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

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The E6 promoters of all genital human papillomaviruses have a characteristic alignment of transcription factor binding sites. Activation of the basic transcription complex at the TATA box depends upon a sequence-aberrant Sp1 site. Repression of E6 promoters is achieved by two binding sites for the viral E2 protein positioned between the Sp1 site and the TATA box. We have purified the human papillomavirus type 16 E2 protein after expression in *Escherichia coli* and studied its binding and repression properties with oligonucleotides representing the homologous promoter sequences. A K_d value of 3×10^{-10} M indicated binding properties expected for a native protein. We found low cooperativity in the binding of two E2 dimers to flanking sites, both when these sites were separated by 3 nucleotides, as in the natural promoter, and when they were further apart. E2 protein, bound close to the distal Sp1 site, displaced the Sp1 factor even when the aberrant sequence was replaced by a typical Sp1 core recognition site. The high affinity of E2 protein for its binding site even led to Sp1 displacement at concentrations of E2 protein nearly 2 orders of magnitude lower than those of Sp1. Functional analyses of mutated E6 promoter sequences showed repression by this distal E2 binding site in the complete absence of binding to the proximal E2 binding site. From our findings and observations published by others, we conclude that each of the E2 binding sites in the E6 promoter of genital human papillomaviruses plays a separate role by displacing the transcription factors Sp1 and TFIID.

It is an idiosyncrasy of papillomaviruses to induce slowly growing or macroscopically stagnant lesions, which permit viral multiplication and persistence for years or even decades. This peculiar form of homeostasis of the infected cell population is achieved through intricate mechanisms of feedback regulation to achieve balanced viral genome copy numbers and levels of transcripts. The products of the E2 gene encoded by papillomavirus genomes are apparently particularly important elements of this strategy.

Most of the research on E2 function has concentrated on bovine papillomavirus type 1 (BPV-1). From its E2 gene, BPV-1 can express a full-length (48-kDa) and two truncated (31- and 28-kDa) proteins. These E2 proteins can bind in the form of dimers to any of 17 sites in a BPV-1 genome, which have the palindromic consensus sequence ACCGN₄CGGT. The full-length E2 protein, which has a DNA binding and a transcription activation domain, turns DNA segments with two of these sites into transcriptional enhancers (35). Either of the two truncated E2 proteins, which lack the transcription activation domain, can compete with the full-length E2 protein for DNA binding or form heterodimers and can thereby annihilate activation (for reviews, see references 14 and 25).

Regulation by E2 proteins may be less complex in human papillomavirus type 16 (HPV-16) and many related HPV types, because these viruses have only four or five E2 binding sites. Two of these sites are close to, and have an influence upon, the function of the E6 promoter, which is instrumental in expression of the transforming genes (9, 10, 33, 38a) and represents a sequence element that is strictly conserved in all genital HPV types, in contrast to dramatic alterations in all

other parts of the viral long control region (5). The other two are located approximately 100 and 500 bp 5' of the E6 promoter. One of these sites is involved in viral replication (6, 32, 37); the other is involved in yet unknown functions, which may include positive modulation of the E6 promoter over large distances (33). Consequently, HPV-16 does not have two flanking E2 binding sites in enhancer configuration, and E2-dependent enhancer activation may not be part of the HPV-16 life cycle, although the HPV-16 E2 protein can transcriptionally activate BPV-1 constructs (30).

The two E2 binding sites at the E6 promoter of HPV-11, HPV-16, and HPV-18 negatively regulate viral gene expression under the influence of the full-length BPV-1 or HPV E2 proteins, as shown in cotransfection experiments (9, 10, 33, 38a). This promoter is activated by an epithelium-specific enhancer (for a review, see reference 2) which is centered 300 bp upstream. Activation of the E6 promoter depends on a Sp1 binding site 32 bp 5' of the TATA box (13). One E2 binding site is 3' from the Sp1 site, and the core binding motif of Sp1, GGGCGT, is separated by 1 bp from that of E2. In most HPVs, the two E2 sites are separated by 3, and in some cases 2 or 4, bp from one another, and the TATA box is positioned another 2 or 3 bp to the 3' side from the second E2 binding site (Fig. 1). In vitro, the heterologous BPV-1 E2 protein can displace the basic transcription factor TFIID (10) and the Sp1 factor (38a) from their binding sites, and either of these mechanisms or the combination of both of them may account for the mechanism of repression.

In this study, we have addressed three questions concerning HPV-16 transcriptional regulation. Firstly, we have asked whether this mechanism can be supported by in vitro experiments with purified HPV-16 E2 protein. An analysis of differ-
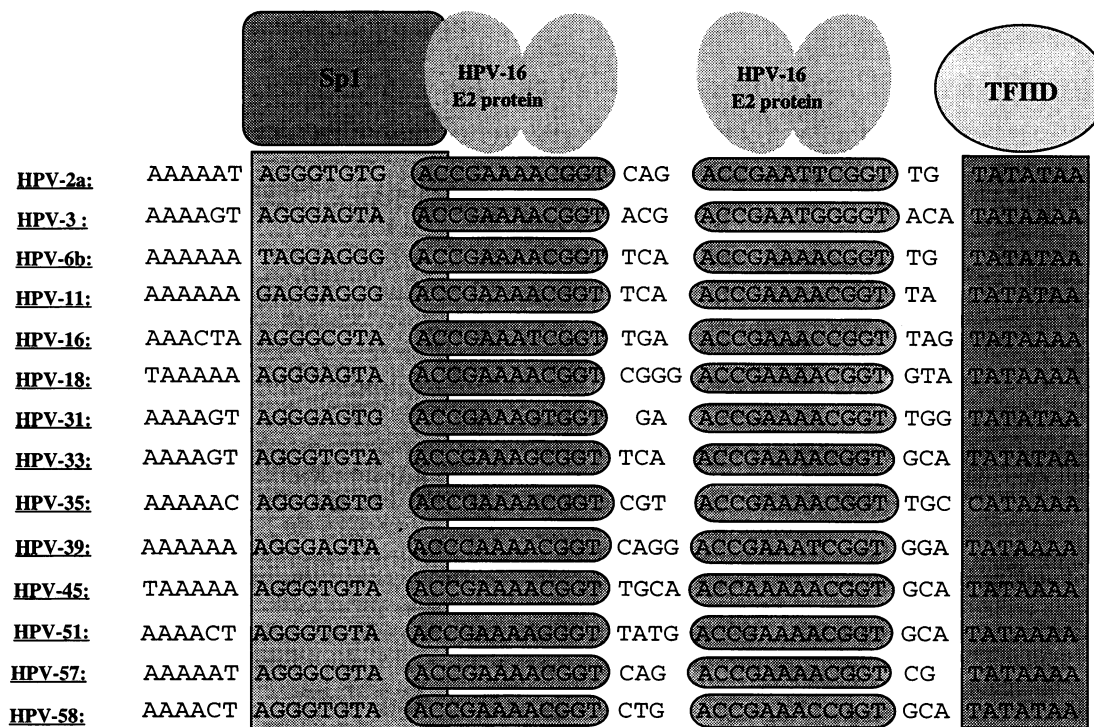
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FIG. 1. Elements of the E6 promoters of 14 HPV types. The Sp1 site has been experimentally determined in the case of HPV-6, -11, -16, and -18 and is inferred in the case of the other viruses. The boundaries of the Sp1 site are indicated according to reference 19 and include 1 nucleotide on the 5' side and 3 nucleotides on the 3' side of the core hexanucleotide, which is GGGCGT in the case of HPV-16.

