JOURNAL OF VIROLOGY, Sept. 1997, p. 6956–6966 Vol. 71, No. 9
0022-538X/97/$04.00+0

Cell-Type-Specific Separate Regulation of the E6 and E7 Promoters of Human Papillomavirus Type 6a by the Viral Transcription Factor E2

B. RAPP,[1] A. PAWELLEK,[1] F. KRAETZER,[1] M. SCHAEFER,[1] C. MAY,[1] K. PURDIE,[2] K. GRASSMANN,[1] AND T. IFTNER[1,3]*

Institut für Klinische und Molekulare Virologie, 91054 Erlangen,[1] and Sektion Experimentelle Virologie, Tübingen,[3] Germany, and Academic Department of Dermatology, Queen Mary and Westfield College, London, United Kingdom[2]

Received 7 March 1997/Accepted 5 June 1997

Gene expression of human papillomaviruses (HPV) is tightly controlled by cellular factors and by the virally encoded E2 protein through binding to distinct sites within the regulatory noncoding region. While for the high-risk genital papillomaviruses a single promoter drives the expression of all early genes, a second promoter present in the E6 open reading frame of the low-risk HPV type 6 (HPV6) would allow an independent regulation of E6 and E7 oncogene expression. In this report, we provide the first evidence that E2 regulates both early promoters of HPV6 separately and we show that promoter usage as well as E2 regulation is cell type dependent. Among the different epithelial cell lines tested, only RTS3b cells allowed an expression pattern similar to that observed in naturally infected benign condylomas. While the E6 promoter was repressed by E2 to 50% of its basal activity, the E7 promoter was simultaneously stimulated up to fivefold. Activation of the E7 promoter was mediated predominantly by the binding of E2 to the most promoter-distal E2 binding site. Repression of the E6 promoter depended on the presence of two intact promoter-proximal binding sites. Mutation of both of these repressor binding sites reversed the effect of E2 on the E6 promoter from repression to activation. In contrast, in HT3 cells we observed an E2-mediated activation of the E6 promoter in the context of the wild-type noncoding region. This indicated that repression of the E6 promoter by binding of E2 to both promoter-proximal binding sites did not function in the cellular environment provided by HT3 cells. These data suggest that the separate regulation of the E6 and E7 promoters of HPV6 is mediated through successive occupation of binding sites with different affinities for E2 depending on the intracellular concentration of E2 and on the cellular environment provided by the infected cell.

Within the last decade, detailed epidemiological studies formed our present understanding that infection of the cervical epithelium with human papillomaviruses (HPVs) is the major determinant for the development of a squamous intraepithelial lesion. A high risk of progression of the initial benign lesion can be clearly assigned to the presence of certain high-risk viruses, like HPV16 and HPV18, whereas lesions containing low-risk viruses, like HPV6 or HPV11, rarely progress (32, 52). Investigations with the aim of identifying conditions related to the process of malignant conversion by descriptive in situ hybridization studies of human biopsy material, by using animal model systems, and by tissue culture experiments identified a permanent upregulation of the transcripts for the viral oncogenes E6/E7 as the corresponding marker event in all cases (3, 5, 14, 15, 22, 51, 56, 57). A tight regulation of the E6 and E7 expression is therefore a crucial requirement for the virus because malignant progression of the infected epithelium counteracts the viral life cycle, which is absolutely dependent on normally differentiated epithelium. One important regulatory protein of the papillomaviruses is the E2 protein, because it influences both transcription of the viral genes and replication of the viral DNA. The E2 protein binds as a dimer to its recognition sequence, $ACCN_6GGT$, a 12-bp palindrome which occurs several times in the noncoding region (NCR) of all papillomaviruses (50). Genital HPVs contain four E2 binding sites (E2BS) within the NCR, which are highly conserved in their relative positions among different types. For the high-risk genital HPV types, E2 binding to one or both of the promoter-proximal binding sites (E2BS-3 and E2BS-4) represses transcription of the single early promoter upstream of the E6 open reading frame (ORF) by preventing formation of the transcription preinitiation complex (8, 12, 49). This experimental finding is indeed reflected by the very low expression of the E6 and E7 genes in benign lesions containing high-risk HPV types in the form of episomal DNA (3). In contrast, up regulation of oncogene transcription following malignant progression occurs because the viral genome is integrated into the host chromosomal DNA in a large number of carcinomas, which leads to disruption of the E2 gene and to a loss of the E2-mediated negative control (57). In the case of episomal persisting viral genomes, deletions or mutations of YY1 repressor binding sites within the NCR were observed in virus isolates from carcinomas or high-grade lesions (1, 13, 33).

Also, a role for the low-risk HPV6 in carcinogenesis was discussed for a prolonged period in the literature. This was based on the fact that HPV6 was frequently found in semimalignant Buschke-Loewenstein tumors (4, 40), in isolated cases of cancer (2, 28, 29, 35–37, 39), and in roughly 1% of 318 invasive cancers of the anogenital tract as described in one study (54). In earlier reports, it was speculated that sequence alterations in the noncoding region of HPV6 isolates from carcinomas may confer an increased malignant potential to the respective isolate by enhancing expression of the viral onco-

* Corresponding author. Mailing address: Institut für Klinische und Molekulare Virologie, Schlossgarten 4, 91054 Erlangen, Germany. Phone: 9131 852104. Fax: 9131 852101. E-mail: tsiftner@viro.med.uni-erlangen.de.

TABLE 1. Oligonucleotides used for PCR mutagenesis and EMSA

Oligonucleotide	Sequence[a]	Application
mt-E2-1	5′-TATTTGCA**TG**CGTTTTC**T**GTTGCCCTT-3′ (nt 7628–7654)	PCR mutagenesis
mt-E2-2	5′-CACCTGCA**TG**CGGTTTC**T**GTTATCCAC-3′ (nt 7961–7987)	PCR mutagenesis
mt-E2-3	5′-TAGGAGGG**C**C2**T**GAAAAC**T**GTTCAACC-3′ (nt 28–53)	PCR mutagenesis
mt-E2-4	5′-GTTCA**T**CCGAAAACG**CG**TGTATATAAACCAG-3′ (nt 46–76)	PCR mutagenesis
P90mt-s	5′-CGAAAACGGTTGTA**G**A**G**A**C**ACCAGCCCT-3′ (nt 53–80)	PCR mutagenesis
P270mt-s	5′-TCATATGCATA**G**A**G**ACACCTAAAGGTC-3′ (nt 235–261)	PCR mutagenesis
E2-1wt	5′-CAGGATCCTTGCAACCGTTTTCGGTTGCCCGGATCCAC-3′ (nt 7631–7652)	EMSA
E2-2wt	5′-CAGGATCCCTGCAACCGGTTTCGGTTATCCGGATCCAC-3′ (nt 7964–7985)	EMSA
E2-3wt	5′-CAGGATCCGAGGGACCGAAAACGGTTCAACGGATCCAC-3′ (nt 31–52)	EMSA
E2-4wt	5′-CAGGATCCGTTCAACCGAAAACGGTTGTATGGATCCAC-3′ (nt 46–67)	EMSA

[a] Nucleotide positions refer to HPV6a (24). Only the coding strand of each double-stranded oligonucleotide is shown. Nucleotides differing from the wild-type sequence are underlined. Oligonucleotides used in EMSA contain, in addition to the HPV6 sequences, two flanking *Bam*HI restriction sites.

genes. However, we have recently been able to show that there is no correlation between the existence of sequence alterations in the NCR and the presence of HPV6 in malignant lesions or an enhanced expression of the viral oncogenes (20).

One important difference between high-risk and low-risk viruses is the presence of an additional promoter within the E6 ORF of HPV6 and HPV11 that would allow a separate regulation of both oncogenes (11, 20, 44). Such independent regulation is indeed suggested by in situ hybridization studies and RNA transcription analyses of HPV6-infected condylomas, which clearly show the presence of different amounts of E6- and E7-specific transcripts in different cell layers of the infected epithelium. In addition, the confined expression of E6 and E7 to the lower epithelial layers of infected condylomas suggests a further level of control (26, 44).

Although E6 mRNA could not be detected in HPV6-positive carcinomas by in situ hybridization, the large amount of E7 transcripts observed in all carcinoma cells points to a similar up regulation of this transcript to that in high-risk HPV-positive malignancies (36). In contrast to the high-risk genital HPVs, however, integration of the viral genome has not been detected in HPV6-positive carcinomas with the exception of a single case (2, 36, 54). The mechanism of this up regulation remains unknown so far, and although the existence of the E7 promoter has been known for some time, the activity of this promoter in cultured tissue cells was demonstrated only recently (11, 20). Any further regulation of this promoter by the viral E2 protein has not been analyzed. We therefore were interested in investigating the activity and regulation of both early promoters of HPV6 in the natural context of the complete NCR under cellular conditions that allow a similar expression pattern to that observed in naturally infected condylomas. For this, we tested a number of cell lines for their ability to support E2-mediated activation of a test construct containing the complete NCR and both early promoters of HPV6a. Two cell lines found to be positive were further used in transient-transfection experiments to perform RNA transcript analyses and luciferase assays with the aim of identifying the role of individual E2 binding sites for promoter regulation. Our data show that E2 has opposite effects on each of the two early promoters of HPV6. Activation of the E7 promoter is mediated mainly by the most distal E2 binding site (E2BS-1). Repression of the E6 promoter by E2 is dependent on the presence of the two intact promoter-proximal binding sites (E2BS-3 and E2BS-4), while mutation of both repressor binding sites reverses the effect of E2 on the E6 promoter from repression to activation.

MATERIALS AND METHODS

Cell culture. Transfection experiments were performed with the following cells: the HPV-negative skin-derived keratinocyte cell line RTS3b (38), the HPV-negative cervical carcinoma cell line HT3 (ATCC HTB32), the HPV16-positive human epithelial cell line KG3 (established from a vulvar intraepithelial neoplasia) (19), the human epithelial cell line MCF-7 (established from an adenocarcinoma of the breast) (ATCC HTB22), the simian virus 40-transformed monkey kidney cell line Cos-7 (ATCC CRL1651), and primary human foreskin keratinocytes (PHFK). HT3, Cos-7, and MCF-7 cells were cultivated in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal calf serum (PAA Laboratories), 2.5 μg of amphotericin B (Fungizone; Gibco BRL) per ml, and 50 μg of gentamicin (Gibco BRL) per ml. RTS3b and KG cells were grown in E-medium (34) containing 10% fetal calf serum, 2.5 μg of amphotericin B per ml, 50 μg of gentamicin per ml, and 5 ng of epidermal growth factor (Sigma) per ml. PHFK were cultivated in KGM medium (Promocell, Heidelberg, Germany).

