The Role of DNA Methylation in Expression of the \( p19/p16 \) Locus in Human Bladder Cancer Cell Lines

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ABSTRACT

Methylation of CpG sites in the control regions of tumor suppressor genes may be an important mechanism for their heritable, yet reversible, transcriptional inactivation. These changes in methylation may impaire the proper expression and/or function of cell cycle regulatory genes and confer a selective growth advantage to affected cells. Detailed methylation analysis using genomic bisulfite sequencing was performed on a series of subclones of a bladder cancer cell line in which a hypermethylated \( p16 \) gene had been reactivated by transient treatment with 5-aza-2'-deoxycytidine. Methylation of the CpG island in the promoter of the \( p16 \) gene in human bladder cancer cells did not stop the formation of a transcript initiated 20 kb upstream by the \( p19 \) promoter but did prevent the expression of a \( p16 \) transcript. Furthermore, we show that reactivant clones that expressed \( p16 \) at varying levels contained heterogeneous methylation patterns, suggesting that \( p16 \) expression can occur even in the presence of a relatively heavily methylated coding region. We also present the first functional evidence that methylation of only a small number of CpG sites can significantly down-regulate \( p16 \) promoter activity, thus providing support for the model of progressive inactivation of this tumor suppressor gene by DNA methylation.

INTRODUCTION

The overall functions of DNA methylation in mammalian cells remain enigmatic, despite the fact that it is essential for development (1). Methylation of CpG islands is clearly important for the regulation of imprinting (2-5) and for reinforcing the transcriptional inactivities of housekeeping genes on the inactive X chromosome (6, 7). Because methylation of CpG-rich promoters has been shown to limit their activities (8-11), it has generally become accepted that methylation of CpG islands is associated with a lack of transcription (12-14).

Not all genes contain CpG islands, but most islands that have been identified are associated with genes (15, 16). The position of CpG islands relative to the transcriptional start sites of genes varies considerably. They are always located in the 5' region of housekeeping genes but are often confined to the coding regions of tissue-specific genes. Additionally, CpG islands associated with parasitic repetitive DNAs are often embedded within the transcriptional units of active genes, and it has been suggested that the primary function of DNA methylation may be related to suppression of parasitic transposable elements (17, 18). Many of these repetitive sequences are observed to be methylated in the genome, presumably as a result of a host-mediated defense mechanism for their inactivation. Indeed, one-third of the human genome consists of interspersed repetitive elements such as Alus and LINEs, many of which fulfill conditions of being CpG islands (16, 19, 20) and can potentially be transcriptionally active (21, 22). Several eukaryotic genes, such as \( Ig2 \) (23, 24) and \( p16 \) (25), have CpG-rich promoters downstream of additional promoters, which can direct transcription through CpG islands. Many transcriptional units, therefore, contain more than one CpG island, and these may be associated with promoters, coding regions, or repetitive elements.

The protein product of the \( p16 \) tumor suppressor gene binds to cyclin-dependent kinases 4 and 6 and inhibits their interaction with cyclin D1, which normally mediates passage through the G1 phase of the cell cycle by phosphorylation of pRB (25). The \( p16 \) gene is located on chromosome 9p21 and has been reported to be a frequent target of homozygous deletion in a variety of human cancer cell lines and tumors (26-28). Intragenic mutation of \( p16 \) in both cell lines and primary tumors has been found to be another mechanism for gene inactivation, albeit at a lower frequency than for other tumor suppressor genes such as \( p53 \) (13, 29, 30). Methylation of the 5' CpG island of \( p16 \) has been proposed as yet another mechanism for inactivation of this tumor suppressor gene in ~20-40% of certain types of human cancers and may account for the low frequency of reported \( p16 \) mutations in primary tumors (31, 32). Numerous studies have documented aberrant methylation of the 5' CpG island of \( p16 \) in various human tumors (11, 31, 33, 34). Furthermore, treatment of human cancer cell lines with 5-Aza-CdR has been correlated with induction of \( p16 \) expression and demethylation of certain CpG sites as determined by restriction enzyme digestion and Southern analysis (11, 35). It has also been suggested that methylation of the 5' CpG island of \( p16 \) may play a role in increasing the metastatic potential of tumor cells, although this has not been definitively proven (36).

