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The human papillomavirus-18 genome is efficiently targeted by cellular DNA methylation

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Abstract

Human papillomaviruses (HPVs) infect epithelia, including the simple and the squamous epithelia of the cervix, where they can cause cancer and precursor lesions. The molecular events leading from asymptomatic HPV infections to neoplasia are poorly understood. There is evidence that progression is modulated by transcriptional mechanisms that control HPV gene expression. Here, we report the frequent methylation of HPV-18 genomes in cell culture and in situ. DNA methylation is generally known to lead to transcriptional repression due to chromatin changes. We investigated two cell lines derived from cervical cancers, namely, C4-1, which contains one HPV-18 genome, and different clones of HeLa, with 50 HPV-18 genomes. By restriction cleavage, we detected strong methylation of the L1 gene and absence of methylation of parts of the long control region (LCR). A 3-kb segment of the HPV-18 genomes downstream of the oncogenes was deleted in both cell lines. Bisulfite sequencing showed that in C4-1 cells and two HeLa clones, 18 of the 19 CpG residues in the 1.2-kb terminal part of the L1 gene were methylated, whereas a third HeLa clone had only eight methylated CpG groups, indicating changes of the methylation pattern after the establishment of the HeLa cell line. In the same four clones, none of the 12 CpG residues that overlapped with the enhancer and promoter was methylated. In six HPV-18 containing cancers and five smears from asymptomatic patients, most of the CpG residues in the L1 gene were methylated. There was complete or partial methylation, respectively, of the HPV enhancer in three of the cancers, and lack of methylation in the remaining eight samples. The promoter sequences were methylated in three of the six cancers and four of the six smears, and unmethylated elsewhere. Our data show that epithelial cells efficiently target HPV-18 genomes for DNA methylation, which may affect late and early gene transcription.

Keywords: HPV-18; Methylation; Cancer

Introduction

Human papillomavirus-18 (HPV-18) is one of the most common HPV types involved in genital neoplasia (Bosch et al., 1995). Together with HPV-6, 11, 16, and 31, it is one of the model systems for transcriptional studies of HPVs. The comparison of these viruses is desirable because they belong to three remotely related HPV groups (HPV-6/11, HPV-16/31, and HPV-18) within the supergroup of genital HPVs (Chan et al., 1995). They also differ in their pathogenicity, as HPV-6 and 11 are most often associated with genital and laryngeal warts. HPV-16/31 and HPV-18 give rise to cervical cancer and its precursors, but among these lesions, HPV-16 and 31 are preferentially associated with squamous carcinoma of the cervix and HPV-18 with adenocarcinoma, respectively (Andersson et al., 2001).

Only a small fraction of HPV infections progresses to neoplastic lesions. While immunological phenomena may determine the clearance of some HPV infections, many infections may lead to persistence of the viral DNA in the absence of symptoms. The mechanisms that determine whether a persistent HPV infection remains free of symptoms or progresses eventually to clinically detectable neoplasia remain enigmatic. Modulation of the transcrip-

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tion of the HPV oncogenes is likely one of the factors that determine the clinical outcome of an HPV infection: There is evidence for the stimulation of HPV oncoprotein expression by physiological stimuli such as steroid hormones (Chan et al., 1989), for major differences in HPV transcription depending on the infected cell type (Sailaja et al., 1999), for the differentiation state of epithelia (O'Connor et al., 2000; Pattison et al., 1997), and for transcriptional heterogeneity in situ in different cell populations within individual lesions (Stoler et al., 1992). This paper describes a part of our effort to describe regulatory phenomena acting through specific transcription factors or through epigenetic mechanisms that affect the biology and pathology of genital HPVs.

The study of the viral gene expression has for many years concentrated on the identification of cis-responsive elements and the sequence-specific transcription factors binding these elements (Bernard, 2002). More recently, it has become clear that epigenetic mechanisms play a major role in modulating the viral gene expression (Stünkel and Bernard, 1999). The term "epigenetics" refers to regulation by higher-order structures of the DNA, such as chromatin conformations that either favor or repress gene expression. The methylation of DNA is one of several mechanisms that influences chromatin conformation (Bird, 1992). Methylation normally occurs at CpG dinucleotides, and the fact that CpGs are present only at 20% of the statistically expected frequency in mammalian genomes (Schorderet and Gartler, 1992) suggests adverse functional consequences of methylation as a cause for their loss during evolution. The same applies to HPV genomes (Badal et al., 2003). With a genome size of 7857 bp and G + C content close to 40%, one may expect to find in the HPV-18 genome about 400 CpG sites, although it actually contains only 172.

It has been known for 20 years that the genomes of HPV-1 and the cottontail rabbit PV can become methylated (Burnett and Sleeman, 1984; Sugawara et al., 1983; Wettstein and Stevens, 1983), and that in vitro methylated HPV-16 genomes are transcriptionally incompetent after transfection into cell culture (Rosl et al., 1993). However, only recently, we and others observed that HPV-16 DNA methylation takes regularly place in vivo in cervical samples from asymptomatic patients, in cervical carcinoma (Badal et al., 2003), and in cell cultures with episomal HPV-16 genomes (Kim et al., 2003). This shows that de novo methylation regularly targets papillomavirus genomes in somatic cells. It raises the questions of where and how HPV genomes are recognized by the cellular DNA methylation mechanism, which enzymatic components are involved in the epithelial de novo methylation, and about the role of this transcription repression mechanism during the normal HPV-16 life cycle and HPV-16-dependent carcinogenesis. The research presented in this paper addresses the question of whether DNA methylation phenomena are, among the genital HPVs, restricted to HPV-16, or also occur in the remotely related type HPV-18. Our data confirm that HPV-18 is as efficiently recognized as HPV-16. We detected methylation preferentially in the late gene L1, but also in the long control region (LCR), which opens the possibility that methylation modulates HPV-18 oncogene as well as L1 transcription.