Plasmids. All numbering of nucleotide positions within the HPV6 genome refers to the HPV6a sequence published by Hofmann et al. (24). For cloning of the HPV6 E2 expression plasmid (pLXSN6a-E2), the E2 ORF of HPV6a (9) was amplified by PCR as described previously (20) with the help of the primer pair 6-E2v and 6-E2r. Primer 6-E2v hybridizes to sequences at positions 2681 to 2699 of the HPV6a genome and contains an additional *Eco*RI restriction site, and primer 6-E2r binds to sequences at positions 3890 to 3906 and contains an additional *Bam*HI restriction site. The amplification product was digested with *Bam*HI and *Eco*RI and subsequently ligated with the *Bam*HI-*Eco*RI sites of the expression vector pLXSN (21). The luciferase reporter plasmid 6aNCR-P2luc consists of the HPV6a NCR including parts of the L1 and the E6 ORFs from nucleotides (nt) 7139 to 446 cloned into the *Sal*I and *Bam*HI sites of the luciferase expression vector pA-luc (13). The insert was generated by PCR amplification of the cloned HPV6a genome (9) with the help of the primer pair 6-1 and 6-2. Primer 6-1 hybridizes to sequences at positions 7139 to 7159 of the HPV6a genome and contains an additional *Sal*I restriction site, and primer 6-2 binds to sequences at positions 446 to 427 and contains an additional *Bgl*II recognition site. The luciferase reporter plasmid 6aNCR-P1luc was constructed by PCR amplification of the HPV6a region from nt 7139 to 158 and cloning it into the *Sal*I and *Bam*HI sites of the vector pAluc. The primer pairs 6-1 (nt 7139 to 7159) and 6-5 (nt 158 to 137) contain an additional recognition sequence for the restriction enzymes *Sal*I and *Bgl*II, respectively. The reporter plasmids 6aNCR-P1*P2luc and 6aNCR-P2*luc consist of the backbone plasmid 6aNCR-P2luc and have a mutation in the TATA box sequences of promoter P1 or P2, respectively. The mutations were created by PCR mutagenesis with the help of the complementary primer pairs P90mt-s and P90mt-as for 6aNCR-P1*P2luc and P270mt-s and P270mt-as for 6aNCR-P2*luc. The sequences of the sense strand primers P90mt-s and P270mt-s are shown in Table 1. Derivatives of 6aNCR-P2luc with mutated E2 binding sites were generated by PCR mutagenesis with the help of appropriate primer pairs (Table 1). All constructs were confirmed by DNA sequencing. For RNA analyses, inserts of plasmids 6aNCR-P2luc, 6aNCR-P1luc, 6aNCR-P1*P2luc, 6aNCR-P2*luc, and E2BS-2/3/4mt were subcloned upstream of the β-globin gene into the *Sal*I and *Kpn*I sites of vector pHM313 (46), a promoterless derivative of vector OVEC-ref (53). The constructs were designated 6aNCR-P2-β, 6aNCR-P1-β, 6aNCR-P1*P2-β, 6aNCR-P2*-β, and E2BS-2/3/4mt-β. The β-galactosidase expression plasmid (CMV-βGal) has been described previously (20). The luciferase reporter plasmid E2tk-luc contains an HPV6 NCR fragment (nt 7756 to 7988) together with the trimeric HPV6 E2 binding site 2 (nt 7964 to 7987) cloned upstream of the herpes simplex virus thymidine kinase (tk) promoter of the luciferase expression vector tk-luc.

In vitro mutagenesis with the help of PCR (PCR mutagenesis). Point mutations were introduced into the HPV6 wild-type sequence by site-directed mutagenesis of PCR fragments by the method of Higuchi et al. (23). Briefly, two

PCRs were performed with cloned HPV6a DNA (9) as a template, using complementary primers and the external primers 6-1 and 6-2 to produce two overlapping DNA fragments which both carry the same desired point mutations in the region of overlap. The mutations were generated by primer mismatches with the help of complementary primer pairs containing the desired nucleotide substitutions (Table 1). These primary PCR fragments were gel purified and combined in the following PCR. The overlap in the sequence of both fragments allows the formation of duplex fragments which serve as templates for amplification with the external primers 6-1 and 6-2. The final amplification products were cleaved with *Sal*I and *Bgl*II and inserted into the *Sal*I and *Bam*HI sites of pA-luc.

Transfection of cells. All transfections were performed on 60-mm plates with subconfluent (60%) cells by two different methods: a modified calcium phosphate coprecipitation method (18) described previously (25) for HT3, KG3, MCF-7, and Cos-7 cells, and a transfection method with Lipofectamine and Lipofectin (Gibco BRL) (17), for RTS3b cells and PHFK, respectively. For transfection of PHFK, up to 10 μg of DNA in 20 μl of H_2O was mixed with 40 μl of Lipofectin (1 μg/μl) and 1 ml of KBM (Gibco) in a polystyrene tube and incubated at room temperature for 15 min. Subsequently, the medium of a 60-mm cell culture plate was replaced by the DNA-Lipofectin mixture and after an incubation of 5 h at 37°C, 1 ml of KGM (Gibco) was added. The next day, the cells were fed with fresh, complete medium. RTS3b cells were transfected with Lipofectamine (2 μg/μl; Gibco BRL). Up to 6 μg of DNA in 300 μl of Opti-MEM (Gibco BRL) was mixed with a Lipofectamine–Opti-MEM solution (5 to 15 μl of Lipofectamine in 300 μl of Opti-MEM) in a polystyrene tube, incubated at room temperature for 30 min, and supplemented with 2.4 ml of Opti-MEM. The cells were rinsed twice with phosphate-buffered saline and overlaid with the DNA-Lipofectamine mixture. After an incubation of 5 h at 37°C, 3 ml of E-medium containing 20% fetal calf serum and 5 ng of epidermal growth factor per ml was added. The next day, the cells were fed with fresh complete medium. They were transfected with 0.5 to 4 μg of luciferase reporter plasmid and different amounts of E2 expression vector (0.1 to 6 μg) as indicated in the respective figures. The amount of DNA added was always standardized by the addition of corresponding amounts of parental vector DNA (pLXSN).

To standardize the transfection efficiency, we cotransfected constant amounts (0.5 μg) of CMV-βGal in all tissue culture dishes and measured the amount of β-galactosidase expressed in a quantitative *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay (41). However, by doing so, we observed a clear E2-mediated activation of the cytomegalovirus (CMV) enhancer promoter in the β-galactosidase reporter construct in every experiment. In the same way, no normalization of the transfection efficiency could be achieved when using expression vectors in which the reporter gene was driven by the simian virus 40 enhancer promoter. We therefore performed at least four independent sets of each transfection experiment with each sample in triplicate to reach a high reproducibility. When the cells were transfected with the β-globin constructs to perform primer extension analyses, CMV-βGal was transfected only in the samples in which no E2 expression vector was cotransfected. Normalization with the ONPG assay showed that the transfection efficiencies were not significantly different. Transfections for RNA analyses were repeated three times with identical primer extension results.

Luciferase assay. At 48 h posttransfection, the cells were rinsed once with phosphate-buffered saline and incubated with 400 μl of lysis buffer (25 mM Tris-phosphate [pH 7.8], 2 mM dithiothreitol [DTT], 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10% glycerol, 1% Triton X-100) at room temperature. Cell lysis was carried out for 30 min at room temperature, the supernatant was briefly centrifuged, and the protein concentration was measured by the bicinchoninic acid protein assay kit (Pierce, Rockford, Ill.) as specified by the manufacturer. The luciferase activity was measured by the method of de Wet et al. (10). Briefly, 40 μl of the protein extract was added to 100 μl of assay buffer (100 mM potassium phosphate [pH 7.8], 15 mM $MgSO_4$, 5 mM ATP). The luciferase activity was measured in relative light units with a luminometer (Berthold LB9051) after injection of 1 mM D-luciferin solution (Boehringer Mannheim) dissolved in assay buffer. The values determined were subsequently standardized to the amount of input protein and are indicated as relative light units per microgram of protein.

RNA isolation and primer extension analysis. Total RNA was isolated from tissue cultures by the acidic guanidinium thiocyanate-phenol-chloroform extraction method (6). For primer extension analysis, the following oligonucleotides were used: primer 6-369, which is complementary to nt 348 to 369 of the HPV6a genome (24), and primer FK-Ovec (5′-CTCACTGGACAGATGCACCATTC-3′), which hybridizes within the β-globin gene of the vector construct Ovec-1 (53). A 50-ng portion of the oligonucleotide was 5′-end labeled with T4 polynucleotide kinase and purified by Sephadex G-50 column chromatography. A 50-μg portion of total RNA of transfected or mock-transfected cells was annealed to 10^5 cpm of labeled primer in a buffer containing 100 mM NaCl, 20 mM Tris-HCl (pH 8.3), and 0.1 mM EDTA by heating the samples for 3 min to 90°C and 10 min to 55°C, followed by cooling to 42°C. The reverse transcription reaction was carried out at 42°C for 1 h with 200 U of Moloney murine leukemia virus reverse transcriptase in the buffer supplied by the manufacturer (Superscript; Gibco BRL) supplemented with 1 mM deoxynucleoside triphosphates, 20 μg of actinomycin D per ml, 4 mM DTT, and 100 μg of bovine serum albumin per ml. After alkaline hydrolysis of the RNA, the extension products were phenol extracted, ethanol precipitated, and separated on a 6% denaturing polyacrylamide gel. Molecular weight markers were a mixture of 5′-end-labeled *Hae*III digestion fragments of ϕX174 and *Msp*I digestion fragments of pBR322. Dried gels were autoradiographed at −80°C with intensifying screens or quantitatively analyzed with the BAS 2000 PhosphorImager and the TINA software program (Fuji).

In vitro transcription and translation of HPV6 E2. A fragment of the HPV6a genome (nt 2711 to 3841) encoding the entire E2 ORF was cloned into the *Bam*HI and *Eco*RI sites of the vector Bluescribe M13+ (Vector Cloning Systems, San Diego, Calif.). Transcription from *Eco*RI-linearized template with T3 RNA polymerase (Stratagene) and translation with nuclease-treated rabbit reticulocyte lysate (Promega) were performed as specified by the manufacturer. The translation mix was stored at −80°C.

