Journal of General Virology (1993), **74**, 791–801. *Printed in Great Britain*

The effect of DNA methylation on gene regulation of human papillomaviruses

Frank Rösl,* Amina Arab, Britta Klevenz and Harald zur Hausen

Forschungsschwerpunkt Angewandte Tumorvirologie, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 242, 69 Heidelberg, Germany

Integration of human papillomaviruses (HPVs) into the host genome is considered to be an early and important event in HPV-linked cervical carcinogenesis. Consequently, the viral DNA potentially becomes a target for cellular control mechanisms normally acting on the corresponding integration site. Besides resulting position effects, host-specific DNA methylation may play a functional role in HPV gene regulation. To elucidate the influence of such a kind of epigenetic modification on viral transcription, *in vitro* methylation studies on HPV-18 upstream regulatory region (URR)-controlled reporter plasmids were carried out. Selective methylation of the viral URR results in a down-regulation of the transcriptional activity, which can be attributed to non-random distribution of methyl-acceptor sites clustered within the constitutive enhancer region. *In vivo* competition experiments show that suppression is not directly mediated by steric hindrance of methyl residues with transcription factors, but rather is due to the association with methyl-CpG DNA-binding proteins. Using a restriction enzyme accessibility assay on both the DNA and chromatin levels, it could be demonstrated that, *in vivo*, extensively methylated viral DNA is nucleosomally organized, characteristic of transcriptionally inactive chromatin. These data suggest that DNA methylation is an important regulatory pathway in the modulation of HPV expression and as a consequence the proliferation rate of virus-infected cells.

Introduction

It has been well documented that particular types of human papillomaviruses (e.g. HPV-16 and HPV-18) are involved in the development of cervical cancer (for review, see zur Hausen, 1989). Detailed investigation of different levels of viral gene expression during the vegetative cycle is presently hampered by the lack of a permissive *in vitro* system for the propagation of infectious virus. Most studies depend on DNA transfections in appropriate cell systems or in cervical carcinoma cells, recultivated from primary biopsies, in order to analyse cis- and trans-acting virus–host cell interactions and their influence on HPV transcription in premalignant or malignant cells.

DNA-mediated gene transfer experiments have revealed that the expression of the open reading frames (ORFs) E6 and E7 is necessary and sufficient to immortalize primary human keratinocytes (Münger *et al.*, 1989). This is consistent with the finding that the corresponding ORFs are well conserved in both fresh biopsy materials and cell lines derived therefrom, implying a still functional selection pressure on E6/E7 transcription even in malignant cells (Schwarz *et al.*, 1985; Yee *et al.*, 1985).

HPV-16/-18 infection and DNA persistence, however, are not sufficient to transform human keratinocytes malignantly (Dürst *et al.*, 1987; Pirisi *et al.*, 1987), indicating that additional modifications are needed to deregulate an evidently balanced equilibrium of host–virus interaction during latency (zur Hausen, 1986, 1991*b*).

An important step towards malignant progression is presumably the recombination between the viral DNA and host cellular sequences. In preneoplastic cells, HPV-16/-18 commonly persist as extrachromosomal elements (Dürst *et al.*, 1985), whereas the DNA is found to be randomly integrated in the majority of cervical cancers (Choo *et al.*, 1987). Integration seems to be an early event in HPV-linked carcinogenesis (Schneider-Maunoury *et al.*, 1987) leading regularly to a disruption of the E2 ORF and to the loss of the corresponding protein, which negatively regulates the upstream regulatory region (URR)-directed expression of the transforming ORFs E6 and E7, via binding to the cognate E2-binding motif (Bernard *et al.*, 1989; Romanczuk *et al.*, 1990). Furthermore, both the use of somatic cell hybrids between cervical carcinoma cells and non-malignant human keratinocytes (Rösl *et al.*, 1988, 1991; Bosch *et al.*, 1990) and studies on partially chromosome 11-deleted human fibroblasts (Smits *et al.*, 1988, 1990) have demonstrated that alterations in the chromosomal

complement of the host cell may result in profound effects on the transcriptional activity of the HPV-16/-18 URR (reviewed in zur Hausen, 1991*a*). Therefore, the URR is not only the target for the virus-specific E2 trans-acting negative regulatory pathway, but also for a host cell control mechanism which negatively interferes with HPV expression and tumorigenicity (zur Hausen, 1986, 1991*a*).

On the other hand, recent studies have shown that the transcriptional activity of HPV-16/-18 can also be controlled by cis-acting mechanisms. Integration of the conserved URR–E6/E7 transcription cassette into the host genome subjects the viral DNA to cellular processes that normally affect the expression of the unoccupied gene or chromosomal domain.

By investigating the nucleosomal organization of the HPV-18 integration locus in the cervical carcinoma cell line HeLa, the location and distribution of DNase I hypersensitive sites were determined; these are known to be hallmarks for regulatory elements at the chromatin level (Elgin, 1984). It has been shown that HPV-18 is integrated in the vicinity of a cellular promoter that cooperates with the viral URR in generating the characteristic HPV-18 transcription pattern (Rösl *et al.*, 1989). Moreover, depending on the chromosomal integration site in different cervical carcinoma cells, cis-acting cellular regulatory elements also modify the glucocorticoid-regulated response of the HPV-18 URR (Chan *et al.*, 1989) in a hierarchical manner, because in addition to up-regulation of viral transcription, E6/E7 expression is also found to be down-regulated or even response-refractory despite the presence of a functional hormone receptor and a corresponding binding site within the URR (von Knebel Doeberitz *et al.*, 1991).

Another possible cis-acting control mechanism is the modulation of the expression of foreign genes integrated into the host cell genome by *de novo* DNA methylation (Holliday, 1987; Doerfler, 1991). Epigenetic modification of particular CpG residues within regulatory regions of eukaryotic genes influence the transcriptional activity, with a correlation between gene expression and hypomethylation (for review, see Razin & Cedar, 1991). DNA methylation seems to be involved in cellular senescence (Klein *et al.*, 1991) and genomic imprinting (Reik *et al.*, 1987) as well as in viral latency as described recently for human immunodeficiency virus (HIV) (Bednarik *et al.*, 1990).

To understand the influence of DNA methylation on transcriptional regulation of HPV, we examined the effect of *in vitro* methylation on HPV URR-controlled reporter constructs in transient transfection assays, as well as the nucleosomal organization of methylated HPV DNA in cervical carcinoma cells. Evidence is provided that *in vitro* methylation selectively down-regulates HPV-18 transcription by an indirect mechanism, mediated by methyl-DNA-binding proteins. Using the *Msp*I/*Hpa*II isoschizomer assay on both the DNA and chromatin levels, it became apparent that methylated DNA is protected against cleavage by the methylation-insensitive enzyme *Msp*I under *in vivo* conditions, suggesting that methyl-DNA-binding proteins also affect the corresponding nucleosomal organization of CpG-methylated DNA. The present study shows that epigenetic modifications of the viral DNA have an effect not only on the transcriptional competence of the regulatory region, but also on the chromatin structure of integrated DNA of pathogenic HPVs. The data indicate that viral DNA methylation is an important additional mechanism in the multi-step process of HPV-linked carcinogenesis.

Methods

Cell culture. Cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 1% penicillin and 1% streptomycin.

Plasmids. pH18URR–CAT contains the URR of HPV-18 cloned in pBLCAT3, a chloramphenicol acetyltransferase (CAT) construct lacking eukaryotic regulatory signals (Lukow & Schütz, 1987). pSV2CAT contains the complete regulatory control region of simian virus 40 (SV40) (Gorman *et al.*, 1982), pBLCAT2 the thymidine kinase (TK) promoter of herpes simplex virus (HSV) cloned in pBLCAT3 (Lukow & Schütz, 1987). pRSV-luc contains a luciferase gene expressed under the control of the Rous sarcoma virus (RSV) long terminal repeat (LTR).

