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E2 Represses the Late Gene Promoter of Human Papillomavirus Type 8 at High Concentrations by Interfering with Cellular Factors

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The late gene promoter P_{7535} of the epidermodysplasia verruciformis-associated human papillomavirus type 8 (HPV8) is regulated by the viral E2 protein. Transfection experiments performed with the human skin keratinocyte cell line RTS3b and P_{7535} reporter plasmids revealed transactivation at low amounts and a repression of basal promoter activity at high amounts of E2 expression vector. This repression was promoter specific and correlated with the amount of transiently expressed E2 protein. Mutational analyses revealed that the negative regulation of P_{7535} activity is mediated by the low-affinity E2 binding site P2, which is separated by one nucleotide from the P_{7535} TATA box. Biochemical and genetic analyses suggested that repression is due to a displacement of the TATA-box binding protein by E2 and an interference of E2 with promoter-activating cellular factors that specifically recognize the P2 sequence. The high conservation of the P2 sequence among several papillomaviruses (epidermodysplasia verruciformis-associated HPVs, HPV1, cottontail rabbit papillomavirus, and bovine papillomavirus type 1) in the vicinity of the late gene promoter cap site suggests that an interplay of E2 and cellular factors at this sequence element is important for the expression of structural proteins.

Gene expression in papillomaviruses is tightly linked to the differentiation state of the keratinocyte. In particular, late genes encoding structural proteins are only transcribed in the uppermost layers of the epithelium. Expression of papillomavirus genes is controlled by a complex interplay of host and viral transcription factors, which mainly act through sequences located in the noncoding regulatory region (NCR) between the L1 and E6 genes (12). The basal promoter activities in the absence of viral proteins are the net result of both positively and negatively acting host transcription factors. These activities can be further modulated by viral proteins, especially by products encoded by the E2 open reading frame (14, 28).

E2 proteins regulate viral gene expression and DNA replication by specifically binding as dimers to the DNA sequence $ACCN_6GGT$, which occurs several times in the NCRs of all papillomaviruses (1, 14, 17, 28). Bovine papillomavirus type 1 (BPV1) expresses three different E2 proteins, of which only the 48-kDa protein (E2TA) is able to transactivate viral promoters (16, 21, 27, 29). The two shorter forms lacking the transactivation domain antagonize the function of E2TA (2, 30). E2TA activates all but one of the BPV1 early promoters to different extents (40). Only the P1 (or P_{7185}) promoter is repressed by E2TA via E2 binding site 1 (BS1), localized immediately downstream of the P1 cap site. Repression seems to occur by interference with a cellular transcription factor whose target sequence overlaps BS1 (37). In the cases of cervical carcinoma-associated human papillomavirus (HPV) types 16 and 18, both positive and negative effects of E2 on the activity of the viral oncogene promoter have been observed under slightly different experimental conditions concerning E2 expression vectors and host cells. HPV16 E2 was shown to transactivate the early promoter in rodent cells (22). According to a recent work, full-length HPV16 E2 protein moderately stimulates viral gene expression in normal and immortalized keratinocytes, but N-terminally truncated E2 protein forms lead to repression (4). Other laboratories described repression of HPV16 and -18 promoter activity by homologous full-length E2 in human keratinocytes (3, 34). Of the four highly conserved E2 binding sites (E2BSs) among mucosotropic HPVs, only the promoter-proximal one turned out to be necessary for E2 repression of the HPV18 E6/E7 promoter (6), whereas both promoter-proximal E2BSs are required for the repression of the HPV16 P_{97} promoter (34, 41, 42). These E2BSs are spaced 1 and 3 bp, respectively, from the recognition sequences for the cellular transcription factors SP1 and the TATA box-binding protein (TBP), the DNA-binding component of the TFIID complex. Binding site competition between E2 and both factors causes repression of HPV16 P_{97} activity, whereas occlusion of TBP alone is sufficient for E2-mediated repression in the case of HPV18 P_{105} (6, 9, 41, 42). This negative modulation of oncogene expression is not restricted to cancer-associated HPVs but also occurs with the "low-risk" HPV11 (7).

Transcriptional regulation of the late promoter has only been studied in the case of HPV8. This type belongs to the group of HPVs associated with the syndrome epidermodysplasia verruciformis (EV) and is regarded as a highly oncogenic virus (32). EV-associated HPVs differ from other papillomaviruses mainly in the structure of the NCR. They all display the sequence motif M33/AP1 and five E2 recognition sequences (P0 to P4) with a highly conserved distribution (10, 20). The late gene promoter P_{7535} of HPV8 is localized in the L1-proximal part of the NCR. The initiated mRNAs are alternatively spliced and may code for the major capsid protein L1 or E2TA (11, 38). Promoter activity is negatively modulated by the negative regulatory element overlapping E2BS P1 and pos-

