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Low-Affinity E2-Binding Site Mediates Downmodulation of E2 Transactivation of the Human Papillomavirus Type 8 Late Promoter

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The constitutively active promoter P_{7535} of the epidermodysplasia verruciformis-associated human papillomavirus type 8 (HPV8) is transactivated by the viral E2 protein. The distribution of potential E2-binding sites ($ACCN_6GGT$) in the viral transcription control region is highly conserved among epidermodysplasia verruciformis-associated human papillomaviruses and differs completely from that of other papillomaviruses. To investigate the role of E2-binding sites P0 to P4 in P_{7535} regulation, we analyzed their binding affinities in gel retardation experiments using a full-length HPV8 E2 protein expressed from a recombinant baculovirus. Binding site P1 within a transcriptional silencer showed the highest affinity, followed by P0 within the L1 gene and P3 downstream of P_{7535}. P2, 33 nucleotides upstream of the mRNA cap site, and P4 were very weak binders. There is some indication that the number of A/T pairs in the nonconserved core of the recognition sequence is critical for the binding of HPV8 E2. Transient transfection experiments were carried out with an HPV8 E2 expression vector and reporter plasmids containing mutated E2-binding sites in the context of the HPV8 regulatory region. The knockout of the strongest binding site P1 sufficed to clearly diminish transactivation. P0, P3, and P4 mutations had little effect on their own, whereas double mutations P01 and P34 strongly reduced E2 inducibility. Both mutations in P2 severely affected constitutive promoter activity but had opposite effects on transactivation. They revealed an inverse correlation between E2-binding strength and the extent of E2 transactivation. This finding suggests that P2 mediates a negative control of P_{7535} by E2, counteracting E2 transactivation exerted via the four distal E2 target sequences.

DNA viruses consistently encode potent transactivators of transcription, allowing the efficient expression of viral genes. These regulator proteins also are key players in switches from latent to active infections and from the early to the late productive phase of the replication cycle. In this context, they modulate their own expression, creating either negative or positive autoregulatory loops (18, 43).

Human papillomaviruses (HPVs) induce various intraepithelial tumors in humans. Viral gene expression is tightly linked to the differentiation state of the infected keratinocyte, with capsid protein synthesis occurring only in the uppermost epidermal layers (29). Replication control sequences and transcriptional regulatory elements such as enhancers, silencers, and promoters are mainly located in the noncoding regulatory region (NCR) between the late and early genes (17, 21). A common feature of all papillomavirus NCRs is the existence of target sequences for viral E2 protein(s) ($ACCN_6$ GGT). E2 proteins regulate viral gene expression and DNA replication by binding as dimers to their specific recognition sequences (25). Bovine papillomavirus type 1 (BPV1) contains 12 target sites within the NCR with binding affinities varying 100-fold (22). The full-length form of E2 (E2TA) activates to different extents all but one of the BPV1 early promoters located throughout the genome (40). Only the P1 (or P_{7185}) promoter is repressed by E2TA (37). The early promoters in front of the E6 genes of HPV type 11 (HPV11), HPV16, and HPV18 are also repressed by homologous E2TA (3, 4, 32, 42). In these cases, E2 seems to prevent the formation of the initiation complex and/or the binding of cellular transcription factors (5, 6, 37, 41). The mRNAs initiated at the HPV promoters encode the oncoproteins E6 and E7 as well as the E2 protein itself (27, 33), which may result in an autoregulatory loop. Loss of the E2-mediated repression is thought to result in overexpression of E6 and E7 proteins, which may contribute to tumor progression and malignant conversion (31).

HPVs associated with the syndrome epidermodysplasia verruciformis (EV) induce macular skin lesions that may progress to cutaneous cancer (28). The NCRs of these viruses differ from those of other papillomaviruses in length (ca. 400 bp versus 1,000 bp) and display a characteristic pattern of highly conserved sequence elements called M33 and M29 (7, 20). Four potential E2-binding palindromes (P1 to P4) occur in the NCR, and a fifth (P0) is located 65 nucleotides (nt) upstream of the stop codon of the L1 gene (Fig. 1). This distribution is completely different from that of other HPV groups and animal papillomaviruses such as BPV1 (17).

The regulatory region of the EV-associated HPV8 contains the late promoter P_{7535}, which is constitutively active in a variety of human epithelial cells (38). The initiated mRNAs are alternatively spliced and may code for the major capsid protein L1 or the regulator protein E2 (9, 38). This promoter is positively regulated by viral E2 and the M33/AP1 element and is silenced by a DNA sequence immediately upstream of this region (Fig. 1), overlapping the E2-binding-site P1 (16, 23, 38). To appreciate the role of the individual E2-binding sites in the E2-dependent control of the P_{7535} promoter, we performed

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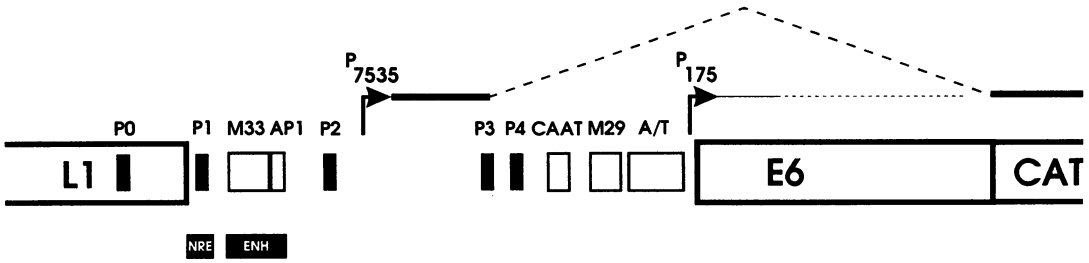

FIG. 1. Schematic representation of the reporter plasmid pNCR8-CAT. The HPV8 fragment driving *cat* gene expression consists of the NCR flanked by parts of the L1 and E6 genes. Sequence motifs specific for EV-associated HPVs (20) are shown by open boxes (M33, AP1, CAAT, M29, and A/T), and E2-binding sites (P0 to P4) as shown by black boxes. The relative amounts of transcripts initiated at promoters P_{7535} and P_{175} are indicated by thick and thin lines, respectively. Negative (NRE) and positive (ENH) transcription modulation sequences are depicted beneath.

a mutational analysis and investigated the effects by transient transfection experiments.

