trans Activation by the Full-Length E2 Proteins of Human Papillomavirus Type 16 and Bovine Papillomavirus Type 1 In Vitro and In Vivo: Cooperation with Activation Domains of Cellular Transcription Factors

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Received 14 March 1994/Accepted 12 July 1994

Papillomaviral E2 genes encode proteins that regulate viral transcription. While the full-length bovine papillomavirus type 1 (BPV-1) E2 peptide is a strong *trans* activator, the homologous full-length E2 product of human papillomavirus type 16 (HPV-16) appeared to vary in function in previous studies. Here we show that when expressed from comparable constructs, the full-length E2 products of HPV-16 and BPV-1 *trans* activate a simple E2- and Sp1-dependent promoter up to ~100-fold in human keratinocytes and other epithelial cells as well as human and animal fibroblasts. Vaccinia virus-expressed, purified full-length HPV-16 and BPV-1 E2 proteins bound a consensus E2 site with high specific affinities ($K_d = \sim 10^{-9}$ M) and stimulated *in vitro* transcription up to six- to eightfold. *In vivo* and *in vitro trans* activation by either E2 protein required cooperation with another activator, such as Sp1, or other factors that interact with papillomavirus promoters, such as AP-1, Oct-1, nuclear factor 1/CTF, transcriptional enhancer factor 1, or USF. The glutamine-rich domain B of Sp1 or the mutually unrelated activation domains of other transcription factors were necessary and sufficient for cooperation with either E2 factor. We conclude that like BPV-1 E2, the HPV-16 E2 protein has the potential to function as a strong activator of viral gene expression in cooperation with cellular transcription factors.

Papillomaviruses induce benign epithelial or fibroepithelial proliferations of the skin and mucosa in humans and in a variety of animals (reviewed in references 13 and 58). Cervical cancer and higher-grade cervical intraepithelial neoplasia are associated with the presence of genital human papillomavirus types 16, 18, and 33 (HPV-16, HPV-18, and HPV-33) and several others in more than 90% of the lesions (63). HPV-16 is the most prevalent virus type found in invasive carcinoma of the cervix. The HPV-16 genome is a 7,903-nucleotide (nt) supercoiled DNA plasmid with a gene organization similar to that of all other papillomaviruses (45). The viral early E6 and E7 gene products of HPV-16 alter cell growth as they antagonize the action of the cellular tumor suppressor gene products, p53 and retinoblastoma proteins, respectively (58, 63). The viral early E1 proteins of human and animal papillomaviruses are required for DNA replication, while the viral E2 open reading frames (ORF) encode factors critical for both

DNA replication (7, 12, 59, 61) and the correct transcriptional regulation of viral genes in infection (reviewed in references 13 and 58).

In the model bovine papillomavirus type 1 (BPV-1), the full-length E2 gene product is a 48-kDa protein that functions as a strong *trans* activator (18, 20, 49). It increases transcription at a major BPV-1 early promoter, P89 or P2, located immediately upstream to the viral E6-E7 region (20, 48), as well as at other viral promoters (reviewed in reference 58). The E2 *trans* activator has an N-terminal transcription activation domain (TAD) with two acidic, amphipathic helices and a C-terminal dimerization/DNA-binding domain (DBD), connected by a central hinge region (21, 35). The E2 DBD recognizes ACC(N)₆GGT or -GTT motifs (E2 sites): two or more E2 sites function cooperatively as strong E2-dependent enhancers. Activation by the BPV-1 E2 further depends on cooperation with other cellular proteins, such as Sp1 (17, 19, 30), USF (19), or other factors (17).

In contrast to the E2 *trans* activator of BPV-1, the functions of the full-length E2 gene products of HPV-16 or other genital HPV types have been more difficult to establish. Although the HPV and BPV-1 E2 genes share extensive sequence homologies in the DNA-binding and *trans*-activating domains (3, 18), previous studies had suggested that the E2 products of genital HPVs may differ from the BPV-1 E2 protein. Full-length HPV E2 expression vectors could *trans* activate reporter clones with viral enhancer fragments containing E2 sites, yet much less effectively than BPV-1 E2 (11, 23, 40). Furthermore, in contrast to the BPV-1 E6 promoter, P89, the E6 promoters of HPV-16 and other genital HPV strains can be repressed by E2

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TABLE 1. Oligonucleotides used in this study

Motif	Source or reference	Sequence ^a
E2 site 1	HPV-16 (42)	GTTGA <u>ACCGAAACCGGT</u> TAGTATAAAAGCAG ^b
E2 site 2	HPV-16 (42)	TATAAACTAAGGGCGTA <u>ACCGAAATCGGT</u> TGAA ^b
E2 site 10	BPV-1 (29)	GGTCAA <u>ACCGTCTTCGGT</u> GCTC ^b
E2 consensus	11, 21	tcg <u>ACCGATATCGG</u> ^{c,d}
E2-Sp1		ggtcg <u>ACCGATATCGGT</u> cgtattCCGGCCCCGCCattc ^f
Sp1x1	HSV-1 IE-3 gene (17)	ctagaaTATTCCGGCCCCCGCCCATtctgtc ^f
Sp1x2	HSV-1 IE-3 gene (17)	ctagaATATCCGGCCCCCGCCCATCCCCGGCCCCGCCATCC ^f
GAL4	17-mer (6)	ctagaatgctCGGAGGACTGTCTCCGga ^f
GAL4x2		ctagaCGGAGGACTGTCTCCGatCGGAGGACTGTCTCCG ^f
Nuclear factor 1/CTF	Adenovirus origin (17)	ctagaATATCTTTGGATTGAAGCCAATGc ^{d,f}
OCT	HSV-1 IE110k (2)	ctagaATGCCGTGCATGCTAATGATATTCTTc ^{d,f}
AP-1 site 3	HPV-16 (10)	ctagaATATTAAAGGTTAGTCATACAc ^{d,f}
USF	Adenovirus major late promoter (44)	ctagagGTAGGCCACGTGACCTGAtctgc ^{d,f}

^a Factor-binding sites are underlined. Lowercase letters represent spacer and cloning end sequences.

^b Sequence shown is the top strand of a blunt-ended double-stranded oligonucleotide.

^c This E2 oligonucleotide self-anneals to yield a double-stranded sequence with *Sal*I-compatible ends for DNA affinity column preparation and cloning (11, 21).

^d Duplicate sites in *tk* (–38)–*cat* clones were generated by repeated subcloning.

^e This E2 oligonucleotide was used in DNA-binding experiments.

^f Top strand of a double-stranded oligonucleotide with *Xba*I- and *Xho*I-compatible ends.

from two proximal E2 operator sites (42, 55, 56). Paradoxically, however, only the heterologous BPV-1 E2 protein was a strong repressor of genital HPV E6 promoters in cotransfections or in vitro whereas the homologous full-length E2 products did not appear to function consistently as activators or repressors in all cell types (5, 8, 15, 18, 52, 55, 56). These effects have been either ascribed to inefficient DNA binding or functional differences between the TADs of genital HPV and BPV-1 E2 proteins.