Results

The restriction enzyme McrBC detects methylated HPV-18 DNA segments in several clones of HeLa and in C4-1 cells

To examine whether methylated CpG dinucleotides (meCpGs) may occur in HPV-18 genomes, we used a strategy successfully employed in studying HPV-16 methylation (Badal et al., 2003), namely, cleavage of the HPV-18 genomes in certain cervical cancer-derived cell lines by the restriction enzyme McrBC, followed by polymerase chain reaction (PCR) amplification of segments of the viral genome and the analysis of the amplicons in agarose gel electrophoresis. McrBC is an unusual restriction enzyme, which recognizes two PumeGpGs closely (50 bp) or even remotely (>1 kb) located relative to one another and cleaves at unspecific positions close to one of these meCpGs (Sutherland et al., 1992); in other words, it recognizes on the average every other meCpG. If, after McrBC cleavage, a DNA segment can be readily detected after PCR amplification, one has to conclude that in the original chromosomal DNA, the sequence was hypomethylated. However, if the amplicon is weakened or absent, the original DNA was hypermethylated. The reaction is powerful in scanning larger genomic segments, but cannot exactly quantify the amount of methylation in each of these segments.

HeLa is a widely used cell line with about 50 genomic copies of HPV-18 recombined with cellular sequences (Ambros and Karlic, 1987; Durst et al., 1987; Mincheva et al., 1987; Popescu et al., 1987; Schwarz et al., 1985). HeLa cells were among the first cell lines ever brought into culture, derived in the middle of the 1950s from an adenocarcinoma of the cervix of an African American patient. Since then, HeLa cells have been cultured separately by countless labs around the world. During this time, they changed genetically by chance as well as by selection, and cell lines called "HeLa" today diverge substantially from one another. Here, we analyzed six different clones of HeLa (see Materials and methods). We present the data for only three of them as the outcome of the McrBC digestion was the same for all six clones. Among these three, we considered HeLa CCl2, a typical representative of the widely used "wildtype" HeLa clones, D98 is a well-known HGPRT negative mutant of HeLa, and 444 is a clone generated during fusion studies between HeLa cells and fibroblast. C4-1 is unrelated to HeLa, but is another widely used cell line derived from a cervical cancer,

which contains only a single HPV-18 genome (Gallego et al., 1994; Kruczek et al., 1981; Schwarz et al., 1985).

The inner circle of Fig. 1 shows a genetic map of the HPV-18 genome, a double-stranded circular DNA with a size of 7857 bp. The LCR is a segment of the genome (position 7134 to 105) with most cis-responsive elements that govern transcription and replication. Transcription of the early genes starts counterclockwise at a promoter at the genomic position 105 (p105) and generates multicistronic transcripts of the early genes E6, E7, E1, E2, and E5 and the late genes L2 and L1. The outer circle of the same figure shows the division of the HPV-18 genome into eight segments that we designed as amplicons, G1–G8, each with an approximate size of 1 kb. The numbers between the amplicons indicate the 5′ ends and the arrows the orientation of the oligonucleotide primers used for generating these amplicons.

Fig. 2 shows the McrBC digestion and PCR amplification analysis of HeLa CCl2, HeLa D98, HeLa444, and C4-1. We could generate the amplicons G1–G4 and G8 from all four clones but not for G5, G6, and G7. This result was expected, as a 2- to 3-kb deletion within HPV-18 of HeLa had been reported (Schwarz et al., 1985). The deletion is fairly exactly defined by the borders given in Fig. 1 (i.e. genomic position 2808 to 5918), as experiments failed to extend the amplicons G4 and G8 even by small increments of 10–50 bp into the direction of the deletion (data not shown). We also confirmed that this region is deleted and not just rearranged since we tried to generate nine different small (200–1000 bp) amplicons throughout the G5–G7 range, without detecting any HPV-18 sequences (data not shown). Strangely, a

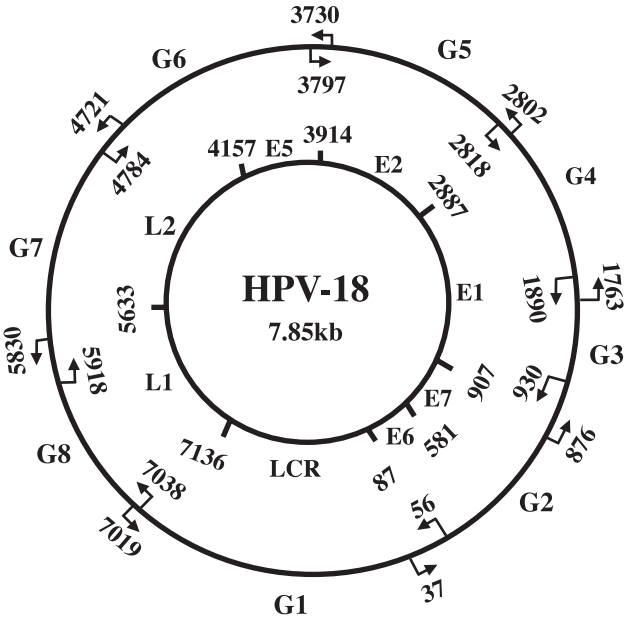

Fig. 1. HPV-18, genome organization (inner circle), and position of PCR primers (outer circle) (see also Table 1A).