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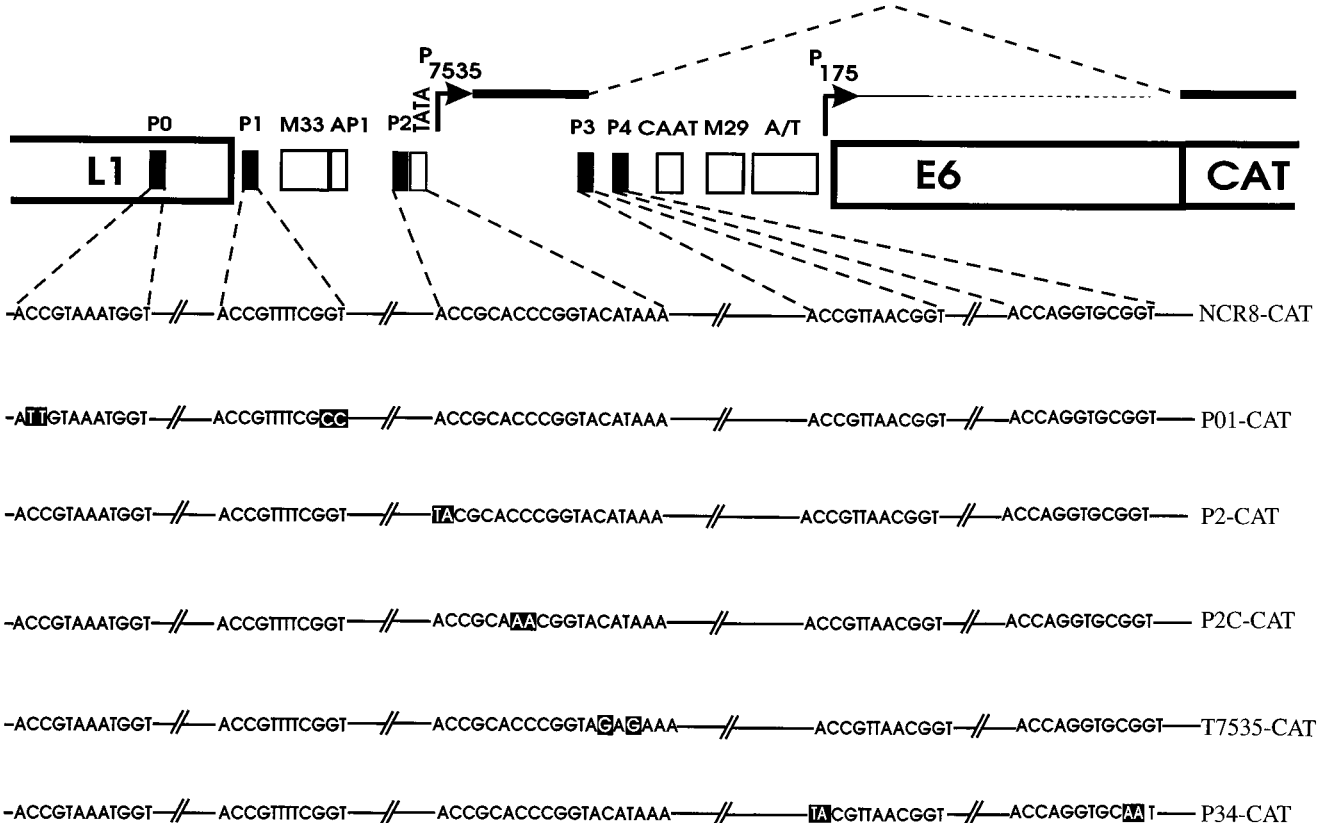


FIG. 1. Schematic representation of the reporter plasmid pNCR8-CAT. The HPV8 fragment driving *cat* gene expression consists of the NCR flanked by parts of the L1 and E6 genes. The relative amounts of transcripts initiated at the P_{7535} and the P_{175} promoters are indicated by thick and thin lines, respectively. Intron sequences of the P_{7535} transcript are shown as dashed lines. Sequence motifs specific for EV-associated HPVs (M33/AP1, CAAT, M29, and A/T [10, 20]) and the P_{7535} TATA box are shown by open boxes; E2BSs P0 to P4 are shown by solid boxes. The wild-type and mutated DNA sequences (white letters black boxes) of the E2BSs and the P_{7535} TATA box in the context of the corresponding reporter plasmids are shown beneath.

itively modulated by the M33/AP1 region, E2BS P2, the TATA box, and viral E2 (19, 25, 38, 39).

The five E2BSs display highly different affinities for the E2 protein. Mutational analyses of E2BSs revealed that four sites (mainly high-affinity sites P0 and P1 and to a smaller extent medium-affinity site P3 and low-affinity site P4) serve to transactivate the promoter, whereas the single low-affinity site close to the promoter, P2, is able to downmodulate E2 transactivation (39). In this report, we present data that late gene promoter activity can be repressed by E2 in the human skin keratinocyte cell line RTS3b (33). This repression seems to be mediated by the simultaneous interference of E2 with TBP and a cellular factor(s) specifically binding to P2.

MATERIALS AND METHODS

Cell culture and transient-transfection assays. HT3 cells (ATCC HTB32) were maintained in Dulbecco's modified Eagle's medium (DMEM) with supplements as described elsewhere (38). The spontaneously immortalized human skin keratinocyte cell line RTS3b contains no papillomavirus sequences (33). It was maintained in F12-DMEM (1:4) supplemented with hydrocortisone (0.4 μg/ml), 10^{-10} M cholera toxine, transferrin (5 μg/ml), 2×10^{-11} M triiodothyronin, 1.8×10^{-4} M adenin, insulin (5 μg/ml), epidermal growth factor (10 ng/ml), gentamicin (10 mg/ml), and 10% fetal calf serum (FCS). Both cell lines were transfected in 60-mm dishes by the calcium coprecipitation method as described before (39). Briefly, cells at 40 to 50% confluency were incubated overnight with 3 μg of reporter plasmid, the indicated amounts of E2 expression plasmid (pCE2), and the empty expression vector pCB6 to standardize the total amount of expression vector to 1 μg. Cells were extracted 24 h after the glycerol shock. Protein extract (5 to 50 μg) was assayed in a 1-h reaction for chloramphenicol acetyltransferase (CAT) activity. Luciferase constructs were introduced into RTS3b cells by liposome-mediated transfection. Cells were seeded 18 to 20 h before transfection at a density of 6×10^5 to 7×10^5 per 60-mm plate. Four micrograms of DNA and 15 μl of Lipofectamin (Gibco BRL, Eggenstein, Germany) were diluted in 0.6 ml of OptiMEM medium (Gibco BRL). After incubation for 30 min at room temperature, 2.4 ml of OptiMEM was added, and the mixture was applied to the cells which had been washed once with OptiMEM. After 5 h, 3 ml of growth medium containing 20% FCS was added and changed after overnight incubation to medium supplemented with 10% FCS. Cells were harvested 48 h after the addition of DNA in 100 mM potassium phosphate buffer (pH 7.8) containing 1 mM dithiothreitol (DTT) and lysed by freeze-thawing. Extract (30 μl) was assayed for luciferase activity in a luminometer (Berthold LB 9501). Transfection experiments were repeated at least three times, and the data in the figures represent averages. For monitoring the expression of E2 in HT3 and RTS3b, both cell lines were transfected by calcium phosphate coprecipitation, but nuclear instead of whole-cell extracts were prepared.