ing properties was warranted, because previous publications have emphasized the effect of the heterologous BPV-1 E2 protein on HPV promoters. The HPV-16 protein may have functions different from those of the BPV-1 protein, because it has a size of 42 kDa and is thus much smaller than the full-length E2 protein of BPV-1 because of a much shorter hinge region between the DNA binding and the transcription activation domain (25). Secondly, we wanted to find out whether the full-length HPV-16 E2 protein alone is sufficient to bring about negative regulation in HPV-16 in spite of its potential transcription activation function. So far, no truncated E2 protein with an intrinsic repressor function could be found in HPV-16-infected cells, although transcripts that could encode such proteins have been cloned in the form of cDNAs from HPV-11- or HPV-16-containing cells (9, 34) and could be shown to function as negative regulators after expression of these cDNAs (9). Thirdly, we asked whether the highly conserved two E2 binding sites are necessary to achieve repression through cooperative binding of E2 proteins or whether these proteins bind independently, each contributing to repression by a separate mechanism.

MATERIALS AND METHODS

Materials. The expression vector pQE-31 and the nickel-nitrilotriacetic acid (Ni^{2+}-NTA) resin were purchased from Qiagen, Inc.; isopropyl-β-D-thiogalactopyranoside (IPTG), aprotinin, and leupeptin were from Sigma Chemical Co.; Bio-Rad protein assay was from Bio-Rad Laboratories; and the Quick-Silver silver stain kit was from Amersham. The preparation of the Sp1 transcription factor was from Promega.

Bacterial strains and media. *Escherichia coli* JM109 ($RecA^-$) was used for the cloning and propagation of the expression plasmid pQE31-E2. For protein expression, the

bacteria containing the expression plasmid were grown in 2× Luria broth medium supplemented with 1% glycerol, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (pH 7.4), and 150 μg of ampicillin per ml.

Construction of pQE-E2 expression plasmid. The plasmid p859 with the HPV-16 E2 gene (30) was obtained from Peter M. Howley. The HPV-16 E2 gene was amplified from this plasmid by PCR with two oligodeoxynucleotide primers which contained the desired restriction endonuclease sites and the human rhinovirus 14 protease ($3C^{pro}$) site SLETLFQGPL (41). Also, the N-terminal primer placed the E2 gene in the appropriate reading frame with respect to the N-terminal histidine tag (16). The sequence of this primer was 5'-AAATCGGATCCCTCCCTAGAAACACTGTTTCAAGGA CCCCTCATGGAGACTCTTTGCCAACGTTTAAATG TGTGT-3', and it contained a *Bam*HI site and the sequence encoding the $3C^{pro}$ cleavage site. The nucleotide sequence of the C-terminal primer was 5'-AAAGAATTCTCATATAGA-CATAAATCCAG-3', containing an *Eco*RI site. The PCR product was digested with restriction endonucleases *Bam*HI and *Eco*RI, purified by agarose gel electrophoresis, and ligated to the homologous ends of the cleaved pGEM-7Zf(+) (Promega) vector. Recombinant plasmids were verified by DNA sequencing. The E2 gene was excised out of pGEM-7Zf(+) with *Bam*HI and *Sph*I, purified, and cloned into the *Bam*HI- and *Sph*I-cleaved plasmid pQE-31. In this final construct, the E2 open reading frame was preceded by an ATG initiator codon, followed by codons for 6 histidine residues, and by the 36 nucleotides which contain sequences coding for a 2-residue spacer and the decameric $3C^{pro}$ target sequence. After digestion by the protease, the E2 protein would have its natural sequence but be preceded by the three amino acid residues GPL, which remained from the peptide sequence essential for cleavage by $3C^{pro}$.

TABLE 1. Oligonucleotides used in the EMSAs

Oligonucleotide name	Oligonucleotide sequence	Size (bp)
2E2D3	TCGACACCGAAATCGGTTGAACCGAAACCGGTG GTGGCTTTAGCCAACTTGGCTTTGGCCACAGCT	37
e2*E2	GATCCAaaGAAATCttTTGAACCGAAACCGGTG GTttCTTTAGaaAACTTGGCTTTGGCCACAGCT	37
2E2D5	GATCCACCGAAATCGGTtTGAgACCGAAACCGGTG GTGGCTTTAGCCAaACTcTGGCTTTGGCCACAGCT	39
2E2D7	GATCCACCGAAATCGGTctTGAggACCGAAACCGGTG GTGGCTTTAGCCAgaACTccTGGCTTTGGCCACAGCT	41
SP1e2*	CTAAGGGCGTAAaaGAAATCttTTG TCGAGATTCCCGCATTttCTTTAGaaAACAGCT	33
SP12E2	GATCCACTAAGGGCGTAACCGAAATCGGTTGAACCGAAACCGGTG GTGATTCCCGCATTGGCTTTAGCCAACTTGGCTTTGGCCACAGCT	49
ConsSP12E2	GATCCACTAAGGGCGgAACCGAAATCGGTTGAACCGAAACCGGTG GTGATTCCCGCcTTGGCTTTAGCCAACTTGGCTTTGGCCACAGCT	49

Expression of recombinant HPV-16 E2 protein. For expression of the fusion protein, 10 ml of an overnight culture was used to inoculate 1 liter of 2× Luria broth. The culture was incubated at 25°C until it reached an optical density of $A_{600} = 0.6$, when IPTG was added to a final concentration of 0.5 mM to induce the T5 promoter. To study the time course of protein expression after induction, 1-ml aliquots of the culture were collected at 0, 1, 2, 3, 4, and 5 h following IPTG induction, centrifuged, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to monitor the expression of the recombinant proteins. In these and other SDS-PAGE experiments, polypeptide bands were detected by staining with either Coomassie brilliant blue or Quick-Silver stain kit.

Purification of the recombinant His-E2 protein. The remaining 1 liter of the induced culture was harvested by centrifugation, and the pellet was resuspended in 25 ml of lysis buffer (20 mM HEPES [pH 7.2], 100 mM KCl, 5 mM MgCl₂, 0.5% Triton X-100, 0.1% Nonidet P-40, 100 U of DNase I per ml, 1 mM phenylmethylsulfonyl fluoride, 1% [vol/vol] aprotinin, 5 μg of leupeptin per ml, and 1 mg of lysozyme per ml) and lysed by sonication. NaCl and imidazole (Sigma) were added to a final concentration of 500 mM and 20 mM, respectively. After centrifugation of the lysate, the supernatant was passed twice over a Ni²⁺-NTA (Qiagen) resin column (2.0-ml bed volume) that had been equilibrated with phosphate-buffered saline (PBS) containing 1 mM dithiothreitol (DTT) and 20 mM imidazole. The column was washed three times with 20 ml of PBS (500 mM NaCl) containing increasing amounts of imidazole (30, 50, and 80 mM, respectively) to remove the nonspecifically bound proteins. The recombinant fusion protein was eluted with 5 ml of PBS containing 120, 150, 180, and 200 mM imidazole and then washed another three times with 1 ml of PBS. All steps of the protein purification were performed at 4°C.