MATERIALS AND METHODS

Plasmids and oligonucleotides. The basic construct pNCR8-CAT contains the 1,133-bp *Eco*RI-*Eco*RV fragment of HPV8 in front of the chloramphenicol acetyltransferase (*cat*) gene (38). Point mutations were introduced with oligonucleotides by the method of Kunkel as described elsewhere (1) or via a PCR-based protocol (15). PCR mutagenesis was carried out as follows. One reaction mixture contained the M13(−20) primer and an oligonucleotide carrying the desired mutation; the other one contained the complementary mutated oligonucleotide and a primer within the *cat* gene (38). Both amplified fragments were gel purified, mixed, and subjected to a PCR with the M13(−20) primer and the CAT primer. Amplification products were cleaved by *Bam*HI and *Eco*47III or by *Eco*47III and *Bgl*II and used to replace the corresponding fragments in pNCR8-CAT. All mutations were confirmed by double-stranded sequencing. The following HPV8-specific oligonucleotides and their derivatives (see Fig. 5A) were used for mutagenesis and gel retardation assays: P0 (nt 7317 to 7348), P1 (nt 7384 to 7421), P2 and T7535 (nt 7486 to 7516), P3 (nt 7638 to 15), and P4 (nt 13 to 44). The numbering of the HPV8 sequence is according to Fuchs et al. (10). The pBLCAT2-derived plasmid pNRE-3U-tk contains three copies of the P1 oligonucleotide in front of the herpes simplex virus thymidine kinase gene promoter (23). CAT expression from plasmid pRR52 is driven by the simian virus 40 enhancer/promoter (30).

The eukaryotic HPV8 E2 expression vector (pCE2) was constructed by inserting the whole E2 open reading frame of HPV8 (nt 2682 to 4222) into pCB6 (a gift from M. Stinski, University of Iowa) between the immediate-early enhancer/promoter of human cytomegalovirus and the termination signals of the human growth hormone gene.

The baculovirus transfer vector for the expression of HPV8 E2 (pBlueBacHisE2$_8$) contains the same E2 DNA fragment as pCE2 cloned into the *Bam*HI site of plasmid pBlueBacHisC (Invitrogen, San Diego, Calif.) downstream of the polyhedrin gene promoter. The resulting E2 fusion gene codes for a total of 539 amino acids, consisting of a short vector-derived peptide (34 amino acids) with an oligohistidine stretch, 7 residues derived from the E2 open reading frame before the first ATG, and the full-length E2 protein.

Cell culture and transient expression assays. The cervical carcinoma cell line HT3 (ATCC HTB 32) was maintained as described previously (38). Cells were split 1:3 to 1:4 24 h before transfection to achieve 30 to 50% confluency in 60-mm-diameter dishes. Cells were refed 2 h before transfection with 2.5 ml of medium. Calcium phosphate-DNA precipitates (0.3 ml) were prepared by standard procedures (1) and contained 3 μg of CAT reporter plasmid and 0.5 μg of HPV8 E2 expression vector (pCE2) or the same amount of the expression vector (pCB6) without insert. Cells were glycerol shocked for 3 min after overnight exposure to the precipitate and further incubated for 24 h in 5 ml of medium. Cells were harvested, and 100 to 200 μg of protein was assayed for CAT activity in a 1-h reaction. Transfections were repeated 8 to 13 times.

The insect cell line *Spodoptera frugiperda* 158 (Sf158 [8]) was maintained at 28°C in TC 100 medium (Gibco BRL, Eggenstein, Germany) supplemented with 350 μg of glutamine per ml, 50 μg of gentamicin per ml, 2.5 μg of amphotericin B (Fungizone; Gibco BRL) per ml, and 10% fetal calf serum (Gibco BRL). All manipulations of Sf158 cells and baculoviruses were done as described by Summers and Smith (39).

Expression and purification of HPV8 E2 protein from insect cells. The recombinant baculovirus vE2$_8$ was recovered after cotransfection of 0.75 μg of transfer vector pBlueBacHisE2$_8$ and 0.25 μg of linearized BaculoGold DNA (Pharmingen, San Diego, Calif.) into Sf158 cells. Recombinant viruses, which express E2 and the *lacZ* gene, were identified as blue plaques after addition of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and purified by a plaque assay. Insect cells were infected at a multiplicity of infection of 10 and harvested 44 to 46 h postinfection. A nuclear extract was made after swelling cells in hypotonic buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride, 5 mg of leupeptin per ml, 10 mg of aprotinin per ml, 1 mg of pepstatin per ml) for 15 min on ice. The cells were then lysed by mechanical shearing in a 25-gauge syringe (six to eight strokes) and centrifuged at 16,000 × *g* (Eppendorf Microfuge) for 30 s. The nuclear pellet was incubated for 30 min on ice in 20 mM HEPES (pH 7.9)–420 mM NaCl–1.5 mM $MgCl_2$–0.2 mM EDTA–0.5 mM DTT–25% (vol/vol) glycerol–protease inhibitors as described above. All following steps were carried out at 4°C. Nuclei were pelleted for 15 min by centrifugation (16,000 × *g*; Eppendorf Microfuge), and the supernatant was diluted to 100 mM NaCl. The purification of E2 via the histidine tag by Ni chelate chromatography was unsuccessful for unknown reasons. Therefore, E2 was enriched by heparin-Sepharose chromatography and DNA affinity chromatography. The nuclear extract was applied to a HiTrap heparin-Sepharose column (Pharmacia, Freiburg, Germany) at a flow rate of 1 ml/min. Active fractions eluted at 0.6 M KCl, as identified by gel retardation assays. They were pooled and

diluted to 0.1 M KCl, and Nonidet P-40 was added to a final concentration of 0.1% (vol/vol). The pooled fractions were applied to a 1-ml DNA affinity column, which was prepared by coupling a modified P1 oligonucleotide, which had an amino group attached at the 5′ end of the upper strand, to a HiTrap *N*-hydroxysuccinimide-activated Sepharose column (Pharmacia, Freiburg) as instructed by the manufacturer. Fractions containing active E2 eluted from 0.5 to 0.8 M KCl and were subsequently stored at −80°C.

Western immunoblot analysis. Thirty micrograms of the cytoplasmic or nuclear protein fraction from wild-type *Autographa californica* multiply enveloped nuclear polyhedrosis virus (Ac*M*NPV)- or $vE2_8$-infected cells was separated in a sodium dodecyl sulfate (SDS)–8% polyacrylamide gel and transferred onto a nitrocellulose filter. The filter was blocked in phosphate-buffered saline (PBS) containing 0.1% (vol/vol) Tween 20 and then incubated overnight at 4°C in PBS–0.1% Tween 20 with a 1:50 dilution of polyclonal rabbit antiserum raised against a procaryotically expressed β-galactosidase–HPV8 E2 fusion protein (36). Bound antibodies were detected by a mouse anti-rabbit immunoglobulin G antibody conjugated to peroxidase (Dako, Hamburg, Germany) and chemoluminescence detection (ECL Western blotting detection system; Amersham, Arlington Heights, Ill.) as instructed by the manufacturer.