Plasmids and oligonucleotides. The basic construct pNCR8-CAT and its mutated derivatives P01-CAT, P34-CAT, P2-CAT, P2C-CAT, and T7535-CAT contain the 1,133-bp *Eco*RI-*Eco*RV fragment of HPV8 in front of the *cat* gene (Fig. 1) (38, 39). Enhancer test plasmid pNCR8-tk-CAT is a derivative of pBLCAT2 (23) and contains the same fragment as pNCR8-CAT cloned in the antisense orientation into the *Bgl*II site upstream of the herpes simplex virus thymidine kinase (tk) gene promoter linked to the *cat* gene.

Luciferase reporter constructs were derived from the promoterless pALuc plasmid (8). *Bam*HI-*Eco*47III fragments containing HPV8 sequences from nucleotides (nt) 7077 to 7628 were excised from plasmids pNCR8-CAT, P01-CAT, P2-CAT, and T7535-CAT and inserted between the *Bam*HI and *Ecl*136II restriction sites of pALuc upstream of the luciferase gene, giving rise to plasmids p7535-Luc, p7535-P01-Luc, p7535-P2-Luc, and p7535-TATA-Luc, respectively. Plasmid p7535-P2+10-Luc is a derivative of p7535-Luc and was constructed by PCR as described before (39). It contains a 10-bp insertion (5′-CATACGCGTA-3′) between HPV8 nt 7503 and 7504 (see Fig. 9).

HPV8 E2 expression vector pCE2 consists of the whole HPV8 E2 gene under the control of the immediate-early promoter-enhancer of human cytomegalovirus (39).

Oligonucleotides P1 (HPV8 nt 7384 to 7421), P2 (HPV8 nt 7486 to 7516), P2mt, P2mtC, and T7535 have been described (39). The oligonucleotide P36 used as a heterologous competitor in gel retardation assays is also derived from the NCR of HPV8 (nt 7544 to 7581). The numbering of the HPV8 sequence is according to Fuchs et al. (13).

Primer extension analysis. For the analysis of transiently expressed *cat* mRNA, RTS3b cells were seeded in 100-mm tissue dishes, and a scaled-up amount of DNA was introduced by liposome-mediated transfection. RNA was extracted 48 h after transfection by the guanidinium isothiocyanate method (5). Total RNA (45 μg) was mixed with an excess of ^{32}P-end-labeled primer 3 (HPV8 nt 7579 to 7590 [38]) in a total volume of 10 μl containing 5 mM Tris-HCl (pH 7.4), 100 mM NaCl, and 0.1 mM EDTA. Hybridization and reverse transcription reactions were performed as described before (24). Primer extension products were separated on a 6% polyacrylamide–urea gel. The dried gel was analyzed by phosphorimaging with a Fujix BAS 2000 (Raytest Strahlenmessgeräte GmbH, Straubenhardt, Germany).

Gel retardation analysis. HPV8 E2 protein was expressed from a recombinant baculovirus and purified as described before (39). Purified human TBP synthesized as a histidine-tagged fusion protein in *Escherichia coli* was a kind gift from G. Steger, Institut Pasteur, Paris. Crude nuclear extracts from nontransfected or transfected RTS3b or HT3 cells were prepared by the method of Sealey and Chalkley (36) with the modifications described below. Briefly, cells were scraped into 2 ml of cold phosphate-buffered saline and pelleted at full speed in a microcentrifuge at 4°C for 30 s. The pellet was incubated for 5 min on ice in 500 μl of lysis buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 300 mM saccharose, 1 mM EDTA, 0.25 mM EGTA [ethylene glycol tetraacetic acid], 1.5 mM $MgCl_2$, 50 mM NaCl, 0.5% [vol/vol] Nonidet P-40 [NP-40], 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml). Nuclei were pelleted at 3,000 rpm for 5 min at 4°C, resuspended in 150 μl of lysis buffer, and repelleted. The nuclear pellet was extracted on ice for 30 min with 50 μl of elution buffer (25% [vol/vol] glycerol, 10 mM HEPES [pH 7.9], 0.1 mM EDTA, 0.1 mM EGTA, 1.5 mM $MgCl_2$, 525 mM NaCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml). The supernatant, representing the crude nuclear extract, was recovered by centrifugation (16,000 × *g*, 5 min, 4°C) in a microcentrifuge. Aliquots were snap-frozen and stored at −80°C.

Binding reactions with TBP and E2 consisted of an aliquot corresponding to 2×10^4 Cerenkov cpm of ^{32}P-end-labeled oligonucleotide in 20 mM HEPES (pH 7.9)–135 mM KCl–4 mM spermidine–0.1 mM EDTA–1 mM DTT–0.05% (vol/vol) NP-40–1 mg of bovine serum albumin per ml–12.5 μg of poly(dG-dC) per ml. Binding reactions with TBP alone were incubated in 60 mM KCl. For E2-TBP binding site competition experiments, the labeled DNA was added after the proteins. For binding site competition experiments that included unlabeled DNA, the protein extract was added last. Reactions were incubated at 30°C for 30 min and then loaded onto a 5% native polyacrylamide gel (19:1) containing 0.5 × TBE (Tris-borate-EDTA). Gels were run at 200 V for 2 h, dried, and then autoradiographed overnight at −80°C with an intensifying screen. Crude nuclear extracts (5 μg) were incubated at room temperature in the same buffer, but 1 mg of poly(dA-dT) per ml was used as the nonspecific competitor.

DNase I footprint analysis. A subcloned HPV8 restriction fragment encompassing nt 7454 to 7602 was labeled with Klenow enzyme and [α-^{32}P]dATP at the coding and noncoding strand, respectively. An aliquot corresponding to 2.5×10^4 Cerenkov cpm was incubated with TBP (240 ng/μl) under the conditions described for gel retardation analysis. Reactions were digested with DNase I and processed as described before (39). An A+G sequencing reaction of the corresponding fragment was used as a molecular size marker.