Cleavage of N-terminal oligohistidine with 3C^Pro. The fusion protein was dialyzed extensively against 50 mM Tris (pH 8.0)–150 mM NaCl–15% glycerol–1 mM DTT. Human rhinovirus 3C protease was then added in the form of a glutathione-S-transferase recombinant protein (GST-3C^Pro) (41) to the purified E2 fusion protein and incubated overnight at 4°C, and pure E2 protein was recovered by passing the mixture sequentially over a Ni²⁺-NTA column and then a glutathione-Sepharose (Pharmacia) column. Microsequencing confirmed that the E2 protein had the expected amino terminus. Protein concentrations were determined with the Bio-Rad protein assay reagent.

Immunoblot analysis. For immunoblots, proteins were electroblotted onto nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) and blocked with PBS with 5% milk powder. The membrane was incubated further in the same buffer containing 0.05% Tween 20 and polyclonal antipeptide antiserum to the HPV-16 E2 protein (at a dilution of 1:500), was washed, and was added to PBS containing ¹²⁵I-protein A (0.1 μCi/ml). After further washing, the filter was autoradiographed. The antipeptide antiserum was obtained by subcutaneous immunization of rabbits with 300 μg of synthetic peptide per ml representing an antigenic domain of the protein (11), QRPRSEPDTGNPC-NH₂, coupled to keyhole limpet hemocyanin (Multiple Peptide Systems, San Diego, Calif.), and emulsified in Freund's complete adjuvant.

Electrophoretic mobility shift assays (EMSAs). Oligonucleotides for the gel shift assay were designed to have 5′ BamHI and SalI overhanging ends to permit cloning and labelling with [α-³²P]dCTP and [α-³²P]dATP. The labelled probes were purified by 12% native PAGE and eluted into TE buffer (10 mM Tris-HCl, 1 mM EDTA) by isotachophoresis. The oligonucleotide sequences are shown in Tables 1 and 2.

Protein extracts were diluted in 20 mM HEPES-KOH (pH 7.9)–20 mM KCl–2 mM MgCl₂–0.2 mM EDTA–1 mM DTT–1

TABLE 2. Promoter and promoter mutant sequences tested in OVEC and CAT vectors

Construct	Sequence			
	Sp1	E2	E2	TATA
SVeHPVp	TATAAAACTAA**GGGCG**TA**ACC**GAAATC**GG**TTGA**ACC**GAAACC**GG**TTAG**TATAAAA**GCAGACATT			
SV3	- **AA**- - - - - - - - - - - - -			
SV5	- - - - - - - - - - - - - - - - **AA**- -			
SV6	- - - - - - - - - - - - - - **AA**- - - - - - - - - **AA**- - - - - - - - - - - -			
SVeHPVp2u*	- - - - - - - **AAAT**- -			

mM phenylmethylsulfonyl fluoride–20% glycerol and mixed with 0.1 pmol of labelled DNA in a total volume of 20 μl containing 6.6 mM HEPES-KOH (pH 7.9), 110 mM KCl, 5 mM spermidine, 0.5 mM MgCl$_2$, 2 mM DTT, 0.1% Nonidet P-40, 0.25 mM phenylmethylsulfonyl fluoride, 0.5 mg of bovine serum albumin (BSA) per ml, and 0.1 mg of poly(dI-dC). After incubation for 30 min on ice, the samples were run on a 5% native polyacrylamide gel buffered with 50 mM Tris–38 mM glycine–2 mM EDTA at 150 V for 2.5 h.

Quantitative analysis. EMSAs were densitometrically analyzed by the integration volume function of the ImageQuant software of the PhosphorImager by applying principles similar to those published for quantification of footprint titration autoradiograms (3, 4, 22). We determined the binding constants and the cooperativity constant through our experiments with equations similar to those previously published in the study of BPV-1 E2 and Oct-2 (22, 28) by quantifying the fraction of free and singly and doubly liganded oligonucleotides for each protein concentration.

In vitro transcription. For in vitro transcription, we used published constructs (38a) based on OVEC cloning vectors (42) that had been previously examined in transfection experiments. These vectors had been termed SVeHPVp-, SV3-, SV5-, or SV6-OVEC and contained, under the influence of the simian virus 40 enhancer, the HPV-16 promoter with natural E2 binding sites, or mutations in the promoter proximal, or promoter distal, or both sites, respectively. They permit the analysis of beta-globin transcripts synthesized from the natural or mutated HPV-16 promoter sequences. These vectors had been designed such that they gave an RNA signal of 179 nucleotides, while a reference vector, OVEC-Ref, provided an endogenous control RNA signal of 160 nucleotides. Vectors for the generation of antisense RNA by SP6 polymerase and processing of the samples have been described previously (38a, 42).

Assays were carried out in a volume of 20 μl of 10 mM HEPES-KOH (pH 7.9)–20 mM KCl–4 mM MgCl$_2$–0.1 mM EDTA–0.25 mM DTT–4 mM spermidine–4 mM phosphocreatine–4 mg of BSA per ml–20 nM E2 dimers–5 mM each ATP, UTP, CTP, and GTP–60 ng of OVEC-Ref vector per ml–50 ng of SVeHPVp-, SV3-, SV5-, or SV6-OVEC vectors per ml (38a) and 8 μl (12 mg/ml) of HeLa cell nuclear extracts (8) as a source of general transcription factors. The HPV-16 E2 proteins were incubated with DNA on ice before addition of nuclear extract. Transcription was started by the addition of nucleotides, followed by incubation at 30°C for 30 min. The reactions were stopped by addition of 20 μl of TE buffer containing 1 μg of tRNA, 25 Units of DNase I per ml, and 1 U of RNase inhibitor per ml (Boehringer), followed by the subsequent incubation at 25°C for 10 min and addition of 160 μl of TE buffer. After extraction with phenol-chloroform, precipitation, and washing with ethanol, the samples were resuspended, treated with DNase I, extracted again with phenol-chloroform, and precipitated with ethanol. The RNA was resuspended in 10 μl of H$_2$O and hybridized to [α-^{32}P]NTP-labelled SP6 polymerase-synthesized probe, which covered the sequence of OVEC1 from position −37 to +179 (38a, 42). The hybridized probes were separated on a 6% urea-polyacrylamide gel. The gel was dried and exposed to PhosphorImager screen, and densitometric analysis of transcription products was performed on the PhosphorImager with the integration volume function of the ImageQuant software, which calculated the ratios between the specific and the control transcripts.