The novel situation that exists with regard to the \( p16 \) locus is the presence of an alternative transcript that is driven by the \( p19 \) promoter originating ~20 kb upstream of the \( p16 \) exon 1a coding domain. The \( p16 \) and \( p19 \) transcripts use alternative first exons (1a and 1b) joined through the same splice acceptor site to exon 2 coding sequences but in different reading frames (37, 38). The \( p19 \) transcript encodes a protein that has also been shown to be capable of suppressing growth in mammalian fibroblasts (39). The 5' CpG island of the \( p16 \) gene, which includes the exon 1a coding domain, is a highly CpG-rich sequence through which transcriptional machinery must pass to generate the \( p19 \) transcript.

In this report, we show that extensive methylation of the CpG island in the promoter of the \( p16 \) gene blocks gene expression but not the expression of a \( p19 \) transcript. The ability of the \( p16 \) promoter to generate a transcript was restored by treatment of a human bladder cancer cell line with 5-Aza-CdR, suggesting that promoters located within other transcriptional units might also be silenced by DNA methylation. One important function of DNA methylation may be to silence the activities of CpG-rich promoters present in actively transcribed regions of mammalian genes. We have also performed the first comprehensive analysis of 67 CpG dinucleotides in the \( p16 \) promoter region in a human bladder cancer cell line before and after treatment with 5-Aza-CdR. Bisulfite genomic sequencing allowed us to char-
acize methylation patterns of the p16 5' CpG island in reactive
clones as a function of gene expression. Furthermore, we have inves-
tigated the effects of site-specific methylation of the p16 promoter as a
potential initiating event in the progressive down-regulation of this
malignant suppressor gene.

MATERIALS AND METHODS

Cell Lines and 5-Aza-CdR Treatment. T24 and J82 human bladder
carcinoma-derived cell lines were obtained from the American Type Culture
Collection (Rockville, MD). DNA and total RNA were isolated from noncon-
fluent, exponentially growing cells. Cell lines were plated at 1 × 10^6 per
75-mm^2 flask and treated 24 h later with a 3 × 10^{-7} M dose of 5-Aza-CdR.
Cell medium was changed 24 h after addition of 5-Aza-CdR, and single cell
subclones were isolated between 48 and 72 h after removal of the drug.
Conditioned media was used for the initial culturing of single-cell subclones.
DNA and RNA were isolated after at least 20 population doublings from each
of the subclones.

RT-PCR Analysis of p16, p19, and GAPDH Expression. Total RNA
(~2.5 μg) was isolated from cells and reverse transcribed using random
hexamers, deoxynucleotide triphosphates (Boehringer Mannheim, Indianapo-
ilis, IN), and Superscript II reverse transcriptase (Life Technologies, Inc.,
Gaithersburg, MD) in a 25-μl reaction. cDNA was amplified using primers
specific for p16, p19, or GAPDH. Briefly, PCR reactions were performed in
25-μl volumes at 94°C for 3 min, 24 cycles at 94°C for 1 min, 56°C for 30 s,
72°C for 40 s (p16 and p19 amplification), and 94°C for 1 min, 20 cycles at
94°C for 1 min, 58°C for 30 s, 72°C for 45 s (GAPDH amplification). Primers
sequences have been described previously (33) and are as follows: p16 sense,
5'-AGG AGC CAG CAG CTA GGG CAG-3'; p19 sense, 5'-TGG CGC TAC TCA CCT
CTG GTG-3'; p19 antisense, 5'-GCG CGT CCG TTC ATC ATG AC-3';
GAPDH sense, 5'-CAT CAC CCT ATC ATC TTC CAT C-3'; and GAPDH antisense,
5'-TCA GGC TGT TGT CAT TCT C-3'. PCR products were resolved on 2% agarose
gels, transferred to Zetaprobe membrane (Bio-Rad, Richmond, CA), and
probed with a digoxigenin-labeled internal oligonucleotide.