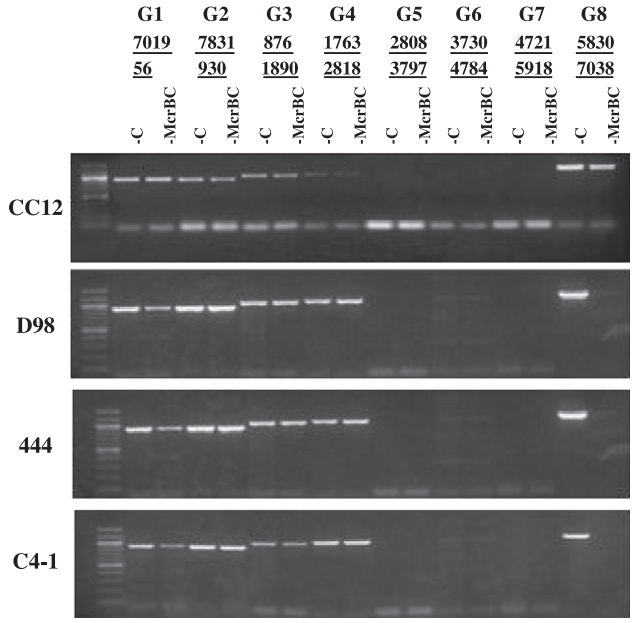

Fig. 2. PCR amplification and McrBC cleavage of the HPV-18 genomes in three different HeLa clones (CCl2, D98, and 444) and the unrelated cell line C4-1. The figure documents lack of cleavage, that is, lack of methylation of amplicons G1–G4, deletion of amplicons G5–G7, and partial or complete cleavage of amplicon G8, overlapping with the L1 gene. Identical data were obtained with the HeLa clones S3, H21, and HD.

seemingly identical deletion of G5 to G7 occurred in C4-1 with approximately the same borders and absence of internal amplicons. We excluded the possibility that C4-1 cells may be a mislabeled HeLa contaminant, as sequencing of the HPV-18 variants confirmed the presence of unrelated HPV-18 variants as published (Ong et al., 1993).

McrBC digestion of the chromosomal DNA of these four cell types showed no change of G1 in HeLa CCl2, but a slight reduction in HeLa D98 and 444 and C4-1. This may indicate, that this region with the enhancer, promoter and replication origin of HPV-18 (Garcia-Carranca et al., 1988; O'Connor et al., 1995), is not methylated in HeLa CCl2, but that possibly some of the 50 genomes in HeLa D98, HeLa444, and C4-1 contain meCpGs. The amplicons G2, G3, and G4, with the early genes E6, E7, and E1, are unaltered, that is, not methylated in all four cell lines. The amplicon G8 is slightly reduced in HeLa CCl2, suggesting that the L1 gene in amplicon G8 is partially methylated in this clone. The complete digestion of G8 in the other three clones suggests heavy methylation of L1.

Six tumor samples lack major deletions of the endogenous HPV-18 genomes

The similarity of the HPV-18 deletions in HeLa and the unrelated C4-1 cells led to a side line question of our methylation research, namely, the question whether this

part of the HPV-18 genome is frequently eliminated during carcinogenesis. To address this possibility, we amplified the HPV-18 DNA from six tumors, two from German (Ge18-1 and 2) and four from Scottish (Sc18-3, 2, 6, 7) patients (Ong et al., 1993). Fig. 3 shows that there is no systematic loss of the G5-G7 segment in these six tumors. All eight amplicons are present in the samples Ge18-1, Sc18-3, Sc18-2, and Sc18-7, and the missing amplicons G3 in samples Ge18-2 and G7 and possibly G5 in sample Sc18-7 may indicate segments of recombination between the viral and the cellular DNA.

Bisulfite sequencing of a 2022-bp L1-LCR-E6 segment of the HPV-18 genomes in HeLa clones and C4-1

Our next objective was to establish a precise map of the meCpGs in HeLa clones and the C4-1 cell line by using the bisulfite sequencing technique. From this, we hoped to learn whether there are precise correlations between methylation patterns, genes, and cis-responsive elements, and whether methylation may occur sporadically or continuously throughout many flanking CpG dinucleotides. We decided to limit this laborious approach to a subsegment of the HPV-18 genome, namely, the genomic position 5940 to 161, which we considered particularly informative. This segment, with a total size of 2022 bp, contains 39 CpG dinucleotides, 26 of these potential McrBC half sites. Nineteen of these CpGs are located in the L1 gene, which ends at position 7133. Three are located in the 5′ part of the LCR (position 7134 to an E2 binding site extending to position 7469). Six are located in the central part of the LCR with the enhancer, which is roughly the region between the two E2 binding sites remote from the promoter, positions 7470 and 7821. Seven are located in the 3′ part of the LCR with the HPV-18 replication origin and promoter (position 7822 to 104), and four downstream of p105, within the transcript encoding the E6 gene (Fig. 4).