Metabolic labeling of cells and immunoprecipitation. Cells were starved 39 h after infection for 1 h in 4 ml of methionine-free Grace's insect medium (Sigma, Deisenhofen, Germany) supplemented with 10% dialyzed fetal calf serum (Gibco BRL). After labeling of the cells for 4 h with 500 μCi of [^{35}S]methionine (>1,000 Ci/mmol [=37 TBq/mmol]; Amersham) in 4 ml of the same medium, nuclear extracts were prepared as described above. One-fifth of the extract was used for each reaction and precleared for 1 h at 4°C with protein A-Sepharose (Sigma) in binding buffer containing 20 mM HEPES (pH 7.9), 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 9% (vol/vol) glycerol, 1% (vol/vol) Nonidet P-40, and protease inhibitors. After centrifugation, the supernatant was incubated overnight at 4°C with E2 antibodies or preimmune serum prebound to protein A-Sepharose. Immune complexes were washed four times at room temperature with binding buffer supplemented with protease inhibitors as described above and once in a buffer containing 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, and 0.2 mM EDTA. Complexes were dissociated by boiling in 20 μl of 2× SDS sample buffer and then separated in an SDS–10% polyacrylamide gel. The fixed gel was treated with Amplify (Amersham), dried, and exposed overnight at −80°C to Kodak X-Omat AR film.

Gel retardation assays. An aliquot of ^{32}P-end-labeled oligonucleotide equivalent to 10^4 Cerenkov cpm was incubated with the indicated amount of affinity-purified E2 in a total volume of 20 μl. The binding buffer consisted of 20 mM HEPES (pH 7.9), 60 to 100 mM KCl, 4 mM $MgCl_2$, 1 mM spermidine, 0.1 mM EDTA, 1 mM DTT, 1 mg of bovine serum albumin per ml, 5 μg of poly(dA-dT) [or 50 μg of poly(dI) · poly(dC) per ml when crude nuclear extracts were used], and 10% (vol/vol) glycerol. For competition experiments, the indicated amounts of wild-type or mutated oligonucleotide were added. After incubation for 15 min at room temperature, reaction products were separated at 200 V in 6 to 8% native polyacrylamide gels (19:1) containing 0.5× Tris-borate-EDTA. The gels were vacuum dried and exposed overnight at −80°C to Kodak X-Omat AR film.

DNase I footprinting experiments. The *Hin*dIII-*Sty*I DNA fragment from pNCR8-CAT-7292 (38) encompassing HPV8 nt 7292 to 7590 was labeled by Klenow enzyme with [α-^{32}P]dATP at the *Hin*dIII site. An aliquot corresponding to 2.5×10^4 Cerenkov cpm was incubated with different amounts of nuclear extracts from $vE2_8$- or Ac*M*NPV-infected cells in binding buffer consisting of 10.5 mM HEPES (pH 7.9), 62 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 3 mM spermidine, 1 mM DTT, 11% (vol/vol) glycerol, 1 mg of bovine serum albumin per ml, and 50 μg of poly(dI) · poly(dC) per ml. After an incubation for 20 min at 20°C, different amounts of DNase I (Boehringer, Mannheim, Germany) were added, and digestion was allowed to proceed for 90 s at 20°C. The reaction was stopped by addition of 200 μl buffer (0.2 M NaCl, 0.03 M EDTA, 1% [wt/vol] SDS, 100 μg of tRNA per ml) followed by phenol extraction. Precipitated reaction products were separated in a 6% urea–polyacrylamide gel. A sequencing reaction was run in parallel as a molecular size marker.

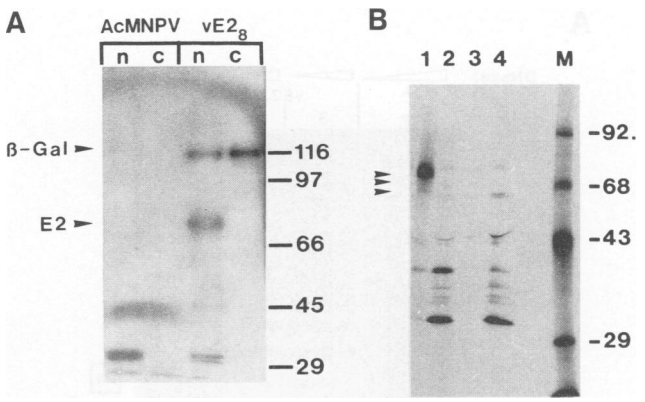

FIG. 2. Expression of a full-length HPV8 E2 protein from $vE2_8$ in insect cells. (A) Western blot analysis of cytoplasmic (c) and nuclear (n) extracts from Ac*M*NPV- or $vE2_8$-infected Sf158 cells. The positions of proteins specifically detected by the anti-E2 serum are indicated by arrowheads. The molecular size markers (in kilodaltons) are shown on the right. β-Gal, β-galactosidase. (B) Immunoprecipitation analysis of nuclear extracts from infected Sf158 cells labeled with [^{35}S]methionine. Extracts from $vE2_8$-infected (lanes 1 and 3) or Ac*M*NPV-infected (lanes 2 and 4) cells were precipitated with the same anti-E2 serum as in panel A (lanes 1 and 2) or preimmune serum (lanes 3 and 4) and separated in an SDS–8% polyacrylamide gel. E2-specific bands are indicated by arrowheads. ^{14}C-labeled proteins served as molecular size markers (M). Sizes are indicated in kilodaltons.

RESULTS

Expression and purification of HPV8 E2. The baculovirus system was chosen for the expression of HPV8 E2 protein. To determine whether a full-length E2 is expressed from the recombinant baculovirus $vE2_8$, Sf158 insect cells were infected with $vE2_8$ or wild-type baculovirus (Ac*M*NPV). Nuclear and cytoplasmic extracts were prepared 44 h postinfection. Western blot analysis with a polyclonal serum raised against a β-galactosidase–E2 fusion protein revealed two specific bands which appeared only in extracts from $vE2_8$-infected cells (Fig. 2A). The upper band corresponded to an apparent molecular mass of 116 kDa and was present in high amounts in the cytoplasm and low amounts in the nuclear extract. This band presumably represents the *lacZ* gene product, which is coexpressed from the recombinant virus. The lower band, which appeared only in the nuclear fraction, is likely to correspond to E2 with an apparent molecular mass of 70 kDa. To further confirm that the 70-kDa protein represents E2, we carried out