Electrophoretic mobility shift assay (EMSA). Equal amounts of complementary single-stranded oligonucleotides (Table 1) were annealed in a buffer containing 1× Tris-borate-EDTA (TBE) by heating the samples for 3 min to 95°C, incubating them for 30 min at 50°C, and cooling them to room temperature. A 150-pg portion of ^{32}P-end-labeled oligonucleotide equivalent to 4×10^4 Cerenkov cpm was incubated with 9 μl of in vitro-translated E2 protein in a total volume of 20 μl. The binding buffer consisted of 10 mM HEPES (pH 7.9), 2 mM $MgCl_2$, 5 mM KCl, 0.1 mM spermidine, 0.1 mM EDTA, 0.0125 mM DTT, 5 μg of bovine serum albumin per ml, 1 μg of poly(dI-dC), and 5% glycerol. For competition experiments, homologous unlabeled oligonucleotides were preincubated with E2 protein for 5 min prior to addition of the labeled oligonucleotide. After incubation for 20 min at room temperature, samples were electrophoresed at 9 V/cm on a 5% native polyacrylamide gel (19:1) containing 0.5× TBE. A quantitative analysis of the radioactive signals was performed with a PhosphorImager.

For off-rate analysis of the different E2 binding sites, individual samples of binding reaction mixtures of the in vitro translated E2 protein and ^{32}P-labeled E2BS-1 to E2BS-4 oligonucleotides were prepared for each time point. After incubation for 20 min at room temperature, the sample for time zero was loaded onto a continuously running polyacrylamide gel and the remaining samples were incubated with a 500-fold excess of unlabeled homologous competitor for different time intervals. The half-life of each individual E2-DNA complex was determined to be the time point at which 50% of the initially present complex could still be detected, as measured with a PhosphorImager.

RESULTS

Cell-type-specific regulation of the HPV6 early promoters by E2. To search for cell lines that allow a similar activity pattern of the E6 and E7 promoters of HPV6 as observed in naturally infected condylomas, we first screened a number of epithelial cell lines with an NCR-promoter test construct. All constructs used in this study are based on HPV6a rather than on the better-known HPV6b subtype. Most of the earlier studies by other groups dealing with the HPV6 enhancer/E6 promoter activity used constructs based on HPV6b, which was believed to represent the prototype of different HPV6 variants (4, 9, 16, 28, 30, 39, 55). Recent data, however, clearly showed that HPV6b seems to be a rare variant usually not found in infected condylomas (29, 40). In addition, we were able to show previously that HPV6b differs from HPV6a in one major and two minor sequence deletions within the NCR, resulting in a 30 to 60% lower basal activity of the early promoters of HPV6b in comparison to HPV6a (20).

To generate the NCR-promoter test construct 6aNCR-P2luc, we cloned the entire NCR of HPV6a (nt 7139 to 446) including the two early promoters, P1 and P2, upstream of the luciferase gene of the promoterless luciferase expression vector pA-luc (13). To be able to differentiate between the activity of the E6 promoter (P1) and the E7 promoter (P2), a second construct, 6aNCR-P1luc, which lacks sequences containing the P2 promoter, was made. To investigate the influence of E2 on the early promoters of HPV6, we constructed the E2 expression vector pLXSN6a-E2, which contains the E2 ORF of HPV6a under the control of the long terminal repeat of the Moloney murine leukemia virus. To identify cell types which allow E2-mediated activation of the HPV6a early promoters, we performed cotransfection experiments of each of the reporter constructs together with the E2 expression vector in different HPV-negative epithelial cell lines (Cos-7, MCF-7, HT3, and RTS3b), in the VIN-derived KG3 cell line and in

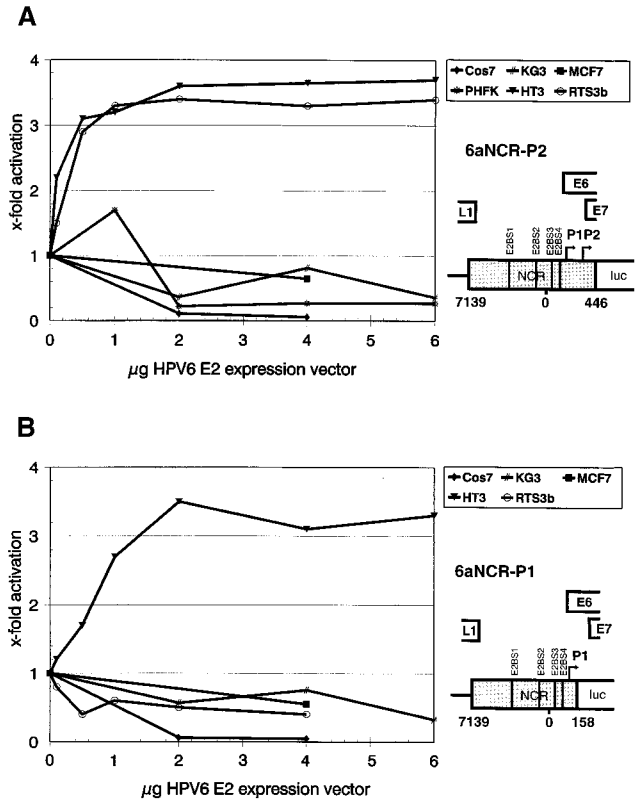

FIG. 1. Influence of the viral transcription factor E2 on the activities of the early HPV6 promoters P1 and P2 in different epithelial cells. Cotransfection experiments were performed with the HPV6 E2 expression vector pLXSN6a-E2 and the reporter construct 6aNCR-P2luc (A) or 6aNCR-P1luc (B). Beside each diagram is a partial map of the HPV6 genome and the reporter constructs. The ORFs for the viral proteins L1, E6, and E7, as well as for the luciferase protein, are indicated by open boxes, and the start sites of the promoters P1 and P2 are shown as black arrows. The E2 binding sites within the NCR are represented as black bars. Nucleotide positions refer to the HPV6a genome (24). The relative luciferase activities of the reporter constructs cotransfected with increasing amounts of pLXSN6a-E2 (0.1 to 6 μg) are shown. For each construct, the basal activity in the absence of E2 protein is set to 1 and the E2-mediated activation is expressed relative to the basal activity. Four to five independent cotransfection experiments with each sample in triplicate were performed with the epithelial cell lines Cos-7, KG3, MCF-7, HT3, and RTS3b and in PHFK.

PHFK (Fig. 1). Increasing amounts of E2 expression vector (0.1 to 6 μg) were cotransfected with constant amounts of 6aNCR-P2luc or 6aNCR-P1luc. In each transfection experiment, the total amount of DNA was kept constant by the addition of corresponding amounts of the parental vector DNA (pLXSN). The basal activities of the luciferase reporter constructs are set to 1, and the fold activation mediated by E2 is shown relative to the basal activity of each reporter construct (Fig. 1). In PHFK and Cos-7, KG3, and MCF-7 cells, cotransfection of 6aNCR-P2luc together with the E2 expression vector resulted in a concentration-dependent repression of the luciferase activities ranging from 80 to 10% of the initial basal activity (Fig. 1A). This is also true for the reporter construct 6aNCR-P1luc (Fig. 1B), which contains only the E6 promoter P1. In contrast, in HT3 cells, cotransfection experiments of both luciferase reporter constructs with E2 resulted in a 3.5-fold-higher luciferase activity, thereby indicating that E2 causes activation of one or both early promoters.

Most interestingly, in the case of the RTS3b cell line, E2 exerted an opposing effect on the two luciferase reporter constructs. While E2 caused a 3.3-fold activation of the construct 6aNCR-P2luc containing both promoters (Fig. 1A), repression of the E6 promoter construct 6aNCR-P1luc of up to 60% was observed (Fig. 1B). This indicated that the two early promoters of HPV6 are differentially regulated by E2 in RTS3b cells, which made this cell line an interesting candidate for the analysis of the E2-mediated regulation in more detail.

Separate regulation of the HPV6 promoters P1 and P2 in RTS3b cells. We next analyzed the effect of E2 on the two promoters directly at the level of transcription. To avoid problems caused by the low stability of the luciferase mRNA, we cloned the insert of 6aNCR-P2luc upstream of the β-globin gene of the promoterless expression vector pHM313 (46) to achieve a longer half-life of the fusion transcripts. This construct, designated 6aNCR-P2-β (Fig. 2A), was cotransfected in RTS3b cells together with the E2 expression vector pLXSN6a-E2 or with parental vector DNA, and total RNA was extracted. Primer extension analysis was performed with 50 μg of total RNA and a 5′-end-labeled oligonucleotide primer (6-369) complementary to the HPV6a sequence between nt 348 and 369. This analysis revealed an extension product of 280 nt corresponding to E6 transcripts initiating at position 90 and two extension products of about 100 nt, indicative of E7 transcripts with closely adjacent start sites around nt 270 (Fig. 2B). This is the same picture as observed in naturally occurring HPV6-infected condylomas and demonstrates that our transient-transfection experiments with RTS3b cells indeed mirror the situation observed in vivo (20, 44). In the absence of E2, the basal activities of the P1 and P2 promoters showed marked differences. E6 was the major transcript, whereas E7 transcripts were detected at only very low levels (Fig. 2B). Quantification of several experiments with a PhosphorImager revealed three- to sixfold-smaller amounts of transcripts initiating at P2 in comparison to those initiating at promoter P1. Cotransfection of the E2 expression vector pLXSN6a-E2 revealed an opposing effect of E2 on each of the early promoters of HPV6. While promoter P1 was clearly repressed to 40 to 60% of its basal activity, promoter P2 was simultaneously activated two- to fivefold (Fig. 2B, right panel).

Differential analysis of each early promoter of HPV6a. To investigate if the observed E2-mediated activation of P2 could be dependent on the simultaneous repression of the upstream promoter P1, we made two derivatives of the construct 6aNCR-P2luc with mutations in the TATA box sequences of either promoter P1 or P2 (Fig. 2A). In the construct 6aNCR-P1*P2luc, the TATA box of the E6 promoter was altered by substituting the nucleotide sequence TATAA with GAGAC. Likewise, the TATA box of the E7 promoter in the construct 6aNCR-P2*luc was mutated from TATAA to TAGAG. Each mutant construct was cotransfected into RTS3b cells together with the E2 expression vector pLXSN6a-E2 and compared in its activity to the wild-type constructs 6aNCR-P2luc and 6aNCR-P1luc. The constructs 6aNCR-P1*P2luc with the mutated promoter P1 and 6aNCR-P2luc containing both promoters were activated by E2 about threefold, whereas 6aNCR-P2*luc as well as the construct 6aNCR-P1luc, which contain only the intact E6 promoter, were repressed in the presence of E2 to up to 50% of the basal activity (Fig. 2C).