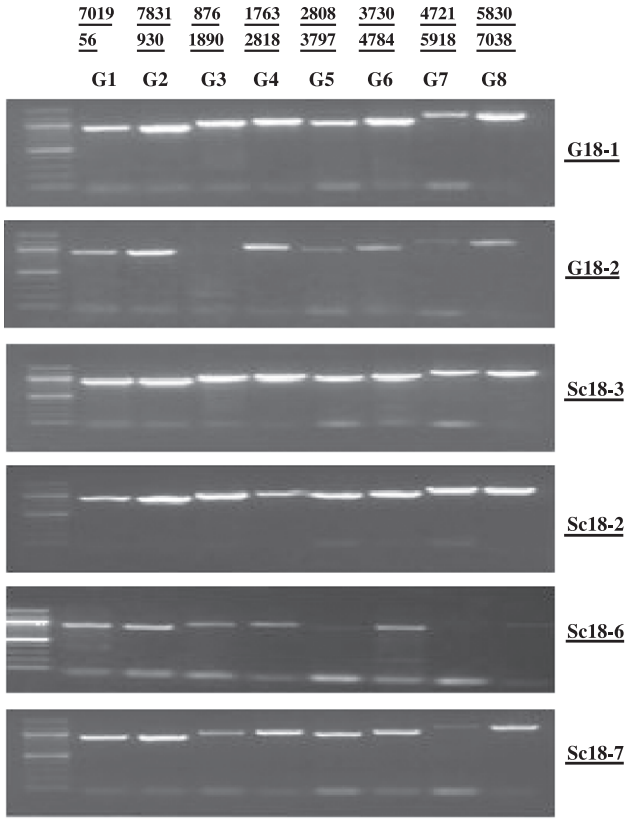

Fig. 3. PCR amplification of the HPV-18 genomes in six tumors, a control experiment to confirm that loss of the amplicons G5–G7 is not a systematic event in HPV-18-dependent carcinogenesis. Lack of other amplicons likely indicates recombinational interruption of the HPV-18 genome in the respective tumor.

Fig. 4 (upper panel) summarizes our findings for three HeLa clones and C4-1 cells. In HeLa D98, HeLa444, and C4-1 cells, all CpGs within the L1 gene were methylated except one at position 6871. In HeLa CCl2, only seven CpGs were methylated in L1, no methylation being detected at position 6039, and all methylation was discontinued after position 6367. This explains the McrBC resistance observed in Fig. 2. From the difference between HeLa CCl2 and HeLa D98 and 444, one has to conclude that methylation of chromosomally integrated HPV-18 copies can change after integration.

Interestingly, the first CpG residue in the 5′ part of the LCR, that is, upstream of known transcriptional regulators (position 7318), was also methylated in three clones, whereas all CpGs downstream of this position overlapping with the enhancer and promoter and the E6 gene were unmethylated. In the case of C4-1 cells, this result had to be expected because the oncogenes of the only HPV-18 genome in this cancer-derived cell line should be transcriptionally active, that is, unmethylated. The data suggest that in HeLa cells, all 50 HPV-18 copies are also potentially transcriptionally competent. This outcome is discrepant from previous observations with CaSki cells, where most of 500 HPV-16 genomes are methylated, and only one or few remain unmethylated and transcriptionally active (Badal et al., 2003; Van Tine et al., 2001).

We confirmed published data (Schwarz et al., 1985) that the HeLa clones and C4-1 cells have an active early promoter by quantifying the E7 mRNA in a reverse transcription experiment (Fig. 5). This was expected due to the absence of methylation of the enhancer and promoter region and shows that the methylation of the L1 gene does repress over a long distance. We also excluded a modulation of the early promoter by methylation of the L1 gene by treatment of the cells with 5-azacytidin (5-azaC), a known antagonist of DNA methylation, since we measured that 5-azaC did not stimulate early HPV-18 transcription in any of the HeLa clones or in C4-1 (data not shown). However, transcription in HeLa cells may be negatively affected by a different mechanism such as the site of genomic recombination, as