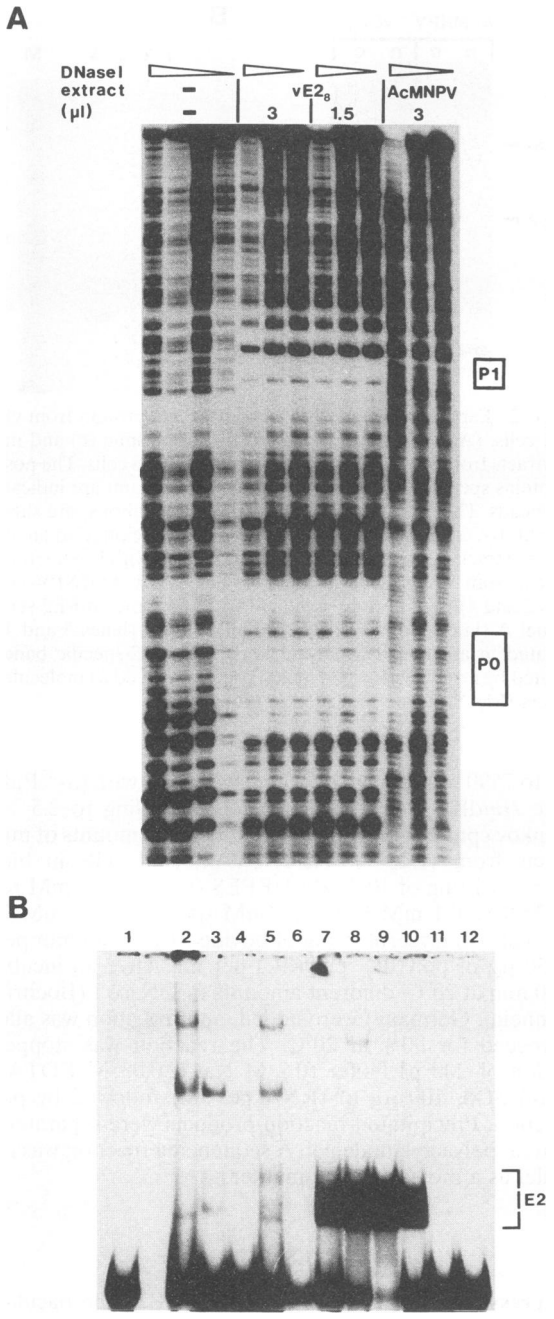

FIG. 3. HPV8 E2 protein expressed in insect cells displays sequence-specific DNA-binding activity. (A) DNase I footprint analysis of crude nuclear extracts (1.5 or 3 μl) from vE2$_8$- or Ac*M*NPV-infected cells and a ^{32}P-end-labeled HPV8 DNA fragment encompassing nt 7292 to 7590. The input marker digested with 16, 8, 4 or 2 ng of DNase I per μl is shown to the left. Binding reaction mixtures were incubated with 100, 40, or 20 ng of DNase I per μl. The positions of the P0 and P1 E2 recognition sequences are indicated by boxes at the right. (B) Gel retardation analysis with aliquots of different fractions from the P1 oligonucleotide-specific DNA affinity column and ^{32}P-labeled P1 oligonucleotide. No protein was added in lane 1. In lanes 2 and 3, 18 μl each of the flowthrough and wash fractions, respectively, were used. Lanes 4 to 12 present binding reactions with 3 μl each of fractions that were eluted by increasing the KCl concentration (0.2 to 1.0 M) in the buffer. E2-specific shifted complexes are indicated by a bracket. The apparently low amount of free oligonucleotide in lane 6 may be due to DNases or phosphatases eluting at this salt concentration.

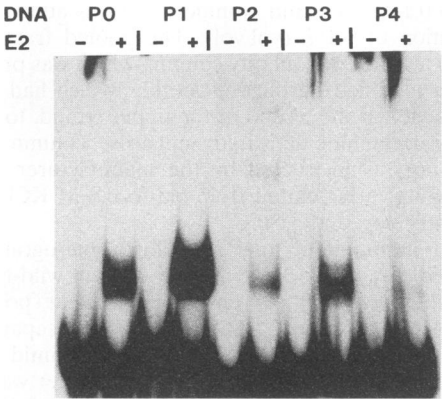

FIG. 4. Different affinities of HPV8 E2-binding sites for E2 protein. Oligonucleotides with a single E2-binding site (P0 to P4) were analyzed for E2 binding in gel retardation assays. Each reaction mixture received an identical aliquot of ^{32}P-labeled oligonucleotides and 2 μl of affinity-purified E2 protein (+) or no protein (−).

an immunoprecipitation assay. Nuclear extracts were prepared from vE2$_8$- or Ac*M*NPV-infected cells labeled with [^{35}S]methionine and incubated with the preimmune or the anti-E2 serum. A major 70-kDa band and two minor 63- to 65 kD bands that were absent from the controls could be detected in extracts from vE2$_8$-infected cells incubated with the anti-E2 serum (Fig. 2B). The different bands may result from degradation or posttranslational modifications. The expected molecular mass for the HPV8 E2 fusion protein expressed from vE2$_8$ is 63 kDa; i.e., there is no evidence for degradation.

To investigate the biological activity of the baculovirus-expressed E2 protein, we performed DNase I footprinting experiments with an HPV8 DNA fragment and crude nuclear extracts (Fig. 3A). Two regions, corresponding to the E2-binding sites P0 and P1, appeared to be specifically protected, thus confirming the specific DNA-binding activity of recombinant E2. The overexpressed E2 protein was further purified via heparin-Sepharose chromatography and DNA affinity chromatography. Figure 3B shows a gel retardation experiment performed with aliquots of the different fractions from the DNA affinity column and ^{32}P-labeled oligonucleotide P1. A broad shifted band appeared with fractions eluted by 0.5 to 0.8 M KCl, which could be specifically competed for with an oligonucleotide comprising an E2-binding site (see Fig. 5B). Shorter exposures revealed that the broad band is actually composed of at least four different complexes, which may be explained by the existence of different forms of E2 as shown above. The same complex pattern could be observed with crude nuclear extracts (data not shown), suggesting that they are not due to protein degradation during the purification procedure.

