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CpG Methylation Directly Inhibits Binding of the Human Papillomavirus Type 16 E2 Protein to Specific DNA Sequences

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CpG methylation of the human papillomavirus upstream regulatory region has previously been shown to reduce virus promoter activity. Here, we demonstrate that methylation of the CpG dinucleotides contained within the binding site of the human papillomavirus type 16 E2 protein has a direct effect on the interaction of this protein with DNA. Methylation of both CpG dinucleotides within the E2 site abolishes the binding of E2.

Human papillomaviruses (HPV) are small, double-stranded DNA viruses that infect a variety of epithelial cells and induce benign hyperproliferative lesions, which are known as warts (27). HPV type 16 (HPV-16) is one of a subset of HPV genotypes that are closely associated with anogenital cancer. Expression of the viral early genes E6 and E7 is generally thought to be causally involved in malignant transformation (1, 10). Transcription of the HPV-16 E6 and E7 genes is under the control of the P97 promoter which lies at the 3′ end of a noncoding upstream regulatory region (URR). Transcription from the P97 promoter is controlled by a number of cellular transcription factors and by the product of the viral E2 gene. The E2 protein binds to four palindromic sites (consensus sequence, 5′-ACCGN_4CGGT-3′) found upstream of the P97 promoter and regulates promoter activity (18, 23). The E2 protein also plays a role in DNA replication; E2 forms a heteromeric complex with the viral replication factor E1 and recruits this protein to the viral origin of replication (7).

In higher eukaryotes, the promoters of transcriptionally active genes are often located within unmethylated CpG islands. In many cases, the methylation of CpG dinucleotides within these islands has been shown to result in gene silencing (6). Although the precise cause of this silencing is not known, it is likely that methylation either directly or indirectly blocks the binding of transcription factors. The methylation of CpG sequences within the binding sites of transcription factors such as c-Myc, c-Myb, and E2F has been shown to directly prevent DNA binding (13, 14, 17). However, CpG methylation alone has little or no effect on the binding of other transcription factors such as Sp1 and YY1 (12, 9). CpG methylation might block the binding of these transcription factors indirectly, either by changing the conformation of chromatin or by interacting with methyl-CpG-specific repressor proteins (2, 24).

The HPV regulatory sequences are located within an unmethylated CpG island (3, 26). Transient transfection studies have shown that the methylation of cytosines within the HPV-18 CpG island results in the repression of HPV-18 promoter activity (19). Similar results were obtained when the HPV-16 URR was methylated prior to transient transfection into cervical carcinoma cells; methylation of the HPV-16 URR resulted in the silencing of a heterologous promoter (15). CpG methylation has been shown to have a direct effect on the binding of at least one cellular transcription factor in the HPV-16 URR (15). Here, we show that CpG methylation also directly inhibits the DNA-binding activity of the HPV-16 E2 protein.

The HPV-16 E2 protein was purified from *Escherichia coli* as a glutathione *S*-transferase (GST) fusion protein. The HPV-16 E2 gene was amplified from HPV-16 DNA by PCR and was cloned into the prokaryotic expression vector pGEX-2T (Pharmacia). This created an in-frame fusion between the full-length E2 protein and GST. The GST-E2 fusion protein was purified from bacterial cell lysate by a modification of the standard purification procedure that incorporates an ATP wash to remove copurifying GroEL (25). Gel retardation assays were then used to quantify the binding of this fusion protein to an unmethylated and a CpG-methylated E2-binding site. The E2-binding sites used in these experiments are indicated in Fig. 1; the consensus E2-binding site contains two CpG dinucleotides. Figure 1 lists sequences for the unmethylated E2 site, the fully methylated E2 site, and E2 sites with methylated cytosines at the −4 and +3 positions. The methylated oligonucleotides were made using the chemically modified nucleoside 5-methyldeoxycytidine at the required position in the synthesis reaction.