In vitro *methylation.* Purified plasmids were treated with the corresponding methyltransferases at 37 °C for 6 to 8 h in the presence of 80 μM-*S*-adenosylmethionine as recommended by the suppliers. Mock-methylated controls were treated as described above, except that the enzyme was omitted. The extent of methylation as well as changes in DNA topology were monitored by digesting with *Hpa*II or *Hha*I restriction endonucleases and by agarose gel analysis of the methylated DNAs in the presence of ethidium bromide (Keller, 1975). Only completely methylated plasmids were used for DNA transfection studies.

DNA transfections and CAT assays. C33a and SiHa cervical carcinoma cell lines were transfected with methylated plasmids and the corresponding controls, together with 0·25 μg of pRSV-luc to correct the transfection efficiencies obtained using the calcium phosphate coprecipitation procedure (Wigler *et al.*, 1978). All transfection experiments were carried out at least four times using different preparations of CsCl-purified plasmids. The cells were harvested 48 h later by three cycles of freezing and thawing to monitor the luciferase activity (deWet *et al.*, 1987). The cellular extracts were standardized for the CAT assay (Gorman *et al.*, 1982) by using equal amounts of luciferase counts. The conversion rate was determined by using a ^{14}C scanner (Tracemaster, Linear analyser, Bertold).

DNA and RNA analysis. Genomic DNA was prepared by standard methods (Sambrook *et al.*, 1989). To monitor the different methylation levels, the DNA was treated with the isoschizomeric enzymes *Msp*I/*Hpa*II (McClelland, 1981) as well as with the methylation-sensitive enzymes *Hha*I and *Sma*I overnight at 37 °C using an enzyme:DNA ratio of 5 units:μg DNA. The digested DNAs were electrophoresed on 1% agarose gels and transferred to GeneScreen Plus filters (NEN) as described (Southern, 1975). Total cellular RNA was isolated according to the method of Chomczynski & Sacchi (1987),

the corresponding poly(A)$^+$ fraction was enriched by oligo(dT)–cellulose chromatography (Aviv & Leder, 1972) and subjected to Northern blot analysis (Khandjian & Meric, 1986). The filters were hybridized with HPV-16/-18 DNA probes labelled by random priming (Feinberg & Vogelstein, 1984) at 42 °C in the presence of 50% formamide. Washing was done at 68 °C in 2 × SSC, 0·1% SDS.

Preparation and digestion of isolated nuclei with restriction enzymes. Nuclei from cervical carcinoma cells (CaSki and SW756) were isolated under isotonic conditions using the non-ionic detergent NP40 for cell lysis (Rösl & Waldeck, 1991). The nuclear DNA concentration was determined by the method of Lawson *et al.* (1980). Nuclei equivalent to 100 µg DNA were resuspended at a concentration of 1 mg/ml in 50 mM-Tris–HCl pH 7·5, 220 mM-sucrose, 5 mM-$MgCl_2$, 1 mM-DTT (Antequera *et al.*, 1989) and separately digested with increasing concentrations of *Msp*I, *Hpa*II and *Hha*I (as indicated in the figure legends) for 30 min at 37 °C. The reaction was stopped by adding an equal volume of 2% SDS, 20 mM-EDTA. After overnight incubation with proteinase K (100 µg/ml), the DNA was extracted and re-suspended in TE buffer (10 mM-Tris–HCl pH 8·0, 1 mM-EDTA).

End-labelling and gel electrophoresis. Digested genomic DNA (0·5 to 1 µg) was end-labelled by Klenow polymerase using 1 µCi [^{32}P]dCTP in the appropriate reaction buffer (Antequera *et al.*, 1989) for 15 min at room temperature. Non-incorporated nucleotides were removed by three consecutive cycles of ammonium acetate–isopropranol precipitations. End-labelled DNA (3000 to 5000 c.p.m.) was separated on 1·8% agarose gels. After drying of the gels on 3MM Whatman paper, the filters were exposed for autoradiography.

Results

In vitro *methylation of HPV-18 URR-directed reporter constructs*

In a previous study we have found that different levels of DNA methylation at the HPV-18 integration locus in HeLa cells and somatic cell hybrids derived therefrom apparently have an inverse relationship to the amount of virus-specific RNA at the steady state level detected in the cytoplasm (Rösl *et al.*, 1988). To investigate the epigenetic modification process on viral transcription in greater detail, we used CAT constructs harbouring the HPV-18 URR in comparison to various control plasmids for *in vitro* methylation experiments. As indicated in Fig. 1, HPV-18 has nine *Hha*I restriction sites (5′ GCGC 3′, at nucleotide positions 108, 110, 1127, 7316, 7572, 7574, 7585, 7596, 7630, respectively) (Cole & Danos, 1987). Six of them are located within the URR and clustered inside the *Rsa*I–*Rsa*I fragment between nucleotide positions 7508 and 7738, defined as the virus-specific enhancer region (Swift *et al.*, 1987).

By *in vitro* methylation of pH18URR-CAT plasmids using the corresponding *Hha*I methyltransferase, an approximately 10-fold decline of the CAT activity in comparison to the mock-treated control can be noted (Fig. 2, lanes 1 and 2). The activity of the HPV-18 URR-directed reporter plasmid was even more reduced than in the pBLCAT2 vector (Fig. 2, lanes 7 and 8), which harbours the TK promoter of HSV, known to be sensitive to *Hha*I methylase (Ben-Hattar *et al.*, 1989). Furthermore, *in vitro* methylation of every CpG residue of pH18URR-CAT using *Sss*I methylase (Boyes & Bird, 1991), reduces the CAT expression to the background level (Fig. 2, lane 3).

To eliminate the possibility that the repressed CAT activity in pH18URR-CAT is due to methylation effects in non-promoter regions, several control experiments were performed. As indicated in Fig. 1, the basic construct pBLCAT3 also possesses a certain number of *Hha*I restriction sites. To exclude any non-specific side effect of the cloning vector on the transcriptional activity of the inserted regulatory region, *Hpa*II methyl-

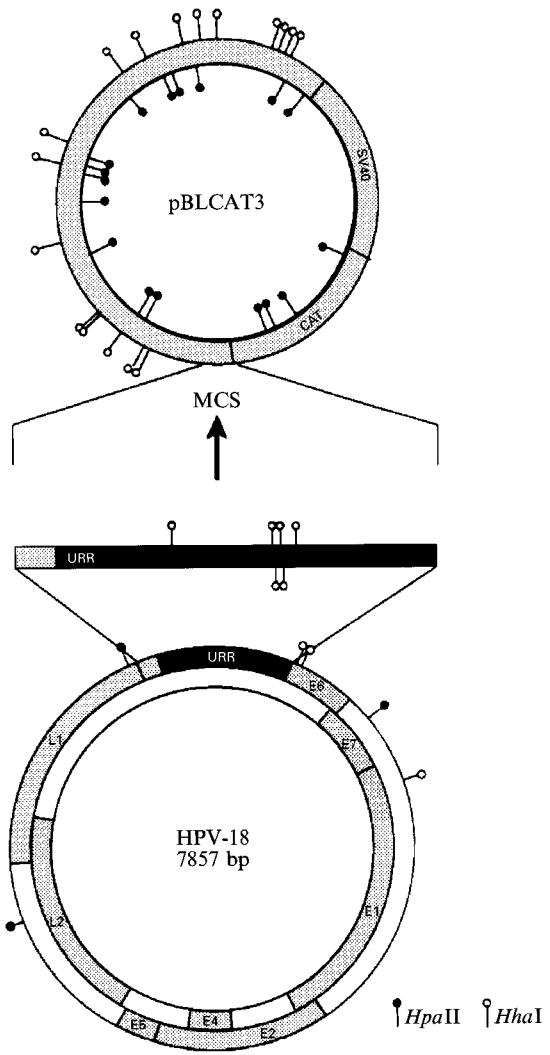

Fig. 1. Location and distribution of the *Hpa*II/*Hha*I recognition sites in the genome of HPV-18 and in pBLCAT3. The URR, positions 6927 to 101, encompassing parts of the late ORF L1 but lacking the virus-specific ATG initiation codon from the early ORF E6, was subcloned as a modified *Bam*HI–*Hin*dIII fragment in sense orientation in the multiple cloning site (MCS) of pBLCAT3, a reporter construct harbouring the CAT gene, as well as the RNA processing/poly(A) of SV40. The positions of the *Hpa*II and *Hha*I sites relative to the early (E) and late (L) ORFs in HPV-18 and pBLCAT3 are indicated.