Examination of the E6 promoter in vivo. For examination in vivo, sequences containing the tandem simian virus 40 72-bp

enhancer and the HPV-16 promoter were excised from OVEC plasmids by cleavage with PstI and inserted into the promoterless vector pBLCAT3ΔHN (38) to generate pSVeHPVp-CAT, pSV3CAT, pSV5CAT, pSV6CAT, and pSVeHPV2U* CAT. The HPV-16 E2 protein was expressed from pXJ-41-E2, which was constructed by cleaving the E2 gene with HindIII and EcoRI out of p859 (30) and insertion in the form of a HindIII-to-XhoI fragment into pXJ-41 (44) after alteration of the 3′ EcoRI site. In this vector, the E2 gene is placed under the control of a strong cytomegalovirus promoter. pBLCAT2-4E2tkCAT has been described previously (38a). HeLa cells were electroporated as described previously (13) with 10 μg of the chloramphenicol acetyltransferase reporter plasmids and 5 μg of the plasmid pXJ-41 with or without the E2 gene as well as 2 μg of the plasmid pCMV-βGAL as an internal control of transcription efficiency. Chloramphenicol acetyltransferase assays were done by standard procedures with 50 to 100 μg of total protein per assay.

RESULTS

Purification and properties of the HPV-16 E2 protein expressed in *E. coli*. The HPV-16 E2 gene was inserted into the vector pQE-31, expressed in *E. coli*, and purified as described above. After the final purification steps, a silver stain gel revealed only a single band in a denaturing polyacrylamide gel running at a position of 42 kDa (Fig. 2). This protein preparation was estimated to be at least 95% pure. Western blotting (immunoblotting) with antiserum against the HPV-16 E2 protein confirmed the identity of the protein (Fig. 2C).

HPV-16 E2 protein binds in the form of one or two dimers to oligonucleotides containing two E2 sites. Papillomavirus E2 proteins have an N-terminal transcription activation domain which is linked by a hinge domain to the C-terminal DNA binding domain. The protein binds DNA in the form of a dimer, and the amino acids involved in dimerization overlap with the DNA binding domain (for reviews, see references 14 and 25). E2 protein exists as a dimer in solution (31), and upon DNA binding, each monomer contacts one ACCG half-palindrome (15).

The sequences of the E6/E7 promoters of all genital HPVs are highly conserved and contain two palindromic E2 binding sites which are normally spaced by 3, or occasionally by 2 or 4, bp (Fig. 1). We speculated that this short distance either could be a prerequisite for the mutual exclusion of two E2 protein dimers from concomitant binding to both sites or, alternatively, could permit cooperative interaction between two dimers to facilitate concomitant binding to both sites.

With an oligonucleotide representing the natural alignment of two E2 binding sites in the HPV-16 promoter (oligonucleotide 2E2 [Table 1]), the HPV-16 E2 protein preparation formed complexes consisting of one or two E2 dimers. The specificity and identity of these complexes were confirmed again by competition experiments and by the generation of a supershift with antiserum raised against HPV-16 E2 protein (Fig. 3).

Weak cooperative binding of E2 proteins to adjacent sites. An initial titration with a fixed amount of protein added to increasing amounts of oligonucleotide indicated that approximately 50% of the E2 protein was in native form and capable of DNA binding. In subsequent titrations, this correction was taken into account. During these quantitative experiments, the assumption was made that a gel shift assay accurately reports the fraction of molecules in the free, singly bound, and doubly bound state.

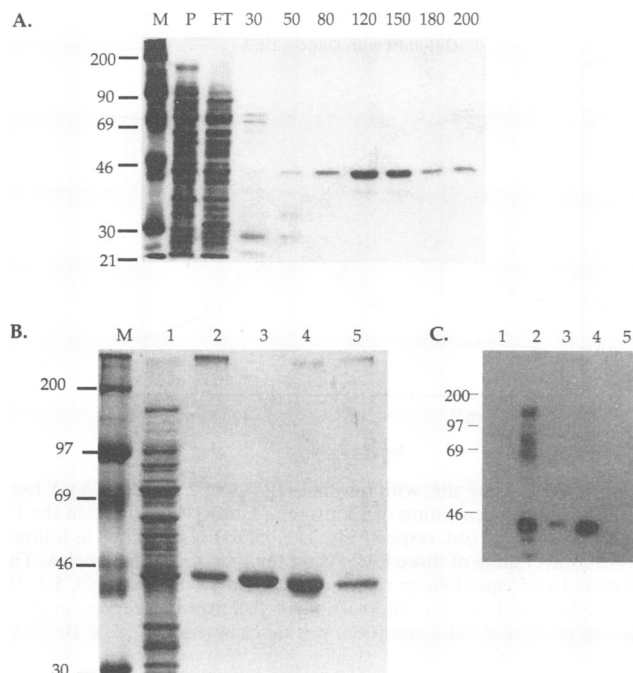Three different titrations of oligonucleotide e2*E2 at a

FIG. 2. Purification of HPV-16 E2 protein after expression in *E. coli* with a polyhistidine N terminus. (A) Purification of HPV-16 E2 protein with an N-terminal hexahistidine by using a Ni^{2+}-NTA affinity column. M, molecular weight marker (numbers at left are in thousands); P, crude extract of induced JM109 transformed with pQE-31-E2; FT, flowthrough fractions; 30, 50, 80, 120, 150, 180, and 200, fractions eluted with PBS buffer containing 30, 50, 80, 120, 150, 180, and 200 mM imidazole. The latter four fractions were pooled for further purification. (B) Proteolytic cleavage of the hexahistidine terminus from the HPV-16 E2 protein. Lane 1, crude bacterial lysate; lane 2, purified HPV-16 hexahistidine E2 fusion protein; lane 3, purified human rhinovirus 3C^{pro} protease (glutathione-*S*-transferase fusion protein) (in the cleavage reaction, the protease was applied at a concentration 100-fold lower than that of the E2 protein); lane 4, aliquot of the cleavage reaction of the HPV-16 E2 fusion protein with GST-3C^{pro}; lane 5, cleaved HPV-16 E2 protein after separation from the protease and the N-terminal peptide; lane M, as for panel A. (C) Western blot processed with antiserum raised against a peptide from HPV-16 E2 protein. Lane 1, lysate of induced JM109 transformed with pQE-31; lane 2, lysate of induced JM109 transformed with pQE-31-E2; lane 3, purified HPV-16 E2 protein with N-terminal hexahistidine; lane 4, cleaved HPV-16 E2 protein after purification; lane 5, human rhinovirus 3C^{pro}. Numbers at left are molecular weights in thousands.

concentration of 0.1 pM against this E2 protein preparation at concentrations ranging from 3 pM to 3 nM gave an average K_d value of 3×10^{-10} M (Fig. 4). This value approximates that determined for the BPV-1 E2 protein (27, 28).