PCR-based Methylation Assay. A PCR-based methylation assay de-
scribed previously (33) was used to analyze various regions of the p16/p19
gene locus. Briefly, 2 μg of genomic DNA was incubated with 2 units of either
HpaII, CfoI, or MspI restriction enzymes prior to PCR amplification. Primers
used for methylation analysis of each region are as follows: exon 1β sense,
5'-GCT CAC CTC TCT TGC TGG CAA AGC GC-3'; exon 1β antisense, 5'-AGG
AGC CAG CAG CTA GGG CAG-3'; 5' Alu sense, 5'-TCA GGC AGT AAG
TTC TTC TTG GTC-3'; 5' Alu antisense, 5'-TGG TTG GGA TAG TAT
CAG TAT TAC-3'; 3' Alu sense, 5'-GTA ATG ATG ATA ATT CTA TCC
CAT CTC TAC-3'; 3' Alu antisense, 5'-AGC ATC TTC TTT TOA GGC ATA
GCA TTA-3'; 5'-GCA ATC TTC TTG TTA GGA ATC TAA-3'; 5'-CAT CAC
CGT CAG GGT GAG GGG G-3'; and 5'-CGC TCT CAC AGG CAC CAT CCT
C-3'. PCR conditions for each region were: exon 1β, 28 cycles at 94°C for 1 min,
57°C for 30 s, 72°C for 1 min; 5' and 3' Alu, 30 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min; and exons 1α and
2, 28 cycles at 94°C for 1 min, 56°C for 45 s, 72°C for 45 s. Sequence
information for the regions upstream of the p16 gene has been deposited in the
GenBank database (accession no. AF222809).

Bisulfite Genomic Sequencing Protocol. The method described by From-
mer's group (40, 41) was used to react ~2.0 μg of DNA isolated from parent
bladder cancer cell lines and subclones with sodium bisulfite. Primary and
secondary PCR reactions were performed on bisulfite-treated DNA for 30
cycles each at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Primers
AB1a (5'-AAT AAT ATT GGA TAA TTT TTT TTT TTT TTT ATT GAT GAG-3')
and AB1b (5'-CTC (A/G)CC AAA ACC AAC (A/G)TT AAC-3') were used to
generate the primary PCR template for regions A and B. Primers C1a (5'-GAG
GGG GTA GAG GGA GGA TAT-3') and C1b (5'-ACC AAT CAA CC (A/G)AA
ACT CCA TAC TA-3') were used for primary PCR amplification for region
C. Primers D1a (5'-GTA GGT GGG GGA GGT TTT ATT TAT-3') and D1b
(5'-CTA CAA ACC CTC TAC TCA ACC AA-3') were used for primary PCR
amplification for region D. Primers AB1a and AB2 (5'-AA AAT ATT CTT
TCC TAA TAA AA -3') were used for secondary PCR amplifica-
tion of region A. Primers B2a (5'-TTT GGT GGG GGT TTT ATT AGG-3') and B2b
(5'-AAA CTA AAC TCC TCC CCA CCT A-3') were used for
secondary PCR amplification of region B. Primers D1a and D2b (5'-CCC
ACC CTC TAA TAA ACA ACC AA-3') were used for secondary PCR
amplification of region C. Primers D2a (5'-AGA GGA GGG GGT TGT TGG
TTA TT-3') and D1b were used for secondary PCR amplification of region D.
P CR products were gel purified and ligated into the pCRII cloning vector
(Invitrogen, San Diego, CA). Individual plasmid molecules were then se-
lected using the Sequenase version 2.0 kit (Amersham Corp., Cleveland, OH).

Construction, Methylation, and Transfection of p16PRO-CAT Vector. A
2.1-kb DNA fragment isolated from a genomic DNA library (Stratagene,
La Jolla, CA) was used as a template for PCR amplification of the region upstream
of the p16 exon 1α ATG initiation codon. The 5' primer included the EcoRI
site at position −869 and the 3' primer replaced the ATG with a HindIII site.
A SacI site was created in the p16 promoter region by changing the nucleotide
at position −341 relative to ATG (GAGCC to GAGCTC) by site-directed
mutagenesis. This nucleotide change did not reduce basal promoter activity
in the CAT assay system. The DNA fragment was ligated into the pSVO-CAT
vector to create p16PRO-CAT. In vitro methylation was performed by incu-
bating the p16PRO-CAT plasmid with either 3 units of M.HpaII or M.Hhal.
A methylation cassette assay was also used to generate additional constructs by
incubating 1 μg of plasmid DNA with either 3 units of M.HpaII (New England
Biolabs, Beverly, MA) followed by digestion with SacI and BstEII or 3 units of
M.Ssfl followed by digestion with EcoRI and SacI. Restricted DNA frag-
ments were separated by agarose gel electrophoresis, purified, and ligated to
either unmethylated or methylated plasmid DNA to produce the desired
construct. Equal amounts of plasmid were introduced into C-33A cells by
liposome-mediated transfection with pRSV-LacZ. CAT activity was measured
48 h after transfection by 14C radioactive quantitative. The relative promoter
activity was calculated and normalized via β-galactosidase activity.