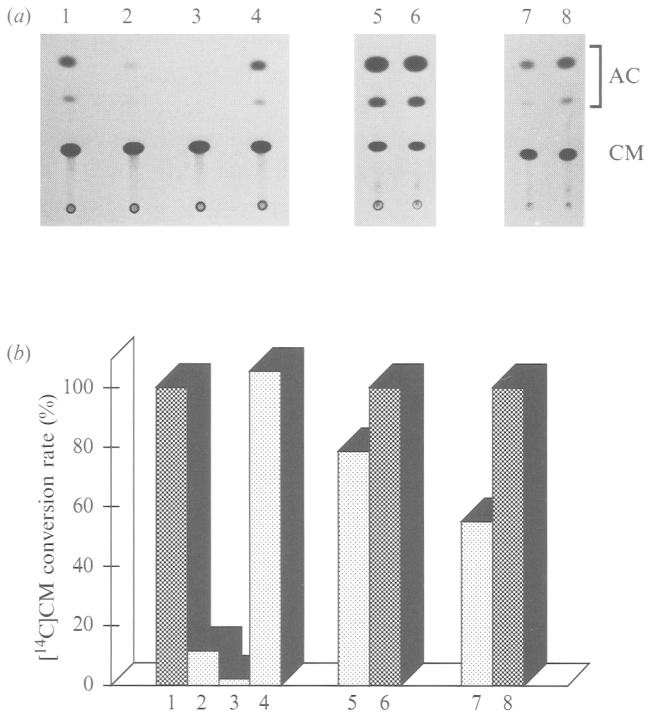

Fig. 2. Effect of *in vitro* methylation in transient transfection assays. (*a*) CAT expression of *in vitro* methylated pH18URR-CAT in comparison with the untreated control (lane 1). Lane 2, *in vitro* methylated with *Hha*I methylase; lane 3, generalized CpG methylation with *Sss*I methylase; lane 4, methylation of non-promoter regions in pBLCAT3 using *Hpa*II methylase. Lane 5, pSV2CAT, harbouring the regulatory region of SV40 (positions −293 to +60) (Gorman *et al.*, 1982) after treatment with *Hha*I methylase; lane 6, untreated control. Lane 7, pBLCAT2, containing the TK promoter of HSV (positions −105 to +51) (Lukow & Schütz, 1987), methylated with *Hha*I methylase; lane 8, control, without any methylation. C33a cells were cotransfected with 5 μg of the corresponding plasmids together with 0·25 μg of pRSV-luc to standardize the transfection efficiencies. Cell extracts equivalent to 10000 luciferase counts were incubated for 2 h at 37 °C to monitor the CAT activity. (*b*) Schematic presentation of the different CAT expression levels. The plates from the thin-layer chromatography were directly measured in a ^{14}C scanner. The values are given relative to the [^{14}C]chloramphenicol conversion rate of the respective untreated control, which was arbitrarily set as 100%. CM, Chloramphenicol; AC, acetylated forms. Lanes 1 to 8 correspond directly to the different CAT assays shown in (*a*). The black bars represent the untreated controls.

transferase was employed. This enzyme has no recognition site within the HPV-18 URR, but the same number of sites within pBLCAT3 as the preceding *Hha*I methylase (see Fig. 1 for comparison). As demonstrated in Fig. 2, lane 4, no reduction can be detected after *Hpa*II methyltransferase treatment. One can therefore conclude that the transcriptional down-regulation of pH18URR-CAT is specific for the viral regulatory region, since pSV2CAT, additionally applied as a negative control for *Hha*I methylase (Kruczek & Doerfler, 1983) and containing only one *Hha*I site in the regulatory region of SV40, is also not significantly affected by *in vitro* methylation under the same experimental conditions (Fig. 2, lanes 5 and 6).

In all transfection experiments described here the HPV-negative C33a cell line was used as the recipient, but the same results were obtained in HPV-16-positive SiHa cells under both transient and stable conditions (data not shown) indicating that endogenous E6/E7 expression does not interfere with the suppressive effect of DNA methylation.

In vivo *competition assays for methyl-DNA-binding proteins*

Transcriptional down-regulation of the HPV-18 URR by DNA methylation can be principally explained by two different mechanisms: the methyl moieties either directly interfere with the binding of transcription factors (Prendergast *et al.*, 1991) or alternatively DNA-binding proteins with high affinities for methylated CpG DNA (Meehan *et al.*, 1989) indirectly prevent a protein–DNA interaction indispensable for the formation of a functional transcription complex (Boyes & Bird, 1991). To gain insight into this question, *in vivo* titration studies were performed. If there is an involvement of proteins capable of binding methylated DNA, it should be possible to compete for them by cotransfecting highly methylated non-specific prokaryotic DNA (Levine *et al.*, 1991) together with *Hha*I-methylated pH18URR-CAT reporter plasmids. This in turn should relieve the transcriptional block and allow partial reactivation of CAT expression. On the other hand, if there is merely a steric hindrance of transcription factors due to the presence of the methyl residues, the addition of increasing amounts of competitor DNA should not alleviate the methylation-mediated inhibition of the viral URR. To discriminate between these two mechanisms methylated CAT plasmids were cotransfected with *Sss*I-methylated Bluescript DNA, keeping the total amount of prokaryotic DNA constant (10 μg) but changing the ratio of methylated and non-methylated competitor. As demonstrated in Fig. 3, the suppressive effect on HPV-18 is indirectly mediated via the binding of proteins specific for methylated DNA, since there is indeed a partial reactivation of the CAT expression after increasing amounts of methylated untranscribable DNA were added. As shown in Fig. 3, there is an almost fourfold increase of the CAT activity after transfection of 10 μg methylated Bluescript (BS-m) DNA in comparison to the CAT signal obtained after introduction of only 2 μg BS-m together with 8 μg unmethylated (BS-u) control DNA.

These findings led to the conclusion that the suppressive effect of DNA methylation on the HPV-18 URR is mediated indirectly by the binding of titratable methyl-

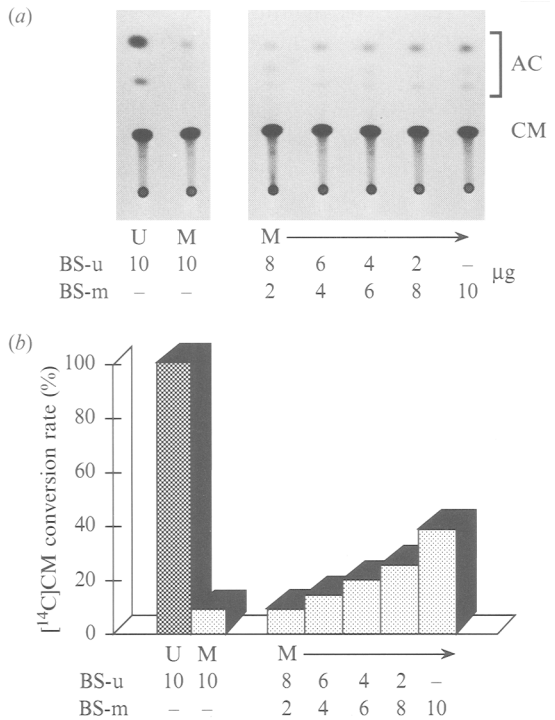

Fig. 3. Reactivation of *Hha*I-methylated plasmids by *in vivo* titration. (*a*) Left: 2·5 µg of both non-methylated (U) and *in vitro* methylated (M) pHPV-18 URR-CAT plasmids were cotransfected with 10 µg unmethylated prokaryotic DNA (Bluescript vector, BS-u). Right: reactivation of CAT expression after changing the ratio between unmethylated (BS-u) and *Sss*I-methylated Bluescript DNA (BS-m) in cotransfection along with constant amounts of 2·5 µg *Hha*I-methylated pH18URR-CAT (M, indicated by an arrow). The plasmids were triple-transfected with pRSV-luc and assayed as described (see Fig. 2). (*b*) Schematic presentation of results as in Fig. 2(*b*).