To verify whether the mutations introduced into the TATA box sequences of the P1 and P2 promoters led to a complete abrogation of the initiation of transcription, we performed primer extension analyses. Both mutant constructs were subcloned upstream of the β-globin gene, and primer extension analysis was performed with primer 6-369 and 50 μg of RNA of RTS3b cells transfected either with 6aNCR-P1*P2-β alone (Fig. 2B, left side) or with 6aNCR-P2*-β in the presence or

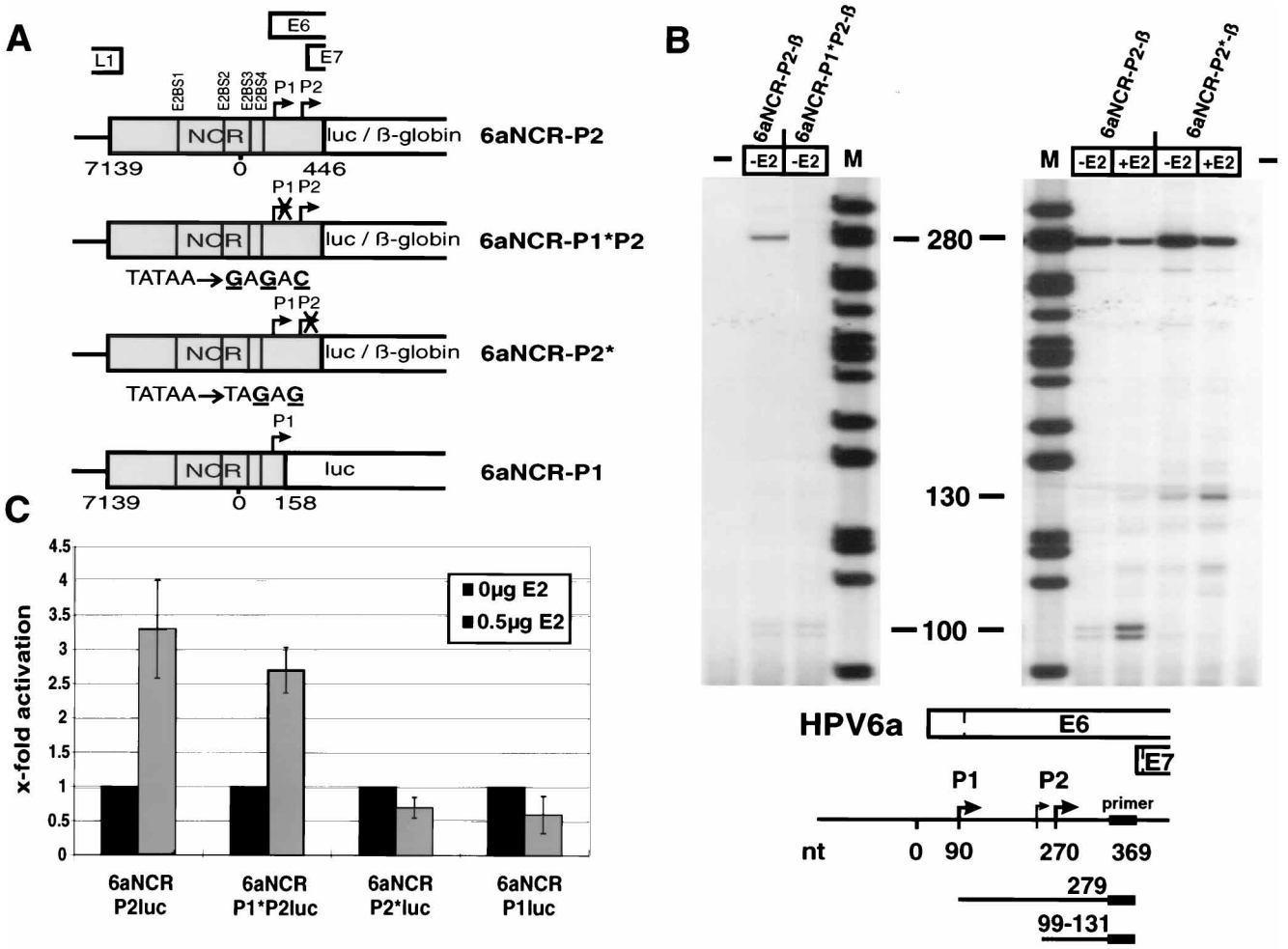

FIG. 2. Opposing effect of HPV6 E2 on the homologous promoters P1 and P2. (A) The constructs 6aNCR-P1*P2 and 6aNCR-P2* contain mutations within the sequences of the TATA boxes of promoters P1 and P2, respectively. The nucleotides substituted within the respective TATA box are underlined. The construct 6aNCR-P1 contains only the P1 promoter. All HPV6-specific inserts were cloned in front of either the luciferase gene or the β-globin gene. For further details, see Fig. 1. (B) The upper part shows two independent primer extension analyses with RNA from RTS3b cells transfected with the wild-type plasmid 6aNCR-P2-β, the mutant construct 6aNCR-P1*P2-β, and the mutant construct 6aNCR-P2*-β cotransfected with pLXSN6a-E2 (+E2) or with parental expression vector pLXSN (−E2). Mock controls (−) contain RNA from untransfected RTS3b cells. A mixture of 5′-end-labeled *Hae*III digestion fragments of the replicative form of ϕX174 and *Msp*I digestion fragments of pBR322 served as the molecular weight marker (M). Numbers indicate the lengths (in nucleotides) of the observed extension products. At the bottom, the locations of the promoters P1 and P2 as well as of the binding site of the primer 6-369 (black box) in the HPV6 genome are outlined. ORFs are shown as open boxes, and the start codons of E6 and E7 are shown as dashed vertical lines. The lengths of the expected primer extension products are indicated below. (C) Relative luciferase activities of wild-type and mutant constructs in the presence of E2. The luciferase constructs 6aNCR-P2luc, 6aNCR-P1*P2luc, 6aNCR-P2*luc, and 6aNCR-P1luc were cotransfected with equal amounts of pLXSN6a-E2 (0.5 μg) into RTS3b cells. At least four independent transfection experiments were performed with each sample in triplicate. Relative luciferase activities are calculated with respect to the basal activities of each construct in the absence of E2 protein, which are set to 1. Standard deviations are indicated by the vertical lines within each bar.

absence of E2 expression vector (Fig. 2B, right side). The results clearly show that the mutation of only 3 nt in the TATA box of the E6-promoter completely abolished promoter activity, as indicated by the lack of an extension product of 280 nt (Fig. 2B, left side). Interestingly, the activity of the E7 promoter remained unaffected by the knockout mutation of the P1 start site. The mutation of the TATA box of the E7 promoter led to the loss of the two major start sites around position 270 and to the appearance of minor transcriptional initiation sites between positions 238 and 270 (Fig. 2B, right side). Such minor E7 transcripts with start sites located upstream of position 270 have already been described in HPV6-positive condylomas (44). Our primer extension analyses therefore confirmed the effectiveness of the mutations of the TATA boxes of P1 and P2 and showed that the observed effect of the E2 protein on the P1 or P2 promoter is not due to a mutual influence of both promoters but occurs independently even in the absence of transcriptional activity of the respective adjacent promoter.

Mutation analysis of the E2 binding sites. Earlier studies dealing with transcriptional control of HPV11, HPV16, and HPV18 early genes investigated only the regulation of the E6 promoter by E2 and mostly reported repression of this promoter that was dependent on the presence of either one or two promoter-proximal E2 binding sites. We were now interested in analyzing which of the four E2 binding sites within the HPV6 NCR mediate the activating effect of E2 onto the E7 promoter. We therefore generated mutant constructs of the wild-type plasmid 6aNCR-P2luc, in which individual E2 binding sites or combinations thereof were altered in their nucleotide sequence. The nucleotide substitutions were introduced

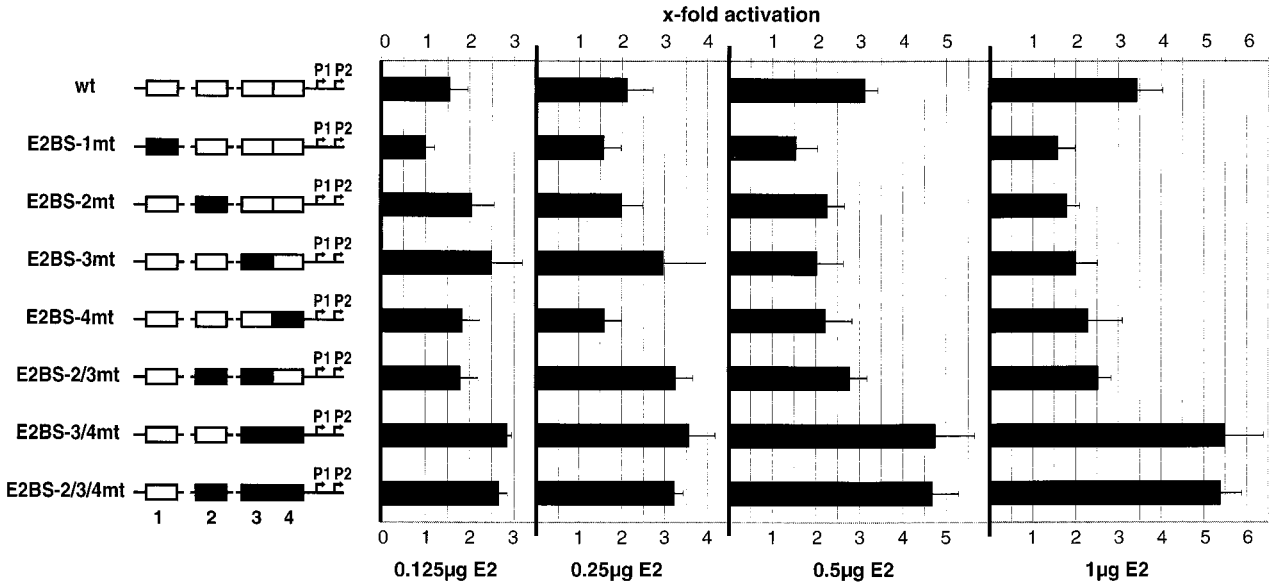

FIG. 3. Relative luciferase activities of the wild-type 6aNCR-P2luc and E2BS mutant constructs in the presence of different amounts of HPV6 E2 expression vector. The reporter constructs are represented schematically, with mutated E2 binding sites shown as black boxes. For each construct, the basal activity in the absence of the E2 protein is set to 1 and the activities in the presence of E2 are expressed relative to the value of the basal activity. At least four independent cotransfection experiments were performed with each sample in triplicate. Standard deviations are indicated by the lines within each bar.