RESULTS

Analysis of p19 and p16 Expression in Subclone Populations. The
correlation between methylation and transcriptional silencing of the
p16 gene has been well established (11, 31, 33) as well as the
modulation of gene expression in culture by the use of demethylating
agents such as 5-Aza-CdR (11, 42, 43). We examined the relationship
between CpG island methylation and expression in the p19/p16 transciptional
unit in the J82 and T24 bladder cancer cell lines that do or do not express a p16 transcript, respectively (Fig. 1). Both cell types
were found to express equivalent levels of the p19 gene. Clones of
T24 cells expressing substantial quantities of p16 mRNA could be obtained following brief treatment with 5-Aza-CdR, and these showed
no changes in the levels of p19 transcript (Fig. 1). The J82 human
bladder carcinoma-derived cell line was used as a positive control for
p16 expression because this cell line normally expresses a p16 transcript at high
levels. A T24 subclone derived from a parent culture that was not
treated with 5-Aza-CdR (clone 1) showed no detectable p16 expres-
sion. Clone 2 was similar to many of the clones derived from parent
cultures treated with 5-Aza-CdR and also showed no detectable p16
expression. Reactivation clone 3 had the least expression of p16 mRNA
followed in increasing order by clone 2, then clone 4. The levels of
p19 mRNA expression were virtually identical for all samples analy-
alyzed, indicating that transcription of this gene was not affected by
differential expression of p16.

Long Range Methylation Analysis of the p19 and p16 Loci. Fig.
2 shows results from PCR-based methylation analysis of p19 exon 1β.
Alu elements located upstream of exon 1α, p16 exon 1α coding
region, and exon 2 in the bladder cancer cell lines described previ-
ously. This assay is qualitative rather than quantitative, and the
presence of bands in the uncut (U) DNA lanes served as a positive
control for PCR amplification, whereas absence of bands in the CfoI
(Č)- and HpaII (H)-digested DNAs indicated that methylation was not
present in at least one of the enzyme sites examined. The methylation
ROLE OF DNA METHYLATION IN EXPRESSION OF P16/WP16

Induction of p16 expression assayed by RT-PCR in T24 subclones. Single-cell subclones were isolated after treatment of parent cultures with 1 \times 10^{-7} M 5-Aza-CdR. J82 cells were used as a positive control for p16 expression. T24 clone 1 was derived from cells that were not treated with 5-Aza-CdR and showed no detectable levels of p16 expression. Clone 2 originated from a parent culture treated with 5-Aza-CdR and showed no p16 expression. Varying levels of p16 expression were observed in subclones derived from T24 parent cultures treated with 3 \times 10^{-7} M 5-Aza-CdR. In increasing order of p16 expression were: clone 7 < clone 3 < clone 4. Parent T24 cultures were treated for 24 h with 5-Aza-CdR, followed by subcloning 48 h after drug removal. Levels of p19 and GAPDH expression were constant for all cell lines examined.

Fig. 1. Induction of p16 expression assayed by RT-PCR in T24 subclones. Single-cell subclones were isolated after treatment of parent cultures with 1 \times 10^{-7} M 5-Aza-CdR. J82 cells were used as a positive control for p16 expression. T24 clone 1 was derived from cells that were not treated with 5-Aza-CdR and showed no detectable levels of p16 expression. Clone 2 originated from a parent culture treated with 5-Aza-CdR and showed no p16 expression. Varying levels of p16 expression were observed in subclones derived from T24 parent cultures treated with 3 \times 10^{-7} M 5-Aza-CdR. In increasing order of p16 expression were: clone 7 < clone 3 < clone 4. Parent T24 cultures were treated for 24 h with 5-Aza-CdR, followed by subcloning 48 h after drug removal. Levels of p19 and GAPDH expression were constant for all cell lines examined.