HPV8 E2-binding sites display different affinities for E2. To analyze the E2-binding capacity of the potential E2 cognate sequences ACCN$_6$GGT (P0 to P4), oligonucleotides with one binding site each were used to perform gel retardation experiments. The binding affinities of E2 to the five different sites varied strongly (Fig. 4). The highest-affinity site is P1, followed by P0 and P3, whereas binding to P2 and P4 is very weak. All of these sites are of the ACCGN$_4$(C/T)GGT type, so that flanking sequences or internal nucleotides have to account for the differences in binding. A comparison between the nonconserved core sequences clearly revealed two groups which correlate well with binding affinities: P1, P0, and P3 have only A/T pairs, whereas both P2 and P4 have identical CACC cores,

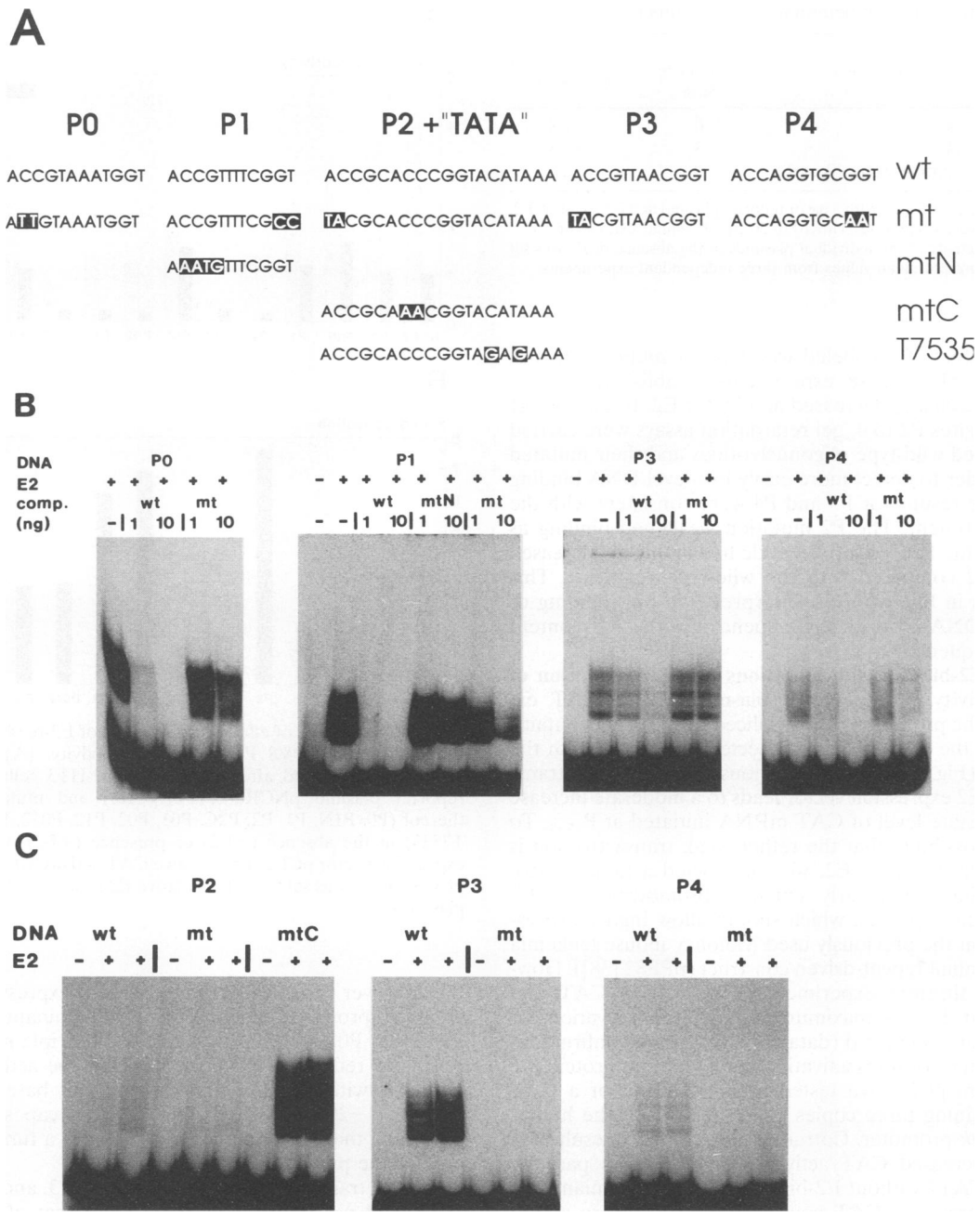

FIG. 5. Analysis of E2 binding to mutated target sequences. (A) Nucleotide sequences of the E2 recognition sites and the P_{7535} TATA box close to P2 are shown at the top (wild type [wt]). The mutated nucleotides are highlighted in black boxes (mt, mtN, mtC, and T7535). (B) Mutations of E2-binding sites P0, P1, P3, and P4 strongly decrease the ability to compete for E2 binding. This was analyzed by adding unlabeled oligonucleotides (1 or 10 ng) that represent individual wild-type (wt) or mutated (mt or mtN) binding sites to the ^{32}P-labeled wild-type oligonucleotide. Control reaction mixtures received no protein or protein and no DNA competitor. (C) Binding of E2 to P2, P3, and P4 or their mutated derivatives was analyzed by incubating different amounts of E2 protein (1 or 2 μl) with aliquots of ^{32}P-labeled wild-type (wt) or mutated (mt or mtC) oligonucleotides.

although in reverse orientations (Fig. 5A). Below we provide further evidence that the number of A/T pairs in the core is critical for the binding of HPV8 E2 to its target sequence.

Analysis of the binding capacity of mutated HPV8 E2-binding sites. To determine the influence of individual E2-binding sites on the regulation of the P_{7535} promoter, the recognition sequences were mutated as shown in Fig. 5A. Two different mutations were designed for the high-affinity site P1 (each destroying one half site) and for the weak binding site P2 (affecting one half site or exchanging two G/C pairs of the core into A/T pairs). Binding sites P0, P1, P3, and P4 and the respective mutants were tested in gel retardation competition experiments. Fixed amounts of labeled wild-type oligonucleotide and affinity-purified E2 protein were incubated with

TABLE 1. Stimulation of heterologous promoters by HPV8 E2[a]

CAT plasmid	Promoter	No. of E2-binding sites	Fold induction by HPV8 E2[b]
pBLCAT2	*tk*	0	1.8
pNRE-3U-tk	*tk*	3	82.3
pRR52	Simian virus 40 early	0	2.5

[a] HT3 cells were transfected with 3 μg of reporter plasmid and 0.5 μg of pCE2, and CAT assays were carried out with 10 to 30 μg of cellular extract for 1 h.
[b] The relative activity of the individual plasmids in the absence of E2 was set at 1. The data represent mean values from three independent experiments.

different amounts of unlabeled wild-type or mutant oligonucleotide (Fig. 5B). These experiments established that all mutants have a clearly decreased affinity for E2. In the case of weak binding sites P2 to 4, gel retardation assays were carried out with labeled wild-type oligonucleotides and their mutated versions in order to detect more easily low-level DNA binding (Fig. 5C). The results for P3 and P4 were consistent with the previous experiment. The P2 mutation weakened binding as expected, but the P2C oligonucleotide had an highly increased affinity for E2 compared with the wild-type sequence. This observation is in line with our interpretation that binding of HPV8 E2 to DNA is drastically influenced by the A/T content of the core sequence.