Oligonucleotides (200 ng) were radiolabelled with [γ-^{32}P] ATP (Amersham), using T4 polynucleotide kinase (Gibco-BRL). After heat inactivation of the kinase (80°C for 10 min),

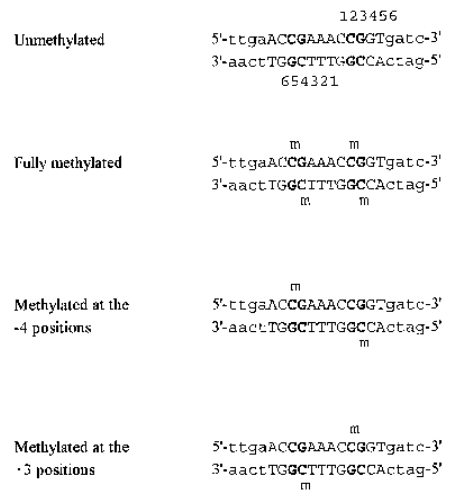

FIG. 1. Oligonucleotides used in this study. Capitals, core E2-binding sites; boldface, CpG dinucleotides; m, methyl group.

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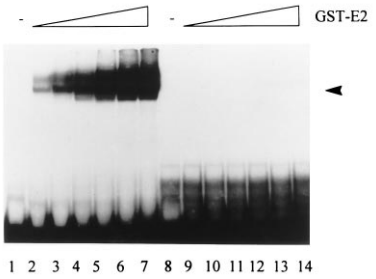

FIG. 2. Binding of E2 to unmethylated and methylated oligonucleotides. Labelled oligonucleotides carrying either the unmethylated E2-binding site (lanes 1 to 7) or the methylated E2-binding site (lanes 8 to 14) were incubated with increasing amounts of purified GST-E2 fusion protein (0.04, 0.08, 0.16, 0.32, 0.64, and 1.05 μM, respectively). Arrowhead, E2-DNA complex.

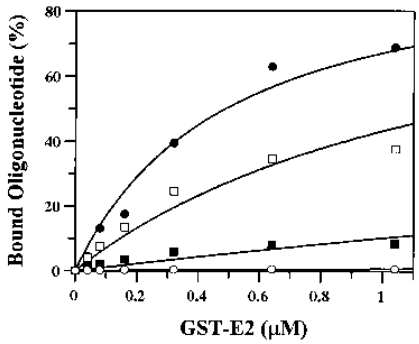

FIG. 3. Binding of E2 to oligonucleotides methylated at different positions. Increasing amounts of GST-E2 protein were added to unmethylated (●), fully methylated (○), and partially methylated (−4 [□], +3 [■]) E2-binding sites exactly as described in the legend to Fig. 2. At each protein concentration, the amounts of free and bound DNA were determined with a PhosphorImager, and weak binding curves were fitted to the data using GraFit. An assay of GST-E2 binding to the unmethylated E2 site was included on each gel as an internal control, and each experiment was repeated at least twice.

the DNA was cooled slowly to room temperature to anneal the strands and radiolabelled DNA was separated from unincorporated nucleotides, using Stratagene G50 size exclusion push columns. Increasing amounts of GST-E2 were added to radiolabelled oligonucleotides carrying the E2 sites. The binding reactions (25-μl mixtures) were carried out in buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.9), 25 mM KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol, 0.5 μg of bovine serum albumin μl^{-1}, and 80 ng of poly(dI · dC) nonspecific carrier DNA μl^{-1}. The reaction mixtures were incubated at room temperature for 20 min before free and bound oligonucleotides were separated by electrophoresis on a 6% native polyacrylamide gel run in 0.5 × Tris-borate-EDTA for 1.5 h at 15 mA. Figure 2 shows the results of a gel retardation assay in which GST-E2 was incubated with the unmethylated oligonucleotide (lanes 1 to 7) or the fully methylated oligonucleotide (lanes 8 to 14). The unmethylated oligonucleotide binds E2 at low protein concentrations, whereas the methylated oligonucleotide binds E2 poorly, if at all, in the presence of nonspecific competitor DNA. These data clearly demonstrate a direct effect of CpG methylation on the binding of the E2 protein.