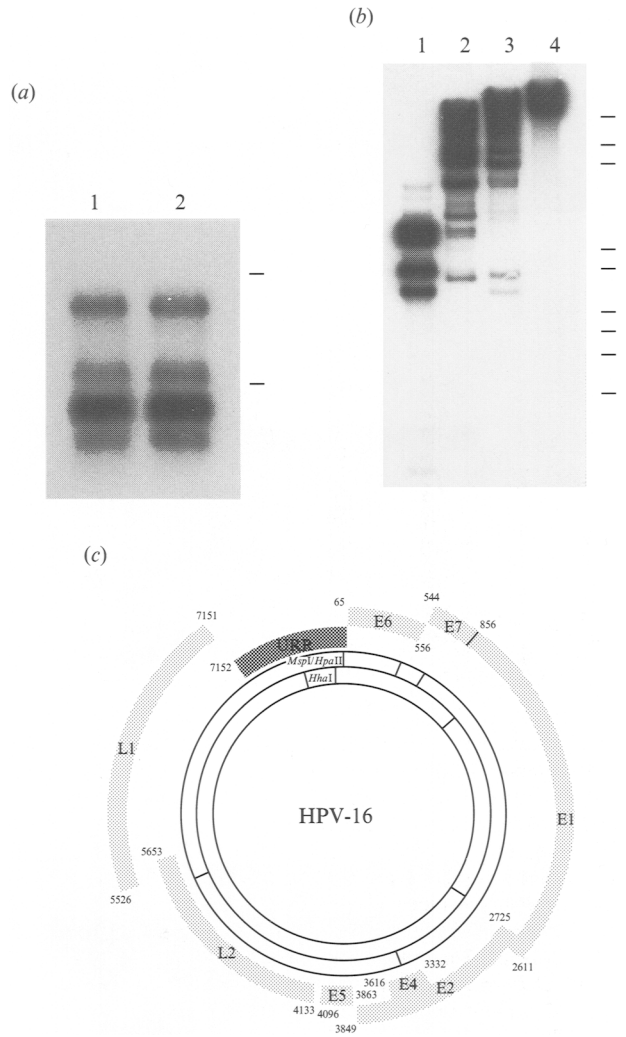

Fig. 4. Hypermethylation of HPV-16 DNA in CaSki cells. (*a*) Northern blot analysis of poly(A)$^+$-selected cytoplasmic RNA (lane 1) and after treatment of the cells for 4 h with 50 µg/ml cycloheximide (lane 2). The positions of the 28S and 18S rRNAs are indicated. (*b*) CaSki DNA was treated with *Msp*I (lane 1), *Hpa*II (lane 2), *Hha*I (lane 3) or *Sma*I (representing a viral no-cut enzyme, lane 4). The bars on the right indicate size markers (23·1, 9·4, 6·6, 2·3, 2·0, 1·35, 1·1, 0·8 and 0·6 kb). Both autoradiographs were made after hybridization with unit-length HPV-16 DNA. (*c*) Schematic overview of the location of the *Msp*I/*Hpa*II and *Hha*I recognition sites relative to the URR, the early (E) and late (L) ORFs in the genome of HPV-16.

CpG-binding proteins (MeCPs) (Meehan *et al.*, 1989). In order to generalize these results, we subsequently determined whether endogenous methylated HPV DNA, integrated into the chromosomal DNA of the host cell, might also be associated with MeCPs on the basis of the accessibility to restriction enzymes on native chromatin in comparison to digestion products at the DNA level (Antequera *et al.*, 1989, 1990).

Viral DNA methylation pattern in cervical carcinoma cells and susceptibility of nucleosomally organized methylated DNA to restriction enzyme digestion

To answer this question we first screened all available cervical carcinoma cell lines for the presence of methylated HPV DNA using the *Msp*I/*Hpa*II isoschizomer assay (McClelland, 1981). This approach is based on the fact that both restriction endonucleases cleave the same recognition site (5′ CCGG 3′). *Msp*I cuts regardless of the degree of methylation, whereas *Hpa*II cannot digest the DNA at this position when the inner cytosine is methylated. In this way a ladder of partially cleaved DNA fragments is created, which migrate as off-size bands and are not present in the corresponding *Msp*I control. Using this strategy, it was found that the cervical carcinoma cell line CaSki, which contains approximately 600 copies of HPV-16 (Baker *et al.*, 1987), has the highest degree of viral DNA methylation. As demonstrated in Fig. 4(*b*), *Msp*I cleavage results in an accumulation of small viral DNA fragments (lane 1), whereas after *Hpa*II digestion the HPV-16 DNA migrates in the high M_r range (lane 2), arguing that most of the viral 5′ CCGG 3′ sites are methylated [see schematic presentation in (*c*)].

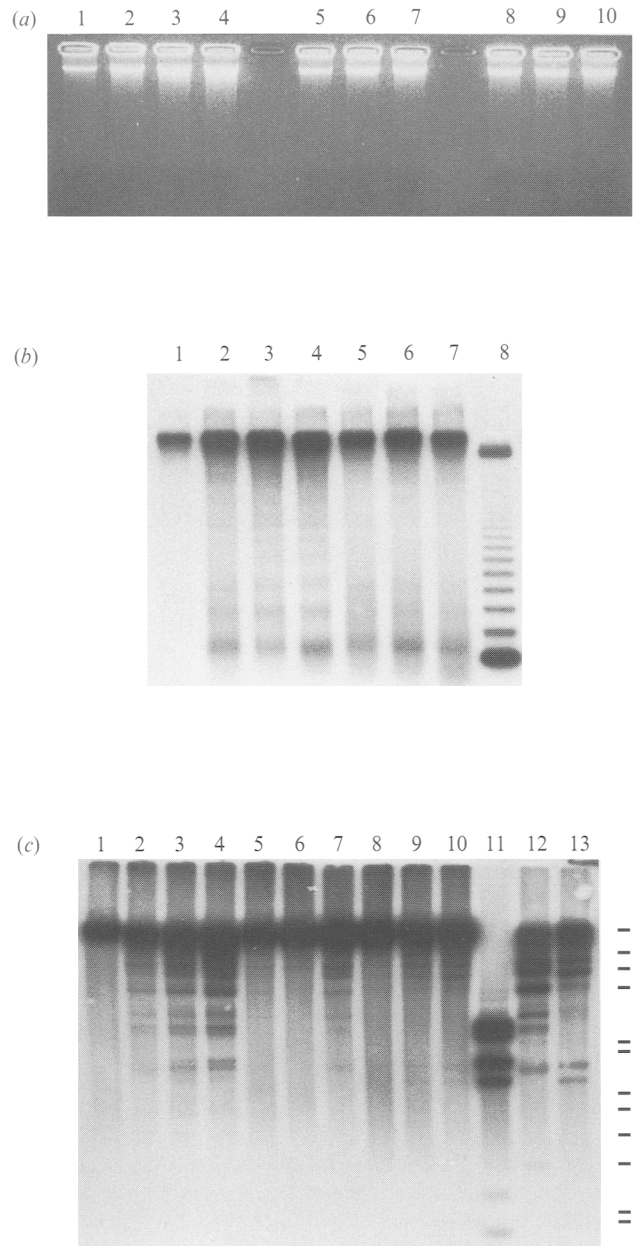

Fig. 5. Effect of restriction enzyme digestion on hypermethylated HPV-16 DNA under *in vivo* conditions. (*a*) Ethidium bromide-stained agarose gel of CaSki DNA isolated from isotonic nuclei corresponding to an equivalent of 100 μg cellular DNA after treatment with 50 units (lane 2), 100 units (lane 3) and 200 units (lane 4) of *Msp*I. Lane 1 represents the untreated control, in which the nuclei were incubated for 30 min at 37 °C without any enzyme. The same amounts of enzyme were applied for *Hpa*II (lanes 5 to 7) and for *Hha*I endonuclease (lanes 8 to 10). (*b*) Autoradiography of the digested DNA after end-labelling with [^{32}P]dCTP. Lane 1, untreated control; lanes 2 to 4, DNA digested with increasing amounts of *Msp*I; lanes 5 to 7, treated with *Hpa*II (see *a*). Lane 8 shows the end-labelled 123 bp ladder. (*c*) Accessibility of nucleosomal organized hypermethylated HPV-16 DNA to *Msp*I (lanes 2 to 4), *Hpa*II (lanes 5 to 7) and *Hha*I (lanes 8 to 10) in comparison with the *Msp*I (lane 11), *Hpa*II (lane 12) and *Hha*I (lane 13) cleavage patterns of purified DNA. The bars represent DNA size markers (23·1, 9·4, 6·6, 4·4, 2·3, 2·0, 1·35, 1·1, 0·8, 0·6, 0·3 and 0·28 kb).