by PCR mutagenesis with the help of four primer pairs that carried three point mutations within each of the four palindromic sequences, which were reported to be critical for E2 binding (31) (Table 1). The constructs with single, double, and triple mutations of E2 binding sites are shown in Fig. 3. All mutations were confirmed by sequencing the complete HPV6a insert. We next performed transient luciferase experiments by cotransfecting the mutant constructs and the wild-type 6aNCR-P2luc together with increasing amounts of E2 expression vector pLXSN6a-E2 (0.125 to 1 μg) in RTS3b cells. All constructs showed a dose-dependent response when cotransfected with the E2 expression vector (Fig. 3). The differences between the individual mutant constructs were most pronounced when 1 μg of E2 expression vector was used. In this case, activation of the wild-type construct 6aNCR-P2luc was about threefold. The mutant constructs E2BS-3mt and E2BS-4mt, which did not significantly differ in their response to E2, were somewhat less activated by E2 than was the wild type. However, when we combined the mutations of E2BS-3 and E2BS-4 in the reporter construct E2BS-3/4mt, a five- to sixfold activation in the presence of E2 was observed, which is twice as much as the wild-type activation level. This suggests that a double mutation of E2BS-3 and E2BS-4 leads to the loss of E6 promoter repression by E2, resulting in a higher level of luciferase expression than was seen with the wild-type construct. When we mutated E2BS-2, we observed only 50% of the wild-type activity in the presence of 1 μg of E2, which points to an activating influence of this binding site at higher intracellular concentrations of E2. The mutation of the promoter-distal E2 binding site 1 resulted in a drastic reduction of luciferase activity, which suggests that activation of promoter P2 appears to be mediated mainly through this binding site. This is further supported by the approximately twofold-higher activation level, compared to the wild type, seen with the mutant construct E2BS-2/3/4mt, which contains E2BS-1 as the only intact binding site. To verify the conclusions drawn from the transient luciferase experiments, we analyzed the activity of one selected construct in RTS3b cells in the presence or absence of E2 with the help of primer extension analysis. For this, we first subcloned the insert of the mutant construct E2BS-2/3/4mt into the β-globin vector pHM313. Total cellular RNA was extracted from transfected RTS3b cells, and primer extension analysis was performed with 50 μg of RNA and the primer 6-369 (Fig. 4). The results obtained show that promoter P2 was activated by E2 two- to threefold in the case of the wild-type construct 6aNCR-P2-β, as well as in the mutant construct E2BS-2/3/4mt-β. In contrast to the wild type, however, the number of transcripts initiating at promoter P1 in construct E2BS-2/3/4mt-β strongly increased (threefold) in the presence of E2, whereas P1 of the wild-type construct was repressed by E2 as shown before. These data indicate that mutation of both promoter-proximal binding sites (E2BS-3 and E2BS-4) not only abolished the E2-mediated repression but even led to activation of P1. This finding also explains the higher luciferase activities observed with the mutant E2BS-2/3/4mt as seen with the wild-type construct 6aNCR-P2luc. Interestingly, the nucleotide substitutions within E2BS-2, E2BS-3, and E2BS-4 also seem to affect the basal activities of P1 and P2, as demonstrated by the weak signals representing the respective extension products (Fig. 4). Such a phenomenon has been observed previously for BPV4, in which distinct E2 binding sites were shown to interact with stimulating host cell transcription factors (27).

E2-mediated activation of the E6 promoter in HT3 cells. The activation of the E6 promoter, which we postulated on the basis of our luciferase experiments with the constructs 6aNCR-P1luc or 6aNCR-P2luc in HT3 cells, is somehow reminiscent of the behavior of the mutant construct E2BS-2/3/4mt in RTS3b cells. The major difference, however, between the two experiments is the presence of the intact promoter-proximal E2 repressor binding sites in the wild-type construct in HT3 cells and the cellular environment provided by the different cell lines. A difference in the transcriptional milieu was already evident by the poor usage of the two major start sites of the E7 promoter

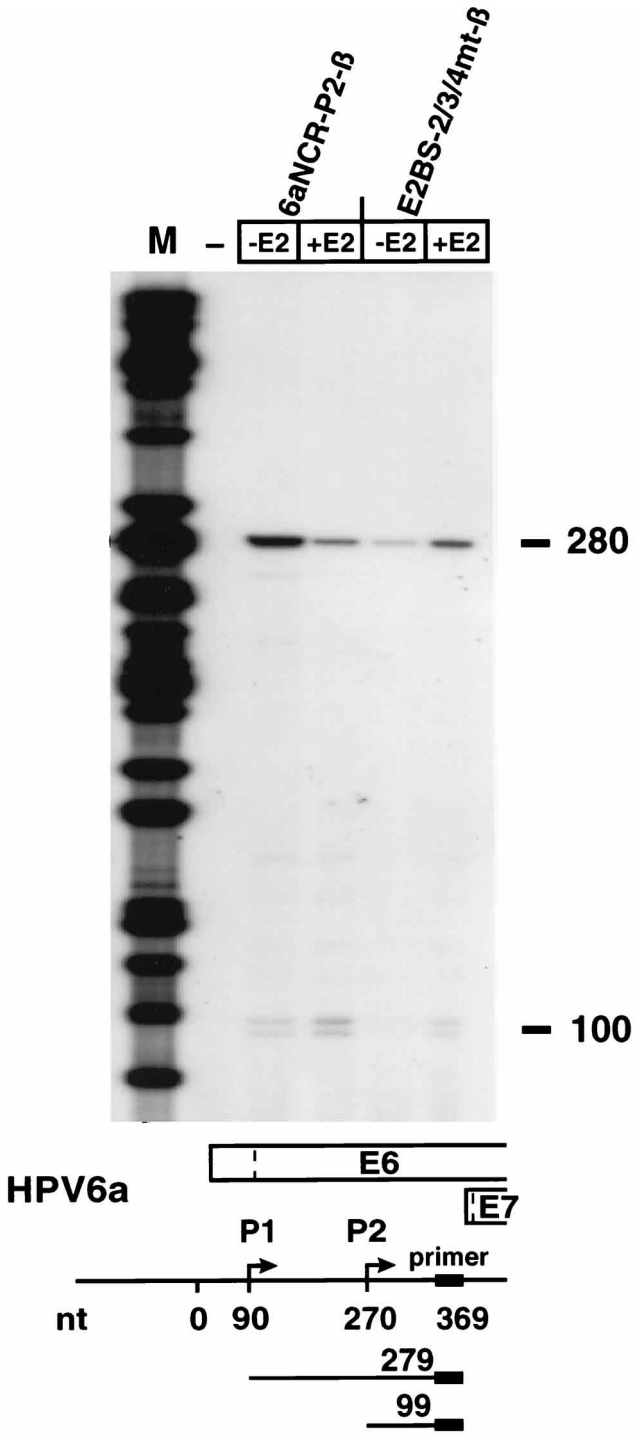

FIG. 4. E2 activates the E6 promoter when prevented from binding to E2BS-3 and E2BS-4. Primer extension analysis with RNA from RTS3b cells transfected with the wild-type construct 6aNCR-P2-β and the E2BS mutant E2BS-2/3/4mt-β cotransfected with pLXSN6a-E2 (+E2) or with the parental expression vector pLXSN (−E2). The mock control (−) contains RNA from untransfected RTS3b cells. For further details, see Fig. 2.

in HT3 cells, resulting in only minor amounts of transcripts with heterogeneous start sites widely dispersed between nucleotide positions 230 to 320 of the HPV6a genome (data not shown). To first address the question how E2 influences the activity of each of the early promoters, we performed luciferase experiments in HT3 cells with the TATA box mutant constructs (Fig. 5A and B). As expected from the former transcription analysis, the construct with the mutations in the TATA box of the E7 promoter 6aNCR-P2*luc showed no significant difference in E2-mediated activation in comparison to the wild type. This observation again confirms the lack of a major E7 promoter start site in HT3 cells. It also indicates that in contrast to RTS3b cells, E2 mediates activation of the E6 promoter in HT3 cells. This unexpected finding is also supported by the 3.5-fold E2-mediated activation of 6aNCR-P1luc containing only promoter P1 in HT3 cells. To confirm these data directly at the RNA level, we next performed primer extension analyses with 50 μg of total RNA from HT3 cells cotransfected with the β-globin derivative of 6aNCR-P1luc (6aNCR-P1-β) and the E2 expression vector. This time we used the primer FK-Ovec, which hybridized to sequences within the β-globin gene, which should result in an expected extension product of 149 nt, if the E6 promoter start site was used. The data presented in Fig. 5C clearly show that correct initiation in HT3 cells takes place at the E6 promoter and that the number of transcripts increases threefold in the presence of E2. This demonstrates that the observed threefold activation of the respective luciferase constructs 6aNCR-P1luc or 6aNCR-P2luc is caused predominantly by an E2-mediated activation of the E6 promoter and that the repression of the E6 promoter by binding of the E2 protein to both promoter-proximal binding sites did not function in HT3 cells. Further experiments are in progress to clarify this mechanism of E6 promoter activation in HT3 cells.