Having established that CpG methylation inhibits binding of the E2 protein, we set out to determine the effects of methylation of individual cytosine bases within the E2-binding site. The results of gel retardation assays in which increasing amounts of GST-E2 were added to labelled oligonucleotides methylated at either the +3 or the −4 positions are shown graphically in Fig. 3. These data were used to estimate the relative dissociation constants for methylated and nonmethylated sites (Table 1). Methylation at the +3 positions has a major effect on the binding of E2, whereas methylation at the −4 positions has a minor effect. However, methylation at all four positions is required for the complete abolition of sequence-specific DNA-binding activity; E2 binds to methylated sites with an affinity comparable to that seen when this protein binds to nonspecific DNA sequences (data not shown). Although the structure of HPV-16 E2 has not yet been determined, the structure of the DNA-binding domain of the bovine papillomavirus E2 protein bound to a specific DNA sequence has been resolved by X-ray crystallography (11). Inspection of the crystal structure reveals that a methyl group at the +3 position might sterically hinder lysine 339 (equivalent to lysine 299 in HPV-16 E2), which is an amino acid that makes two direct hydrogen bonds to the DNA. In contrast, methyl groups at the −4 position do not seem to hinder any of the protein-DNA interactions. The modest effects on E2 binding seen when the −4 cytosines are methylated might be due to subtle alterations in DNA structure induced by the addition of methyl groups (8).

TABLE 1. Relative dissociation constants for the binding of E2 to unmethylated and CpG-methylated DNA

E2-binding site	$K_{d(\mathrm{rel})}$[a]	$\Delta\Delta G$(kJ mol^{-1})[b]
Unmethylated	1	
Fully methylated	269	11.9 ± 2.2
Methylated at −4	3	1.6 ± 0.8
Methylated at +3	18	6.2 ± 1.0

[a] The relative dissociation constant ($K_{d(\mathrm{rel})}$) was obtained by the following equation: $K_{d(\mathrm{rel})} = K_{d(\text{test binding site})}/K_{d(\text{unmethylated binding site})}$.

[b] $\Delta\Delta G$ was determined by the following equation: $\Delta\Delta G = -\mathrm{RT} \ln K_{d(\text{unmethylated binding site})}/K_{d(\text{test binding site})}$.

The HPV genome exists as an extrachromosomal element in preneoplastic cells but is often integrated into the host chromosome in cervical carcinomas (5). Chromosomal integration frequently results in disruption of the E2 open reading frame, with consequent loss of the E2 protein (21, 22). However, in malignant lesions in which the DNA has integrated, viral DNA can also persist extrachromosomally (16). Furthermore, it has been reported that many HPV-16-positive cervical cancers contain only episomal viral DNA (4). The methylation states of both episomal and integrated HPV sequences might have a significant effect on viral gene expression. Integrated HPV-18 genomes in the CaSki cervical cancer cell line have been shown to be CpG methylated (20). In addition, nontumorigenic HeLa-fibroblast hybrid cells have been shown to contain methylated HPV-18 sequences, whereas tumorigenic HeLa-fibroblast hybrid cells were found to contain unmethylated HPV-18 sequences (20). Thus, the degree to which integrated HPV DNA is CpG methylated might correlate with the tumorigenic potential of the host cells.

Transient transfection experiments with HPV-18 and HPV-16 sequences have shown that methylation of the viral URR results in a repression of transcriptional activity (15, 19). The addition of highly methylated prokaryotic DNA was found to partially reactivate promoter activity, indicating that the repression is, at least in part, caused by a methyl-CpG-binding protein (19). However, our data clearly demonstrate that CpG methylation also has a direct effect on the binding of the E2

protein and, therefore, a direct effect on viral gene expression and DNA replication.

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