DNA segments with two E2 binding sites provide a strong synergistic enhancement when activated by the large BPV-1 E2 protein, although binding studies involving two E2 dimers have identified only a low or moderate cooperativity in the binding reaction (12, 20, 21, 28). In our initial studies of HPV-16 E2 proteins binding to an oligonucleotide with two palindromic E2 binding sites (Fig. 3), we observed that complexes with one or two E2 dimers formed with approximately similar efficiencies. This observation suggested that the binding of the first dimer does not significantly increase the propensity for the binding of a second dimer. Starting with this observation, we decided to examine the potential for cooperative binding of two HPV-16 E2 dimers in a systematic way. As an additional variable, this study aimed to alter the natural promoter se-
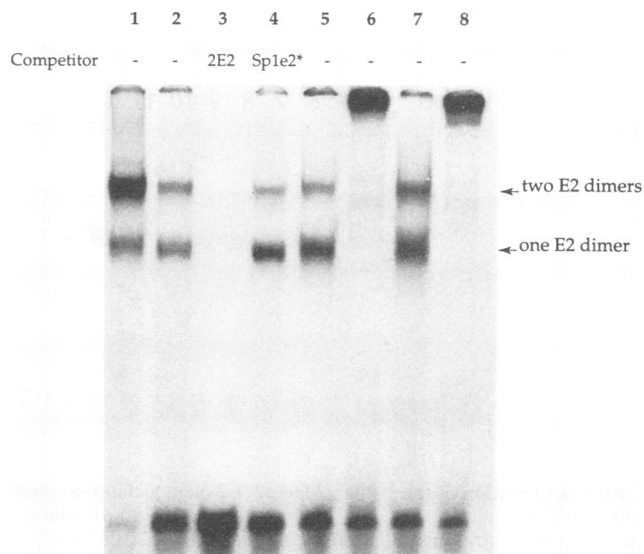

FIG. 3. EMSA with oligonucleotide 2E2D3, which carries the two E2 binding sites of the E6 promoter of HPV-16 separated by 3 nucleotides. Lane 1, bandshift with the HPV-16 E2 protein before cleavage of the hexahistidine N terminus; Lanes 2 to 8 contain the purified HPV-16 E2 protein free of this N-terminal leader; lane 3, competition with homologous oligonucleotide; lane 4, competition with the oligonucleotide Sp1e2*, which has point mutations in its only E2 binding site; lane 5, addition of preimmune serum before addition of probe; lane 6, supershift resulting from the addition of anti-E2 antiserum before addition of probe; lane 7, addition of preimmune serum after addition of probe; lane 8, supershift from the addition of anti-E2 antiserum after addition of probe.

quences of HPV-16, which exhibit a spacing of the two E2 binding sites by 3 bp, to address the question of whether or not conservation of this sequence reflects a distance dependence of cooperativity. Toward this objective, we used the oligonucleotides 2E2D3, 2E2D5, and 2E2D7 with a spacing of 3, 5, and 7 bp between the two E2 binding site palindromes, respectively.

Figure 5 shows the data of bandshift experiments with the oligonucleotides 2E2D3, 2E2D5, and 2E2D7 with purified HPV-16 E2 protein together with the quantitative analyses. The data were analyzed with the assumption that the binding constants describing the interaction of the E2 dimer with either of its binding sites in the oligonucleotide are identical. This assumption allows the determination of the binding and the cooperativity constants from the graphs of the titration curves. The curves predicted by the best-fitting binding constants (Table 3) agree with the datum points over an entire range of titration (Fig. 5). This suggests that the model (28) is used adequately to describe the results and to calculate cooperativity constants of 1.7, 2.0, and 2.9 for the three oligonucleotides 2E2D3, 2E2D5, and 2E2D7 (data not shown). We conclude from these values that there is only minimal cooperativity in the binding between two E2 dimers, which slightly increases as one expands the natural distance between the E2 binding sites.

HPV-16 E2 protein can displace Sp1 from adjacent sequence-aberrant as well as consensus Sp1 binding sites. We have previously published data to show that the E6 promoter of HPV-16, and probably all genital HPVs, contains a sequence-aberrant Sp1 binding site. Sp1 bound to this site is important in the activation of the E6 promoter in vivo and in vitro (13, 38a), and in the natural genomic environment it is apparently mediating the activation by the epithelium-specific enhancer of HPV-16, a finding that has recently been extended
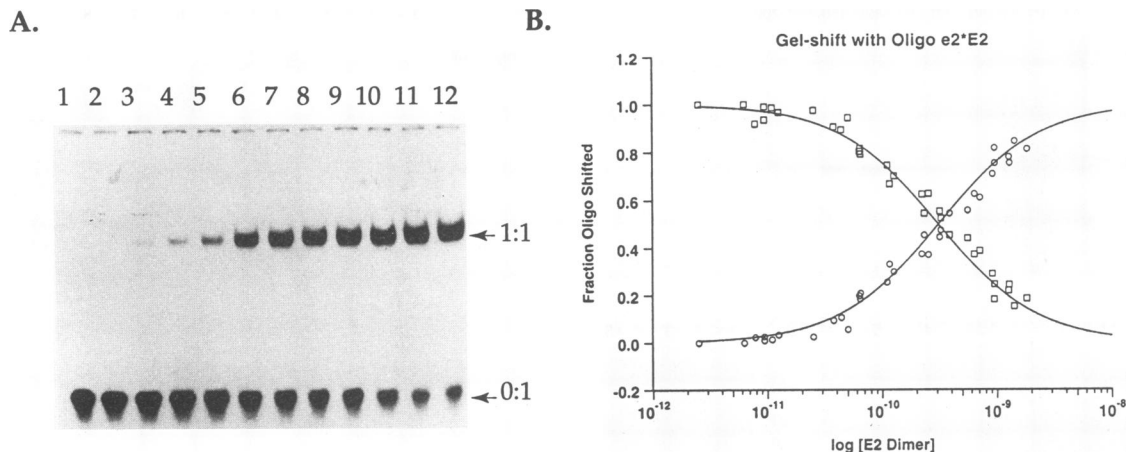
FIG. 4. Titration of the ^{32}P-label led e2*E2 oligonucleotide, containing a single E2 binding site, with purified HPV-16 E2 protein. (A) A fixed amount of oligonucleotide (10^{-11} M) was titrated in a 20-μl volume with an increasing concentration of E2 protein; lanes 1 to 12 contain the E2 protein at concentrations of 0, 0.003, 0.006, 0.012, 0.025, 0.062, 0.125, 0.25, 0.5, 1.0, 2.0, and 3.0 nM, respectively. The bands are identified as follows: 0:1, free probe; 1:1, E2 dimer-oligonucleotide complex. (B) Quantitative analysis by averaging of three EMSAs o f the type shown in panel A. The best fit was determined by the Fig.P software from Biosoft according to the model and equations in references 22 and 28. Symbols: O, 1:1, E2 dimer-oligonucleotide complex; □, free probe. The binding constant, k_1, was obtained from the intersection of the titration curves, and the dissociation constant, K_d ($= 1/k_1$), which indicates the concentration of the active protein at half-saturation, was determined to be 3×10^{-10} M.

to the homologous elements of HPV-11 and HPV-18 (9, 18). The strict conservation of the spacing of the Sp1 core binding site, which in HPV-16 has the sequence GGGCGT, by 1 bp from the palindromic E2 binding site (Fig. 1) suggests a highly specific mechanism for the interaction between Sp1 and E2 proteins at this site.