RESULTS

E2-specific repression of HPV8 promoter activity in RTS3b cells. To test for differences in E2 modulation between mucosal keratinocytes (HT3), which were used in the previous study (39), and keratinocytes of cutaneous origin, we performed transfection assays with the human skin keratinocyte cell line RTS3b. Low input levels (10 ng) of E2 expression vector pCE2 slightly activated CAT expression (3.5-fold) from the reporter plasmid pNCR8-CAT (Fig. 2). Higher amounts of pCE2 reduced transactivation and finally repressed basal promoter activity. In HT3 cells, CAT expression was not further stimulated but was never diminished by increasing amounts of E2 (39). The repression observed with increasing amounts of E2 expression vector may theoretically be due to squelching or dominantly expressed N-terminally truncated E2 repressor forms. To control for the ability of pCE2 to produce transactivation-competent E2 even when high amounts of expression vector were transfected in RTS3b cells, cotransfection experiments were carried out with an HPV8 enhancer construct (Fig. 2).

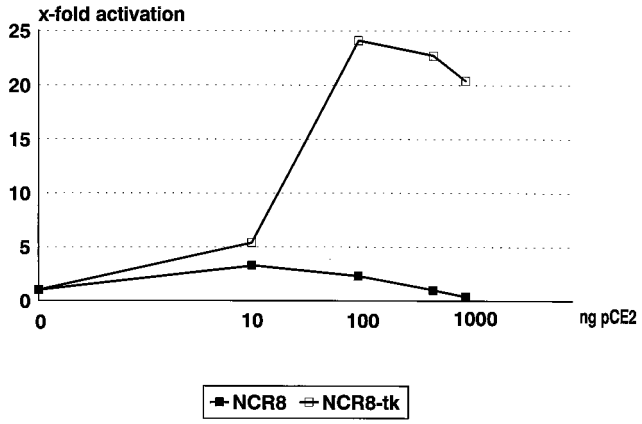


FIG. 2. Repression of HPV8 promoter activity by E2 is promoter specific. RTS3b cells were transfected with CAT reporter plasmids (NCR8 or NCR8-tk) and different amounts of HPV8 E2 expression vector pCE2, adjusted with the parental expression plasmid pCB6 to a total of 1 μg. Cell extracts were prepared after 36 h, and CAT activity was determined with 50 μg (NCR8) or 5 μg (NCR8-tk) of total cellular protein for 1 h at 37°C. The basal activities of pNCR8-CAT and pNCR8-tk-CAT were set to 1.

Plasmid pNCR8-tk-CAT contains the same HPV8 fragment as pNCR8-CAT but in the antisense orientation relative to the tk promoter-*cat* gene fusion. Titration experiments with this plasmid and pCE2 showed a 24-fold stimulation of CAT activity at 100 ng of transfected pCE2 and no significant drop at higher amounts of HPV8 E2 expression vector. This confirms that the repression of pNCR8-CAT by E2 is specific for the promoter configuration.

Transfected HT3 and RTS3b cells contain different amounts of E2 protein. The apparent difference between HT3 and RTS3b cells in the response of pNCR8-CAT to E2 might be due to different amounts of E2 protein after transfection. We therefore isolated nuclear extracts from HT3 and RTS3b cells which had been transfected under conditions identical to those for the analysis of transient expression of CAT.

Gel retardation assays were performed with equal amounts of nuclear extracts and ^{32}P-labeled oligonucleotide P1, carrying the highest-affinity E2BS of the HPV8 NCR (39). The two cell lines displayed the same pattern of three shifted bands when extracts isolated from the control transfection with the parental expression vector pCB6 were used (Fig. 3). Increasing amounts of transfected pCE2 resulted in a new complex specific for E2. The E2-DNA complex obtained with nuclear extracts from transfected RTS3b cells was much more prominent than that obtained with HT3 extracts. This experiment strongly suggests that the difference in the response to E2 between HT3 and RTS3b cells is mainly due to different amounts of E2 protein after transfection.

E2 repression is mediated by binding site P2. Experiments in HT3 cells indicated that P2 is responsible for the down-modulation of E2 transactivation (39). To test for the possibility that P2 also mediates the E2 repression of promoter activity in RTS3b cells, different amounts of pCE2 and pNCR8-CAT or mutated derivatives thereof (Fig. 1) were transfected, and CAT activity was determined (Fig. 4). Mutations in P2 or in the putative TATA box of P_{7535} decreased basal activity to 20 to 30%, which is similar to the reduction of promoter activity in HT3 cells (39). The mutation of P0 and P1 led to a clear drop in E2 transactivation, confirming that the weak transactivation is binding-site dependent. The promoter activity of all plasmids that contained an intact binding site P2 was repressed below

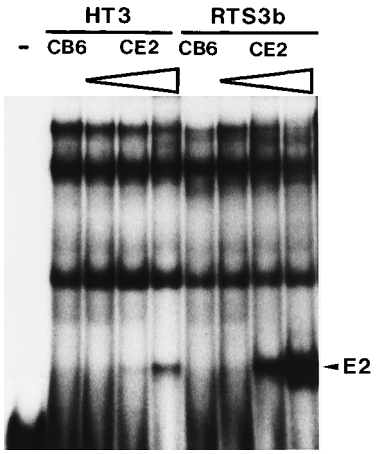


FIG. 3. RTS3b cells express higher amounts of E2 than HT3 cells. Both cell lines were transfected in 100-mm tissue culture dishes with 9 μg of reporter plasmid and 30, 300, or 3,000 ng of pCE2 (CE2) or 3,000 ng of pCB6 (CB6). Nuclear extracts were prepared 36 h after addition of the DNA. Gel retardation assays were carried out with ^{32}P-labeled oligonucleotide P1 and 15 μg of nuclear extract. The E2-DNA complex is indicated by an arrow.

the basal level at higher amounts of cotransfected E2 expression vector. Knockout of binding site P2 (P2-CAT) resulted in higher induction by E2 than with pNCR8-CAT and in a release from E2 repression. The notion that P2 mediates E2 repression was further substantiated by analysis of plasmid P2C-CAT, which contains a mutated P2 site with a higher affinity for E2 than the wild type (see Fig. 7) (39). It displayed a reduced E2 induction and was repressed more strongly than all other constructs. This confirms that E2 not only downmodulates E2 transactivation via P2 but is able to repress basal promoter activity if present in sufficient amounts.