Fig. 2. PCR-based methylation analysis of exon 1β, Alu elements, exon 1α, and exon 2 in human bladder cancer cell lines. Top, relative positions of exon coding regions, Alu elements, CpG sites (tick marks), and restriction enzymes sites used for PCR-based methylation analysis. Lanes marked U (undigested), C (CfoI), H (HpaII), and M (MspI) indicate that DNAs were digested with the corresponding restriction enzyme before PCR amplification using primers flanking the region of interest. The presence of bands indicates methylation at all monitored CpG sites within the region of PCR amplification. The absence of bands indicates lack of methylation at least one site within the region amplified by PCR. Uncut (U) DNA served as a control for PCR amplification; digestion of DNA with MspI (M) before PCR served as a control for restriction enzyme digestion.

mRNA

GAPDH mRNA

p16 mRNA

p19 mRNA

status of seven CfoI sites and one HpaII site in exon 1β of the p19 gene was monitored using the PCR-based assay. Analysis of both 5' and 3' Alu elements located approximately 1.3 kb upstream of the p16 exon 1α translation start codon revealed methylation at all sites (two CfoI sites and one HpaII site in each Alu) as indicated by the presence of bands in the CfoI- and HpaII-digested lanes for all samples analyzed. Analysis of two CfoI sites and one HpaII site in the exon 1α coding region showed methylation in T24 cell line DNA, absence of methylation in J82 cell line DNA, and partial methylation in clone 4 DNA. Exon 2 was found to be methylated at seven CfoI sites and four HpaII sites in all of the samples. These findings demonstrated that a p19 transcript can be formed, even in the presence of heavily methylated CpG-rich sequences located downstream of its promoter.

Methylation Status of T24 and J82 Cell Lines. Bisulfite genomic sequencing (40, 41) was used to determine the methylation patterns in bladder cancer cell lines in greater detail by dividing a 1-kb region that included p16 exon 1α and upstream sequences into 4 regions (A to D) defined by the PCR primers used to amplify bisulfite-treated DNA. DNA sequences extending to position -869 relative to the p16 ATG initiation codon were included in this study because this region has been reported previously to be sufficient for basal p16 promoter activity (44). PCR products were cloned into the TA vector (Invitrogen, San Diego, CA) after amplification of each region, and individual plasmid molecules were sequenced. Sequencing of 80 plasmid clones revealed a complete absence of methylation at all 67 CpG sites in the J82 cell line (0%) and a close to fully methylated pattern at all CpGs in the T24 cell line (97%; data not shown).

Effects of 5-Aza-CdR on Methylation Patterns in T24 Subclones. Typical results from sequencing individual plasmid molecules for determination of CpG methylation status in a p16-expressing subclone (clone 4) isolated after treatment of the parent T24 cells with 5-Aza-CdR are shown in Fig. 3. Because 5-Aza-CdR only acts as an inhibitor of methylation of newly synthesized DNA (45), we were not surprised to find heterogeneous mixtures of molecules in which some DNA molecules were completely unaffected by the drug, whereas other DNA molecules contained areas of demethylation consistent with the incorporation of 5-Aza-CdR into parental DNA strands during S phase of the first cell division after drug treatment. We have defined 5-Aza-CdR-affected molecules as those which contained at least two unmethylated CpGs in tandem or a total of three unmethylated CpG sites within a region amplified by PCR of bisulfite-treated DNA, because these methylation patterns were never observed in molecules sequenced from the untreated parent T24 culture. 5-Aza-CdR-unaffected molecules were defined as having less than three unmethylated CpG sites for each region amplified by PCR and were presumably derived from parent DNA strands that did not incorporate 5-Aza-CdR during S phase of the first division after drug treatment. Interestingly, 105 of 245 (43%) of the total number of individual molecules examined for all p16-expressing subclones were classified as being 5-Aza-CdR unaffected, and 140 of 245 (57%) were defined as being 5-Aza-CdR-affected molecules. Our hypothesis was that DNA molecules that were methylated (5-Aza-CdR-affected) did not express p16, whereas molecules that were demethylated by the effects of 5-Aza-CdR (5-Aza-CdR-affected) were competent for p16 expression. This is also supported by the finding that clone 2, which was derived from a parent culture treated with 5-Aza-CdR but showed no detectable p16 expression, contained an almost fully methylated 5' CpG island similar to the untreated T24 cultures (data not shown). Consensus methylation patterns at each CpG site were, therefore, generated for each of the T24 subclones that expressed p16, considering the data from 5-Aza-CdR-affected molecules to characterize
Fig. 3. Genomic sequencing data from a T24 subclone that expressed p16 at high levels. Methylation status at CpG dinucleotides: ○, unmethylated; ●, methylated. Horizontal rows of circles indicate individual plasmid molecules that were sequenced after PCR amplification and cloning of bisulfite-treated DNA. The entire 1-kb region analyzed was divided into four regions (designated A–D) defined by the primers used for PCR amplification; therefore, the horizontal rows do not represent contiguous 1-kb molecules. Tick marks, relative positions of CpG dinucleotides; △, location of HpaII sites in the p16 promoter region; ▲, location of HhaI sites in the p16 promoter region; arrows, putative transcription initiation sites; brackets, 5-Aza-CdR-unaffected molecules excluded from compilation of consensus demethylation patterns.

methyltion patterns that were associated with various levels of gene activity.