The same degree of methylation can be also detected using other methylation-sensitive enzymes, such as *Hha*I (lane 3) or *Sma*I (lane 4).

Although CaSki cells contain a high copy number of HPV-16 DNA, the virus-specific mRNA is poorly expressed and only reliably detectable by Northern blot analysis after poly(A)$^+$ selection (Fig. 4*a*, lane 1). Treatment of the cells with the protein synthesis inhibitor cycloheximide (CHX) is not accompanied by an increase of the signal (lane 2) favouring the interpretation that the low transcriptional activity might be due to extensive DNA methylation rather than to trans-repressing CHX-labile proteins. Since DNA methylation promotes the nucleosomal assembly into a configuration characteristic of a heterochromatin structure (Keshet *et al.*, 1986; Buschhausen *et al.*, 1987), we used CaSki cells (together with HPV-18-positive SW 756 cervical carcinoma cells, see below) as a model to study the chromatin structure in terms of accessibility to *Msp*I and other methylation-sensitive restriction enzymes. If the *in vivo* situation is the same as that detected by the titration assay described above, one might predict that methylation-binding proteins should protect nucleosomally organized methylated DNA from cleavage by *Msp*I, which normally cleaves protein-free DNA independently of the degree of methylation (see Fig. 4*b*, lane 1, for comparison).

Isotonic nuclei from CaSki cells were prepared, digested with increasing amounts of *Msp*I, *Hpa*II and *Hha*I endonucleases and the cleavage patterns were compared with those of purified DNA. Fig. 5(*a*) shows the effect of *Msp*I (lanes 2 to 4) on the accessibility of bulk cellular chromatin. In contrast to *Hpa*II (lanes 5 to 7) and *Hha*I (lanes 8 to 10), increasing amounts of *Msp*I induce a slight smear after electrophoretic separation of purified DNA; this can mainly be attributed to the higher cleavage efficiency of the enzyme owing to its insensitivity to DNA methylation. Since *Msp*I and *Hpa*II (but not *Hha*I) produce 3′-OH sticky ends, the accessibility of the cellular chromatin to a methylation-insensitive (*Msp*I) and a methylation-sensitive (*Hpa*II) restriction endonuclease can be directly compared by end-labelling the digestion products using Klenow polymerase and [^{32}P]dCTP. Lane 1 (*b*) shows the untreated control, which represents the DNA from nuclei incubated in the corresponding digestion buffer without any restriction endonuclease. Lanes 2 to 4 reveal the end-labelled probes after *Msp*I digestion, lanes 5 to 7 those after *Hpa*II treatment, where ladders of single and multiple copies of the nucleosomal repeat are accumulated.

To monitor the nuclease accessibility of nucleosomally organized HPV-16 DNA, the probes were restricted with the viral non-cutting enzyme *Hin*dIII and hybridized with unit-length viral DNA. Fig. 5(*c*) allows a direct

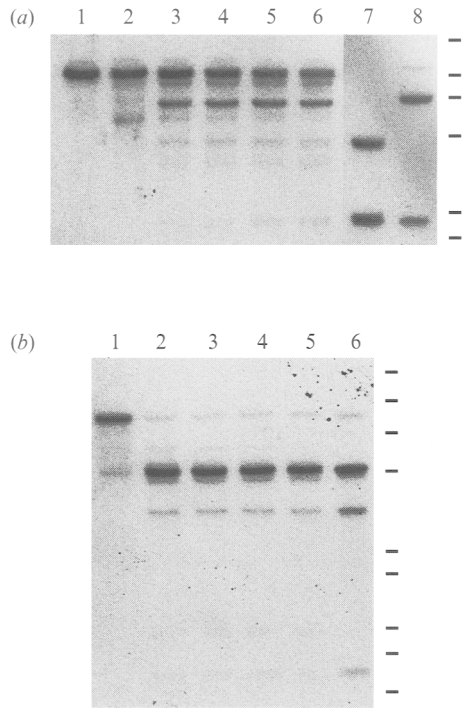

Fig. 6. Restriction enzyme digest of methylated HPV-18 DNA in SW 756 cells. (*a*) Cleavage pattern of HPV-18 chromatin with increasing amounts of *Msp*I. Lane 1, DNA from nuclei lysed immediately after preparation. Lane 2, DNA obtained from nuclei after incubation in digestion buffer without any enzyme. Lane 3, 25 units of *Msp*I; lane 4, 50 units; lane 5, 100 units; lane 6, 200 units. Lane 7, purified DNA after *Msp*I digestion; lane 8, SW 756 DNA treated with *Hpa*II. The bars indicate *Hin*dIII size markers. (*b*) Lanes 2 to 5, isotonic nuclei treated with the same amount of *Hha*I, in comparison with the cleavage pattern obtained at the DNA level, lane 6. Lane 1, incubated control nuclei (see lane 2 in *a*). Both filters were hybridized with unit-length HPV-18 DNA. The size markers are indicated by bars (23·1, 9·4, 6·6, 4·4, 2·3, 2·0, 1·35, 1·1 and 0·8 kb).

comparison of the cleavage properties of *Msp*I/*Hpa*II and *Hha*I at both the chromatin and DNA levels. In contrast to *Hpa*II (lane 7) and *Hha*I (lane 10), which show fragment patterns similar to those detected after cutting purified DNA (lane 12, *Hpa*II; lane 13, *Hha*I), increasing amounts of *Msp*I (lanes 2 to 4) did not create a digestion pattern similar to that obtained after cleavage of protein-free DNA (lane 11), but rather one resembling the *Hpa*II fragment pattern obtained at the DNA level (compare lanes 4 and 12). This finding indicates that under *in vivo* conditions *Msp*I exclusively cleaves at positions also accessible to *Hpa*II on naked DNA. In other words, only the non-methylated *Hpa*II recognition sequences are accessible to *Msp*I, whereas methylated *Hpa*II sites are obviously protected in native chromatin (see Discussion).

A less complex fragment pattern can be detected in SW 756 cells, in which only 15 to 20 copies of HPV-18 are integrated (Fig. 6). Here, the viral DNA is not so extensively methylated as in CaSki cells, generating only two off-size bands after *Hpa*II digestion (*a*, lane 8) in contrast to the corresponding isoschizomer *Msp*I (lane 7). By monitoring the effect of increasing amounts of *Msp*I on viral chromatin (lanes 3 to 6), essentially the same situation is revealed as shown in the previous experiment using CaSki nuclei as the substrate. The prominent 6·8 kb off-size fragment detected after *Hpa*II digestion at the DNA level (lane 8) is also visible after *Msp*I cleavage of native chromatin (lanes 3 to 6), again indicating that methylated 5′ CCGG 3′ sites in nuclei are protected against *Msp*I digestion. Also of interest is the finding that the treatment of isotonic SW 756 nuclei with *Hha*I endonuclease (Fig. 6*b*) results in the same cleavage pattern in native chromatin (lanes 2 to 5), which is also detectable for purified DNA (lane 6), strongly suggesting that the corresponding *Hha*I recognition sites are not associated with nucleosomes.