Effects of E2-binding site mutations on E2 modulation of promoter activity. The reporter plasmid pNCR8-CAT expresses from the promoter P_{7535} a spliced mRNA that contains an exon from the HPV8 NCR connected to an exon with the *cat* gene (38) (Fig. 1). We have previously shown that cotransfection of an E2 expression vector leads to a moderate increase of the steady-state level of CAT mRNA initiated at P_{7535}. To exclude the possibility that the rather weak transactivation is due to low expression of E2, we constructed a human cytomegalovirus immediate-early enhancer/promoter-driven E2 expression vector (pCE2), which should allow higher expression levels than the previously used Moloney mouse leukemia virus long terminal repeat-driven construct (pPF82 [38]). However, several titration experiments with pNCR8-CAT and pCE2 revealed that the maximum level of transactivation did not exceed four- to fivefold (data not shown). To confirm that sufficient amounts of transactivation-competent E2 protein are expressed from pCE2, we tested for stimulation of a CAT plasmid containing three copies of P1 in front of the herpes simplex virus *tk* promoter. Cotransfection of pCE2 resulted in a 82.3-fold increased CAT activity (Table 1). The parental plasmid pBLCAT2 without E2-binding sites and a simian virus 40 enhancer/promoter-CAT construct (pRR52) were stimulated only 1.8- and 2.5-fold, respectively. This result demonstrates that the HPV8 E2 protein is principally a potent binding-site-dependent transactivator with weak nonspecific transactivation properties. The weak transactivation of pNCR8-CAT in spite of five functional E2-binding sites therefore suggests a more complex regulation.

To evaluate the role of individual recognition sequences, the above-described mutated versions of E2-binding sites were introduced alone (P0, P1N, P1, P2, P2C, P3, and P4) or in combination (P01, P02, P12, P012, and P34) into the background of the reporter plasmid pNCR8-CAT. Plasmid pNCR8-CAT and its derivatives were transfected alone or with the E2 expression vector pCE2 into the human cervical carcinoma cell line HT3, and CAT activity was determined (Fig. 6A). The basal activity of P_{7535} was not or only slightly affected by mutations in P0, P1, P3, and P4. Both mutations in

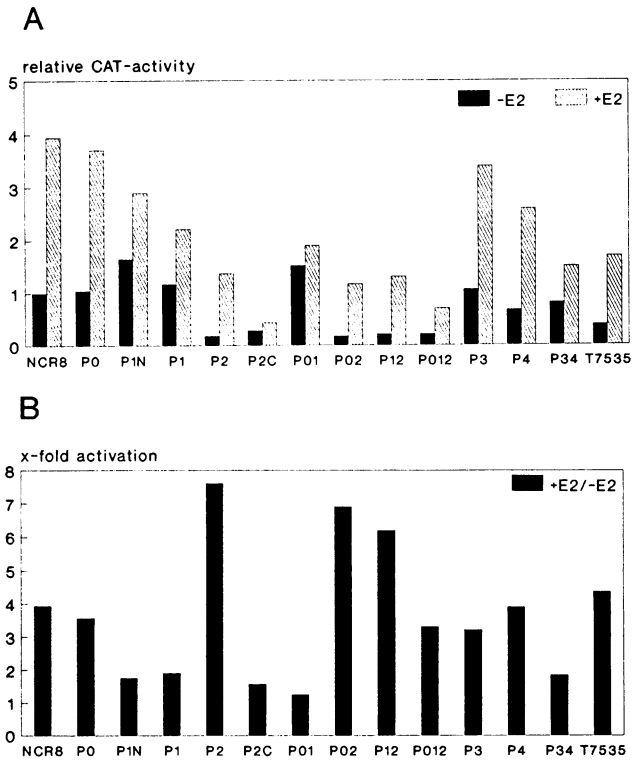

FIG. 6. Analysis of effects of mutations of E2-binding sites and the TATA box on HPV8 P_{7535} promoter activity. (A) Relative CAT activities determined after transfection of HT3 cells with wild-type reporter plasmid pNCR8-CAT (NCR8) and mutated derivatives thereof (P0, P1N, P1, P2, P2C, P01, P02, P12, P012, P3, P4, P34, and T7535) in the absence (−E2) or presence (+E2) of the HPV8 E2 expression vector pCE2. The relative CAT activity after transfection of pNCR8-CAT was set to 1. (B) Relative E2 transactivation of individual plasmids.

P2, however, drastically reduced CAT expression. This decrease of promoter function was also dominant in the double mutations P02 and P12 as well as in the triple mutation P012. A similar reduction (50% of the wild-type activity) could be observed with the T7535 mutation, with base exchanges at positions −29 and −31 relative to the cap site (Fig. 5A), indicating that the sequence CATAAA is a functional TATA box for the promoter.

The E2 transactivation of mutants P0, P3, and P4 or mutant T7535 did not significantly differ from that of pNCR8-CAT (fourfold). Transactivation was diminished to almost the same extent by both mutations in P1 (P1N and P1). Interestingly, the P2 mutant could be more efficiently activated than pNCR8-CAT (eightfold versus fourfold), pointing to a negative effect of E2 mediated by P2. This suggestion could be confirmed by analysis of the P2C mutant, which has a higher E2-binding capacity than the wild-type sequence (Fig. 5C) and allowed almost no E2 transactivation (1.5-fold). The double mutant P34 showed a reduced response to E2 in contrast to the single mutants (P3 and P4), which indicates that these close binding sites can compensate for one another. A similar compensation effect seems to exist between binding sites P0 and P1 when combined with the P2 mutation. Whereas E2 inducibility was only slightly decreased in the cases of mutants P02 and P12, a clear drop from 8- to 3.3-fold transactivation by E2 could be seen with the triple mutant P012.

DISCUSSION

To gain insight into the E2 modulation of the HPV8 P_{7535} promoter activity, we first evaluated the binding affinity of recombinant E2 protein to five target sites. High (P1, P0, and P3) and low (P2 and P4) affinities to E2 are obviously not due to variations of the palindromic target sequence, as P1, P3, and P2 all represent a perfect $ACCGN_4CGGT$. Instead, they seem to correlate with the content of A/T pairs in the nonconserved core. This could be confirmed by exchanging two C/G pairs in the core region of the low-affinity site P2 into A/T pairs, thereby creating a high-affinity site (P2C). The binding requirements for HPV8 E2 are in line with the observation that HPV16 E2 displays a strong preference for A/T-rich cores, whereas the binding affinity of BPV1 E2 is almost independent of the base composition of the core (2, 22). According to the crystallization data from Hedge et al. (14), the four bases of the core in a BPV1 E2/DNA complex are bent toward the minor groove. As protein-induced DNA bending is facilitated by A/T pairs (11), the different binding requirements of HPV and BPV1 E2 proteins might reflect differences in the ability to bend DNA.