To define the potential of the full-length HPV-16 E2 protein as a transcription factor, we have reexamined its DNA-binding and *trans*-activation properties in vitro and in vivo in comparison to those of BPV-1 E2. Both full-length E2 proteins recognized a consensus E2 DNA-binding site with similar high affinities and were comparably efficient as *trans* activators in a variety of cell types in vivo as well as in in vitro transcription assays. In both cases, E2 activity required cooperation with an additional activator. Experiments with chimeric GAL4 constructs revealed that unrelated activation domains of heterologous transcription factors were both necessary and sufficient for cooperation with E2. These results establish the full-length HPV-16 E2 protein as a strong potential activator of viral transcription. Possible roles of the HPV-16 E2 protein as a *trans* activator during viral infection are discussed.

MATERIALS AND METHODS

Molecular constructions. The clone design is shown in the figures. The BPV-1 pCG-E2 was a kind gift of M. Ustav and A. Stenlund (59). pCG-E2 carries a point mutation in the AUG at nt 3091 and thus cannot synthesize the C-terminal sE2 repressor form (27, 59). The clone pCG (16)-E2 contains the HPV-16 E2 ORF with the native ATG replaced by a synthetic translation start site identical to that in pCG-E2. (Nucleotide numbers refer to the corrected HPV-16 DNA sequence [10]). The constructs pGAL4-VP16, pGAL4 (amino acids [aa] 1 to 147), pGAL4 (aa 1 to 881), and pGAL4-E1A were gifts from M. Green and M. Ptashne, and pGAL4-Sp1 domain B was a gift from S. Liang. pGAL4-TEF-1 has been described previously (24). The pGAL4-Sp1 clone was made by linking the coding sequence of Sp1 cDNA (from W. Jackson and R. Tjian [9]) to GAL4 (aa 1 to 147) after the addition of an *Eco*RI site at aa 84 and an *Xba*I site past the C terminus. Chimeric constructs

linking Sp1 fragments to the GAL4 DBD were made with PCR primers to match GAL4-Sp1 clones described previously (39). pGAL4-E2-TAD expresses the BPV-1 TAD (aa 4 to 283; nt 2617 to 3456) in frame with the GAL4 DBD.

The *tk* (–38)–*cat* plasmids contain the herpes simplex virus type 1 (HSV-1) *tk* core promoter (nt –38 to +56) linked to the *cat* sequence and inserted between the *Xba*I and *Bam*HI sites in the pUC18 polylinker. An *Xho*I site was introduced immediately upstream of the *tk* sequence. Synthetic double-stranded oligonucleotides of known factor-binding specificities (Table 1) were inserted between the polylinker *Xba*I site and the *Xho*I site. The consensus E2-binding motifs were inserted into the adjacent upstream polylinker *Sal*I site. Some clones with duplicate motifs were prepared by additional subcloning (Table 1). All new molecular constructions were verified by DNA sequencing on an Applied Biosystems, Inc., automated sequencer. Preparative quantities of most new plasmid DNAs were purified on DNA affinity columns (Maxiprep; Promega, Madison, Wis.).

Cells and transfections. Cells were grown, transfected, and analyzed by RNase protection or in enzymatic chloramphenicol acetyltransferase (CAT) assays as described previously (10, 11, 21, 25). Primary human and HaCaT cells were transfected by lipofection (Life Technologies, Bethesda, Md.). Optimal plasmid DNA levels were determined empirically for each cell type. For RNase protection assays, all RNA samples were prepared from pooled duplicate 150-mm-diameter dish cultures (5×10^6 cells per dish) 22 to 24 h after transfection.

RNase protections. Antisense RNA probes for RNase protection assays were synthesized with T7 RNA polymerase from DNA templates. DNA templates were synthesized by PCR by using a primer, 5'-CCTAATACGACTCACTAGAGGGAGACGGTGGTATATCCAGTG-3', that contained the T7 promoter linked 5' to *cat* sequences from nt +69 to +52 and another primer that annealed in the adjacent pUC vector, 5'-GTTTCCCAGTCACGAC-3'. An aliquot (1 μ l) was transcribed directly with T7 RNA polymerase in a volume of 20 μ l to yield a uniformly ³²P-labeled RNA probe that extended from +69 in the *cat* gene to upstream of the *tk* promoter in the *tk* (–109)–*cat* plasmid construction. As internal control, the SV2N-*cat*- Δ 13 construction containing the strong simian virus 40 enhancer/promoter (20, 21) and a deletion at the 5'-untranslated end of the *cat* gene cassette, resulting in tran-

scripts that protect 56 nt of probe *cat* RNA, was used. Protected fragment sizes were determined from sequencing ladders run in parallel. 32 P-labeled DNA size markers were included in the gels.

Recombinant vaccinia virus vectors. Recombinant vaccinia viruses expressing E2 proteins were produced by using the protocols developed by Moss (38). The complete coding sequences for E2 proteins were inserted in frame with the efficient translation start site of the bacterial plasmid pTM-1 which contains a cassette including the T7 promoter, the encephalomyocarditis virus 5'-untranslated region, and downstream mRNA termination signals flanked by vaccinia virus thymidine kinase gene sequences (16).

Isolation of recombinant vaccinia viruses. HeLa cells infected with a low multiplicity of wild-type vaccinia virus were transfected with the E2-pTM-1 plasmid constructs. At a low frequency, homologous recombination occurs between the plasmid *tk* sequences and virus *tk* sequences, resulting in an insertionally inactivated *tk* gene. After 48 h of infection, the E2-coding *tk*-deficient viruses were selected by plaque assay on *tk* cells in the presence BrUdR. A high proportion of *tk*-deficient viruses selected in this way were recombinants that contained the E2 coding sequences. The plaques were isolated and resuspended in 300 μ l of saline. Aliquots (about 1/300th of one plaque) were assayed for the presence of papillomavirus E2 sequences by PCR followed by agarose electrophoresis and ethidium bromide staining. Recombinant viruses were plaque purified an additional time. Viral stocks were prepared in HeLa cells and titrated by plaque assay before further use.

Extraction of proteins from recombinant vaccinia virus-infected cells. Confluent HeLa cultures were coinfecting with 3 to 10 PFU per cell of both vaccinia virus-E2 recombinants and vTF73, a vaccinia virus that contains the bacteriophage T7 RNA polymerase under the control of a strong vaccinia virus promoter (16). After 14 h, the cells were harvested and washed with 150 mM NaCl–10 mM Tris-HCl (pH 8.0). The E2 proteins were extracted with 4 volumes of 800 mM NaCl–20 mM Tris-HCl, and the insoluble residue was discarded. E2 proteins were then purified by sequential chromatography on heparin agarose for BPV-1 E2 or BioRex 70 (Bio-Rad) for HPV-16 E2, followed by two cycles of absorption and elution from a DNA affinity resin containing E2-binding sequences. The synthetic E2 motif oligonucleotide TCGACCGATATCGG was made as a 5'-phosphorylated derivative directly on an ABI, Inc., synthesizer. This oligonucleotide self anneals to form a double strand with an internal *EcoRV* restriction site and with *SalI* sticky ends. Ligated, multimeric chains with E2 consensus binding sites were covalently linked to Sepharose 4B. Chromatography was performed as described for transcriptional enhancer factor 1 by Ishiji et al. (25). The purified fractions were concentrated approximately sevenfold on a Centricon ultrafiltration device (molecular mass cutoff of 30 kDa; Amicon, Waltham, Mass.) and stored in a vapor-phase liquid nitrogen freezer. The purified HPV-16 or BPV-1 E2 preparations contained 75 to 90% pure E2 proteins of the correct apparent sizes at concentrations of 0.3 to 1.2 ng/ μ l (6 to 25 fmol/ μ l) as estimated on silver-stained sodium dodecyl sulfate (SDS)-polyacrylamide gels. E2 concentrations were confirmed by determining the quantity of purified E2 fractions required to complex 50% of probe E2 site DNA in mobility shift experiments. Approximately 0.3 to 0.6 ng of HPV-16 E2 (~7 to 15 fmol) sufficed to fully protect an E2 site in footprinting experiments. Purified recombinant human Sp1 expressed in a vaccinia virus vector was from Promega.