Methylation Patterns of T24 Subclones Reveals Areas of Demethylation Associated with Increasing p16 Expression. The overall methylation status throughout the 1-kb region analyzed by genomic sequencing for three T24 subclones derived from parent cultures treated with $3 \times 10^{-7}$ M 5-Aza-CdR and expressing p16 is shown in Fig. 4. The percentage of methylation at each CpG site was determined by sequencing multiple plasmid molecules and considering only those molecules that were classified as being 5-Aza-CdR-affected. There was no direct correlation between the degree of methylation over the entire 1-kb region and gene expression in the subclones. In general, regions A–C appeared to be less methylated than region D. However, a good association between the overall levels of methylation in region C and p16 expression was found. A direct correlation between decreasing levels of methylation in region C and increasing p16 expression in subclone populations derived from parent cultures treated with 5-Aza-CdR was also observed. Clone 7 had the highest overall percentage of methylation in region C (75%), followed by clone 3 (55%), and then clone 4 (31%). The methylation...
levels of CpGs located at the HpaII and SmaI sites in the region of putative transcription start in T24 subclones was also confirmed by methylation-specific PCR (Ref. 46; data not shown).

The methylation patterns generated by bisulfite genomic sequencing of reactivant clones also suggested that p16 expression could occur in the presence of a relatively heavily methylated coding domain (region D). In general, region D contained a higher proportion of 5-Aza-CdR-unaffected molecules than other regions, but even the 5-Aza-CdR-affected molecules used to generate the composite methylation profiles for this region still contained a large number of methylated CpGs. No other correlation between the number of 5-Aza-CdR-affected alleles and gene expression was found among the reactivant clones, suggesting that the proportion of 5-Aza-CdR-affected alleles in each clone is not responsible for the observed differences in the levels of p16 expression.

We have also generated a total of four additional subclones of clone 4 to further investigate the relationship between methylation patterns and p16 transcriptional activity. Three of the four subclones derived from the clone 4 culture did not express p16 by RT-PCR analysis, whereas one subclone was found to express p16 at a level that was similar to the original clone 4 culture (data not shown). The proportion of methylation of the p16 5' CpG island was monitored at three CpG sites in each of these subclones using the quantitative M.SsSI method as described previously (47, 48). M.SsSI analysis showed that the single subclone that expressed p16 had an average of 52% methylation, whereas the three nonexpressing subclones had an average of between 96 and 99% methylation at the monitored CpG sites. These results showed that the clone 4 culture, which was originally generated from a single cell, had diverged into a more heterogeneous population of cells after drug treatment that contained either low levels of methylation and expressed p16 or contained virtually fully methylated promoter regions and did not express the p16 gene.

**Methylation of HpaII Sites Is Capable of Down-Regulating p16 Promoter Activity.** A plasmid containing the upstream region of p16 exon 1a was used in a CAT reporter assay system to determine the effect of CpG methylation on p16 promoter activity. The p16PRO-CAT construct was made by inserting a fragment that contained sequences between the EcoRI site at position −869 and a HindIII site that replaced the normal ATG translational start site by PCR-directed mutagenesis into the pSV0-CAT plasmid vector. This fragment has been characterized as being sufficient for basal p16 promoter activity in pRB-negative cell lines (44) and contained 49 CpG dinucleotides. A total of 13 HpaII sites and 11 Hhal sites are located in the vector sequences flanking the EcoRI-HindIII p16 promoter fragment. Methylation of specific regions of the p16PRO-CAT construct was accomplished using a cassette assay as described previously (49).