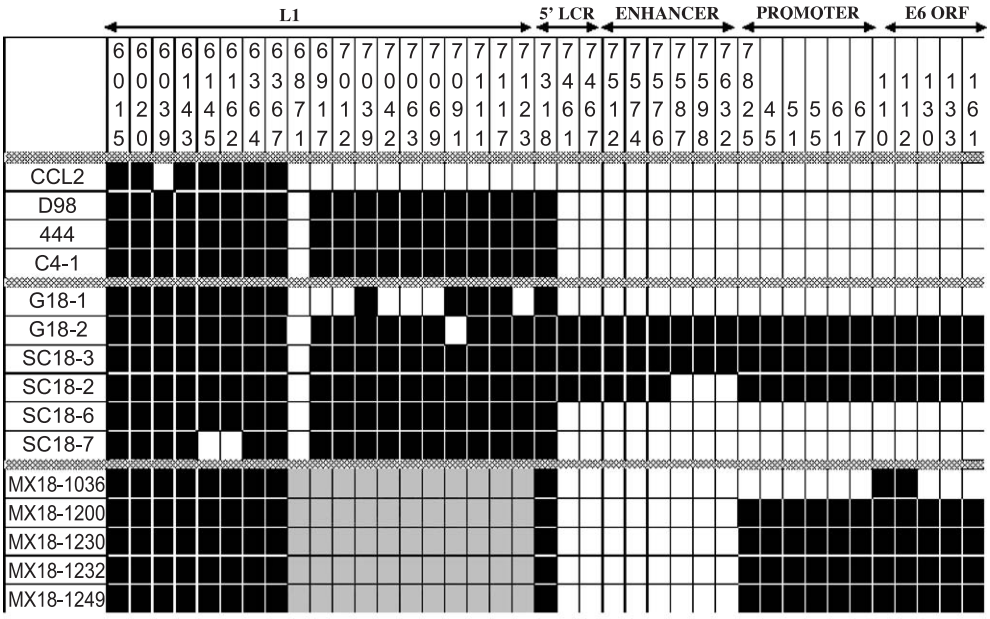

Fig. 4. Methylated CpG residues (black boxes) in a 2022-bp genomic segment of HPV-18, stretching over 1.2 kb of the L1 gene (position 5940 to 7133), the LCR, and the 5′ end of E6 (downstream of position 105). The segment contains 39 CpG dinucleotides. The top of the figure shows the overlap with genetic elements, the vertically listed numbers the genomic positions of CpGs. White boxes: unmethylated CpGs. Grey boxes: not analyzed due to lack of material.

suggested for HPV-16 genomes in CaSki cells (Van Tine et al., 2001), as the amount of E7 mRNA was only slightly higher in HeLa than in C4-1.

Bisulfite sequencing of a 2022-bp L1 gene-LCR segment of the HPV-18 genomes from six cervical cancers

The central panel of Fig. 4 shows a methylation analysis of the tumors discussed above. Some of these data resembled the observations made with the cell lines, others diverged. Throughout L1, most CpGs were methylated just like in the HeLa clones and in C4-1, strangely, the only consistent exception being a lack of methylation at the CpG at position 6871. Among the remaining 18 CpGs, G18-1, G18-2, and Sc18-7 had six, one, and two unmethylated sites. Just as in three of the four HeLa cells, the first CpG in the 5′ -LCR at position 7318 was methylated. We conclude that in cervical cancers, just like in cancer-derived cell lines, the L1 sequences and the 5′ -LCR are preferentially methylated.

The LCR and E6 sequences of these six tumors showed a less consistent pattern. All CpGs were unmethylated in Ge18-1, Sc18-6, and Sc18-7. All enhancer, promoter, and E6 sequences were methylated in Ge18-2, Sc18-3, and Sc18-2, except three neighboring CpGs overlapping with the enhancer of Sc18-2. We conclude that the LCR can become methylated, although by far not as consistently as the L1 gene. It has to be stressed that the detection of methylated transcription units in the LCR does not necessarily mean that all HPV-18 genomes in these tumors were transcriptionally repressed, as exemplified in the previously studied CaSki cell line and one HPV-16-containing tumor (Badal et al., 2003), where bisulfite sequencing detected a majority of methylated and transcriptionally repressed genomes, although some other HPV genomes remained transcriptionally active and thereby maintained the tumor phenotype.

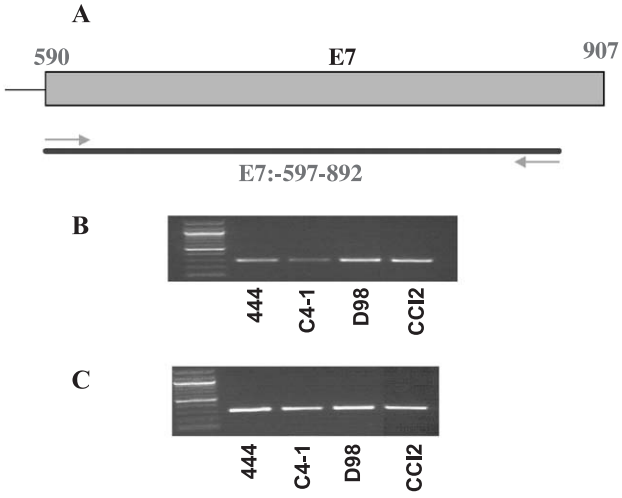

Fig. 5. Transcription of the HPV-18 E7 gene in four cell lines demonstrates that methylation of the L1 gene and the 5′ part of the long control region does not repress the early promoter. (A) E7 gene of HPV-18 and the amplicon assayed by reverse transcription PCR. (B) Reverse transcription PCR of three HeLa cell clones and the cell line C4-1. The weaker expression of E7 in C4-1 likely results from a gene dosage effect. No stimulation of transcription occurred under the influence of 5-aza-C. (C) Transcription control of a cellular gene, glyceraldehyde-3-P dehydrogenase (GAP).

Bisulfite sequencing of an L1 and an LCR segment of the HPV-18 genomes from five smears from asymptomatic patients

We also addressed the question of whether methylation could be found in HPV-18 genomes in cervical smears from asymptomatic patients. Toward this, we studied five samples from a recently published analysis of HPV-18 genome variants found in a cohort in the city of Monterrey, Mexico (Calleja-Macias et al., 2004), Mx18-1036, 1200, 1230, 1232, and 1249. Unfortunately, due to the low amounts of sample, we could not sequence the complete 2022-bp L1-LCR-E6 segment, but only a segment from L1 with eight CpGs and a promoter-E6 segment with 11 CpGs. The bottom panel of Fig. 5 shows that the L1 sequences of all four samples were completely methylated. This methylation might continue through the unsequenced part of the 3′ side of the LCR, as the neighboring position 7318 was methylated, just as in most the previously described samples. Interestingly, the enhancer of all five samples was unmethylated, although the promoter and E6 sequences were methylated.