Mobility shift assays. All DNA-binding reactions with purified proteins included 0.2 to 0.5 μ g of bovine serum albumin

per μ l. Binding was performed in a 25- μ l reaction mixture volume with 15,000 cpm (~5 fmol) of 32 P-labeled oligonucleotide probes (Table 1) in the presence of 30 ng of synthetic poly(dI)-poly(dC) polynucleotide competitor (Pharmacia) at 30°C for 30 min as described previously (10, 25). The dissociation constants (K_d) were estimated from three or more quantitative competition assays containing 32 P-labeled consensus E2 oligonucleotide probe with increasing concentrations of unlabeled double-stranded competitor oligonucleotides in the binding mixtures prior to E2 protein addition. Protein-DNA complexes were resolved by electrophoresis in nondenaturing acrylamide gels at 4°C, visualized on X-ray films, and quantitated by densitometric scanning. The K_d values were equal to the unlabeled competitor concentrations that depleted bound complex formation by 50%.

DNase I footprinting. The DNA probe for footprinting was 32 P labeled at a single 5' end by PCR with an end-labeled primer. The labeled fragments were purified on a polyacrylamide gel. Binding conditions were optimized for simultaneous binding of Sp1 and E2 proteins. Reaction mixtures contained 20,000 cpm of probe, 25 mM Tris-HCl (pH 8.0), 10% glycerol, 0.5 mM EDTA, 6 mM MgCl₂, 40 μ g of bovine serum albumin per ml, 5 mM dithiothreitol, 50 mM KCl, 15 nM poly(dG)-poly(dC), and binding proteins, in a final volume of 20 μ l. DNase digestion (Pharmacia) was initiated by the addition of CaCl₂ to 2.5 mM.

In vitro transcription assays. Recombinant vaccinia virus-expressed E2 proteins were tested in in vitro transcription assays with the reporter and control G-free constructs (see Fig. 3A). The test and control promoters were assayed for transcription activity in 25- μ l reaction mixtures containing 50 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 0.1 mM EDTA, 0.5 mM dithiothreitol, 50 mM KCl, 8.7% glycerol, and 0.1% Nonidet P-40 detergent, in the presence of 10 μ l of the pretested HeLa cell extract prepared as described previously (62). In those reaction mixtures receiving exogenous E2, the DNA templates were preincubated with up to fivefold molar excess of E2 over DNA template (final template concentration at 50 ng per reaction mixture was ~0.6 nM) at 25°C for 15 min before the addition of the HeLa extract. After an additional preincubation at 25°C to allow the formation of preinitiation complexes, 32 P-labeled CTP and other nucleoside triphosphates except GTP were added to final concentrations of 0.5 mM to start RNA synthesis. Incubation was continued at 25°C for 45 min and then stopped by the addition of 200 μ l of 0.2% SDS. The reaction mixtures were extracted with phenol-chloroform and chloroform and ethanol precipitated with 50 μ l of 5 M ammonium acetate and 10 μ g of wheat germ tRNA as the carrier. One-half of the sample was then resolved on a denaturing polyacrylamide gel. Transcripts were visualized on X-ray films and quantitated by scanning.

RESULTS

The full-length HPV-16 E2 product is a strong *trans* activator. In previous studies, the E2 gene activities of genital HPV strains were examined in transfections with E2 plasmids driven by diverse heterologous promoters and including various portions of 5'-untranslated sequences (11, 18, 23, 40). To minimize these differences, we subcloned the HPV-16 E2 ORF downstream from the cytomegalovirus enhancer in the pCG vector backbone (53, 59). In the resulting clone pCG (16)-E2, translation of the HPV-16 E2 protein begins at an efficient consensus translation start site in the pCG plasmid to maximize protein expression. The pCG ATG site is also used in the BPV-1 pCG-E2 construct (59). In addition, the BPV-1

A

Expression Vector:

HPV-16:

pRSV (16)-E2

pCG (16)-E2 (2709)

pCG (16)-E2

BPV-1:

pRSV-E2

pCG-E2

control:

pCG-*neo*

Reporter Plasmid:

pE2x2-Sp1x2-*tk* (-38) *cat*

C

FIG. 1. HPV-16 E2 is a strong *trans* activator comparable to BPV-1 E2. (A) E2 expression vectors, the reporter *cat* clone, and the design for reporter RNA detection by RNase protection. The expression of all pCG constructions was directed by the human cytomegalovirus enhancer-promoter and 3' rabbit globin poly(A) sequences in the parent pCG plasmid (59). In pCG-E2 and pCG (16)-E2, the native ATG codon was replaced with a synthetic translation initiation site. In addition, the BPV-1 E2 clone pCG-E2 contains a point mutation that inactivates the ATG codon for sE2 synthesis. The pRSV clones were controlled by the Rous sarcoma virus long terminal repeat (RSV LTR) promoter and simian virus 40 (SV40) early poly(A) signal (11, 20). The reporter plasmid pE2x2-Sp1x2-*tk* (-38)-*cat* contains two consensus E2 sites and two Sp1 sites in *tk* (-38)-*cat*; site sequences are given in Table 1. Hybridization of reporter mRNA with a T7 antisense probe yields a 127-nt protected fragment. (B) RNase protection assays. The reporter plasmid pE2x2-Sp1x2-*tk* (-38)-*cat* (10 µg) was cotransfected with 0.45, 1.5, 4.5, or 10 µg of pCG (16)-E2 (lanes 2 to 5) or 0.045, 0.15, 0.45, or 1.5 µg of pCG-E2 (lanes 7 to 10) in duplicate 150-mm-diameter HeLa cell cultures. pCG-*neo* was used as control (lanes 1 and 6) and was also added to a total of 10 µg (lanes 1 to 5) or 1.5 µg (lanes 6 to 10) to keep the pCG vector concentration constant. RNAs were extracted 24 h later, and RNase protection analysis was performed with a probe spanning the *tk* promoter. The signals derived from the correctly initiated *tk* promoter and from the internal control are designated by the arrowheads labeled *tk* and *cat*Δ13. (C) Quantitative CAT enzyme analysis. Duplicate HeLa cell cultures were cotransfected with the pE2x2-Sp1x2-*tk* (-38)-*cat* plasmid (1.0 µg per 35-mm well) and increasing amounts of pCG (16)-E2 or pCG-E2 with the negative control plasmid pCG-*neo* as the carrier DNA. Relative CAT enzyme activity was determined 72 h later in comparison to pCG-*neo*-transfected controls in two to four independent experiments.

pCG-E2 clone contains a mutation in the ATG codon for the translation of the sE2 repressor at nt 3091 to exclude the possibility that potential negative regulation could be due to sE2 synthesis (Fig. 1A).