We had previously made the observation that the addition of in vitro-translated BPV-1 E2 proteins to nuclear extracts can reduce the binding of Sp1 to the HPV-16 promoter sequences. Therefore, we examined whether titration of the HPV-16 E2 protein against Sp1 can alter bandshifts in a similar manner with oligonucleotides of HPV-16 promoter sequences. Figure 6 shows that increasing amounts of HPV-16 E2 protein first weaken and then eliminate a bandshift obtained with Sp1 protein, while high concentrations of Sp1 protein can only reduce, not eliminate, DNA-bound HPV-16 E2 protein.

A partial quantification of the experiments in Fig. 6 was possible because the HPV-16 E2, as well as the Sp1 preparations, was highly purified and the protein concentrations of both preparations were known. The binding reaction applied to slot 3 in Fig. 6A, which shows displacement of most Sp1 factor from DNA, contained E2 protein at a concentration of 0.25 nM, and 30 nM Sp1 protein, with the oligonucleotide at a concentration of 40 pM. These observations suggest that repression by HPV-16 E2 is a very sensitive process because of an affinity of E2 for its binding site higher than that of Sp1. We believe that our experiments reflect a behavior of these factors close to the situation in vivo as both of these protein preparations were nearly pure, with a major part in an active conformation, as confirmed by our binding studies for the E2 protein and as suggested by the affinity purification of Sp1. It is not known, however, how close the buffer conditions in vitro mimic the cellular environment of these transcription factors in vivo.

High-affinity Sp1 binding sites contain the core consensus sequence GGGCGG, and the affinity of Sp1 is reduced by alteration of this sequence (19). In each of the E6/E7 promoter sequences of genital papillomaviruses (Fig. 1 and our unpublished observation), this sequence deviates by at least 1 nucleotide. One may therefore hypothesize that the biology of genital HPV types selects for sequences that provide Sp1

binding sites with low affinity. To examine this possibility, we have carried out bandshift experiments with HPV-16 promoter sequences, whose Sp1 binding sites were altered from GGGCGT to GGGCGG. Rather than drastic differences between these two sequences being observed, Fig. 6B shows that only slightly higher amounts of HPV-16 E2 protein are needed for the displacement of Sp1 from consensus sites. It must be pointed out, however, that nucleotides outside the 6-bp core sequence can influence the affinity of Sp1 (19).

The E2 binding site adjacent to the Sp1 binding site participates in transcriptional repression of the HPV-16 E6 promoter in vitro and in vivo. Transfection studies of the E6 promoters of HPV-16 and HPV-18 have pointed to the requirement of both E2 binding sites for repression by the heterologous BPV-1 E2 proteins (10, 33). In principle, this could be explained by either of two mechanisms. Both E2 dimers may bind, but only one of them may have a repression function, the other facilitating the binding of this dimer, or alternatively, both DNA-bound E2 dimers may exert a repression function, either by the displacement of cellular transcription factors from their DNA binding sites or through some other mechanism.

Figure 7 shows an analysis of in vitro transcription from HPV-16 promoter sequences cloned into OVEC vectors. These vectors permit the monitoring of a beta-globin transcript synthesized from HPV-16 promoters which contain either the natural or mutated E2 binding sites. The vector OVEC-Ref provides an endogenous control for each reaction and monitors the synthesis of a homologous transcript, independent of E2 binding sites.

Figure 7B shows 57% repression of promoter activity when the distal E2 site is unaltered and the proximal E2 site is mutated, a situation which correlates mechanistically with displacement of Sp1 but binding of TFIID (lanes 3 and 4). The alternative mechanism, displacement of TFIID (10), which correlates with alteration only of the distal E2 site, leads to 80% repression (lanes 5 and 6). The combination of both mechanisms reduces promoter activity by 87% (lanes 1 and 2). Our data provide evidence that each of the two E2 binding sites achieves partial repression in the presence of the HPV-16
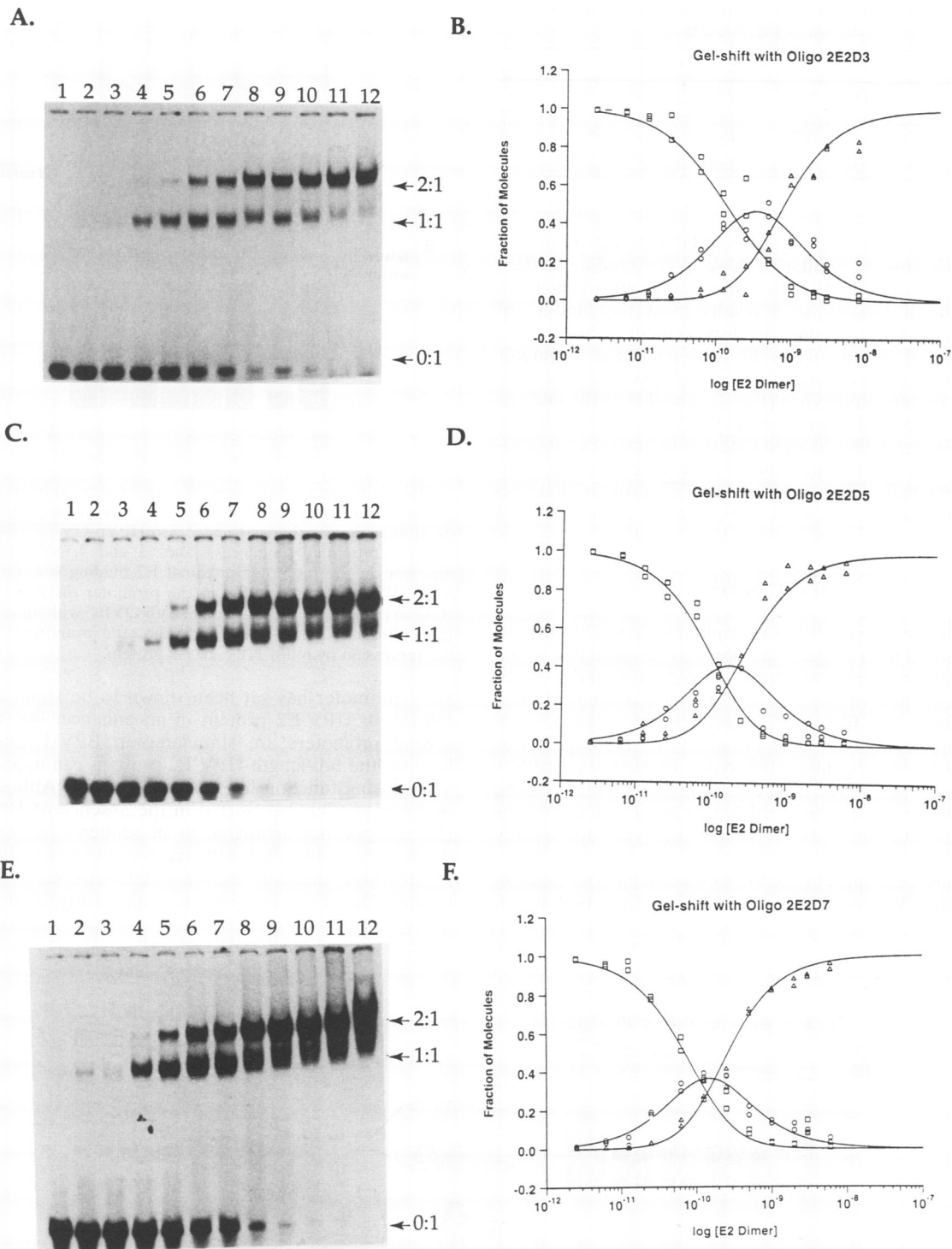
FIG. 5. Titration of the ^{32}P-labelled 2E2D3, 2E2D5, and 2E2D7 oligonucleotides containing two E2 binding sites, spaced 3, 5, and 7 bp apart, respectively, with an increasing amount of HPV-16 E2 protein (A, C, and E). A fixed amount of oligonucleotide (10^{-11} M) was titrated in a 20-μl volume against an increasing concentration of E2 protein dimers; lanes 1 to 12 contain the E2 protein at concentrations of 0, 0.006, 0.012, 0.025, 0.062, 0.125, 0.25, 0.5, 1.0, 2.0, 3.0, and 6 nM, respectively. The bands are identified as follows: 0:1, free probe; 1:1, E2 dimer-oligonucleotide complex; 2:1, complexes with two E2 dimers bound (B, D, and F). Quantitative analysis of two sets of data for the fraction of free and singly and doubly bound molecules for each of those represented in panels A, C, and E was best fitted by the Fig.P software of Biosoft to the model and equations in references 22 and 28. Symbols: □, free probe; ○, 1:1, complexes with one E2 dimer; △, complexes with two E2 dimers. The cooperativity constants, k_{12}, of the binding of two HPV-16 E2 protein dimers are tabulated in Table 3.