It should be pointed out that all bisulfite sequencing data analyzed directly the amplification product rather than individual clones. As a consequence, we detected only the predominant methylation pattern, but not microheterogeneities between different HPV genomes in the same clone or sample as recently observed (Kim et al., 2003; our unpublished observations).

Discussion

The expression ‘‘DNA methylation’’ refers to the fact that a methyl group can be added to cytosine residues. This can occur in any sequence constellation, but is maintained typically at CpG dinucleotides, as the palindromic nature of this site, with methylation of the C on the upper and the lower strand, is a prerequisite for maintenance of methylation following replication. DNA methylation has two consequences, namely, (i) interference with the binding of some, but not all, transcription factors (Harrington et al., 1988; Thain et al., 1996), and (ii) being one step in a complex network of chromatin modifications, which also includes the activities of histone methylases and histone deacetylases (Jaenisch and Bird, 2003). De novo DNA methylation plays a major role in reprogramming of genetic information in early embryogenesis where it has been intensely studied (Jaenisch and Bird, 2003). It is enzymatically under the control of the DNA methylases DNMT3A and B, which can also target viral DNA that enters embryonic stem cells (Dodge et al., 2002). It also occurs during tumorigenesis, as methylation of the promoters of tumor suppressor genes is found in cancer as a mechanism with similar consequences as the deletion of tumor suppressor genes (Paulsen and Ferguson-Smith, 2001). Very little is known about de novo methylation in somatic tissue, which may occur rarely or not at all, although DNMT3A and B could be detected in several cell types. Under in situ conditions, the Epstein–Barr Virus (EBV) was until recently thought to be the only virus targeted by de novo methylation. Here, methylation may have the function to repress the EBNA promoters, thereby reducing the availability of immune targets during EBV-induced carcinogenesis (Robertson, 2000).

Against this background it was a surprise when we recently found that HPV-16 DNA is frequently methylated in cell lines and in clinical material (Badal et al., 2003). This observation was complemented by the report that HPV-16 DNA is frequently methylated in undifferentiated cells in culture, but becomes demethylated in differentiated cells (Kim et al., 2003). These data revived historic reports that one can detect DNA methylation in HPV-1 and the cottontail rabbit papillomavirus (CRPV) (Burnett and Sleeman, 1984; Sugawara et al., 1983; Wettstein and Stevens, 1983). From these observations, it has to be concluded that mucosal and cutaneous epithelia are competent to perform de novo DNA methylation, and that HPV genomes can be efficiently detected as targets of this process. It has to remain the objective of future research to identify, which of the many diverse methylation patterns have transcriptional consequences. But there is no doubt that methylation will correlate with repression, as suggested by the study of methylated cellular genes, by the observation that in vitro methylated HPV DNA is transcriptionally incompetent in vivo (Rosl et al., 1993), and in CaSki cells, most of the 500 endogenous HPV-16 copies are transcriptionally repressed correlating with their extensive methylation (Badal et al., 2003; Van Tine et al., 2001). The phenomenon of HPV DNA methylation leads to many obvious and fascinating research questions regarding the cell populations and the enzymatic mechanisms that perform this reaction, the recognition of HPV genomes, and the role of methylation in the normal HPV life cycle and during neoplasia. To study these phenomena, we decided to firstly determine in several different HPV types the patterns of DNA methylation. Here, we report that we found methylation in each of 18 samples with HPV-18, namely, in six cell lines, seven cervical cancers, and five cervical smears from asymptomatic patients.

As a sideline of our research, we confirmed reports that HeLa cells have about 3 kb of their genomes deleted, and these genomic arrangements apparently did not change over five decades of separate cloning. HeLa was reported by one group to have HPV-18 genomes integrated in a single chromosomal site, chromosome 8, band q24 (Durst et al., 1987; Mincheva et al., 1987; Schwarz et al., 1985), whereas two other groups (Ambros and Karlic, 1987; Popescu et al., 1987) reported integration into four different chromosomal regions, one of which confirmed as 8q24. These integration events are apparently even more complex, as amplification of HeLa

at one locus has been reported to involve the cellular flanking sequences (Lazo et al., 1989). We did not follow up on these contradictory findings, but we point out the puzzling consequence of the latter reports with possibly identically deleted HPV-18 genomes in four different loci. We also report that the similar deletion in C4-1 cells was apparently a fortuitous event, as no such deletion was observed in six cancers. Contamination of C4-1 cells by HeLa cells could be excluded due to the different HPV-18 variants in the two cell lines.

In spite of the limited diagnostic power of the McrBC analysis of clones of the six HeLa cell lines and C4-1 cells, one can conclude that methylation is absent or rare throughout the early genes and the LCR (amplicons G1–G4) and only detectable in the late gene L1 (G8). No conclusions could be drawn about L2, which would have been part of the deleted G7 segment. While McrBC is a useful tool to scan larger genomic segments, we continued this study by bisulfite sequencing technique of a partial genomic segment, as only few amplification experiments were possible due to the small amounts of DNA from clinical material.

In spite of the small number of samples analyzed by bisulfite sequencing, our data lead us to the following conclusions.