Discussion

Comparative studies have demonstrated a close functional correlation between DNA methylation and gene regulation in eukaryotic cells (for review, see Razin & Cedar, 1991). Not only endogenous genes are subjected to this kind of epigenetic modifications but also certain human DNA tumour viruses such as Epstein–Barr virus (Masucci *et al.*, 1989) and the hepatitis B virus (Korba *et al.*, 1985) which take advantage of this host cell control mechanism as a regulatory pathway to modulate virus-specific gene expression at the transcriptional level. In the present investigation, evidence is provided that the activity of the URR of pathogenic HPV-18 can also be altered by DNA methylation. Sequence-specific *in vitro* methylation by using *Hha*I methyltransferase results in a significant down-regulation of CAT expression in HPV-18 URR-controlled reporter plasmids (Fig. 2), which seems to be specific for several *Hha*I acceptor sites within the viral control region (see Fig. 1). In contrast, *Hpa*II methylase, which has no recognition sites in the HPV-18 URR but the same number of acceptor sites as *Hha*I methylase within the basic vector, has no effect on CAT expression (Fig. 2, lane 4). This suggests that the suppressive effect is not region- (Murray & Grosveld, 1987) but sequence-specific, depending on a non-random distribution of six *Hha*I sites enriched in the viral control region and of which five are located within the constitutive enhancer element (Swift *et al.*, 1987). This is in accordance with other studies (Boyes & Bird, 1992) also showing that the density of methylated CpG sites is an important parameter for the degree of repression at particular regulatory regions.

The application of sequence-specific methyltransferases, however, is often limited, because further

CpG dinucleotides, normally representing targets for the eukaryotic methylase (Rachal *et al.*, 1989), cannot be reached unless they are embedded in a corresponding recognition site. Inspection of the HPV-18 sequence reveals that the regulatory region has 20 CpG dinucleotides among which only 11 coincide with *Hha*I methylase acceptor sites. Another stretch of five CpGs is located near the TATA box within the palindromic sequence of the E2-binding site [5′ ACCG$(A)_4$CGGT-CGGG-ACCG$(A)_4$CGGT 3′] (Romanczuk *et al.*, 1990). The use of *Sss*I methylase (Boyes & Bird, 1991), which mimics the activity of the eukaryotic counterpart in methylating CpG residues, completely abolishes the activity of the HPV-18 URR-driven reporter construct, emphasizing the functional significance of these sites in the transcriptional competence of the regulatory region. The effect of *Sss*I *in vitro* methylation can be considered to be specific for the viral URR, since it has been reported that the same basic vector harbouring the LTR of RSV as a regulatory insert results in only a twofold reduction after general CpG methylation (Rachal *et al.*, 1989). A possible explanation is that the strong enhancer configuration of the RSV LTR appears to be sufficient to overcome the methylation-imposed repression effect (Knebel-Mörsdorf *et al.*, 1988; Boyes & Bird, 1991). Mutational dissection of the HPV-18 URR in its natural context (Hoppe-Seyler & Butz, 1993) in parallel with *in vitro* methylation studies should clarify the role of the CpG cluster at this position.

A compilation of experiments analysing the mechanism of gene repression by DNA methylation has shown that methylated DNA becomes assembled in the nucleus into a nucleosomal conformation which is reminiscent of transcriptionally inactive chromatin (Keshet *et al.*, 1986; Buschhausen *et al.*, 1987). Furthermore, recent studies have described different classes of DNA-binding proteins with high affinity for methylated DNA, which are distinguishable by their binding specificities. While the methylated DNA-binding protein requires additional recognition sequences surrounding the methylated CpG site (Zhang *et al.*, 1989), the properties of MeCP can be regarded as non-specific, depending only on a symmetrical distribution of a certain number of methylated CpG residues (Meehan *et al.*, 1989). Since *Hha*I *in vitro* methylated pHP18URR-CAT expression vectors can be reactivated by cotransfection of increasing amounts of *Sss*I methylase-treated untranscribable prokaryotic DNA, this result strongly indicates the involvement of the latter class of methyl-DNA-binding proteins in the repression mechanism of the HPV-18 regulatory region (Fig. 3). Moreover, reactivation by *in vitro* titration also supports the notion that the chromatin structure cannot solely account for the transcriptional down-regulation of *in vitro* methylated plasmids. The MeCPs might function either as stabilizing components of the nucleoprotein complex (Antequera *et al.*, 1989) or as phasing proteins on methylated CpG residues during the process of assembly into a nucleosomal composition typical of transcriptionally inactive chromatin (Meehan *et al.*, 1989). Although the first possibility cannot be ruled out conclusively at the moment, the results obtained by the restriction enzyme accessibility assays of cervical carcinoma cells rather favour the assumption that MeCPs are involved in the process of positioning nucleosomes on methylated DNA templates into a heterochromatin conformation.

The analysis of susceptible or protected regions in native chromatin is thought to be determined mainly by two parameters. Recognition sites coinciding with condensed chromatin domains or with nucleosomal core regions are resistant, whereas linker regions in transcribed stretches or nuclease-hypersensitive sites are vulnerable to restriction enzyme digestion (Almer & Hörz, 1986). As already shown for endogenous genes (Antequera *et al.*, 1989), *Msp*I endonuclease, normally cleaving purified DNA whether methylated or not, is unable to cut the corresponding HPV-16/-18 sequences if the DNA is organized as chromatin (Fig. 5 and 6). The *Msp*I fragment pattern is similar or even identical to the cleavage products obtained after *Hpa*II digestion at the DNA level, implying that methylated recognition sites are protected under *in vivo* conditions. Since it is unlikely that every methylated CpG site in both investigated cell lines consistently coincides with a nucleosomal core region, the resistance can be explained either by the assumption that the MeCP binding pattern is retained even after integration of viral DNA, or a supranucleosomal structure is assembled which is not accessible to exogenously added nucleases.

The role of DNA methylation in HPV-linked carcinogenesis can be considered in the context that viral integration into the host genome seems to be an early event during the different progression steps to anogenital cancer (Schneider-Maunoury *et al.*, 1987; zur Hausen, 1991*b*). Consequently, the viral DNA is subjected to epigenetic modification mechanisms which would normally affect the expression of the unoccupied chromosomal domain. *Msp*I/*Hpa*II isoschizomer assays on both premalignant and malignant biopsy material have revealed that episomal HPV DNA is not methylated, but is found to be methylated if integrated (F. Rösl & H. zur Hausen, unpublished results). It is therefore reasonable to assume that integrated HPV-18 DNA can potentially be methylated *de novo* at the same positions shown to be sensitive to *Hha*I or *Sss*I methyltransferases under *in vitro* conditions. This could result in viral latency caused by negative interference

with HPV gene expression, which is reminiscent of conditions described for Moloney murine leukaemia virus in mice (Jähner & Jaenisch, 1985) or for HIV in human cells (Bednarik *et al.*, 1990). A possibly analogous system is found in transgenic mice carrying integrated SV40 DNA. Here, the viral DNA was found to be demethylated exclusively in tumorigenic cells, but hypermethylated in non-malignant tissues (Van Dyke *et al.*, 1985) suggesting that a decrease in methylation maintenance is either the cause or a consequence of cellular transformation (Feinberg & Vogelstein, 1983). Consistent with this interpretation is the finding that bovine papillomavirus DNA also shows a reduced transforming potential after *in vitro* methylation and transfection into heterologous rodent cells (Christy & Scangos, 1986).

A detailed comparison of the methylation levels of HPV-16/-18 DNA in premalignant and malignant lesions in relation to viral transcription should provide insight into the role of viral methylation in the different steps leading to cervical cancer. Moreover, the utilization of cell lines containing integrated *Hha*I methylase-suppressed HPV-18 URR–CAT reporter constructs should be helpful in clarifying the question of whether certain carcinogens, known to induce hypomethylation (Wilson *et al.*, 1987; Barr *et al.*, 1992) and considered to be cofactors in HPV-linked carcinogenesis (zur Hausen, 1991*b*), are capable of overcoming methylation-imposed repression. A cell system could thus be developed which should allow the screening and identification of substances that may act in an either additive or synergistic manner with HPV infection.