The plasmid pNCR8-CAT harbors the late promoter P_{7535} and the E6 promoter P_{175}, which was shown to be only marginally active in HT3 cells (38). In view of the residual activity of the TATA box mutant (T7535-CAT), one might be concerned that P_{175} contributes to CAT expression in RTS3b cells.

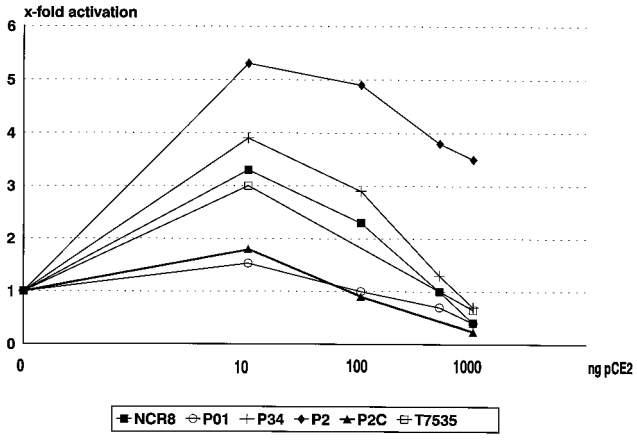


FIG. 4. E2 repression is mediated by P2. RTS3b cells were transfected with pNCR8-CAT (NCR8) or mutated reporter plasmids (P01-, P2-, P2C-, P34-, and T7535-CAT; see Fig. 1) and different amounts of pCE2, and CAT activity was determined in 50 μg of extract. The activation or repression by E2 is given relative to the basal activity of the individual constructs. The relative basal activities of the constructs compared with NCR8 (set at 1) are 1.8 for P01, 1.2 for P34, 0.2 for P2, 0.3 for P2C, and 0.3 for T7535.

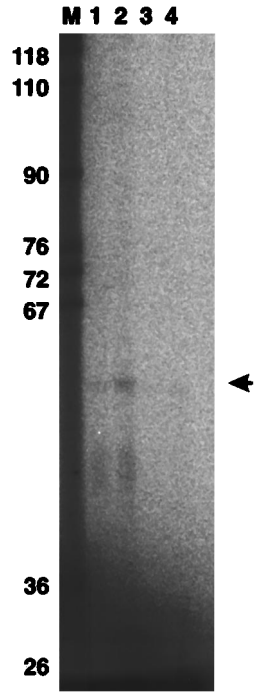


FIG. 5. Primer extension analysis of RNA isolated from RTS3b cells transfected with 9 μg of HPV8 reporter plasmid pNCR8-CAT (lanes 1 and 2) or T7535-CAT (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 30 ng of HPV8 E2 expression vector pCE2. Equal amounts of total cellular RNA (45 μg) were reverse transcribed after addition of ^{32}P-end-labeled primer 3. Extension products were separated in a 6% polyacrylamide–urea gel, and the gel was analyzed after drying by phosphorimaging. Specific extension products are marked by an arrow. A mixture of ^{32}P-end-labeled fragments of *Hae*III-digested ϕX174 and *Msp*I-digested pBR322 DNA served as molecular size markers (lane M). Sizes are shown in nucleotides.

We therefore performed primer extension experiments to map RNA initiation sites and constructed reporter plasmids in which the P_{175} sequences were deleted. RNA was isolated from cells transfected with plasmid pNCR8-CAT or T7535-CAT in the presence or absence of E2 expression vector. An extension product of 56 nt could be observed in all cases, confirming a correct initiation at position 7535 (Fig. 5). This indicates that for P_{7535}, TBP binding is important for promoter strength but not essential for precise initiation. The intensities of the bands correlated well with the results of the CAT assays, suggesting

TABLE 1. Modulation of P_{7535}-driven luciferase expression by HPV8 E2[a]

Reporter	Luciferase expression[b] with pCE2 at:				
	0 ng	10 ng		100 ng	
	Relative activity	Relative activity	Fold activation	Relative activity	Fold activation
p7535-Luc	1	2.9	2.9	1.7	1.7
p7535-P01-Luc	1.8	2.3	1.3	1.3	0.7
p7535-P2-Luc	0.3	1.8	6	1.8	6
p7535-TATA-Luc	0.5	1.8	3.7	1.4	2.9

[a] RTS3b cells were transfected with 3 μg of reporter plasmid and the indicated amount of HPV8 E2 expression vector pCE2 plus pCB6 to keep the amount of expression vector added constant. Luciferase assays were done with 30 μl of cellular extract.

[b] Relative activity reflects expression relative to that with p7535-Luc. These baseline values are then used to calculate fold activation for each reporter.