Data obtained from CAT reporter gene expression after in vitro methylation and transfection of plasmid DNA into the pRB-negative human cervical carcinoma-derived cell line C-33A is shown in Fig. 5. The unmethylated p16PRO-CAT plasmid was defined as having 100% CAT activity. Methylation of the entire construct with M.SsSI significantly reduced CAT expression by 96%, whereas methylation of all 24 CpG sites located within the EcoRI-Sacl fragment of the p16 promoter reduced CAT activity by 74%. Promoter activity was further reduced by 97% when all CpG sites located outside of the EcoRI-Sacl fragment were methylated. For technical reasons, we were unable to generate a cassette with all CpGs methylated by SsSI in region C; however, methylation of the p16PRO-CAT plasmid with M.HpaII reduced promoter activity by 75%. Interestingly, methylation of only three HpaII sites located within the SacI-BstEII fragment (which corresponds to sequences located within region C) resulted in approximately a 67% decrease in CAT reporter gene expression. On the other hand, methylation of 1 HpaII site located outside of region C and 13 HpaII sites within the plasmid resulted in a 48% decrease in promoter activity. Methylation of all Hhal sites in the plasmid, which included three CpG sites located within the p16 promoter, reduced CAT activity by only 4%.

The in vitro methylation experiments indicate that methylation of only a few CpG sites in the p16 promoter can significantly reduce transcriptional activity. In fact, methylation of a single HpaII site located in the p16 promoter reduced CAT activity by 48%. Presumably, this repression is not attributable to methylation of the HpaII sites in the vector backbone because the number and location of HpaII sites in the vector is almost identical to that of the Hhal sites (13 HpaII sites compared to 11 Hhal sites), which did not reduce gene activity. Furthermore, previous studies have demonstrated no effect of methylation at HpaII or Hhal sites on CAT gene expression in plasmids containing the SV40 promoter (50, 51). Methylation of three HpaII sites in the p16 promoter reduced transcriptional activity by 67%, whereas methylation of all HpaII sites, which included four
The J82 human bladder carcinoma cell line was methylated in only the Alu elements and exon 2. This cell line also showed detectable expression of both p19 and p16. The T24 cell line was shown to be extensively methylated in all regions examined except for exon 1β. This clone was found to be extensively methylated in the Alu elements, exon 1α coding domain, and exon 2. No methylation was detected in exon 1β, and lower levels of methylation were detected in the p16 promoter region. Clone 4 was capable of expressing both p19 and p16 transcripts.

CpGs in the promoter, caused a 75% reduction in CAT reporter activity. These results suggest that the degree of transcriptional down-regulation may be dependent upon the density of CpG methylation. However, the location of methylated CpG sites may also be an important factor because methylation of 24 CpG sites in regions A–B reduced promoter activity by 74%, which was similar to the degree of transcriptional repression caused by methylation of only three to four HpaII sites in the promoter (67–75%). Although it is difficult to distinguish whether down-regulation of p16 promoter activity is dependent upon the location or density of methylated CpG sites, the large reduction in promoter activity by methylation of HpaII site(s) located within the p16 promoter provides the first functional evidence that site-specific methylation may potentially act as an initiating event in the progressive inactivation of this tumor suppressor gene.

DISCUSSION

A summary of the relationship between the methylation status of regions examined in this report and transcriptional activities of p19 and p16 is shown in Fig. 6. The J82 cell line was unmethylated at sites in exon 1β, methylated in each of the Alu elements, unmethylated in the 5' CpG island of p16 including exon 1α, and methylated in exon 2. The expression of both p19 and p16 mRNA was detected by RT-PCR. The T24 cell line was methylated in all regions examined except for exon 1β, and only the p19 transcript was detected. The situation observed in the T24 cell line demonstrated that extensive methylation of repetitive sequences and CpG islands downstream of the p19 promoter did not block expression of the p19 transcript but prevented expression of a p16 transcript. Finally, the data for clone 4 is summarized showing absence of methylation in exon 1β, presence of methylation in each of the Alu sequences, variable (partial) methylation of the p16 promoter, more extensive methylation of the exon 1α coding region, and methylation of exon 2. Interestingly, clone 4 showed expression of both p19 and p16 transcripts. Similar methylation patterns were also observed for the other subclones (clones 2 and 7), which expressed p19 at the same level as clone 4, but showed higher levels of p16 promoter methylation and lower levels of p16 expression.

Methylation of repetitive sequences such as Alus and L1s may be the result of a host defense system that is aimed at abrogating the potential transcriptional activities of such elements. Alu sequences have been found previously to be located in close association with growth-regulatory genes such as p53 (52, 53), and the presence of repetitive sequences near gene coding regions or promoters may affect the methylation patterns of these adjacent regions of DNA (18, 52, 54). De novo methylation of sequences associated with repetitive elements may also result in increased mutation rates such as those observed for certain regions of the p53 gene (52).