Activities of the pCG (16)-E2 and pCG-E2 constructs were tested in cotransfections with a defined E2-dependent reporter clone, pE2x2-Sp1x2-*tk* (-38)-*cat*. This construct contains two consensus E2 and two Sp1 sites spaced by approximately one turn of DNA from each other and from the TATA box of the HSV-1 *tk* (-38) promoter (Fig. 1A). The transfections were performed in the cervical carcinoma cell line, HeLa, in which the full-length HPV-16 or HPV-18 E2 products had been reported to function inconsistently either as *trans* activators or as repressors (42, 52, 55, 56). Figure 1B shows the results of an RNase protection assay. Compared with the negative control clone, pCG-*neo*, the full-length E2 products of either HPV-16

(lanes 2 to 5) or BPV-1 (lanes 7 to 10) at increasing concentrations augmented steady-state levels of correctly initiated *tk* mRNA up to 15- to 30-fold in a concentration-dependent fashion.

Surprisingly, *trans* activation by both the HPV-16 pCG (16)-E2 and the BPV-1 pCG-E2 clones was extremely efficient (Fig. 1C). Both constructs activated the reporter clone 10- to 20-fold at 0.1 to 1.0 ng of DNA per culture and to comparable maximum levels (~100-fold) at 30 to 100 ng per culture. Higher E2 concentrations resulted in lower relative *trans* activation (Fig. 1C and data not shown), most likely because of intracellular competition for limiting cofactors (squenching [36, 41, 54]). These results indicate that the HPV-16 E2 product can *trans* activate a defined promoter in cooperation with Sp1 as effectively as BPV-1 E2.

The pCG (16)-E2 plasmid required ~3- to 10-fold more

DNA per culture to achieve equivalent levels of activation as pCG-E2 (Fig. 1C). We do not know whether this reflects a subtle difference in the expression or function of the E2 proteins. Although we cannot formally exclude that the pCG (16)-E2 clone also directs the synthesis of a shorter, C-terminal product analogous to the sE2 of BPV-1, the maximum *trans* activation reached with pCG (16)-E2 was similar to BPV-1 pCG-E2 with a mutated sE2 ATG, whereas an sE2-like repressor would competitively inhibit activation (11, 21, 27, 35). An sE2-like peptide is thus unlikely to be a major product of the pCG (16)-E2 clone.

In contrast to pCG (16)-E2, a second HPV-16 E2 clone which contains additional viral sequences 5' to the E2 initiation codon, pCG (16)-E2 (nt 2709) required 30-fold-higher DNA concentrations to reach activation levels comparable to those of pCG (16)-E2. We previously characterized another full-length HPV-16 E2 clone, pRSV (16)-E2, which also includes these 5' sequences and is driven by the long terminal repeat of Rous sarcoma virus (11). This construction was another order of magnitude less efficient as a *trans* activator. Similarly, the pRSV-E2 construct of BPV-1 (20, 21) required a concentration 300-fold-higher than that of pCG-E2 to activate the reporter 50-fold, and its activity peaked more than 50% below that of either pCG (16)-E2 or pCG-E2. These results suggest that the reported variation in HPV-16 E2 function may have been due to inefficient expression rather than to the intrinsic activity of the HPV-16 E2 protein itself and demonstrate that the full-length HPV-16 E2 product functions as a strong activator comparable to the BPV-1 E2 protein.

The HPV-16 E2 protein functions in a wide variety of cell types. To determine whether HPV-16 E2 activation is cell type dependent, the pCG (16)-E2 and pCG-E2 constructs were cotransfected with the E2-dependent reporter clone E2x2-Sp1x2-*tk* (-38)-*cat* (Fig. 1A) into a number of different cell lines (Table 2). Although the extent of maximal *trans* activation varied from cell to cell, both the HPV-16 and BPV-1 E2 clones activated the target promoter not only in primary and established human keratinocytes and cervical carcinoma cell lines but also in an epithelial cell type that does not support papillomavirus replication or transcription, the hepatic carcinoma cell line HepG2, and in fibroblasts of human or animal origin. Any cellular factors required by either the HPV-16 or the BPV-1 E2 protein are thus present and active in all these cell types.

DNA-binding properties of vaccinia virus-expressed, purified E2 proteins. To obtain sufficient quantities of correctly modified HPV-16 and BPV-1 E2 proteins for biochemical studies, E2 coding sequences were inserted into recombinant vaccinia viruses under the control of a T7 promoter. The E2 proteins were expressed in HeLa cells coinfecting with the recombinant strains and an additional vaccinia virus coding for the T7 RNA polymerase, vTF73, extracted, and purified on an ion-exchange column followed by two cycles of sequence-specific DNA chromatography. E2 purification was monitored in mobility shift assays. The final protein fractions were analyzed on a denaturing SDS-polyacrylamide gel followed by silver staining (Fig. 2A). The HPV-16 E2 preparation showed a single prominent band of ~42 kDa consistent with the expected protein size (lane 1), whereas the BPV-1 E2 fraction contained an ~48-kDa band (lane 2).

Although the HPV-16 E2 protein is known to contain a DNA-binding domain (4, 28), the DNA-binding properties of the full-length peptide have not been characterized. We therefore estimated the dissociation constants (K_d) and off-rates of the purified HPV-16 and BPV-1 E2 proteins at selected E2 motifs in mobility shift assays (Fig. 2). The K_d values reflect the

TABLE 2. Full-length E2 gene products of HPV-16 and BPV-1 *trans* activate gene expression in keratinocytes as well as in other epithelial cells and fibroblasts^a

Cell type and cell line	Relative CAT activity with:	
	pCG (16)-E2	pCG-E2
Keratinocyte derived		
Primary human keratinocytes	25	9
HaCaT (immortalized human)	49	78
Cervical carcinoma		
HeLa (HPV-18 positive)	108	105
C33A (HPV negative)	77	38
Hepatocyte derived HepG2	69	108
Fibroblast-like		
Primary human fibroblasts	12	18
CV-1 (monkey kidney)	29	76
NIH-3T3 (murine)	12	31
B1 (bovine)	245	532

^a The reporter clone E2x2-Sp1x2-*tk* (-38)-*cat* was cotransfected with the HPV-16 E2 clone, pCG (16)-E2, or the BPV-1 E2 vector, pCG-E2, into duplicate cultures of the indicated cells as described (10, 11, 25), and relative *trans* activation compared with that of pCG-*neo*-cotransfected controls was evaluated 48 to 72 h later. The data are the averages for two independent experiments.

affinity of each E2 protein for specific E2 motifs (Fig. 2B and Table 3) and represent the concentrations of unlabeled specific E2 site oligonucleotides that depleted bound complex formation by 50% in repeated competition assays (10, 25). The off-rates measure the stability of preformed E2 protein-DNA complexes at specific E2 motifs in the presence of excess consensus E2 site DNA (Fig. 2C and Table 3) and are expressed as dissociation half time ($T_{1/2}$), that is, the time required to exchange 50% of labeled, bound E2 motif with unlabeled E2 DNA.