TABLE 3. Binding constants (k_1), dissociation constants (K_d), and cooperativity constants (k_{12}) as determined from the titrations shown in Fig. 5 and 6

Probe	k_1 (M^{-1})	K_d (M)	k_{12}
2e2*E2	$(3.3 \pm 0.6) \times 10^9$	$(3.0 \pm 0.2) \times 10^{-10}$	
2E2D3	$(2.2 \pm 0.6) \times 10^9$	$(4.5 \pm 0.2) \times 10^{-10}$	1.7
2E2D5	$(4.0 \pm 0.1) \times 10^9$	$(2.5 \pm 0.1) \times 10^{-10}$	2.0
2E2D7	$(3.9 \pm 0.1) \times 10^9$	$(2.6 \pm 0.1) \times 10^{-10}$	2.9

E2 protein. Since the concentrations of HPV-16 E2 proteins in these experiments are similar to those in our binding studies, we conclude that the partial repression that occurs through the promoter distal binding sites results from the binding of HPV-16 E2 and the concomitant displacement of Sp1.

When tested in vivo (Fig. 8), the particular promoter mutations that we had examined behaved quantitatively similarly to the repressions in vitro (Fig. 7) as well as to the findings of previous studies of repression in vivo (9, 10, 33, 38a). These similarities suggest that in vitro data are indicative of mechanisms operative in vivo and that the presence of three additional N-terminal amino acids in the E2 protein that was examined in vitro is unlikely to have affected the behavior of this factor.

DISCUSSION

Research into the properties of papillomavirus E2 transcription factors and their target sequences has revealed one of the most intricate transcriptional feedback systems found so far in eukaryotes or their viruses. Most efforts have concentrated on the analysis of the BPV-1 E2 system, which is particularly complex, because the genome contains 17 E2 binding sites as opposed to 4 in most genital HPV types. Furthermore, BPV-1 gives rise to at least three different E2 proteins with opposing functions, while the question of whether or not shorter forms of HPV E2 proteins exist in vivo is not yet satisfactorily resolved.

It does not seem appropriate to extend observations made in the study of BPV-1 to HPV gene regulation, firstly because no
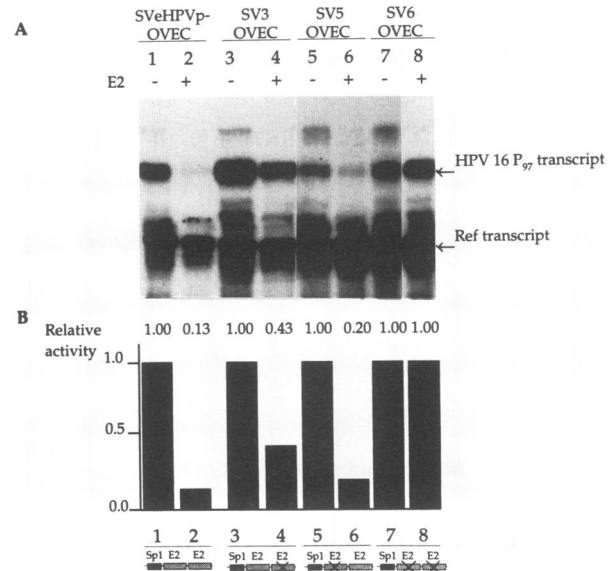
FIG. 7. Repression of the HPV-16 E6 promoter by the HPV-16 E2 protein during transcription in vitro. (A) RNase protection; (B) densitometric scan. Lanes 1 and 2, SVeHPVp-OVEC with the natural HPV-16 promoter sequences; lanes 3 and 4, SV3-OVEC with a mutation in the promoter proximal E2 binding site; lanes 5 and 6, SV5-OVEC with a mutation in the promoter distal E2 binding site close to the Sp1 site; lanes 7 and 8, SV6-OVEC with mutations in both E2 binding sites. Lanes 2, 4, 6, and 8 show the promoter function under the repression by 6 nM HPV-16 E2 protein.