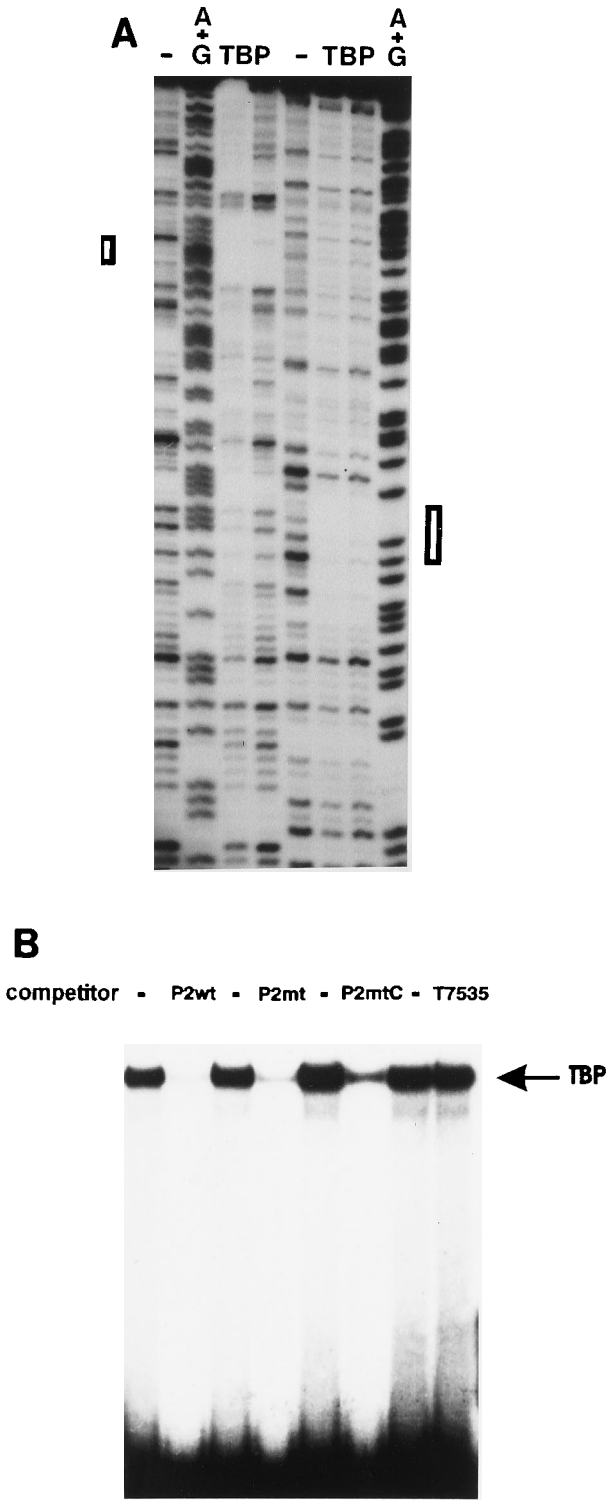


FIG. 6. TBP specifically recognizes the P_{7535} TATA box. (A) DNase I footprinting analysis of the P_{7535} promoter region. An HPV8 DNA fragment (nt 7454 to 7602) was labeled at the coding (left half) or the noncoding (right half of the autoradiogram) strand and then incubated with recombinant TBP (TBP) or with buffer only (lanes —). Binding reactions were digested with 40 ng (left TBP lane), 10 ng (right TBP lane), or 2 ng (lanes —) of DNase I. Reaction products were separated in a 6% polyacrylamide–urea gel. An A+G Maxam-Gilbert sequencing reaction of the labeled fragments served as molecular size markers. The positions of the P_{7535} TATA box on both strands are indicated by open boxes. (B) Gel retardation competition analysis of TBP binding to the P2 TATA region (nt 7486 to 7516). The ^{32}P-end-labeled P2 oligonucleotide was incubated with TBP and without (lanes —) or with 100 ng of unlabeled wild-type or mutant oligonucleotides as competitors. Reaction products were separated in a 5% polyacrylamide gel containing 0.5× TBE, dried, and then autoradiographed.

that these data reflect P_{7535} activity. This was further confirmed by the analysis of HPV8 luciferase reporter plasmids p7535-Luc, p7535-P01-Luc, p7535-P2-Luc, and p7535-TATA-Luc, which contain sequences from −457 to +93 relative to the P_{7535} cap site and lack the P_{175} promoter, the splice donor site of the P_{7535} transcripts, and E2BSs P3 and P4. Plasmid p7535-Luc displayed a 50-fold-higher basal activity than the promoterless parental vector pALuc after transfection into RTS3b cells. Cotransfection of luciferase reporter plasmids with different amounts of E2 expression vector resulted in activity profiles almost identical to those with the corresponding CAT reporter plasmids that include the P_{175} promoter (Table 1). Taken together, these experiments clearly demonstrate that the observed E2 regulation acts on the late gene promoter P_{7535}.

E2 and TBP binding to the P2/TATA region is mutually exclusive. To test for the possibility that binding site competition between HPV8 E2 and TBP is involved in E2 repression of the late promoter, we investigated the ability of TBP to bind to the P_{7535} TATA box. A DNase I footprint experiment was carried out with purified recombinant TBP and an HPV8 fragment encompassing the −81 to +68 region of the P_{7535} promoter (Fig. 6A). The specifically protected region is centered over the TATA box sequence at −31 to −26. To further elucidate the specificity of TBP binding to the TATA box, band shift competition experiments were performed (Fig. 6B). ^{32}P-labeled P2 oligonucleotide was incubated with TBP alone or with an excess of unlabeled oligonucleotides carrying the P2 wild-type, P2mt, P2mtC, or T7535 sequence. Only the T7535 oligonucleotide, with mutations in the CATAAA sequence, was no longer able to compete for TBP binding. These results, in conjunction with data from the transfection experiments, strongly suggest that binding of TBP to the HPV8 late promoter is important for its function.

The sequences protected by TBP partially overlap the E2 recognition sequence, indicating that the simultaneous binding of both proteins might not be possible for steric reasons. To test for this, gel retardation experiments were carried out (Fig. 7). Aside from the labeled P2 wild-type oligonucleotide, labeled P2mtC was used, as this oligonucleotide displays a higher affinity to E2 than the wild type, while its affinity for TBP is unchanged (Fig. 6B) (39). Furthermore, in the background of the reporter plasmid P2C-CAT, this mutant displays the strongest repression by E2. Incubation of both oligonucleotides with TBP or E2 protein alone resulted in clearly distinguishable shifted complexes. Binding reactions with both proteins gave rise to two shifted bands corresponding to TBP-DNA and E2-DNA complexes. Increasing the amount of E2 in the presence of a fixed amount of TBP decreased the intensity of the TBP-DNA complex with both the P2 wild-type and P2mtC oligonucleotides. This strongly suggests that E2 represses P_{7535} activity by displacement of TBP.