We would like to thank Dieter Lewe for his help with computer work, Kerstin Kleine for helpful comments on the manuscript and Tobias Bauknecht for providing the pH18URR-CAT vector. We are grateful to Renate Webler for the photographic work and Beate Hornung for her help with the ^{14}C scanner. The plasmid pRSV-luc was kindly provided by Martin Rentrop.

References

ALMER, A. & HÖRZ, W. (1986). Nuclease hypersensitive regions with adjacent positioned nucleosomes mark the gene boundaries of the PHO3/PHO5 locus in yeast. *EMBO Journal* **5**, 2681–2687.

ANTEQUERA, F., MACLEOD, D. & BIRD, A. (1989). Specific protection of methylated CpGs in mammalian nuclei. *Cell* **58**, 509–517.

ANTEQUERA, F., BOYES, J. & BIRD, A. P. (1990). High levels of *de novo* methylation and altered chromatin structure at CpG islands in cell lines. *Cell* **72**, 503–514.

AVIV, H. & LEDER, P. (1972). Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose. *Proceedings of the National Academy of Sciences, U.S.A.* **64**, 1408–1412.

BAKER, C. C., PHELPS, W. C., LINDGREN, V., BRAUN, M. J., GONDA, M. A. & HOWLEY, P. M. (1987). Structural and transcriptional analysis of human papillomavirus type 16 in cervical carcinoma cell lines. *Journal of Virology* **61**, 962–971.

BARR, F. G., DAVIS, R. J., EICHENFIELD, L. & EMANUEL, B. S. (1992). Structural analysis of a carcinogen-induced genomic rearrangement event. *Proceedings of the National Academy of Sciences, U.S.A.* **89**, 942–946.

BEDNARIK, D. P., COOK, J. A. & PITHA, P. M. (1990). Inactivation of the HIV LTR by DNA CpG methylation: evidence for a role in latency. *EMBO Journal* **9**, 1157–1164.

BEN-HATTAR, J., BEARD, P. & JIRICNY, J. (1989). Cytosine methylation in CTF and Sp1 recognition sites of an HSV *tk* promoter: effects on transcription *in vivo* and on factor binding *in vitro*. *Nucleic Acids Research* **17**, 10179–10190.

BERNARD, B. A., BAILLY, C., LENOIR, M.-C., DARMON, M., THIERRY, F. & YANIV, M. (1989). The human papillomavirus type 18 (HPV 18) E2 gene product is a repressor of the HPV 18 regulatory region in human keratinocytes. *Journal of Virology* **63**, 4317–4324.

BOSCH, F. X., SCHWARZ, E., BOUKAMP, P., FUSENIG, N. E., BARTSCH, D. & ZUR HAUSEN, H. (1990). Suppression *in vivo* of human papillomavirus type 18 E6-E7 gene expression in non-tumorigenic HeLa-fibroblast hybrid cells. *Journal of Virology* **64**, 4743–4754.

BOYES, J. & BIRD, A. (1991). DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell* **64**, 1123–1134.

BOYES, J. & BIRD, A. (1992). Repression of genes by DNA methylation depends on CpG density and promoter strength: evidence for involvement of a methyl-CpG binding protein. *EMBO Journal* **11**, 327–333.

BUSCHHAUSEN, G., WITTIG, B., GRAESSMANN, M. & GRAESSMANN, A. (1987). Chromatin structure is required to block transcription of the methylated herpes simplex virus thymidine kinase. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 1177–1181.

CHAN, W.-K., KLOCK, G. & BERNARD, H.-U. (1989). Progesterone and glucocorticoid response elements occur in the long control regions of several human papillomaviruses involved in anogenital neoplasia. *Journal of Virology* **63**, 3261–3269.

CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium–thiocyanate–phenol–chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

CHOO, K. B., PAN, C. C. & HAN, S. M. (1987). Integration of human papillomavirus type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. *Virology* **161**, 259–261.

CHRISTY, B. A. & SCANGOS, G. A. (1986). *In vitro* methylation of bovine papillomavirus alters its ability to transform mouse cells. *Molecular and Cellular Biology* **6**, 2910–2915.

COLE, S. T. & DANOS, O. (1987). Nucleotide sequence and comparative analysis of human papillomavirus type 18 genome. *Journal of Molecular Biology* **193**, 599–608.

DE WET, J. R., WOOD, K. V., DELUCA, M., HELINSKI, D. R. & SUBRAMANI, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. *Molecular and Cellular Biology* **7**, 725–737.

DOERFLER, W. (1991). Patterns of DNA methylation – evolutionary vestiges of foreign DNA inactivation as a host defence mechanism. *Biological Chemistry Hoppe-Seyler* **372**, 557–564.

DÜRST, M., KLEINHEINZ, A., HOTZ, M. & GISSMANN, L. (1985). The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumours. *Journal of General Virology* **66**, 1515–1522.

DÜRST, M., PETRUSSEVSKA, R. T., BOUKAMP, P., FUSENIG, N. E. & GISSMANN, L. (1987). Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus 16 DNA. *Oncogene* **1**, 251–256.

ELGIN, S. C. R. (1984). Anatomy of hypersensitive sites. *Nature, London* **309**, 213–214.

FEINBERG, A. & VOGELSTEIN, B. (1983). Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature, London* **301**, 89–91.

FEINBERG, A. & VOGELSTEIN, B. (1984). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **137**, 266–267.

GORMAN, C. M., MOFFAT, L. F. & HOWARD, B. (1982). Recombinant genomes which express chloramphenicol acetyl transferase in mammalian cells. *Molecular and Cellular Biology* **2**, 1044–1051.

HOLLIDAY, R. (1987). The inheritance of epigenetic defects. *Science* **238**, 163–170.

HOPPE-SEYLER, F. & BUTZ, K. (1993). A novel cis-stimulatory element maps to the 5′ portion of the human papillomavirus type 18 upstream regulatory region and is functionally dependent on a sequence-aberrant Sp1 binding site. *Journal of General Virology* **74**, 281–286.

JÄHNER, D. & JAENISCH, R. (1985). Retrovirus-induced *do novo* methylation of flanking host sequences correlates with gene inactivity. *Nature, London* **315**, 584–587.

KELLER, W. (1975). Determination of the number of superhelical turns in simian virus 40 DNA by gel electrophoresis. *Proceedings of the National Academy of Sciences, U.S.A.* **72**, 4876–4880.

KESHET, I., LIEMAN-HURWITZ, J. & CEDAR, H. (1986). DNA methylation affects the formation of active chromatin. *Cell* **44**, 535–543.

KHANDJIAN, E. W. & MERIC, C. (1986). A procedure for Northern blot analysis of native RNA. *Analytical Biochemistry* **159**, 227–232.

KLEIN, C. B., CONWAY, K., WANG, X. W., BHAMBRA, R. K., LIN, X., COHEN, M. D., ANNAB, L., BARRETT, J. C. & COSTA, M. (1991). Senescence of nickel-transformed cells by an X chromosome: possible epigenetic control. *Science* **251**, 796–799.

KNEBEL-MÖRSDORF, D., ACHTEN, S., LANGNER, K.-D., RÜGER, R., FLECKENSTEIN, B. & DOERFLER, W. (1988). Reactivation of the methylation-inhibited late E2A promoter of adenovirus type 2 by a strong enhancer of human cytomegalovirus. *Virology* **166**, 166–174.

KORBA, B. E., WILSON, V. L. & YOAKUM, G. H. (1985). Induction of hepatitis B virus core gene in human cells by cytosine demethylation in the promoter. *Science* **228**, 1103–1106.

KRUCZEK, I. & DOERFLER, W. (1983). Expression of the chloramphenicol acetyl transferase gene in mammalian cells under the control of adenovirus type 12 promoters. Effect of promoter methylation on gene expression. *Proceedings of the National Academy of Sciences, U.S.A.* **80**, 7586–7590.

LAWSON, G. M., TSAI, M. J. & O'MALLEY, B. W. (1980). Deoxyribonuclease I sensitivity of the nontranscribed sequences flanking the 5′ and 3′ ends of the ovomucoid gene and the ovalbumin and its related X and Y genes in hen oviduct nuclei. *Biochemistry* **19**, 4403–4411.