1. As HeLa and C4-1 cells express the E6 and E7 oncogenes, no long distance repression results from the methylated region around L1 to the enhancer and promoter elements located 250–600 bp 3′ from the last methylated site at position 7318.
2. Changes of the methylation status of the HPV-18 genome can occur after clonal integration, as seen by the differences between CCl2 and D98/444.
3. Selected sites can be resistant to methylation as documented by site 6871, which is not only unmethylated in the cell lines, but also in six tumors.
4. In many samples, methylation ends at position 7318. This site is nearly 200 bp downstream of the 3′ end of L1, and we conclude that methylation does not strictly correlate with the L1 gene.
5. Lack of methylation may correlate with transcription factor binding sites since all CpGs 3′ of position 7318 overlap with cis-responsive elements of the enhancer or promoter.
6. In the cell lines and in most tumors, HPV-18 genomes are integrated, and sequences upstream of the HPV-18 promoters are only transcribed, if they happened to come under the control of cellular elements. This lack of transcription, however, cannot explain the methylated status of L1, as L1 and site 7318 are also methylated in five smears of asymptomatic patients, likely to contain circular HPV-18 genomes and competent to transcribe L1. This leads to the speculation that methylation of L1 may be involved in the early-late switch of HPV-18 transcription.
7. The enhancer and promoter are completely or partially methylated in three tumors, likely indicating transcriptional repression of these HPV-18 genomes. It is likely, however, that some of the numerous HPV genomes in these tumor cells are unmethylated and express the HPV-18 oncogenes, as observed in CaSki cells for HPV-16.
8. In seven samples, we observed methylation of the promoter and the E6 gene. These sequences might be repressed by the chromatin configuration, but methylation would not affect the transcription factors directly, as Sp1, which activates the E6 promoter, is not affected by methylation (Harrington et al., 1988), and E2, which is affected (Thain et al., 1996), functions in this position as a repressor of transcription, and its displacement would activate the promoter (Tan et al., 1994).

Our study confirms HPV genomes as fascinating models to study DNA methylation and makes it likely that this mechanism has profound effects on the HPV life cycle and carcinogenesis.

Materials and methods

Origin of cell lines

Since its original cultivation in the 1950s, different cultures of the HeLa have diverged substantially. We included in this study six HeLa clones of different origins. HeLa H21 (Defilippi et al., 1987) was obtained from A.G. Porter at the Institute of Molecular and Cell Biology (IMCB), Singapore, who originally received it from Walter Fiers, Rijksunivesiteit Te Gent University. HeLa D98 was also obtained from A.G. Porter. It is a hypoxanthine guanine phosphoribosyltransferase (HGPRT) negative, 8-azaguanin resistant mutant of HeLa constructed more than 30 years ago (Bengtsson et al., 1975). HeLa CCl2 (Chou et al., 1978) was received from the American Type Culture Collection (ATCC). HeLa S3 is an methylguanine DNA methyltransferase (MGMT)-positive cell line (Painter and Robertson, 1959), which was obtained from Peter Karran (Imperial Cancer Research Fund, UK), the original source cited to be the American Type Culture Collection. Yet another line with unknown pedigree was obtained by one of us (H.U.B.) in 1985 at the German Cancer Research Center in Heidelberg and maintained in our lab since that time. HeLa444 cells were constructed during a study of the properties of fusion cells between HeLa D98 and fibroblasts (Srivatsan et al., 1986), and were made available to us by Dr. E. Stanbridge (University of California Irvine).

The cell line C4-1, also derived from a cervical carcinoma (Kruczek et al., 1981), has been reported to contain one or few HPV-18 genomic copies (Gallego et al., 1994; Schwarz et al., 1985) and has been made available by E. Schwarz, Heidelberg, in 1985, and was maintained in our Singapore laboratory since then.

Clinical specimens

Samples from cervical cancers were obtained during a published study of the genomic diversity of HPV-18 genomes from German and Scottish patients (Ong et al., 1993). HPV-18 smears were identified during an epidemiological study in Monterrey, Mexico, part of which has been published (Calleja-Macias et al., 2004).

Purification of DNA

DNA derived from cell lines or smears was purified with Qiagen genomic tips following protocols suggested by the supplier. The purification of tumor DNA has been described (Ong et al., 1993).

McrBC cleavage

The restriction enzyme McrBC cuts close to the sequence purine-meC (PumeC) in the context of a second, arbitrarily spaced PumeC (Sutherland et al., 1992). As a consequence, McrBC recognizes pairs of PumeCpG residues, and, on the average, cleaves every other meCpG. For digestions with McrBC (New England Biolabs), 250 ng of chromosomal DNA was digested with 3 units of enzyme for 1 h at 37 °C in 25 μl NE buffer 2 [50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), pH 7.9].