Consistent with their ability to *trans* activate, the HPV-16 and BPV-1 E2 proteins bound to the consensus E2 site, TCGACCGATATCGGTCGA, with the nearly identical K_d values of 1.3×10^{-9} and 1.0×10^{-9} M, respectively, and both had a dissociation $T_{1/2}$ of greater than 90 min. We found, however, that the E2 proteins differed in their binding affinities and off-rates at other E2 motifs. HPV-16 E2 bound the homologous HPV-16 E2 site 1 or 2 approximately two- to threefold more weakly than BPV-1 E2, yet these interactions were much more stable (with $T_{1/2}$ of 4- and >10-fold greater.) In contrast, BPV-1 E2 had an ~60-fold-higher affinity for BPV-1 E2 site 10 than did HPV-16 E2, but only a slightly greater $T_{1/2}$. Taken together, the data indicate that each value stems from specific interactions between each E2 protein and individual DNA motifs. Nevertheless, the HPV-16 E2 protein can bind to at least some DNA motifs with affinity and DNA-protein complex stability similar to those of BPV-1 E2.

Purified HPV-16 E2 protein functions as a transcriptional activator in vitro. To determine whether the purified E2 proteins retained their functional activity, the HPV-16 and BPV-1 E2 preparations were tested in an in vitro transcription assay in HeLa cell extracts. The target promoter for *trans* activation was identical to that of pE2x2-Sp1x2-*tk* (-38)-*cat*, but the *cat* reporter gene was replaced with a 380-nt G-free cassette (Fig. 3A, clone *a*). The *tk* (-38) G-free (250) template (clone *b*) served as an internal control. Both supercoiled plasmid transcription templates were incubated with HeLa cell extracts in the presence of [³²P]UTP and unlabeled ATP and CTP. GTP was omitted from the reaction mixtures so that only

FIG. 2. DNA-binding properties of BPV-1 and HPV-16 E2 proteins. (A) Purification of vaccinia virus-expressed recombinant E2 proteins. Recombinant vaccinia virus-infected HeLa cells were extracted, and the E2 proteins were purified by ion exchange and two cycles of sequence-specific DNA chromatography. Aliquots of purified proteins were electrophoresed on SDS-10% acrylamide gels and visualized by silver staining. Molecular weight markers (in thousands) are indicated by arrows. (B) Binding of purified HPV-16 and BPV-1 E2 proteins to consensus E2 motif and selected HPV-16 and BPV-1 E2 sites. HPV-16 E2 (lanes 1 to 6) or BPV-1 E2 (lanes 7 to 12) was tested for binding to a 32 P-labeled consensus E2 site (Table 1) in mobility shift assays in the presence of increasing concentrations of unlabeled E2 competitors (indicated on the left). Upper band, bound protein-DNA complex; lower band, free probe. (C) Off-rates of HPV-16 and BPV-1 E2 proteins for HPV-16, BPV-1, and synthetic consensus E2 sites. 32 P-labeled E2 oligonucleotide probes (shown on the left) were incubated with HPV-16 (lanes 1 to 7) or BPV-1 (lanes 8 to 14) E2 proteins. After 30 min of binding, excess consensus E2 site competitor (300 nM) was added to each reaction mixture. Binding reaction mixtures in lanes 2 and 9 were loaded immediately, while those in lanes 3 to 7 and 10 to 14 were electrophoresed 1, 5, 10, 30, or 90 min after the addition of excess competitor. Controls (labeled "C") in lanes 1 and 8 received 300 nM consensus E2 site competitor prior to the addition of the E2 proteins.

G-free stretches of the DNA templates would give rise to continuous transcripts. The resulting 32 P-labeled RNA fragments were resolved on denaturing urea-acrylamide gels (Fig. 3B).

In the absence of E2, the E2x2-Sp1x2-*tk* promoter template (clone *a*) had higher in vitro activity than the *tk* control (clone *c*) because of endogenous Sp1 in HeLa extracts interacting with the Sp1 motifs of the promoter (lanes 1 and 2). The baseline activity could be abolished by competition with excess Sp1 oligonucleotide (data not shown; see also Fig. 3D). The addition of increasing concentrations of HPV-16 E2 (1 to 5 μ l, corresponding to ~5 to 25 ng or 100 to 500 fmol) increased the reporter template signal six- to eightfold (lanes 3 to 5), that is, to a similar extent as the addition of comparable quantities of purified BPV-1 E2 (lanes 6 to 8). Activation required E2 binding because control promoters without E2 sites, *tk* (-38) (clone *c* in Fig. 3B) or the adenovirus major late promoter (clone *d* in Fig. 3C and D), were not activated by the addition of either E2 protein under these conditions. However, the in vitro activation also depended on cooperation with Sp1 since a promoter containing E2 sites only (clone *b*) was not stimulated (Fig. 3C). Furthermore, the response to either E2 protein was blocked by excess Sp1 site competitor (Fig. 3D, lane 1, and data not shown) but not by the addition of an unrelated, nonspecific oligonucleotide corresponding to a short stretch of the BPV-1 URR (nt 7798 to 7819; lane 2). Activation was due

TABLE 3. DNA-binding constants and off-rates of HPV-16 and BPV-1 E2 proteins at different E2 sites^a

Virus or motif	E2-binding site	Positions	Sequence ^b	HPV-16 E2		BPV-1 E2	
				K_d (nM)	$T_{1/2}$ (min)	K_d (nM)	$T_{1/2}$ (min)
HPV-16	1	50-61	TGAACCGAAACCGGTTAG	3.9	63	2.5	14
HPV-16	2	35-46	GTAACCGAAATCGGTTGA	2.5	>90	0.9	7
BPV-1	10	7781-7792	CAAACCGTCTTCGGTGCT	29.0	8	0.5	18
Synthetic consensus motif			TCGACCGATATCGGTCGA	1.3	>90	1.0	>90

^a The K_d values were equal to the unlabeled competitor concentrations that depleted bound complex formation by 50% in three or more experiments. Off-rates are given as dissociation $T_{1/2}$, that is, the time required to replace 50% of labeled E2 motif in the bound complex with excess unlabeled site DNA as determined from the slope of log (complex) versus time plot. The positions of E2-binding sites in BPV-1 and HPV-16 are from the studies by Li et al. (29) and from Romanczuk et al. (42), respectively. The consensus E2 motif (Table 1) was also used in all *cat* and G-free reporter clones in this study.

^b Italics denote consensus nucleotides of E2 recognition sites.