Binding affinity and off-rate analysis of individual E2 binding sites within the NCR of HPV6a. To this point, we have analyzed the influence of each of the four E2 binding sites within the NCR on the E2-mediated regulation of the E6 and E7 promoters of HPV6. While E7 promoter activation is dependent mainly on an intact E2BS-1, the binding sites E2BS-3 and E2BS-4 together are responsible for repression of the E6 promoter. However, besides the binding ability alone, the affinity of E2 to the individual E2 binding sites might play a further role in promoter regulation. We therefore performed comparative EMSA to estimate the relative binding affinities of E2 to each of the four E2BSs (Fig. 6). The oligonucleotides contain, in addition to the 12-bp E2 recognition site, 10 nt of the wild-type HPV6a sequence (Table 1), which have been reported to be critical for the binding strength (31). As a source of E2 protein, we used in vitro-translated homologous HPV6a E2 protein. We tested the four E2BSs for the ability to compete for binding of E2. A fixed amount of E2 protein was incubated with ^{32}P-labeled E2BS-1 in the presence of increasing amounts of unlabeled oligonucleotides encoding each of the four E2BSs. The plot of the percentage of E2BS-1 bound by E2 against the amount of competitor DNA revealed no strong differences between the affinities of E2 to the individual E2 binding sites. E2BS-2 showed the strongest binding affinity, followed by E2BS-3, whereas E2BS-1 and E2BS-4 revealed a lower affinity to E2. We then tested the relative stability of the E2-protein oligonucleotide complex for each of the four E2BSs used in this study (data not shown). Off-rate analyses revealed that the E2 complex with E2BS-1 had a half-life of 35 min, followed by the complex with E2BS-4 (29 min), whereas the E2 complexes with E2BS-3 and E2BS-2 were less stable (21 and 13 min, respectively).

DISCUSSION

Regulation of gene expression seems to be the major pathway to control different restriction points of the viral life cycle

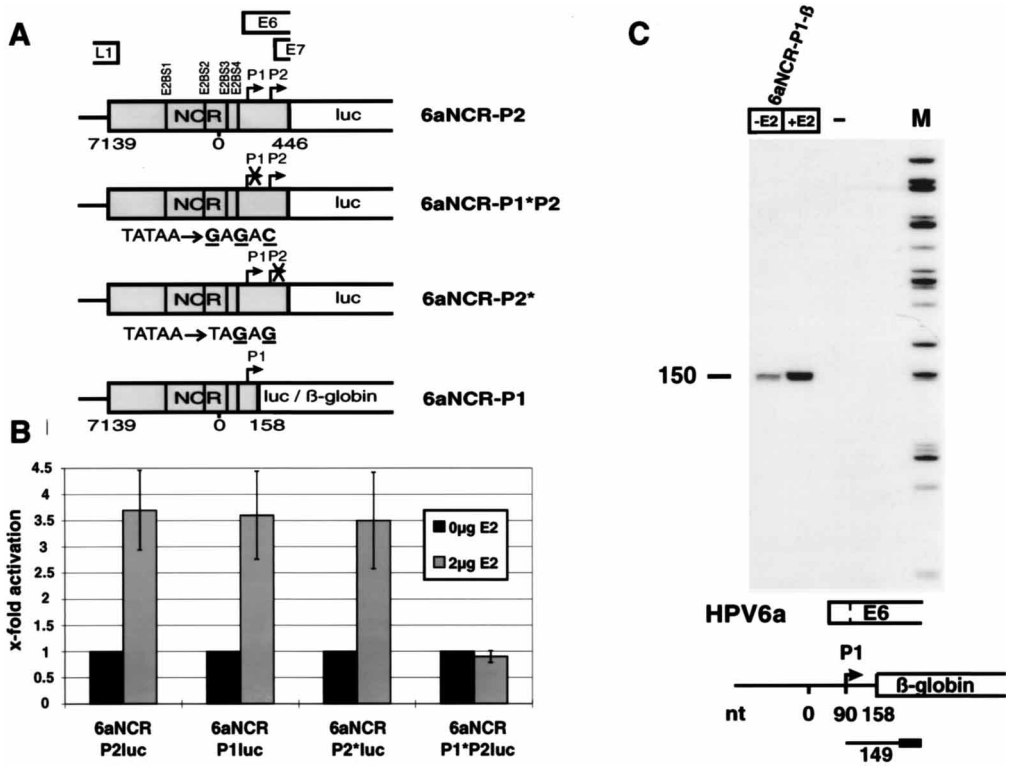

FIG. 5. Activation of promoter P1 by E2 in HT3 cells. (A) Schematic representation of the reporter plasmids. For further details, see Fig. 2. (B) Relative luciferase activities of the wild-type and mutant luciferase constructs in the presence of E2. The luciferase constructs 6aNCR-P2luc, 6aNCR-P1*P2luc, 6aNCR-P2*luc, and 6aNCR-P1luc were cotransfected with equal amounts of pLXSN6a-E2 (2 μg) into HT3 cells. At least four independent transfection experiments were performed with each sample in duplicate. Relative luciferase activities are calculated with respect to the values of the basal activities of each construct in the absence of E2 protein, which are set to 1. Standard deviations are indicated by the vertical lines within each bar. (C) The upper part shows the primer extension analysis with RNA from HT3 cells transfected with the construct 6aNCR-P1-β cotransfected with pLXSN6a-E2 (+E2) or with parental expression vector pLXSN (−E2). The mock control (−) contains RNA from untransfected HT3 cells. At the bottom, a partial map of the construct 6aNCR-P1-β is shown, with the positions of the binding site of the primer FK-Ovec (black box) and the start site of the promoter P1 in the HPV6 genome (black arrow) indicated. The length of the expected primer extension product is indicated below. For further details, see Fig. 2.

of HPVs. Already at the very first step, the ability to infect primary target cells seems to be controlled by unique factors provided by the transcription machinery of the permissive host cells (42). After the infection became established, a differentiation-dependent transcription pattern of early- and late-gene expression can be observed within the infected epithelium. The expression pattern of the early genes in productively infected benign tumors, however, appears to be different between high- and low-risk genital HPV types. High-risk HPV16 reveals restricted expression of the viral oncogenes within the less highly differentiated layers of the epithelium and only moderate expression of E7 in highly differentiated keratinocytes (3, 22). In contrast, in HPV6-infected condylomas, low levels of E6 are expressed exclusively in the basal cell layer and the moderate amounts of E7 transcripts present in the lower third of the epithelium decrease with ongoing differentiation of the keratinocytes (26). Both risk groups, however, have a common transcription pattern regarding the expression of the viral oncogenes E6 and E7 in malignant tumors. The overwhelming majority of cancers harboring HPV16 revealed small amounts of E6 but contained E7 as the major transcript, exactly as in the few HPV6-associated cancers analyzed so far (3, 7, 15, 36, 43, 44). This up regulation of oncogene transcription during malignant conversion is for the high-risk types believed to be caused mainly by the loss of the viral E2 repressor protein due to integration of the viral genome into the host chromosome (57). The resulting derepression of the single early promoter, however, does not fully explain the specific up regulation of the spliced E7 transcripts, because the unspliced transcripts with a coding potential for a complete E6 protein initiating at the same promoter do not seem to be upregulated (3, 45).

In contrast, in the case of the low-risk HPV6 and HPV11, the presence of two early promoters allows a separate regulation of E6 and E7 expression. Such an independent regulation of each of the early promoters is indeed indicated by the different levels of E6 and E7 transcripts in naturally occurring tumors (11, 20, 26, 44). Earlier studies by other groups investigated solely the mechanism of E6 promoter repression by the E2 protein or the influence of *cis* elements on the enhancer and E6 promoter activity and completely neglected the existence of the E7 promoter. These data therefore did not sufficiently explain the observed transcription pattern in HPV6-infected human biopsy specimens containing E7 as the major transcript in the lower third of the epithelium. To be able to investigate the regulation of the complete transcription unit of HPV6a in detail, we first screened a number of epithelial cell lines with the aim of identifying permissive cells that allow an expression pattern similar to that observed in naturally occurring lesions (Fig. 1). The results presented here provide the first evidence that E2 is able to separately regulate two adjacent promoters at the same time. Furthermore, our data clearly show that the correct initiation of transcription at the E7 promoter and the E2-mediated repression of the E6 promoter are cell type dependent. Only RTS3b cells provided a

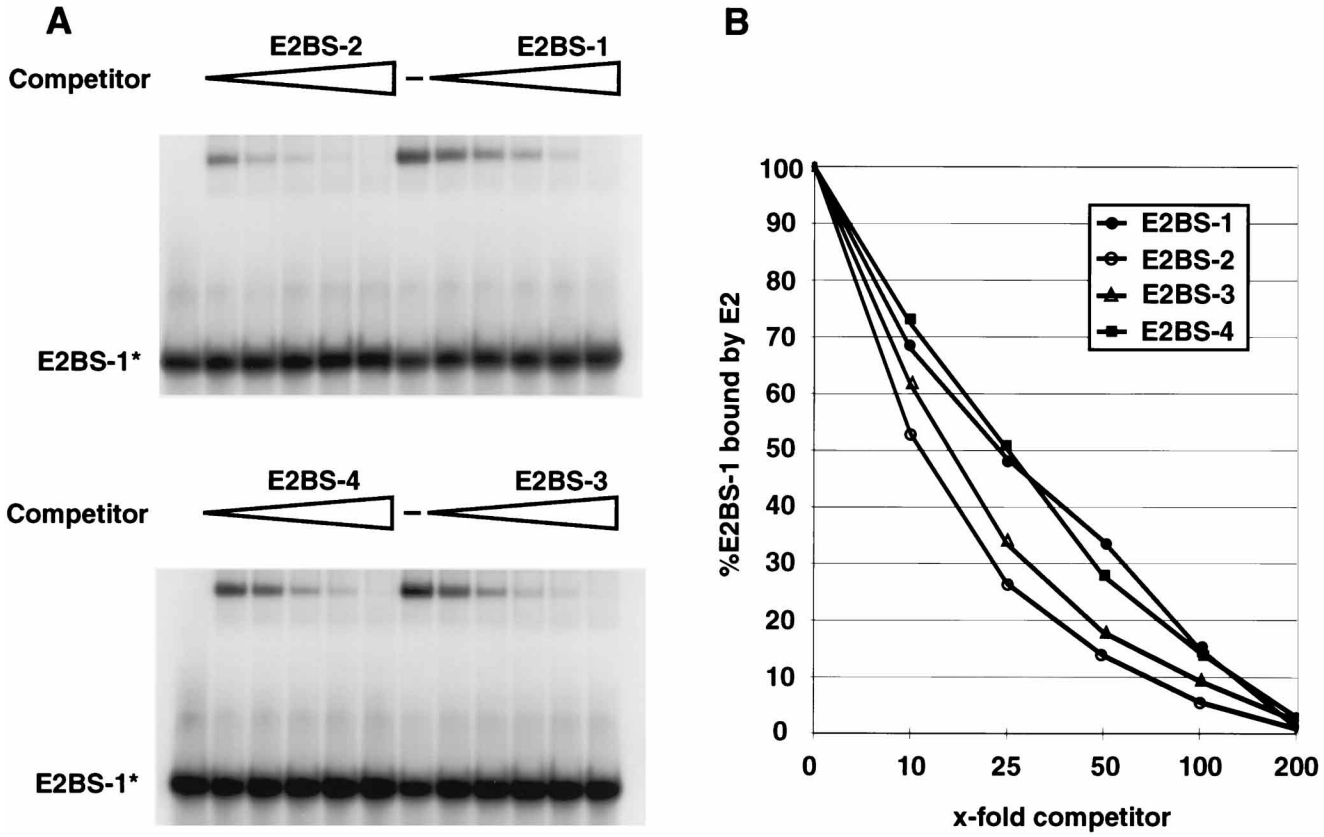

FIG. 6. Binding affinities of in vitro-translated HPV6a E2 to each of the E2 binding sites present within the NCR of HPV6a. (A) The leftmost lane of each panel shows the ^{32}P-labeled oligonucleotide encoding the sequence of E2BS-1 in the absence of E2. ^{32}P-labeled oligonucleotide E2BS-1 (150 pg) was incubated with E2-programmed reticulocyte lysate in either the absence (−) or presence of increasing amounts of unlabeled oligonucleotides encoding E2BS-1, E2BS-2, E2BS-3, or E2BS-4 as indicated. (B) The percentages of E2BS-1 bound by HPV6 E2 were calculated with the help of a PhosphorImager and are given relative to the value without competitor, which was set to 100%. The diagram shows the respective values plotted against the amount of unlabeled oligonucleotide added as competitor DNA.