HPV promoter has yet been shown to be stimulated by the full-length HPV-E2 protein in an enhancer-like manner, as several promoters are stimulated in BPV-1, and secondly because the full-length HPV E2 proteins can apparently have a repressing function for the E6 promoter. Although repression can possibly be exerted in the absence of DNA binding (1), the peculiar arrangement of sequence elements at the promoter of all genital HPV types suggests a highly sterically specific mechanism at the level of DNA-bound transcription factors. Part of this mechanism is apparently the displacement
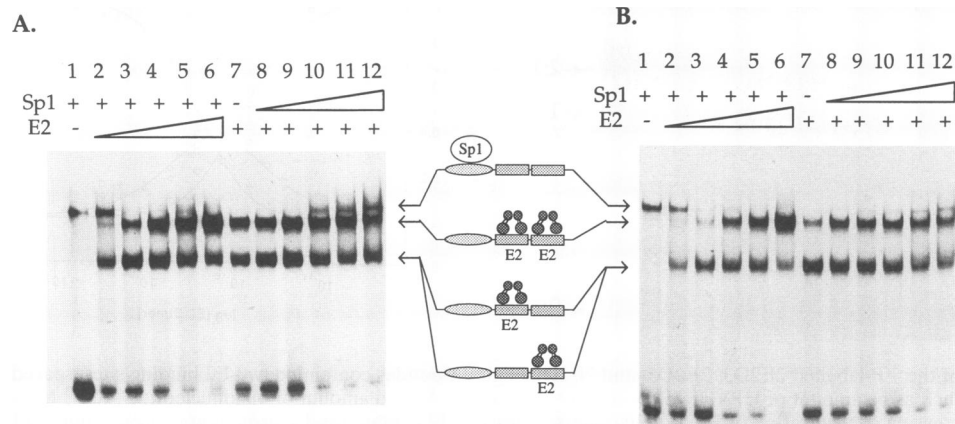
FIG. 6. EMSA to examine the mutual exclusion of Sp1 and E2 protein from overlapping binding sites in the HPV-16 E6 promoter with oligonucleotides containing one Sp1 and two E2 sites. (A) Sp12E2 oligonucleotide with the natural aberrant Sp1 sequence of HPV-16. In lanes 1 to 6, the Sp1 concentration is 30 nM, and the E2 protein concentration rises from 0.125 nM (lane 2) to 2.0 nM (lane 6). In lanes 7 to 12, the E2 protein is held constant at 0.5 nM, and the Sp1 protein concentration rises from 3.75 nM (lane 8) to 45 nM (lane 12). (B) As in panel A but with an oligonucleotide (ConsSp12E2) representing the HPV-16 E6 promoter sequences but having the core of the Sp1 binding site replaced by the consensus sequence GGGCGG instead of GGGCGT. All binding reactions are as in panel A.
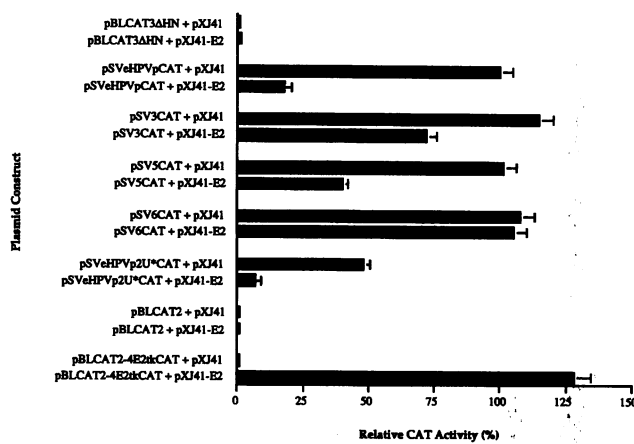
FIG. 8. Repression of the HPV-16 E6 promoter by the HPV-16 E2 protein in vivo. pSVeHPVpCAT documents repression of the wild-type promoter sequences, and pSV3CAT, pSV5CAT, and pSV6CAT show partial repression or absence of repression after alteration of the distal, the proximal, or both E2 sites, respectively. In the absence of SP1 binding (pSVeHPVp2U*CAT), there is strongly reduced promoter activity, which is further negatively affected by E2 protein. The construct pBLCAT2-4E2tkCAT shows the strong activation potential of HPV-16 E2 protein, when this factor targets a promoter construct with four E2 binding sites in enhancer position. The activity of pSVeHPVpCAT in these experiments was 350 pmol of [^{14}C]chloramphenicol per min per mg of protein and was set to be 100%. The activity shown is the average from three to six experiments.

of TFIID, a factor of the basic transcription complex, through E2 binding to the promoter proximal binding site of HPV-18, and probably other genital HPV types, as it has been documented by Dostatni et al. (10). The function of the distal E2 binding site in repression could be (i) to stabilize the proximally bound E2 factor cooperatively; (ii) to interfere with the mechanism of interaction of cellular transcription factors bound to the HPV genome with the basic transcription complex in some indirect manner, e.g., through DNA bending (29), or through some other interaction between Sp1 and E2 (24); or (iii) to displace the Sp1 factor from the distal promoter element through competition for overlapping binding sites.

The experiments whose results are presented in this paper strongly support our previous suggestion (38a) that repression by the distal E2 binding site occurs via displacement of the Sp1 factor. A similar mechanism may also play a role in the regulation of a functionally unrelated promoter in BPV-1 (36, 40).

One aim of our studies was to resolve the question of whether the HPV-16 E2 protein may have properties differing from those of the BPV-1 E2 protein in the cooperative binding to DNA by two E2 dimers. This possibility existed since HPV-16 E2 protein is significantly smaller than the BPV-1 E2 protein, and cooperative binding to closely spaced sites may be favored as opposed to steric hindrance due to bulkiness. We observed only very minor cooperativity, similar to that observed in most studies of the BPV-1 E2 protein (27, 28). These values were small compared with cooperativity values of other transcription factors, which were also determined by gel shift analysis. For example, Oct-2 has been observed to bind to adjacent octamer sites with 10-fold cooperativity (22), and the paired-class homeodomain proteins bind to palindromic DNA sequences with up to 300-fold cooperativity (43). We conclude that cooperativity plays only a minor part in the repression of HPV E6 promoters. It is interesting to note that in constructs with a functional distal, but with a mutated proximal, E2

binding site (Fig. 7, lanes 3 and 4) E2 protein replaces Sp1 and is then positioned to act as a promoter proximal transcriptional activator. However, this position may be too close to the TATA box, because E2 fails to activate the E6 promoter and partial repression is observed.

Competition binding studies were performed with HPV-16 E2 protein and commercially obtained HeLa Sp1 transcription factor, by both of them being enriched close to purity and, in the case of Sp1, by inclusion of affinity chromatographic steps. We conclude that our findings were based on an Sp1 protein preparation containing mostly native factors and an E2 protein preparation, which had been determined to contain 50% native factors. Surprisingly, HPV-16 E2 protein could displace DNA-bound Sp1 factor at concentrations differing by nearly 2 orders of magnitude. Sp1 may have a greater affinity for its binding site at differing ionic strengths, and for higher-affinity binding sites that may not exist in papillomaviruses, because the nucleotides that flank the core Sp1 element in papillomaviruses do not match published consensus binding sites (19). However, we think that it is likely that our competition experiments reflect natural differences of affinity, as our and published (28) binding constants place E2 proteins among other high-affinity DNA-binding proteins. While no binding studies have been done with Sp1, TFIIIA, which like Sp1 has a zinc finger DNA binding domain, has approximately a 10-fold lower affinity for DNA than E2 (7).

One may speculate whether Sp1 displacement is a prerequisite not only for transcriptional repression but also for replication initiation. Apparently, all genital HPVs contain a binding site for the replication initiation protein E1 centered 25 bp 5' of the Sp1 site (17). E1 binding is stimulated by flanking E2 binding sites (26, 39, 45), and the E6 promoter distal E2 site is one of two that can exert this function (6, 26). Replication may thus be favored after binding of E2 to this site and the concomitant displacement of Sp1. Since E1 seems to have a negative effect on transcriptional stimulation by E2 (23, 33a), one may envisage a mechanism in which viral genomic function alternates between transcription of the transforming genes and replication of the genome.

ACKNOWLEDGMENTS

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