FIG. 3. Purified HPV-16 E2 protein functions as transcriptional activator in vitro. (A) G-free templates used in assays. (B) E2 concentration-dependent activation in vitro. The E2x2-Sp1x2-*tk* (-38) promoter linked to a 380-nt G-free cassette (clone *a*) and control *tk* (-38) promoter linked to a 250-nt G-free cassette (clone *c*) were transcribed in vitro in HeLa cell extracts with ATP, UTP, [³²P]CTP, but no GTP, without added proteins (lanes 1 and 2) or in the presence of purified E2 proteins from HPV-16 (lanes 3 to 5) or BPV-1 (lanes 6 to 8). The resulting G-free products were resolved on denaturing gels, visualized on X-ray film, and quantitated by densitometry. The *tk* (-38)-G-free promoter controls were overexposed on autoradiograms to visualize their activity. (C) E2-stimulated in vitro transcription dependence on Sp1 binding. The E2x2-*tk* (-38) promoter (clone *b*) served as activation target and the MLP-G-free clone (clone *d*) served as the control. (D) Sp1 depletion. The E2x2-Sp1x2-*tk* (-38) G-free template was transcribed in a HeLa extract with BPV-1 E2 and 300 nM Sp1-binding site oligonucleotide (lane 1) or 300 nM control nonspecific oligonucleotide (lane 2). MLP G-free clone (clone *d*) was used as a control.

to the action of the E2 TAD since a C-terminal E2 DBD protein elicited no response in these assays (data not shown). Taken together, these results indicate that the purified preparations of the HPV-16 E2 as well as the BPV-1 E2 *trans*-activator proteins are active and functionally equivalent in vitro.

Cooperation with both HPV-16 and BPV-1 E2 is mediated by the glutamine-rich Sp1 activation domain. The HPV-16 E2 protein activates transcription in vivo and in vitro in cooperation with cellular factor Sp1. One possible mechanism of HPV-16 E2 and Sp1 cooperation could be cooperative binding of both proteins to DNA. To test this possibility, we examined the binding of vaccinia virus-expressed, purified recombinant Sp1 in the absence and presence of HPV-16 E2 in DNase I footprinting assays. The E2x2-Sp1x2-*tk* (-38) promoter served as target DNA for protein binding (Fig. 4). In the presence of 0.3 footprinting unit (fpu) of recombinant Sp1 protein, the dual Sp1 sites were only partially protected from DNase I digestion (lanes 2 and 3). As seen in lane 5, 2 fpu of HPV-16 E2 (~0.6 to 1.2 ng of protein) sufficed to completely protect both E2 motifs on the DNA. The addition of 2.0 fpu, but not of 0.3 fpu, of HPV-16 E2 to binding reaction mixtures containing 0.3 fpu of Sp1 resulted in complete protection of both the E2 and the Sp1 sites (lanes 4 versus 3). Additional titration experiments revealed that both the BPV-1 E2 and the HPV-16 E2 potentiated Sp1 binding approximately threefold (data not shown), consistent with the observed approximately threefold

FIG. 4. Joint HPV-16 E2 and Sp1 binding in vitro. A DNase I protection assay was performed, using a 5'-end-labeled DNA fragment containing two tandem Sp1 sites adjacent to two tandem E2 consensus sites derived from the E2x2-Sp1x2-*tk* (-38)-*cat* plasmid. Lanes: 1 and 6, no added proteins; 2 to 4, purified Sp1 (0.3 fpu); 3, 4, and 5, purified HPV-16 E2 protein (0.3, 2.0, and 2.0 fpu, respectively).

cooperative binding between Sp1 and BPV-1 E2 expressed in a baculovirus (30). However, it is apparent that these results cannot account for the up-to-100-fold cooperative activation of target promoters in vivo.

The Sp1 factor consists of several well-defined functional domains (9, 39). The carboxy-terminal half comprises a C-terminal domain, D, and an adjacent domain, C, that together contain a zinc-finger-like DNA-binding segment (Fig. 5). Two glutamine- and serine/threonine-rich domains, A and B, in the N-terminal portion are required for full activation. The very N-terminal aa 1 to 84 can be deleted without impairing Sp1 function. To map the domains of the Sp1 protein required for cooperative activation, we have linked the active part of the Sp1 protein (aa 84 to 778) or its functional domains ABC (aa 84 to 612), domain A alone (aa 84 to 346), domain B alone (aa 340 to 500), or domain D (aa 612 to 778) to the DNA-binding domain of the yeast factor GAL4 (aa 1 to 147). The ability of the chimeric GAL4-Sp1 products to cooperatively activate transcription in the presence of either HPV-16 E2 or BPV-1 E2 was tested in cotransfections with a GAL4-dependent reporter construction, E2x2-GAL4x5-*tk* (-38)-*cat*. This clone contains five 17-mer GAL4-binding sites upstream of the *tk* TATA box (Fig. 5).

In the absence of E2, cotransfection with GAL4-Sp1 (clone *b*) and GAL4-ABC (clone *c*) activated the reporter clone approximately two- and threefold, respectively, compared with the GAL4 DBD alone (clone *a*). Other GAL4-linked Sp1

FIG. 5. Glutamine-rich activation domain of Sp1 is necessary and sufficient for cooperative *trans* activation by HPV-16 and BPV-1 E2 gene products. Peptide-coding domains of Sp1 were linked to the DNA-binding domain of GAL4, and the resulting chimeric plasmid constructions (0.5 μ g per well) were cotransfected in duplicate HeLa cell cultures together with the pE2x2-GAL4x5-tk (-38)-cat reporter clone (0.5 μ g per well) and pCG (16)-E2 or pCG-E2. Reporter activity was determined in enzymatic CAT assays (11, 21). Relative CAT activities compared with those of pCG-neo and GAL4 DBD-cotransfected controls (clone a) were calculated from two to three independent experiments.

segments increased the CAT activity less than 1.5-fold. Cotransfection with either HPV-16 E2 or BPV-1 E2 constructs resulted in a further ~6- to 12-fold-increased activation with GAL4-ABC (clone c), indicating that the Sp1 ABC domain was capable of synergistic cooperation with either E2 factor. GAL4-Sp1 (clone b) was relatively inert, similar to its low activity in cooperation with the adenoviral E1A protein (32). The glutamine-rich Sp1 domain B in GAL4-B (clone f) was sufficient to support four- to eightfold cooperative activation with either E2 factor. These results demonstrate that synergistic cooperation between Sp1 and the HPV-16 or the BPV-1 E2 proteins is mediated by the transcriptional activation domain of Sp1.

HPV-16 and BPV-1 E2 cooperate with several unrelated types of activation domains. To determine whether the HPV-16 and BPV-1 E2 factors differ in cooperation with cellular transcription factors other than Sp1, we examined the E2 response of molecular constructions containing defined sites for other cellular transcription factors in addition to two E2 motifs (Fig. 6A). They included binding sites for nuclear factor-1/CTF, Oct-1, and AP-1 as motifs that are present in HPV regulatory regions, and USF as another common promoter motif. Constructions with one or two Sp1 sites served as positive controls, while a construct containing a GAL4 17-mer and another clone with no additional binding sites for cellular factors were included as negative controls. While the E2x2-Sp1x2-tk reporter promoter containing two Sp1 motifs was activated most strongly by both HPV-16 and BPV-1 E2, a single Sp1 site in E2x2-Sp1-tk sufficed for 10- to 12-fold activation under these conditions. Similar levels of cooperative activation (6- to 12-fold) were observed with reporter clones containing USF, Oct-1, and AP-1 sites, while the NF-1 motifs conferred a three- to fivefold response. In contrast, E2 alone did not suffice to activate the promoter either from sites