Specific binding of cellular factors to P2. Plasmids T7535-CAT and p7535-TATA-Luc are also repressed by E2 despite the fact that the mutated TATA box is no longer able to bind to TBP in vitro (Fig. 6B). This suggests that E2 might also interfere with the function of other factors that are important for promoter activity. Mutations in E2BS P2 (P2mt and P2mtC) reduce promoter activity (Fig. 4 and Table 1) (39), which indicates that it is an important element required for the

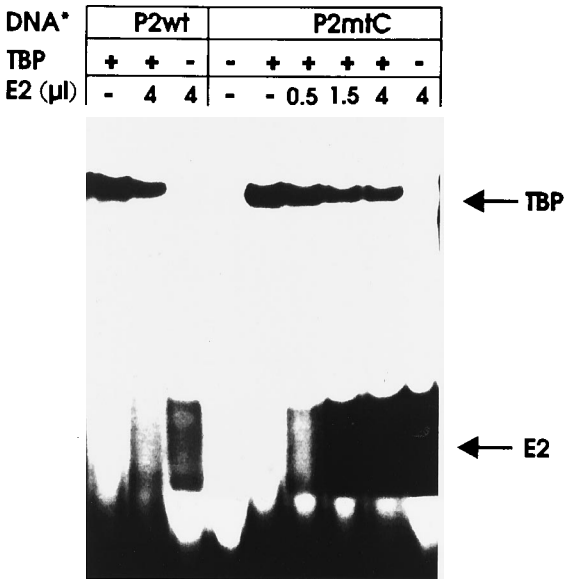


FIG. 7. E2 occludes TBP from binding to the P_{7535} TATA box. Gel retardation assays were carried out with the ^{32}P-labeled P2wt or P2mtC oligonucleotide, which were added to the reaction after the protein(s). Binding reactions were incubated with TBP (25 ng) and the indicated amounts of recombinant HPV8 E2 protein affinity purified from insect cells. The TBP-DNA and E2-DNA complexes are indicated by arrows.

basal activity of P_{7535}. The gel retardation assays suggested that the reduced activity of these mutants is not due to a decrease in TBP binding affinity (Fig. 6B). Specific binding of cellular factors to P2 has been demonstrated with HeLa nuclear extracts (26). We carried out gel retardation experiments with labeled P2 wild-type oligonucleotide and crude nuclear extracts from RTS3b cells and HT3 cells, which yielded identical patterns (Fig. 8 and data not shown). Five different complexes (A to E) could be detected with extracts from both cell lines.

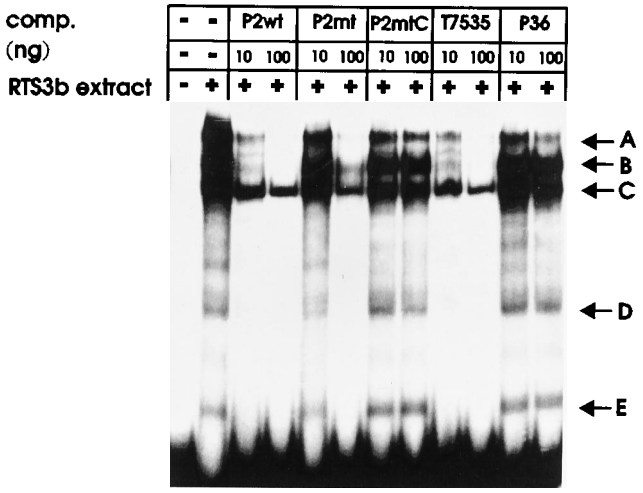
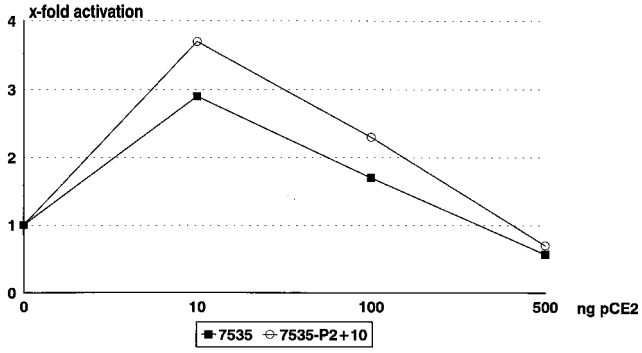


FIG. 8. Nuclear proteins from RTS3b cells specifically interact with the P2 sequence. Gel retardation assays were carried out with ^{32}P-labeled P2 oligonucleotide and crude nuclear extracts isolated from RTS3b cells. Reactions received no protein (−) or 5 μg of extract (+). Prior to the addition of extract, 0, 10, or 100 ng of unlabeled wild-type P2, mutated P2 (P2mt or P2mtC), mutated TATA box (T7535), or unrelated P36 oligonucleotide was added. Reactions were incubated at room temperature for 20 min and then separated in a 5% polyacrylamide gel containing 0.5× TBE. Complexes A to E are indicated by arrows.



P2 ACCGCACCCGGTACATAAA
P2+10 ACCGCACCCGGTACATACGCGTACATAAA

FIG. 9. E2 represses a modified P_{7535} promoter (P2+10) in which the E2BS P2 is separated by 11 nt from the TATA box. RTS3b cells were transfected with p7535-Luc (7535) or p7535-P2+10-Luc (P2+10) and different amounts of HPV8 E2 expression vector pCE2, and luciferase activity was determined. The activation or repression by E2 is given relative to the basal activity of the individual constructs. The relative basal activity of P7535-P2+10-Luc is 4.0, with that of p7535-Luc set at 1. The DNA sequences of the wild-type (P2) and the mutated (P2+10) P2 are shown below; the insertion is underlined.

Complexes B to E seem to be specific, as they cannot be eliminated by an unrelated oligonucleotide (P36). Complex C was only weakly inhibited by specific oligonucleotides, indicating that the responsible factor might be present in high amounts or might be of low specificity. The three specific complexes B, D, and E are not TBP or TFIID related, as they were eliminated by the T7535 oligonucleotide. They were not or only weakly inhibited by oligonucleotides P2mtC and P2mt, respectively. On principle, the significantly lower binding affinity correlates with reduced promoter activity of P2mt and P2mtC, suggesting that the protein(s) involved is important for promoter function. However, the P2mt oligonucleotide seemed to bind slightly better than the P2mtC oligonucleotide to the specific factors, although the basal activity of P2-CAT is lower than that of P2C-CAT. This may be due to the fact that the gel retardation assay does not correctly reflect the in vivo binding affinities of these factors or may point to the involvement of additional factors that could not be detected in gel retardation assays. It was not yet possible to demonstrate competition between P2-binding cellular factors (P2Fs) and increasing amounts of purified E2 in vitro with crude nuclear extracts (data not shown). These experiments have to await the purification of the P2Fs.

