to the four distal Sp1 sites of the SV40 Pr, three of which are high-affinity sites and one a moderate-affinity site, as opposed to the proximal sites, which have only moderate or low affinity for Sp1 (43,44).

DNA demethylation, which is most prevalent very early in embryogenesis, can be mediated by demethylating activities associated especially (45), but not exclusively (46–48), with embryonic cells. Demethylation can also be mediated passively

Table 3. Demethylation of the SV40 promoter and the immediate downstream region in methylated pSVneo stably transfected into HeLa cells

Transfectant colony	CpG methylation at the given position in the SV40 promoter region of individual DNA clones[a]												
	-106 High Sp1	-94 High Sp1	-85 Mod. Sp1	-73 High Sp1	-63 Mod. Sp1	-52 Low Sp1	-12	-6	-1	+63	+84	+113	+118
pSVmeth 1	u	u	u	u	M	M	M	M	M	M	M	M	M
	u	u	u	u	M	M	M	M	M	M	M	M	M
	u	u	u	u	M	M	M	M	M	M	M	M	M
	u	u	u	u	M	M	M	M	M	M	M	M	M
	u	u	u	u	u	M	u	u	u	u	u	u	u
pSVmeth 2	u	u	u	u	M	u	u	u	u	M	u	u	u
	u	u	u	u	u	M	u	u	u	M	M	u	M
	u	u	u	u	u	u	u	u	u	M	u	u	M
pSVmeth 3	u	u	u	u	M	M	M	u	u	M	M	u	u
	u	u	u	u	M	u	u	M	M	u	u	u	u
	u	u	u	u	u	u	u	u	u	u	u	u	u
	u	u	u	u	u	u	u	u	u	u	u	u	u
pSVmeth 4	u	u	u	u	M	u	u	u	u	u	u	u	M
	u	u	u	u	M	u	u	u	u	u	u	u	u
pSVmeth 6	M	M	M	M	M	M	M	M	M	M	M	u	M
	M	M	M	M	M	M	M	M	M	M	M	u	M
	u	u	u	u	u	M	M	u	u	u	u	u	u
% demeth.[b]	88	88	88	88	35	41	53	59	59	41	53	76	47

[a]By bisulfite-based genomic sequencing, the methylation status of all C residues on the transcribed strand of the SV40 promoter and downstream sequences was determined for two to five molecular clones from PCR-amplified bisulfite-treated DNA from each of five transfectant clones. The DNA subjected to this genomic sequencing was obtained from HeLa colonies stably transfected with M.*Sss*I-methylated pSVneo that expressed the SV40 Pr/E-driven *neo* gene. Essentially all m^5C residues were at m^5CpG sites, as expected. The positions of the CpGs in this region relative to the major transcription start site of the early SV40 transcription unit and to three high-affinity, two moderate-affinity and one low-affinity Sp1 sites in the SV40 promoter are indicated. CpGs from position –106 to +84 are from SV40 sequences. Those at +113 and +118 (in the sequence 5′-ATGAACGTTTTTCGAGGG-3′, in which the palindrome overlapping the CpG at +113 is underlined) are from the 5′ end of the *neo* gene. M, methylated; u, unmethylated at the given CpG.
[b]The percentage of analyzed molecular clones that were demethylated at a given site is given.

by inhibition of maintenance methylation or by a combination of active and passive processes (19,40). That we observed a high degree of demethylation at Sp1 sites in the SV40 promoter of transfectants generated with a CpG-methylated plasmid introduced into HeLa cells suggests that targeted demethylation may occur *in vivo* in normal postnatal cells more frequently than realized and may restore function in genes subjected to aberrant methylation-induced silencing. Such demethylation might serve as an epigenetic repair system to compensate for some physiologically inappropriate methylation of certain CpG islands subject to background levels of adventitious *de novo* methylation. However, this epigenetic repair may fail or be overwhelmed by selection for fast-growing cells during carcinogenesis so that tumor suppressor genes in cancers are often inactive due to aberrant CpG island methylation.

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