The functional relevance of the E2-binding sites P0 to P4 for regulation of the P_{7535} promoter was evaluated by analyzing CAT expression from reporter plasmids with mutated palindromic sites. The two mutations in P2 were the only ones to strongly decrease basal CAT expression, thus defining another essential promoter element besides the TATA box CATAAA, whose mutation reduced promoter activity to 50%. The P2 sequence is highly conserved among EV-associated HPVs (7, 20) and was shown to interact specifically with cellular protein(s) in the case of HPV8 (24). It resembles an Sp1 recognition site, but we could not detect any Sp1 binding in DNase I footprinting experiments (24) or gel retardation assays (data not shown). We previously found evidence for the existence of P2-like binding sites in the vicinity of the late promoters of HPV1, cottontail rabbit papillomavirus, and BPV1 (38). The corresponding BPV1 E2-binding site BS1 is part of the constitutive enhancer and a promoter element of P_{7185} (or P1) and/or the late promoter P_L (37, 44). We suggest that this element and its cognate factor(s) play an important role in the transcriptional regulation of skin-associated papillomaviruses.

E2 displays a general stimulatory effect on a variety of promoters (13), but our mutational analysis confirmed that the moderate fourfold stimulation of P_{7535} by E2 is binding site dependent. Single mutations of P0, P3, or P4 had no effect on transactivation, but mutations in P1 (P1N and P1) reduced stimulation by E2 to 1.6- and 1.9-fold, respectively, which is in line with P1 having the highest affinity for E2 in gel retardation assays. The additional mutation of P0 showed a particularly strong effect in the triple mutant P012, thus suggesting some cooperativity between P0 and P1. This is in line with previous results showing that both sites are constituents of the major E2-dependent enhancer of HPV8 (30). The cooperativity between two E2-binding sites can be explained to some extent by facilitating E2 binding to DNA (19, 26) and mainly by allowing the synergism of two E2 dimers in transactivation (12, 34, 35). The double mutation P34 reduced transactivation less than the P01 double mutation. This finding suggests a minor contribution to transactivation, which correlates with the binding affinities of E2 to the individual sites. Mutations in the weak E2-binding site P2 not only affected the basal activity of P_{7535} but also significantly changed E2 inducibility. The mutant which allowed almost no E2 binding to P2 could be induced eightfold by E2, which is twice as high as the wild-type level. In contrast, creation of a high-affinity E2-binding site at P2 (P2C) blocked transactivation. This inverse correlation between E2 binding to P2 and the extent of transactivation suggests that the P2 site mediates a negative control of P_{7535} by E2. In analogy to BPV1 binding site 1 and the promoter proximal E2-binding sites of HPV11, HPV16, and HPV18, inhibition might be achieved by competition between E2 and promoter recognition factors or TFIID (5, 6, 37, 41). As HPV8 P2 and the TATA box are only 1 bp apart, an interference of E2 and TFIID seems very likely (6).

The different E2 affinities observed in gel retardation assays suggest that P2 would become less often occupied by E2 than P0, P1, and P3. This may explain why E2 has an overall activating effect on P_{7535}. The downmodulation via P2 may be one reason why transactivation does not exceed rather moderate levels of four- to fivefold. In summary, E2 would exert a self-limited transactivation of P_{7535}. A further complication of regulation by E2 may be envisioned by cellular proteins interfering with E2 binding at the high-affinity site P1 (23), which would impede the major positive site. Vice versa, proteins that bind to P2 may counteract the negative role of E2 (24). These multifarious possibilities for regulation could account both for the autoregulation of E2 and the switch to the late productive phase.

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REFERENCES

1. **Ausubel, F. A., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology. Greene Publishing and Wiley-Interscience, New York.
2. **Bedrosian, C. L., and D. Bastia.** 1990. The DNA-binding domain of HPV-16 E2 protein interaction with the viral enhancer: protein-induced bending and role of the nonconserved core sequence in binding site affinity. Virology **174**:567–575.
3. **Bernard, B. A., C. Bailly, M.-C. Lenoir, M. Darmon, F. Thierry, and M. Yaniv.** 1989. The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J. Virol. **63**:4317–4324.
4. **Dollard, S. C., T. R. Broker, and L. T. Chow.** 1993. Regulation of the human papillomavirus type 11 E6 promoter by viral and host transcription factors in primary human keratinocytes. J. Virol. **67**:1721–1726.
5. **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.
6. **Dostatni, N., P. F. Lambert, R. Sousa, J. Ham, P. M. Howley, and M. Yaniv.** 1991. The functional BPV-1 E2 *trans*-activating protein can act as a repressor by preventing formation of the initiation complex. Genes Dev. **5**:1657–1671.
7. **Ensser, A., and H. Pfister.** 1990. Epidermodysplasia verruciformis associated human papillomaviruses present a subgenus-specific organization of the regulatory genome region. Nucleic Acids Res. **11**:3919–3922.
8. **Frech, B., U. Zimber-Strobl, K. O. Suentzenich, O. Pavlish, G. M. Lenoir, G. W. Bornkamm, and N. Müller-Lantzsch.** 1990. Identification of Epstein-Barr virus terminal protein 1 (TP1) in extracts of four lymphoid cell lines, expression in insect cells, and detection of antibodies in human sera. J. Virol. **64**:2759–2767.
9. **Fuchs, P. G., S. Horn, T. Iftner, M. May, F. Stubenrauch, and H. Pfister.** 1993. Molecular biology of epidermodysplasia verruciformis-associated human papillomaviruses. *In* W. Doerfler and P. Böhm (ed.), Virus strategies. Verlag Chemie, Weinheim, Germany.