transcriptional milieu which allowed promoter usage and regulation by E2 in a similar way to that observed in benign condylomas. In the presence of the homologous E2 protein, a simultaneous two- to fivefold stimulation of the E7 promoter together with a twofold repression of the E6 promoter was observed (Fig. 2). This activity of E2 could be the reason for the lack of detectable E6 mRNA in the suprabasal cell layers of the infected epithelium of condylomas, which contain, in addition to large amounts of E7 mRNA, sufficient levels of transcripts with a coding potential for a full-length E2 protein (26). All the other cell lines we analyzed did not allow E7 promoter activity, which explains the difficulties experienced in demonstrating this promoter in tissue culture systems in earlier studies.

To investigate the role of the individual binding sites in the regulation of both HPV6 promoters by E2, we introduced mutations in single E2 binding sites within the context of the complete early transcription unit or made constructs that contained different combinations of mutated binding sites and analyzed their effect on the promoter activities (Fig. 3). This analysis clearly showed that the single most promoter-distal E2BS-1 is sufficient to enhance transcription from both the E6 and the E7 promoters, as indicated by the E2 responsiveness of the E2BS-2/3/4mt construct (Fig. 3 and 4). This is in contrast to an earlier study with HPV18, in which both promoter-distal binding sites were not sufficient to allow BPV1 E2-mediated activation of the early P105 promoter (8). In the meantime, however, a more recent report suggested a role for E2BS-1 in counteracting repression of the HPV18 E6 promoter (47), and studies with BPV4 clearly demonstrate an important role of the most distal E2 binding site in promoter activation (27). Whereas activation of the E7 promoter of HPV6a could already be observed in the wild-type construct, activation of the E6 promoter was visible only after mutating both promoter-proximal binding sites (E2BS-3 and E2BS-4). The constructs with single mutations introduced in E2BS-3 or E2BS-4 point to a cooperative effect in binding to the E2 protein, compared to the double mutant E2BS-3/4mt. Mutation of only E2BS-3 led to a loss of repression at a low concentration of E2, as is the case with mutant E2BS-3/4mt, whereas at higher levels of E2 ($>0.5\mu g$), the intact low-affinity E2BS-4 was still able to repress the E6 promoter. In contrast, if E2BS-4 is mutated alone, no derepression of P1 can be observed, which further supports a role for E2BS-3 in E6 promoter repression. Binding studies with the different oligonucleotides containing each of the E2 binding sites present in the NCR of HPV6a (Fig. 6) indeed demonstrated that the E2 protein has a lower affinity for E2BS-4 than for E2BS-3. This could explain the need for a cooperativity between E2BS-3 and E2BS-4 to efficiently bind E2. Interestingly, although E2BS-1 is the predominant site in mediating activation by E2, all other binding sites seem to participate in E7 promoter activation, as indicated by the reduced level of transactivation at larger amounts of E2 seen with the respective single-mutant constructs (Fig. 3).

Surprisingly, in HT3 cells, the E2 protein was able to activate the E6 promoter in the context of the wild-type construct harboring all four intact E2 binding sites whereas the E7 promoter was not functional (Fig. 5). This difference between HT3 and RTS3b cells, however, is not simply due to distinct levels of E2 in each cell line, because of the presence of different transfection efficiencies. Experiments with a reporter construct (E2tk-luc) containing multiple E2BSs in the enhancer configuration upstream of a tk promoter show the same kinetics of activation when increasing amounts of E2 expression vector were cotransfected in both cell lines (data not shown). A cell-specific influence is further demonstrated by the fact that the transcriptional milieu of the host cell not only determines the correct initiation of transcripts at the E7 promoter but also influences the effect of E2 on each of the promoters. At present, we cannot distinguish if the binding of E2 to the two promoter-proximal binding sites in HT3 cells is prevented or if the E2 protein itself is modified, resulting in the abolition of its repressor function. Such an alteration in the cellular environment may also have a potential effect on the fate of the lesion in terms of malignant progression.

In summary, we have shown that both early promoters of HPV6a can be regulated by the homologous E2 protein. Whereas E2 represses the E6 promoter through both proximal binding sites (E2BS-3 and E2BS-4), all binding sites seem to contribute to the activation of the E7 promoter, although the major activity is clearly mediated by the most distal binding site, E2BS-1. These data help us to understand earlier observations in HPV6-infected condylomas, in which E6 expression was restricted to the basal cell layer and E7 transcripts were found predominantly in the lower third of the epithelium (26). Based on our findings, one could postulate a model of early promoter regulation of HPV6, in which the intracellular concentration of E2 and unknown cellular factors are the major actors. In the absence of E2, the E6 promoter represents the major transcript initiation site, and the infected cell therefore contains mainly transcripts for E6, which are bicistronic and also have a coding potential for a full-length E2 protein. This basal level of transcription leads to small intracellular amounts of the E2 protein that cause up regulation of both promoters by binding to the binding sites E2BS-1 and E2BS-2 with either a long half-life of E2 binding or a high binding affinity, respectively. As a consequence, the levels of E7-specific bicistronic transcripts will preferentially increase, and these transcripts again can encode E2. This leads to high intracellular levels of E2, with the consecutive binding of E2 protein to the promoter-proximal binding sites, resulting in down regulation of the E6 promoter. Such a situation can indeed be observed in the suprabasal cell layers of HPV6-infected condylomas. However, with increasing differentiation, the infected keratinocytes may undergo changes in their transcriptional milieu, which could be responsible for the down regulation of early transcription and concomitant up regulation of late promoters, as observed in the upper third of the infected epithelium of a condyloma (26, 48).

In the rare cases of malignant progression, a continuous expression of E6 does not seem to be necessary for the maintenance of the transformed phenotype. In HPV6-associated cancers, the expression pattern was comparable to that of the suprabasal cell layer of a condyloma, with undetectable levels of E6 and high levels of E7 and E2 expression (36). This does not, however, necessarily exclude a role for E6 in the process of malignant conversion, which can be an important determinant of progression, as suggested by data from the cottontail rabbit papillomavirus animal model system (56).

At present, all available data clearly indicate that HPV6 normally does not escape from cellular control and is only very rarely found in malignancies. Thus, the low-risk phenotype may occur because HPV6 has, as an additional safety feature, two promoters for the regulation of E6 and E7. This allows better control of the individual levels of each oncogene compared to the case for the high-risk viruses, which have only one early promoter that drives the expression of both viral oncogenes.

ACKNOWLEDGMENTS

We thank A. Schmitt and K. Lorz for excellent technical assistance, Thomas Stamminger for the gift of the pHM313 plasmid, F. Stubenrauch for the gift of CMV-βGal, and Michaela Elbel for critical reading of the manuscript.

This research was supported by a grant from the Doktor Robert Pfleger-Stiftung and by the Deutsche Forschungsgemeinschaft DFG IF 1/4-1 to T.I.

REFERENCES

1. **Bauknecht, T., F. Jundt, I. Herr, T. Oehler, H. Delius, Y. Shi, P. Angel, and H. zur Hausen.** 1995. A switch region determines the cell-type-specific positive or negative action of YY1 on the activity of the human papillomavirus type 18 promoter. J. Virol. **69:**1–12.
2. **Bercovich, J. A., C. R. Centeno, O. G. Aguilar, S. Grinstein, and T. Kahn.** 1991. Presence and integration of human papillomavirus type 6 in a tonsillar carcinoma. J. Gen. Virol. **72:**2569–2572.
3. **Boehm, S., S. P. Wilczynski, H. Pfister, and T. Iftner.** 1993. The predominant mRNA class in HPV16-infected genital neoplasias does not encode the E6 or the E7 protein. Int. J. Cancer **55:**791–798.
4. **Boshart, M., and H. zur Hausen.** 1986. Human papillomaviruses in Buschke-Loewenstein tumors: physical state of the DNA and identification of a tandem duplication in the noncoding region of a human papillomavirus 6 subtype. J. Virol. **58:**963–966.
5. **Brandsma, J. L.** 1994. Animal models of human-papillomavirus-associated oncogenesis. Intervirology **37:**189–200.
6. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
7. **Crum, C. P., G. Nuovo, D. Friedman, and S. J. Silverstein.** 1988. Accumulation of RNA homologous to human papillomavirus type 16 open reading frames in genital precancers. J. Virol. **62:**84–90.
8. **Demeret, C., M. Yaniv, and F. Thierry.** 1994. The E2 transcriptional repressor can compensate for Sp1 activation of the human papillomavirus type 18 early promoter. J. Virol. **68:**7075–7082.
9. **de Villiers, E. M., L. Gissmann, and H. zur Hausen.** 1981. Molecular cloning of viral DNA from human genital warts. J. Virol. **40:**932–935.
10. **de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani.** 1987. Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. **7:**725–737.
11. **DiLorenzo, T. P., and B. M. Steinberg.** 1995. Differential regulation of human papillomavirus type 6 and 11 early promoters in cultured cells derived from laryngeal papillomas. J. Virol. **69:**6865–6872.
12. **Dong, G., T. R. Broker, and L. T. Chow.** 1994. Human papillomavirus type 11 E2 proteins repress the homologous E6 promoter by interfering with the binding of host transcription factors to adjacent elements. J. Virol. **68:**1115–1127.
13. **Dong, X. P., F. Stubenrauch, E. Beyer Finkler, and H. Pfister.** 1994. Prevalence of deletions of YY1-binding sites in episomal HPV 16 DNA from cervical cancers. Int. J. Cancer **58:**803–808.
14. **Duerst, M., F. X. Bosch, D. Glitz, A. Schneider, and H. zur Hausen.** 1991. Inverse relationship between human papillomavirus (HPV) type 16 early gene expression and cell differentiation in nude mouse epithelial cysts and tumors induced by HPV-positive human cell lines. J. Virol. **65:**796–804.
15. **Duerst, M., D. Glitz, A. Schneider, and H. zur Hausen.** 1992. Human papillomavirus type 16 (HPV 16) gene expression and DNA replication in cervical neoplasia: analysis by in situ hybridization. Virology **189:**132–140.
16. **Farr, A., H. Wang, M. S. Kasher, and A. Roman.** 1991. Relative enhancer activity and transforming potential of authentic human papillomavirus type 6 genomes from benign and malignant lesions. J. Gen. Virol. **72:**519–526.
17. **Felgner, P. L., T. R. Gadek, M. Holm, R. Roman, H. W. Chan, J. P. Northrop, G. M. Ringold, and M. Danielsen.** 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. USA **84:**7413–7417.
18. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52:**456–467.
19. **Grassmann, K., B. Rapp, H. Maschek, K. U. Petry, and T. Iftner.** 1996. Identification of a differentiation-inducible promoter in the E7 open reading frame of human papillomavirus type 16 (HPV-16) in raft cultures of a new