LEVINE, A., CANTONI, G. & RAZIN, A. (1991). Inhibition of promoter activity by methylation: possible involvement of protein mediators. *Proceedings of the National Academy of Sciences, U.S.A.* **88**, 6515–6518.

LUKOW, B. & SCHÜTZ, G. (1987). CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Research* **15**, 5490.

MCCLELLAND, M. (1981). The effect of sequence specific DNA methylation on restriction endonuclease cleavage. *Nucleic Acids Research* **12**, 5859–5866.

MASUCCI, M. G., CONTRERAS-SALAZAR, B., RAGNAR, E., FALK, K., MINAROVITS, J., ERNBERG, I. & KLEIN, G. (1989). 5-Azacytidine up regulates the expression of Epstein–Barr virus nuclear antigen 2 (EBNA-2) through EBNA-6 and latent membrane protein in the Burkitt's lymphoma line Rael. *Journal of Virology* **63**, 3135–3141.

MEEHAN, R. R., LEWIS, J. D., MCKAY, S., KLEINER, E. & BIRD, A. P. (1989). Identification of mammalian protein that binds specifically to DNA containing methylated CpGs. *Cell* **58**, 499–507.

MÜNGER, K., PHELPS, W. C., BUBB, V., HOWLEY, P. M. & SCHLEGEL, R. (1989). The E6 and E7 genes of the human papillomavirus type 16 are necessary and sufficient for transformation of primary human keratinocytes. *Journal of Virology* **63**, 4417–4423.

MURRAY, E. J. & GROSVELD, F. (1987). Site-specific demethylation in the promoter of human γ-globin gene does not alleviate methylation mediated suppression. *EMBO Journal* **6**, 2329–2335.

PIRISI, L., YASUMOTO, S., FELLER, M., DONINGER, J. & DIPAOLO, J. (1987). Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *Journal of Virology* **61**, 1061–1066.

PRENDERGAST, G. C., LAWE, D. & ZIFF, E. B. (1991). Association of Myn, the murine homolog of Max, with c-Myc stimulates methylation-sensitive DNA binding and ras cotransformation. *Cell* **65**, 395–407.

RACHAL, M. J., YOO, H., BECKER, F. F. & LAPEYRE, J.-M. (1989). *In vitro* DNA cytosine methylation of *cis*-regulatory elements modulates c-Ha-ras promoter activity *in vivo*. *Nucleic Acids Research* **13**, 5135–5147.

RAZIN, A. & CEDAR, H. (1991). DNA methylation and gene expression. *Microbiological Reviews* **55**, 451–458.

REIK, W., COLLICK, A., NORRIS, M. L., BARTON, S. C. & SURANI, M. A. (1987). Genomic imprinting determines methylation of parental alleles in transgenic mice. *Nature, London* **328**, 248–251.

ROMANCZUK, H., THIERRY, F. & HOWLEY, P. M. (1990). Mutational analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. *Journal of Virology* **64**, 2849–2859.

RÖSL, F. & WALDECK, W. (1991). Topological properties of bovine papillomavirus type 1 (BPV-1) DNA in episomal nucleoprotein complexes: a model system for chromatin organization in higher eukaryotes. *Molecular Carcinogenesis* **4**, 248–256.

RÖSL, F., DÜRST, M. & ZUR HAUSEN, H. (1988). Selective suppression of human papillomavirus transcription in non-tumorigenic cells by 5-azacytidine. *EMBO Journal* **7**, 1321–1328.

RÖSL, F., WESTPHAL, E.-M. & ZUR HAUSEN, H. (1989). Chromatin structure and transcriptional regulation of human papillomavirus type 18 DNA in HeLa cells. *Molecular Carcinogenesis* **2**, 72–80.

RÖSL, F., ACHTSTÄTTER, T., BAUKNECHT, T., FUTTERMAN, G., HUTTER, K.-J. & ZUR HAUSEN, H. (1991). Extinction of the HPV 18 upstream regulatory region in cervical carcinoma cells after fusion with non-tumorigenic human keratinocytes under non-selective condition. *EMBO Journal* **10**, 1337–1345.

SAMBROOK, J., FRITSCH, E. & MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory.

SCHNEIDER-MAUNOURY, S., CROISSANT, O. & ORTH, G. (1987). Integration of human papillomavirus type 16 DNA sequences: a possible early event in the progression of genital tumors. *Journal of Virology* **61**, 3295–3298.

SCHWARZ, E., FREESE, U. K., GISSMANN, L., MAYER, W., ROGGENBUCK, B., STREMLAU, A. & ZUR HAUSEN, H. (1985). Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature, London* **314**, 111–114.

SMITS, H. L., RAADSHEER, E., ROOD, J., MEHENDALE, S., SLAZER, R. M., VAN DER NOORDA, J. & TER SCHEGGET, J. (1988). Induction of anchorage-independent growth of human embryonic fibroblasts with a deletion in the short arm of chromosome 11 by human papillomavirus type 16. *Journal of Virology* **62**, 4538–4543.

SMITS, P. H. M., SMITS, H. L., JEBBINK, M. F. & TER SCHEGGET, J. (1990). The short arm of chromosome 11 likely is involved in the regulation of the human papillomavirus type 16 early enhancer–promoter and in the suppression of the transforming activity of the viral DNA. *Virology* **176**, 158–165.

SOUTHERN, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology* **98**, 503–517.

SWIFT, F. V., BHAT, K., YOUNGHUSBAND, H. B. & HAMADA, H. (1987). Characterization of a cell-type specific enhancer found in the human papillomavirus type 18 genome. *EMBO Journal* **6**, 1339–1344.

VAN DYKE, T., FINLAY, C. & LEVINE, A. J. (1985). A comparison of several lines of transgenic mice containing the SV 40 early genes. *Cold Spring Harbor Symposia on Quantitative Biology* **50**, 671–678.

VON KNEBEL DOEBERITZ, M., BAUKNECHT, T., BARTSCH, D. & ZUR HAUSEN, H. (1991). Influence of chromosomal integration on glucocorticoid-regulated transcription of growth-stimulating papillomavirus genes E6 and E7 in cervical carcinoma cells. *Proceedings of the National Academy of Sciences, U.S.A.* **88**, 1411–1415.

WIGLER, M., PELLICER, A., SILVERSTEIN, A. & AXEL, R. (1978). Biochemical transfer of single-copy eukaryotic genes using total cellular DNA as donor. *Cell* **14**, 725–731.

WILSON, V. L., SMITH, R. T., LONGORIA, J., LIOTTA, M. A., HARPER, C. M. & HARRIS, C. C. (1987). Chemical carcinogen-induced decreases in genomic 5-methyldeoxycytidine content of normal human bronchial epithelial cells. *Proceedings of the National Academy of Sciences, U.S.A.* **84**, 3298–3301.

YEE, C., KRISHNAN-HEWLETT, I., BAKER, C. C., SCHLEGEL, R. & HOWLEY, P. M. (1985). Presence and expression of human

papillomavirus sequences in human cervical carcinoma cell lines. *American Journal of Pathology* **119**, 361–366.

ZHANG, X.-Y., SUPAKAR, P. C., KHAN, R., EHRLICH, K. C. & EHRLICH, M. (1989). Related sites in human and herpesvirus DNA recognized by methylated DNA-binding protein from human placenta. *Nucleic Acids Research* **17**, 1459–1474.

ZUR HAUSEN, H. (1986). Intracellular surveillance of persisting viral infections: human genital cancer results from deficient cellular control of papillomavirus gene expression. *Lancet* ii, 489–491.

ZUR HAUSEN, H. (1989). Papillomaviruses in anogenital cancer as a model to understand the role of viruses in human cancers. *Cancer Research* **49**, 4677–4681.

ZUR HAUSEN, H. (1991*a*). Viruses in human cancer. *Science* **254**, 1167–1173.

ZUR HAUSEN, H. (1991*b*). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* **184**, 9–13.

(*Received 17 November 1992; Accepted 8 January 1993*)