Prior investigation of apo-E and Igf2r have shown that downstream methylation of CpG-rich sequences is permissive for gene expression (55, 56). In the case of Igf2r, the parental allele that expressed the gene was shown to be methylated in downstream sequences. The effect of regional DNA methylation has also been studied in the hamster aprt gene, demonstrating that methylation at the 3' end did not interfere with transcription (57). The hamster aprt gene, however, has a high density of CpGs at its 5' end but is relatively CpG depleted at the 3' end. On the other hand, methylation of both the 5' and 3' ends of the hamster tk gene, which contains a high number of CpGs over its entire length, inhibited transcriptional activity (57). We have shown that methylation of the p16 5' CpG islands blocks expression of the p16 transcript but not p19 expression, as demonstrated by our analysis of methylation patterns and gene activity in human bladder cancer cell lines. Methylation of Alu repetitive elements and gene coding sequences downstream of the p19 promoter also did not inhibit p19 expression. Therefore, methylation of CpG islands, including the p16 promoter region and p16 exon 2 which are located within the transcriptional unit of the p19 gene, do not block p19 expression.

A number of previous studies correlating CpG methylation with transcriptional silencing of p16 in cell lines and tumors have examined sites in the exon 1α coding domain (31–33). These reports have used Southern analysis or PCR-based methylation assays and were limited to the possible CpG sites analyzed. Although methylation of
the p16 exon 1α coding region has been correlated previously with a lack of gene expression (11, 31–34, 36), we have shown the presence of extensive methylation of the exon 1α coding domain in reactive clones expressing p16. Our data also suggest that localized areas of demethylation in the p16 promoter, including CpGs associated with putative transcriptional start sites, may be permissive for p16 expression and may also serve as a better marker for methylation-associated transcriptional silencing of p16.

Although it is not surprising that in vitro methylation of the p16PRO-CAT construct reduced transcriptional activity, it is remarkable that methylation of only a few HpaII sites was sufficient to confer such a high degree of transcriptional down-regulation. Interestingly, methylation of three HhaI sites in the p16 promoter did not have any significant effect on promoter activity. Although it is difficult to determine whether density or location of CpG methylation is responsible for the observed reduction in CAT expression, it appears that each of these components may play some role in decreasing promoter activity. The presence of methylation at a small number of CpG sites may, therefore, be an initiating event resulting in progressive inactivation of the p16 gene. The partial methylation of previously unmethylated CpG islands located in the promoters of tumor suppressor genes may result in transcriptional down-regulation and thus lead to an increased rate of cellular growth. The aberrant methylation of CpG islands associated with such growth-regulatory genes may also result in a clonal selection of cells that possess increased growth potential because of decreased tumor suppressor gene activity. The use of S-Aza-CdR for possible treatment of certain cancers may be feasible because demethylation of only a few CpG sites could be capable of restoring tumor suppressor gene function. Indeed, it has been shown previously that demethylation at one critical CpG site is sufficient for transcriptional activation of the EBV latency C promoter (49).

The genomic sequencing data from single-cell subclones in conjunction with the methylation cassette experiments using the p16PRO-CAT plasmid illustrate several important points that can be made about the role of DNA methylation in regulating p16 promoter activity: (a) methylation of CpG islands within the p19 transcriptional unit does not block expression of the p19 gene. Previous reports that have investigated the effects of downstream methylation on transcriptional activity have not considered the effects of methylation of an internal CpG island-containing promoter. Additionally, methylation of other CpG-rich sequences, including Alu elements and p16 exon 2, did not block p16 expression; (b) methylation of certain regions upstream of the p16 exon 1α coding domain, particularly region C, may be critical for transcriptional activity. The consensus methylation patterns of the reactive clones that expressed p16 showed a direct correlation between decreased levels of methylation in region C and increased gene expression and also suggested that p16 expression can occur in the presence of a relatively heavily methylated coding domain; and (c) methylation of HpaII site(s) within the p16 promoter can significantly down-regulate transcriptional activity. These results suggest that a relatively low number of methylation errors may be sufficient to initiate a reduction of gene expression and thus provide support for the model of progressive inactivation of this tumor suppressor gene by DNA methylation.

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REFERENCES


The Role of DNA Methylation in Expression of the \textit{p}19\slash\textit{p}16 Locus in Human Bladder Cancer Cell Lines

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