To obtain genetic evidence that E2 repression of P_{7535} activity also involves an interference of E2 with the P2Fs, we created plasmid p7535-P2+10-Luc, in which E2BS P2 is separated from the TATA box by 11 instead of 1 bp (Fig. 9). Steric hindrance between E2 and TBP should be relieved with this plasmid, because it has been shown for the HPV18 P_{105} promoter that increasing the distance between the E2BS and the TATA box from 3 to 8 bp results in vitro in simultaneous binding of both proteins and in vivo in transactivation instead of repression by E2 (9, 15). Plasmid p7535-P2+10-Luc displayed fourfold-higher basal activity than p7535-Luc (Fig. 9). Cotransfection with different amounts of pCE2 revealed that this plasmid responds to E2 as p7535-Luc does. It was transactivated to a slightly higher degree at 10 ng of pCE2, but this transactivation dropped at higher amounts of E2 expression vector (Fig. 9). The E2-mediated negative modulation of promoter activities in the context of plasmids p7535-P2+10-Luc

and p7535-TATA-Luc makes it likely that the interference of E2 with the cellular P2Fs is involved in transcriptional repression. However, for maximum repression, interference with both TBP and the P2Fs is needed.

DISCUSSION

The E2 proteins of papillomaviruses are multifunctional proteins involved in regulation of viral transcription and replication. One intriguing aspect is that the transactivation-competent E2 protein is also able to repress promoters dependent on the promoter sequence (28). We have previously described a moderate (fourfold) binding-site dependent transactivation of the HPV8 P_{7535} promoter by E2 which was limited by E2 binding to the low-affinity recognition sequence P2 (39). This negative effect, observed in the cell line HT3, turned out to be more pronounced in RTS3b cells, finally leading to repression of the basal promoter activity. The obviously high levels of E2 in RTS3b cells might interfere with E2 transactivation by squelching. This was discussed for HPV16 E2 and the HPV16 P_{97} promoter (4), whereas no squelching was detectable with HPV16 E2 and an E2-dependent minimal promoter construct (43). Only a slight reduction in E2 transactivation, from 24- to 20-fold, at high levels of HPV8 E2 expression vector could be noted with the HPV8 enhancer construct, which could indeed be due to squelching. However, this cannot account for the repression of P_{7535} starting at 100 ng of pCE2. Transactivation of the binding site mutant P2mt (P2-CAT and p7535-P2-Luc) was slightly reduced at high E2 levels (Fig. 4 and data not shown), similar to the enhancer construct, but repression could not be achieved. This binding-site dependence of the negative control argues strongly against squelching mechanisms.

The difference in the response of P_{7535} to E2 between HT3 and RTS3b cells seems to be due to different amounts of E2 protein after transfection. This can be explained by a higher transfection efficiency of RTS3b cells than of HT3 cells, as judged by in situ staining after transfection of a β-galactosidase-expressing plasmid (data not shown). Other mechanisms, such as differences in the expression level of the cytomegalovirus-E2 construct or different stabilities of the HPV8 E2 protein might also be involved.

The E2BS P2 is localized at positions −44 to −33 relative to the P_{7535} cap site. It overlaps recognition sequences for a cellular factor(s) (P2F [Fig. 8]) which is important for promoter function and is separated by only one nucleotide from the P_{7535} TATA box. Mutational and biochemical analyses strongly suggest that E2 represses promoter activity by interference with the binding and/or function of cellular transcription factors. We have shown on the one hand that E2 is able to occlude TBP from binding to the promoter in vitro. This has also been reported to be relevant for the repression of HPV18 P_{105} activity by BPV1 E2TA (9). E2 repression of plasmids with a mutated TATA box and p7535-P2+10-Luc, with an increased spacing between P2 and the TATA box, indicated that there is an interference with promoter activity that is independent of the occlusion of TBP binding. The TATA box mutant was negatively regulated by E2 despite the fact that TBP is not able to bind in vitro to the mutated promoter. The sequence arrangement of p7535-P2+10-Luc, in which E2BS P2 is separated by 11 bp from the TATA box, should allow the simultaneous binding of E2 and TBP, as shown for the E6/E7 promoter of HPV18 after introduction of only 5 bp (9, 15), but it is still repressed by high amounts of pCE2 and not transactivated, as in the case of HPV18 (15). This suggests that interference of E2 with the P2Fs is also involved in repression of P_{7535}. Repression of viral promoters by competition of E2 with cellular factors other than TBP has been described for HPV11, HPV16, and BPV1 (7, 37, 42), but this is the first report of such regulation for a late papillomavirus promoter.

The P2 sequence is highly conserved among EV-associated HPVs and can also be found in the vicinity of other papillomavirus late gene promoters (HPV1, cottontail rabbit papillomavirus, and BPV1 [38]), suggesting that the interplay of cellular and viral proteins is in general important for the regulation of late gene expression. The homologous E2BS (=BS1) of BPV1 has already been shown to mediate negative regulation by E2TA (37, 44). It has been discussed that E2 represses the early promoter P_{7185} (or P1), but the proximity of the late promoter cap site makes it likely that P_L is also influenced. These data suggest that repression of viral promoters by E2 is a common regulatory aspect of papillomaviruses and may also play a role in the expression of structural proteins. Even the strategy for transcriptional repression by E2 seems to be very similar among early and late promoters.

The HPV8 late gene promoter is activated at low E2 protein concentrations and repressed at higher amounts, which correlates with the observation that P2 is a low-affinity E2BS (39). The biological significance of this complex regulation is still unclear, but cDNA analyses suggest that P_{7535} may drive the expression of both the L1 and E2 genes (11, 38). It is tempting to speculate that P_{7535} might be used at early times of infection as an autoregulated E2-specific promoter. The observed regulatory loop would then serve to maintain a low but constant level of E2. E2 autoregulation has been described for BPV1 (18) and might also occur with HPV11 and -16, as mRNAs transcribed from the respective E2-regulated E6 promoters may encode the E2 gene (31, 35). In the uppermost layers of the epithelium, transcription of the L1 gene could be achieved by reduced E2 expression and/or by upregulation of the P2Fs, preventing E2 from binding. On the other hand, the P2-E2 interaction may be weakened by modification of the E2 protein or of the P2 sequence, e.g., by DNA methylation.

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