cell line containing high copy numbers of episomal HPV-16 DNA. J. Virol. **70:**2339–2349.
20. **Grassmann, K., S. P. Wilczynski, N. Cook, B. Rapp, and T. Iftner.** 1996. HPV6 variants from malignant tumors with sequence alterations in the regulatory region do not reveal differences in the activities of the oncogene promoters but do contain amino acid exchanges in the E6 and E7 proteins. Virology **223:**185–197.
21. **Halbert, C. L., G. W. Demers, and D. A. Galloway.** 1992. The E6 and E7 genes of human papillomavirus type 6 have weak immortalizing activity in human epithelial cells. J. Virol. **66:**2125–2134.
22. **Higgins, G. D., D. M. Uzelin, G. E. Phillips, P. McEvoy, R. Marin, and C. J. Burrell.** 1992. Transcription patterns of human papillomavirus type 16 in genital intraepithelial neoplasia: evidence for promoter usage within the E7 open reading frame during epithelial differentiation. J. Gen. Virol. **73:**2047–2057.
23. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. **16:**7351–7367.
24. **Hofmann, K. J., J. C. Cook, J. G. Joyce, D. R. Brown, L. D. Schultz, H. A. George, M. Rosolowsky, K. H. Fife, and K. U. Jansen.** 1995. Sequence determination of human papillomavirus type 6a and assembly of virus-like particles in *Saccharomyces cerevisiae*. Virology **209:**506–518.
25. **Iftner, T., S. Bierfelder, Z. Csapo, and H. Pfister.** 1988. Involvement of human papillomavirus type 8 genes E6 and E7 in transformation and replication. J. Virol. **62:**3655–3661.
26. **Iftner, T., M. Oft, S. Boehm, S. P. Wilczynski, and H. Pfister.** 1992. Transcription of the E6 and E7 genes of human papillomavirus type 6 in anogenital condylomata is restricted to undifferentiated cell layers of the epithelium. J. Virol. **66:**4639–4646.
27. **Jackson, M. E., and M. S. Campo.** 1995. Both viral E2 protein and the cellular factor PEBP2 regulate transcription via E2 consensus sites within the bovine papillomavirus type 4 long control region. J. Virol. **69:**6038–6046.
28. **Kasher, M. S., and A. Roman.** 1988. Characterization of human papillomavirus type 6b DNA isolated from an invasive squamous carcinoma of the vulva. Virology **165:**225–233.
29. **Kitasato, H., H. Delius, H. zur Hausen, K. Sorger, F. Roesl, and E. M. de Villiers.** 1994. Sequence rearrangements in the upstream regulatory region of human papillomavirus type 6: are these involved in malignant transition? J. Gen. Virol. **75:**1157–1162.
30. **Kulke, R., G. E. Gross, and H. Pfister.** 1989. Duplication of enhancer sequences in human papillomavirus 6 from condylomas of the mamilla. Virology **173:**284–290.
31. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3:**510–526.
32. **Lorincz, A. T., R. Reid, A. B. Jenson, M. D. Greenberg, W. Lancaster, and R. J. Kurman.** 1992. Human papillomavirus infection of the cervix: relative risk associations of 15 common anogenital types. Obstet. Gynecol. **79:**328–337.
33. **May, M., X. P. Dong, E. Beyer Finkler, F. Stubenrauch, P. G. Fuchs, and H. Pfister.** 1994. The E6/E7 promoter of extrachromosomal HPV16 DNA in cervical cancers escapes from cellular repression by mutation of target sequences for YY1. EMBO J. **13:**1460–1466.
34. **McCance, D. J., R. Kopan, E. Fuchs, and L. A. Laimins.** 1988. Human papillomavirus type 16 alters human epithelial cell differentiation in vitro. Proc. Natl. Acad. Sci. USA **85:**7169–7173.
35. **Mevorach, R. A., L. R. Cos, P. A. di Sant'Agnese, and M. Stoler.** 1990. Human papillomavirus type 6 in grade I transitional cell carcinoma of the urethra. J. Urol. **143:**126–128.
36. **Oft, M., S. Boehm, S. P. Wilczynski, and T. Iftner.** 1993. Expression of the different viral mRNAs of human papilloma virus 6 in a squamous-cell carcinoma of the bladder and the cervix. Int. J. Cancer **53:**924–931.
37. **Okagaki, T., B. A. Clark, K. R. Zachow, L. B. Twiggs, R. S. Ostrow, F. Pass, and A. J. Faras.** 1984. Presence of human papillomavirus in verrucous carcinoma (Ackerman) of the vagina. Immunocytochemical, ultrastructural, and DNA hybridization studies. Arch. Pathol. Lab. Med. **108:**567–570.
38. **Purdie, K. J., C. J. Sexton, C. M. Proby, M. T. Glover, A. T. Williams, J. N. Stables, and I. M. Leigh.** 1993. Malignant transformation of cutaneous lesions in renal allograft patients: a role for human papillomavirus. Cancer Res. **53:**5328–5333.
39. **Rando, R. F., D. E. Groff, J. G. Chirikjian, and W. D. Lancaster.** 1986. Isolation and characterization of a novel human papillomavirus type 6 DNA from an invasive vulvar carcinoma. J. Virol. **57:**353–356.
40. **Ruebben, A., S. Beaudenon, M. Favre, W. Schmitz, B. Spelten, and E. I. Grussendorf Conen.** 1992. Rearrangements of the upstream regulatory region of human papillomavirus type 6 can be found in both Buschke-Lowenstein tumours and in condylomata acuminata. J. Gen. Virol. **73:**3147–3153.
41. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
42. **Schmitt, A., J. B. Harry, B. Rapp, F. O. Wettstein, and T. Iftner.** 1994. Comparison of the properties of the E6 and E7 genes of low- and high-risk cutaneous papillomaviruses reveals strongly transforming and high Rb-binding activity for the E7 protein of the low-risk human papillomavirus type 1. J. Virol. **68:**7051–7059.
43. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314:**111–114.
44. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and non-oncogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. **63:**1441–1447.
45. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83:**4680–4684.
46. **Stamminger, T.** 1997. Unpublished data.
47. **Steger, G., and S. Corbach.** 1997. Dose-dependent regulation of the early promoter of human papillomavirus type 18 by the viral E2 protein. J. Virol. **71:**50–58.
48. **Stoler, M. H., S. M. Wolinsky, A. Whitbeck, T. R. Broker, and L. T. Chow.** 1989. Differentiation-linked human papillomavirus types 6 and 11 transcription in genital condylomata revealed by *in situ* hybridization with message-specific RNA probes. Virology **172:**331–340.
49. **Tan, S. H., L. E. Leong, P. A. Walker, and H. U. Bernard.** 1994. The human papillomavirus type 16 E2 transcription factor binds with low cooperativity to two flanking sites and represses the E6 promoter through displacement of Sp1 and TFIID. J. Virol. **68:**6411–6420.
50. **Turek, L. P.** 1994. The structure, function, and regulation of papillomaviral genes in infection and cervical cancer. Adv. Virus Res. **44:**305–356.
51. **Vousden, K. H.** 1995. Regulation of the cell cycle by viral oncoproteins. Semin. Cancer Biol. **6:**109–116.
52. **Walboomers, J. M., A.-M. De Roda Husman, A. J. Van Den Brule, P. J. Snijders, and C. J. Meijer.** 1994. Detection of genital human papillomavirus infections: critical review of methods and prevalence studies in relation to cervical cancer, p. 41–69. *In* P. L. Stern and M. A. Stanley (ed.), Human papillomaviruses and cervical cancer, biology and immunology. Oxford University Press, Oxford, United Kingdom.
53. **Westin, G., T. Gerster, M. M. Müller, G. Schaffner, and W. Schaffner.** 1987. OVEC, a versatile system to study transcription in mammalian cells and cell-free extracts. Nucleic Acids Res. **15:**6787–6798.
54. **Wilczynski, S. P., M. Oft, N. Cook, S. Y. Liao, and T. Iftner.** 1993. Human papillomavirus type 6 in squamous cell carcinoma of the bladder and cervix. Hum. Pathol. **24:**96–102.
55. **Wu, T. C., and P. Mounts.** 1988. Transcriptional regulatory elements in the noncoding region of human papillomavirus type 6. J. Virol. **62:**4722–4729.
56. **Zeltner, R., L. A. Borenstein, F. O. Wettstein, and T. Iftner.** 1994. Changes in RNA expression pattern during the malignant progression of cottontail rabbit papillomavirus-induced tumors in rabbits. J. Virol. **68:**3620–3630.
57. **zur Hausen, H.** 1994. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types, p. 131–156. *In* H. zur Hausen (ed.), Human pathogenic papillomaviruses. Springer-Verlag KG, Berlin, Germany.