A

cat Reporter Plasmids:		Relative CAT Activity		
		neo	HPV-16 E2	BPV-1 E2
a	E2x2-Sp1x2-tk (-38)	0.8	46.8	32.0
b	E2x2-Sp1x1-tk (-38)	0.8	11.5	12.2
c	E2x2-USF2-tk (-38)	0.9	7.5	6.8
d	E2x2-USF1-tk (-38)	1.1	16.6	13.1
e	E2x2-Oct-1x2-tk (-38)	1.3	11.4	10.9
f	E2x2-AP-1x2-tk (-38)	0.8	9.9	8.7
g	E2x2-NF-1x2-tk (-38)	1.2	2.7	5.3
h	E2x2-GAL4x2-tk (-38)	0.7	1.8	1.8
i	E2x2-tk (-38)	1.0	1.4	1.9
j	E2x2 tk (-109) cat	1.8	81.9	72.7
k	E2x2 SVE cat	0.7	31.0	42.7

B

Chimeric Activator Clones:		Relative CAT Activity		
		neo	HPV-16 E2	BPV-1 E2
a	GAL4	2.4	22.2	33.3
b	GAL4-VP16	299.0	593.0	666.0
c	GAL4-(BPV-1)-E2	1.2	1.2	1.3
d	GAL4-Sp1-ABC	3.3	19.5	37.9
e	GAL4-Sp1-B	0.9	4.5	8.0
f	GAL4-TEF-1Δ55-121	3.2	7.8	6.7
g	GAL4-E1a	3.7	26.6	22.5
h	GAL4-ER (A,B)	1.0	1.3	1.8
i	GAL4-ER (E,F)	1.6	6.7	13.8
j	GAL4 DBD	1.0	1.2	1.0

FIG. 6. Activation domains of unrelated transcription factors are necessary and sufficient for cooperation with HPV-16 or BPV-1 E2. (A) HPV-16 and BPV-1 E2 products cooperate with unrelated cellular factors. Reporter *cat* clones containing E2-binding sites upstream of cellular factor-binding sites and the tk (-38) promoter (1.5 μ g per 35-mm well) were cotransfected in duplicate cultures with pCG (16)-E2 or pCG-E2 in two independent experiments as described in the legend to Fig. 5. Activation was calculated relative to the CAT enzyme levels obtained with E2x2-tk (-38)-cat (clone i) in cotransfections with pCG-neo. (B) HPV-16 and BPV-1 E2 proteins cooperate with a broad range of activation domains. Transcription activation domains of the listed transcription factors linked to the GAL4 DNA-binding domain were tested for *trans* activation of an E2 and GAL4-dependent promoter clone, E2x2-GAL4x5-tk (-38)-cat. Relative activation was determined after cotransfection with pE2x2-GAL4-tk (-38)-cat and pCG (16)-E2, pCG-E2, or the pCG-neo control as described in the legend to Fig. 5. TEF-1, transcriptional enhancer factor 1.

adjacent to the TATA box (at a distance of approximately one DNA turn) or separated from the TATA box by a single or duplicate GAL4 17-mer binding sites (approximately three or six DNA turns, respectively). These results indicate that similar to BPV-1 E2, the HPV-16 E2 protein requires the cooperation of at least one additional cellular transcription factor for the activation of a minimal TATA box promoter.

To define the types of transcription activation domains that the HPV-16 and BPV-1 E2 proteins can cooperate with, we also tested their activity in cotransfection experiments with chimeric GAL4 proteins containing the TADs of other cellular and viral activators. As representative acidic amino acid-rich TADs, we used the full-length GAL4 protein (aa 1 to 881) (33, 60), the negatively charged TAD of HSV-1 VP16 (Vmw65; aa 411 to 490) (43), and the TAD of BPV-1 E2 itself (aa 1 to 210). In addition to the ABC and B fragments of the glutamine-rich TAD of Sp1, we also tested other nonacidic TADs: transcriptional enhancer factor 1 (aa 1 to 55 and 121 to 426) (62), adenoviral E1A (aa 121 to 222) (31), and the constitutive A/B (aa 1 to 184) and the estrogen-dependent E/F (aa 148-282) TAD fragments of the estrogen receptor (54, 57).

The results are shown in Fig. 6B. As expected, the entire activation domain ABC of Sp1 as well as the glutamine-rich domain B synergized with both E2 activators (clones *d* and *e*). The constitutive TAD segment of the estrogen receptor ER-AB (clone *h*) exhibited little activity under these conditions while the hormone-responsive ER-EF domain (clone *i*) cooperated with the E2 factors approximately four- to eight-fold. The TAD of adenovirus E1A (31) and the GAL4 TAD as part of the entire GAL4 activator also led to synergistic cooperation with the E2 factors (clones *g* and *a*). Neither the VP16 activation domain (clone *b*) nor the BPV-1 E2 TAD (clone *c*) synergized with either E2 factor, although the GAL4-VP16 clone strongly activated the target promoter alone, while the GAL4-E2 TAD construct did not. Interestingly, the GAL4-transcriptional enhancer factor 1 construction showed only a modest cooperation with the E2 vectors under these conditions (clone *f*). The HPV-16 or BPV-1 E2 proteins thus are not restricted to cooperation with Sp1 or with glutamine-rich TADs but function with a variety of different cellular transcription factors.

DISCUSSION

This study demonstrates that the full-length HPV-16 E2 protein can function as a strong transcriptional activator. Its activity depends on cooperation with at least one of a broad range of cellular transcription factors, including those that bind to HPV-16 regulatory sequences. In addition to its postulated role as repressor of the HPV-16 E6-E7 region promoter, P97, the HPV-16 E2 protein thus also has the potential to activate viral gene transcription.

DNA-binding function of HPV-16 E2. The HPV-16 E2 protein bound a synthetic consensus E2 site with an affinity and stability equivalent to those of BPV-1 E2, in agreement with its comparable ability to *trans* activate reporter clones with such motifs in vitro and in vivo. The E2 protein preparations used here were purified from HeLa cells infected with E2-expressing vaccinia virus vectors. It is not apparent why the observed K_d values of $\sim 10^{-9}$ M for both E2 proteins were higher than those reported for full-length BPV-1 E2 expressed in a baculovirus (37). Possible explanations include differences between E2 sites and experimental protocols used (for example, the presence of nonspecific competitor DNA in our assays). In addition, E2 proteins expressed in mammalian cells may

undergo functionally important posttranslational modifications that modulate DNA binding.