10. **Fuchs, P. G., J. Weninger, T. Iftner, and H. Pfister.** 1986. Epidermodysplasia verruciformis-associated human papillomavirus type 8: genomic sequence and comparative analysis. J. Virol. **58:**626–634.
11. **Gartenberg, M. R., and D. M. Crothers.** 1988. DNA sequence determinants of CAP-induced bending and protein binding affinity. Nature (London) **333:**824–829.
12. **Gauthier, J.-M., N. Dostatni, M. Lusky, and M. Yaniv.** 1991. Two DNA-bound E2 dimers are required for strong transcriptional activation and for cooperation with cellular factors in most cells. New Biol. **3:**498–509.
13. **Haugen, T. H., L. P. Turek, F. M. Mercurio, T. P. Cripe, B. J. Olson, R. D. Anderson, D. Seidl, M. Karin, and J. T. Schiller.** 1988. Sequence-specific and general transcriptional activation by the bovine papillomavirus-1 E2 *trans*-activator require an N-terminal amphipathic helix-containing E2 domain. EMBO J. **7:**4245–4253.
14. **Hedge, R. S., S. R. Grossman, L. A. Laimins, and P. B. Sigler.** 1992. Crystal structure at 1.7Å of the bovine papillomavirus-1 E2 DNA-binding domain bound to its DNA target. Nature (London) **359:**505–512.
15. **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.
16. **Horn, S., H. Pfister, and P. G. Fuchs.** 1993. Constitutive transcriptional activator of *Epidermodysplasia verruciformis*-associated human papillomavirus 8. Virology **196:**674–681.
17. **Iftner, T.** 1990. Papillomavirus genomes: sequence analysis related to functional aspects, p. 181–202. *In* H. Pfister (ed.), Papillomaviruses and human cancer. CRC Press, Boca Raton, Fla.
18. **Kieff, E., and D. Liebowitz.** 1990. Epstein-Barr virus and its replication. *In* B. N. Fields and D. M. Knipe (ed.), Virology, vol. 2. Raven Press, New York.
19. **Knight, J. D., R. Li, and M. R. Botchan.** 1991. The activation domain of the bovine papillomavirus E2 protein mediates association of DNA-bound dimers to form DNA loops. Proc. Natl. Acad. Sci. USA **88:**3204–3208.
20. **Krubke, J., J. Kraus, H. Delius, L. T. Chow, T. R. Broker, T. Iftner, and H. Pfister.** 1987. Genetic relationship among human papillomaviruses associated with benign and malignant tumours of patients with epidermodysplasia verruciformis. J. Gen. Virol. **68:**3091–3103.
21. **Lambert, P. F.** 1991. Papillomavirus DNA replication. J. Virol. **65:**3417–3420.
22. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. R. 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.
23. **May, M., K. Grassmann, H. Pfister, and P. G. Fuchs.** 1994. Transcriptional silencer of the human papillomavirus type 8 late promoter interacts alternatively with viral E2 or a cellular protein. J. Virol. **68:**3612–3619.
24. **May, M., V. Helbl, H. Pfister, and P. G. Fuchs.** 1991. Unique topography of DNA-protein interactions in the non-coding region of epidermodysplasia verruciformis-associated human papillomaviruses. J. Gen. Virol. **72:**2989–2997.
25. **McBride, A. A., H. Romanczuk, and P. M. Howley.** 1991. The papillomavirus E2 regulatory proteins. J. Biol. Chem. **266:**18411–18414.
26. **Monini, P., S. R. Grossman, B. Pepinsky, E. J. Androphy, and L. A. Laimins.** 1991. Cooperative binding of the E2 protein of bovine papillomavirus to adjacent E2-responsive sequences. J. Virol. **65:**2124–2130.
27. **Nasseri, M., J. R. Gage, A. Lorincz, and F. O. Wettstein.** 1991. Human papillomavirus type 16 immortalized cervical keratinocytes contain transcripts encoding E6, E7 and E2 initiated at the P97 promoter and express high levels of E7. Virology **184:**131–140.
28. **Orth, G.** 1987. Epidermodysplasia verruciformis, p. 199–243. *In* N. P. Salzman and P. M. Howley (ed.), The Papovaviridae, vol. 2. Plenum Publishing Corp., New York.
29. **Pfister, H.** 1984. Biology and biochemistry of papillomaviruses. Rev. Physiol. Biochem. Pharmacol. **99:**111–181.
30. **Reh, H., and H. Pfister.** 1990. Human papillomavirus type 8 contains cis-active positive and negative transcriptional control sequences. J. Gen. Virol. **71:**2457–2462.
31. **Romanczuk, H., and P. M. Howley.** 1992. Disruption of either the *E1* or the *E2* regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. Proc. Natl. Acad. Sci. USA **89:**3159–3163.
32. **Romanczuk, H., F. Thierry, and P. M. Howley.** 1990. Mutational analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. J. Virol. **64:**2849–2859.
33. **Rotenberg, M. O., C.-M. Chiang, M. L. Ho, T. R. Broker, and L. T. Chow.** 1989. Characterization of cDNAs of spliced HPV-11 E2 mRNA and other HPV mRNAs recovered via retrovirus-mediated gene transfer. Virology **172:**468–477.
34. **Sowden, M., S. Harrison, R. Ashfield, A. J. Kingsman, and S. M. Kingsman.** 1989. Multiple cooperative interactions constrain BPV-1 E2 dependent activation of transcription. Nucleic Acids Res. **17:**2959–2972.
35. **Spalholz, B. A., J. C. Byrne, and P. M. Howley.** 1988. Evidence for cooperativity between E2 binding sites in E2 *trans* regulation of bovine papillomavirus type 1. J. Virol. **62:**3143–3150.
36. **Steger, G., M. Olszewsky, E. Stockfleth, and H. Pfister.** 1990. Prevalence of antibodies to human papillomavirus type 8 in human sera. J. Virol. **64:**4399–4406.
37. **Stenlund, A., and M. R. Botchan.** 1990. The E2 *trans*-activator can act as a repressor by interfering with a cellular transcription factor. Genes Dev. **4:**123–136.
38. **Stubenrauch, F., J. Malejczyk, P. G. Fuchs, and H. Pfister.** 1992. Late promoter of human papillomavirus type 8 and its regulation. J. Virol. **66:**3485–3493.
39. **Summers, M. D., and G. E. Smith.** 1987. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agricultural Experiment Station Bulletin no. 1555.
40. **Szymanski, P., and A. Stenlund.** 1991. Regulation of early gene expression from the bovine papillomavirus genome in transiently transfected C127 cells. J. Virol. **65:**5710–5720.
41. **Tan, S.-H., B. Gloss, and H.-U. Bernard.** 1992. During negative regulation of the human papillomavirus-16 E6 promoter, the viral E2 protein can displace Sp1 from a proximal promoter element. Nucleic Acids Res. **20:**251–256.
42. **Thierry, F., and P. M. Howley.** 1991. Functional analysis of E2-mediated repression of the HPV18 P_{105} promoter. New Biol. **3:**90–100.
43. **Tjian, R.** 1981. T antigen binding and the control of SV40 gene expression. Cell **26:**1–2.
44. **Vande Pol, S. B., and P. M. Howley.** 1990. A bovine papillomavirus constitutive enhancer is negatively regulated by the E2 repressor through competitive binding for a cellular factor. J. Virol. **64:** 5420–5429.