PCR

Table 1A summarizes the primers for dissection of the HPV-18 genome. PCR was carried out in 25 μl containing 0.2 mM of each of the four dNTPs, 10 pmol primers, 2.5 μl of buffer B supplied by the producer (Promega), 2 mM $MgCl_2$, and 0.75 units Taq (Promega) with 25 or 0.25 μl of C4-1 and HeLa DNA, respectively, uncleaved or cleaved by McrBC. The PCR started at 94 °C for 1 min followed by 35 amplification cycles (denaturation 94 °C, 10 s, annealing 58 °C, 30 s, extension 68 °C, 45 s increasing by 10 s per cycle), final extension at 68 °C, 7 min. PCR with TaqGold was carried out in 25 μl containing template DNA, 5 mM dNTPs, 10 pmol primers, 2.5 μl of magnesium ion free buffer supplied by the manufacturer, 2 mM $MgCl_2$, and 1.25 units AmpliTaqGold (Applied Biosystems) at 94 °C, 9 min followed by 40 amplification cycles (denaturation at 94 °C, 10 s, annealing 55 °C, 30 s, extension 68 °C, 45 s increasing by 10 s per cycle), final extension at 68 °C, 7 min.

Bisulfite modification, PCR, and direct sequencing

For mapping of methylated residues, DNAs were modified by the CpGenomeTm DNA modification kit of Intergen Inc. or by modifications adjusted in our laboratory as follows: 50–1000 ng sample DNA supplemented with 1 μg of salmon sperm DNA in a total volume of 18 μl in water were denatured with 2 μl of 3 M NaOH and incubated at

Table 1
PCR primers used in this study (A) for amplification of larger genomic segments (see Figs. 1–3) and (B) for amplification of bisulfite-treated DNA (Fig. 4)

Amplicon	Primer	Position	Sequence
A			
G1	G1F	7019–7038	TTTGGTTCAGGCTGGATTGC
	G1R	56–37	CCGACCGTTTTCGGTTACTC
G2	G2F	37–56	GAGTAACCGAAAACGGTCGG
	G2R	930–911	CCTTCTGGATCAGCCATTGT
G3	G3F	876–895	TTGTGTGTCCGTGGTGTGCA
	G3R	1890–1871	AGTGCTGCAACACTACTTCG
G4	G4F	1763–1782	GCCCTGTTGCGTTACAAATG
	G4R	2818–799	ATCTTCCTCTTCCTCGTGCA
G5	G5F	2802–2821	ACGAGGAAGAGGAAGATGCA
	G5R	3797–3778	ATTGCCTGCACCTGTCCAAT
G6	G6F	3730–3750	GATTGCGAAAACATAGCGACC
	G6R	4784–4765	ATACATTACCTGCCACCTCC
G7	G7F	4721–7740	CCTGCATTTTCTGATCCGTC
	G7R	5918–5899	ACAGGCCCACACTAAACGTT
G8	G8F	5830–5849	TTAGGGTGCAGTTACCTGAC
	G8R	7038–7019	GCAATCCAGCCTGAACCAAA
B			
Msp4–5	Msp4F	5940–5960	GGTTAGTTTTTAGGTGTTGGT
	Msp5R	6482–6461	CACACAACTACCAAATAAAACA
Msp6	Msp6F	6847–6871	TTATTAGTTTGGTGGATATATATTG
	Msp6R	7186–7161	AAAACATACAAACACAACAATAAATA
Msp10	Msp10F	7282–7293	TAAAATATGTTTTGTGGTTTTGTG
	Msp10R	7747–7721	ATAATTATACAAACCAAATATACAATT
Msp8	Msp8F	7753–7781	TGTTTAATATTTTGTTTATTTTTAATATG
	Msp8R	186–161	TATCTTACAATAAAATATTCAATTCC

37 °C. After denaturing, 278 μl of 4.8 M sodium bisulfite and 2 μl 100 mM hydroquinone were added and incubated in a thermal cycler for 20 cycles each at 55 °C for 15 min and 95 °C for 30 s. The modified DNA was desalted with the QIAquick PCR purification protocol. The modified DNA was desulfonated by adding 5.5 μl of 3 M NaOH, 5 μg glycogen, and incubation at 37 °C for 15 min. The DNA was precipitated with 5.6 μl of sodium acetate and 150 μl of 100% ethanol. The pellet was washed with 70% ethanol and dissolved in 30–50 μl TE buffer (10 mM Tris–HCl, pH 8, 1 mM EDTA). The reaction products amplified with primers specific for modified HPV-18 DNA (Table 1B) and sequenced directly. Since bisulfite-treated DNA is partially degraded, large amplicons, it cannot be generated, and we divided this segment in four amplicons (5940–6482, 6847–7186, 7282–7747, 7753–186).

Reverse transcription and PCR

RNA was prepared using the QIAGEN RNA kit. One microgram of RNA was primed with 5 pmol of oligodT primer, topped up to 11 μl with H_2O, heated at 70 °C for 10 min, and placed on ice for 5 min. A mastermix containing 2 μl of 10× New England Biolab (NEB) M-MuLV reverse transcriptase buffer, 100 mM DTT, 0.5 mM of each of the four dNTPs, 100 units NEB Reverse transcriptase enzyme, and 20 units of RNAse Inhibitor (Roche) was added to each sample and incubated for 50 min at 42 °C. The enzyme was heat inactivated at 70 °C for 15 min. PCR was carried out in 25 μl containing 0.2 mM of each of the four dNTPs, 10 pmol primers, 2.5 μl of buffer B (supplied by the producer, Promega), 2 mM $MgCl_2$ and 0.75 units Taq polymerase (Promega) and 1.5 μl of the cDNA preparation. The PCR started at 94 °C for 2 min followed by 25 amplification cycles (denaturation 94 °C, 10 s, annealing 55 °C, 30 s, extension 68 °C, 45 s), final extension at 68 °C, 7 min.

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