In contrast to the consensus E2 sequence, binding to selected E2 motifs from HPV-16 and BPV-1 varied widely. While differences between sites could be accounted for by nucleotides within and outside the ACC(N)₆GGT palindrome (4, 29), the two E2 DBDs also diverged in their affinities and complex stabilities on the same motif despite their peptide homologies. HPV-16 E2 bound with somewhat lower affinities and higher stabilities (that is, higher K_d and longer $T_{1/2}$ values) than BPV-1 E2 at the homologous HPV-16 E2 sites 1 and 2. Furthermore, the HPV-16 E2 protein recognized a BPV-1 E2 motif only poorly while BPV-1 E2 did not bind well to an HPV-16 E2 site (data not shown). As no clear rules have emerged, the K_d and $T_{1/2}$ values at each site therefore can be arrived at only experimentally. The specific amino acid-DNA contacts responsible for DNA recognition and binding stability will need to be determined by a comparison of the crystal structure of HPV-16 E2-DNA complexes with that formed by BPV-1 E2 (22).

trans-activation function of HPV-16 E2. In agreement with the conserved peptide structure of papillomaviral E2 proteins (3, 18), *trans* activation by HPV-16 E2 in vivo and in vitro was equivalent to that of BPV-1 E2. When expressed from comparable pCG-based clones, both E2 proteins activated transcription at defined promoters up to 100-fold in a wide range of cell types. It is difficult to reconcile these results with the inconsistent HPV E2 activities reported in previous studies (5, 8, 11, 15, 18, 23, 40, 52, 55, 56). E2 clones used previously by us and others utilized diverse promoters and also included additional viral sequences upstream to the E2 ORF that may contain viral promoter elements and untranslated ORF. Such differences in E2 clone design would be expected to affect E2 protein expression in transfected cells, and the lower activity of Rous sarcoma virus long terminal repeats or other cytomegalovirus-driven E2 constructs compared with that of pCG (16)-E2 or pCG-E2 in this study is consistent with this explanation. Furthermore, the data indicate that neither E2 protein requires a cellular factor(s) limited to specific cell types, in agreement with the previously observed ability of the BPV-1 E2 *trans* activator to function not only in cells of mammalian origin but also in insect cells (30) and yeasts (15).

Activation of a minimal TATA box promoter, *tk* (−38), by both the HPV-16 and BPV-1 E2 proteins also required at least one additional DNA-binding proximal factor. Consistent with previous observations on the action of BPV-1 E2 in transfected cells (17, 19, 30), neither E2 protein alone stimulated transcription when positioned directly 5' to the TATA box in vivo or in vitro. Furthermore, in vitro activation was abolished by depletion of the cooperating factor. Using chimeric GAL4 activators, we show that the activation domains of additional factors are both necessary and sufficient for cooperation with either HPV-16 or BPV-1 E2. While both E2 proteins functioned with the glutamine-rich TAD of Sp1 (9, 39), cooperation was not limited to a specific class of TADs. For example, the ligand-dependent TAD, E-F, of the estrogen receptor, and the E1A TAD are not glutamine rich and appear to utilize different subsets of cellular coactivators on the basis of squelching experiments (34, 54). Neither E2 protein cooperated with the acidic, amphipathic helical TAD of VP16 or with the BPV-1 E2 TAD itself, yet the TAD of GAL4 also cooperated with the E2 factors despite the fact that much of its activity also resides in acidic helix domains (33). Taken together, these results argue that E2 *trans* activation stems from functional synergy with a broad range of TADs rather than direct protein-protein interactions with the cooperating fac-

tors. HPV-16 E2 thus has the potential to activate viral transcription in cooperation with one or more of the many cellular transcription factors that bind viral regulatory elements (58).

Role of HPV-16 E2 in viral infection. In BPV-1, E2 coordinately activates a subset of viral early region promoters during infection (20, 48, 51). Our results demonstrate that the HPV-16 E2 protein is a *trans* activator functionally analogous to BPV-1 E2, yet its role as a transcription factor in HPV-16 infection remains unclear. So far, the only well-characterized HPV-16 promoter is the E6-E7 oncogene region promoter, P97 (11, 46, 47); other genital HPV strains have E6-E7 promoters in the same location. In contrast to the BPV-1 E6 promoter, P89 or P2, the HPV-16 P97 as well as the E6 promoters of other genital HPVs can be repressed by E2 proteins (5, 8, 38a, 42, 55, 56). The repression of genital HPV E6 promoters by E2 is thought to be due to displacement of cellular factors by E2 binding to two proximal sites located in an operator position adjacent to the TATA box (5, 8, 14, 15, 38a, 42, 55, 56). This effect, however, does not explain the conserved structure and function of the E2 TADs since the TAD is unnecessary for E6 promoter repression (8, 14, 38a, 55).

Together with the E1 gene product, the full-length E2 protein is required for viral replication (7, 12, 59, 61). It is therefore possible that the E2 TAD structure was conserved to serve in replication. This is a compelling concept since the replication and *trans*-activation requirements could not be separated by mutagenesis of the N-terminal portion of the BPV-1 E2 protein (61). It is, however, also possible that the E2 activator in genital HPVs functions to activate other viral promoters. Karlen and Beard have recently described potential promoters in HPV-18 that were active in *in vitro* transcription assays and may correspond to P3, P4, and P5 of BPV-1 (26). Smotkin and coworkers defined an additional transcription start site in the E6-E7 gene region of HPV-6 (46) that was absent from HPV-16-positive cervical carcinoma cells (46, 47). However, this or other HPV-16 promoters would have escaped detection in E2-negative cervical carcinoma cells if their activity depended on the presence of E2.

Alternatively, other viral promoters may depend on at least partial keratinocytic differentiation of the host cell and thus would be expected to be active mostly in the suprabasal cells of the epithelium. We speculate that the *trans*-activation function of the full-length E2 protein of HPV-16 may become critical at later stages of viral infection that occur in suprabasal keratinocytes. First, we would expect E2 synthesis to be increased at the DNA replication stage of papillomavirus infection as E2 is absolutely required for the replication of either BPV-1 or HPV genomes (7, 12, 59). This hypothesis is consistent with relatively high levels of E2 region transcripts observed by *in situ* hybridization in the upper epithelial layers of cervical warts (50). Second, the levels of cellular transcription factors that are active in basal keratinocytes are likely to become lower as the terminally differentiating cells undergo programmed cell death. A very strong activator such as the E2 protein not only could substitute for cell factors that are no longer available but also may efficiently utilize diminishing levels of those DNA-binding factors and coactivators that are required for its own function. It is also quite possible that in this situation, E2 activates transcription in cooperation with other factors that are not present in basal cells, such as the POU factor Skn-1 (1). Finally, negative cellular factors that may block the activity of specific viral promoters in basal cells would also be expected to decrease in activity and concentration and thus permit E2-directed reprogramming of viral transcription. These possibil-

ities will need to be tested in differentiating cell systems and by modulating the functions of individual positive and negative cellular factors in transfection and *in vitro* transcription experiments.

ACKNOWLEDGMENTS

We thank B. Moss for vaccinia virus vectors and protocols, M. Ustav and A. Stenlund for BPV-1 pCG-E2 clones, S. Liang and M. Green for many GAL4 constructs, W. Jackson and R. Tjian for Sp1 cDNA, A. Stenlund, M. Stinski, M. Stoltzfus, and P. Winokur for comments on the manuscript, J. Carl for photography, and S. McConnell for editorial help.

This work was supported by the Department of Veterans Affairs, the National Institutes of Health (CA-49912), the American Cancer Society (VM-69), the Diabetes and Endocrinology Research Center and the DNA Core at the University of Iowa (L.P.T. and T.H.H.), and INSERM and CNRS (I.D.). S.P. was supported in part by funds from the University of Kuopio and the Finnish Cancer Society. L.P.T. was a Clinical Investigator and T.H.H. was a Research Associate of the Veterans Affairs Research Career Development program.

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