JOURNAL OF VIROLOGY, Feb. 1994, p. 1115–1127
0022-538X/94/$04.00+0

Vol. 68, No. 2

Human Papillomavirus Type 11 E2 Proteins Repress the Homologous E6 Promoter by Interfering with the Binding of Host Transcription Factors to Adjacent Elements

GANG DONG,† THOMAS R. BROKER,‡ AND LOUISE T. CHOW*

Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642

Received 23 July 1993/Accepted 12 November 1993

The E6 promoter of human papillomaviruses (HPVs) trophic for epithelia of the lower genital tract and the upper respiratory tract is regulated in vitro by homologous and heterologous papillomaviral E2 proteins that bind to a consensus responsive sequence (E2-RS) ACCN$_6$GGT. When HPV type 11 (HPV-11) expression is examined in epithelial cell lines, the HPV-11 E2-C protein, which lacks the amino-terminal transactivating domain of the full-length E2 protein, invariably represses the homologous viral E6 promoter. In contrast, when the novel constitutive enhancer (CE) CE II is deleted, not only is the basal promoter activity much reduced, it is further repressed by the intact HPV-11 E2 protein (M. T. Chin, T. R. Broker, and L. T. Chow, J. Virol. 63:2967–2976, 1989). Here, we demonstrate that, when expressed from a stronger surrogate promoter, the HPV-11 E2 protein represses the E6 promoter effectively, regardless of CE II. By performing systematic mutational analyses of the four highly conserved copies of the HPV-11 E2-RS and of the adjacent enhancer-promoter elements, we show that the furthest upstream, promoter-distal E2-RS copy 1 plays no apparent role in E6 promoter regulation. Repression by the homologous HPV-11 E2 proteins is mediated through each of the three promoter-proximal copies of the E2-RS, but the presence of CE II abrogates the full-length E2 protein repression exerted at E2-RS copy 2. Repression is alleviated when the two (for E2) or three (for E2-C) promoter-proximal copies of E2-RS are mutated. We specifically demonstrate that repression exerted at E2-RS 3 is due to preclusion of binding of the host transcription factor Sp1 or Sp1-like proteins to a nonconsensus sequence AGGAGG located 1 bp upstream of the tandem E2 protein binding sites 3 and 4. A 3-bp insertion between the adjacent Sp1 and E2-RS 3 sites permits both Sp1 and E2 proteins to bind, with a concomitant relief of E2-RS 3-mediated repression. Similar mutational analyses show that proteins that bind to the GT-1 motif near the upstream E2-RS 2 help abrogate repression by the E2 protein in the presence of CE II. The implications of these results with respect to the viral infectious cycle and during viral oncogenesis are discussed.

Infections of genital and oral epithelia by human papillomaviruses (HPVs) cause a broad spectrum of epithelial lesions ranging from benign condylomata and papillomas associated with types 6 and 11 to intraepithelial dysplasias associated with types 16 and 18 and other closely related types. Infections by the latter group of viruses pose a risk for neoplastic progression to cervical and penile cancers (64). Studies in vitro and in vivo have identified the E6 and E7 gene products as viral oncoproteins (2, 22, 25, 35, 44, 58). Thus, the regulation of the E6 promoter responsible for their transcription has been the subject of intense investigation. Several investigations show that the E6 promoter is under the control of viral E2 proteins or host factors that bind to the upstream regulatory region (URR), also known as the long control region (for reviews, see references 23, 43, and 54).

We have previously identified two constitutive enhancer (CE) domains, CE I and CE II, in the HPV type 11 (HPV-11) URR which are active in several types of cell (8, 16, 28) (Fig. 1). Multimerized copies of CE I, which contains several functional NF-1 sites, can activate surrogate promoters in monkey CV-1 cells and the homologous E6 promoter in the cervical carcinoma cell line C-33A and in primary human keratinocytes and fibroblasts. CE II includes a novel palindrome (CCTGGCGCCAGG) with an adjacent AP-1-like motif. Host proteins that bind to CE II impart transcriptional activation in cervical carcinoma cell lines but not in primary keratinocytes.

Papillomaviral E2 proteins regulate both viral mRNA transcription (for reviews, see references 23, 43, and 54) and viral DNA replication (7, 14, 48). There are three HPV-11 E2 proteins: the full-length E2, carboxyl-half-length E2 (E2-C), and an E1M^E2C fusion protein containing the amino-terminal quarter of the E1 protein fused to the E2-C domain (5, 51, 52). They share a carboxyl-terminal domain essential for protein dimerization and DNA binding. E2 proteins of all animal papillomaviruses and HPVs recognize the consensus sequence ACCN$_6$GGT, designated the E2-responsive sequence (E2-RS) or binding site (E2BS) (1, 28, 40). The E2-C and E1M^E2C proteins are transcription repressors. The full-length HPV-11 E2 protein activates the HPV-11 URR linked to the minimal simian virus 40 (SV40) promoter in monkey CV-1 cells (27, 28). In the cervical carcinoma-derived cell line C-33A, a very weak activation of the homologous E6 promoter is observed only when CE II is present. When CE II is deleted, basal activity of the E6 promoter is greatly reduced and the residual E6 promoter activity is further repressed by

* Corresponding author. Mailing address: Department of Biochemistry and Molecular Genetics, The University of Alabama at Birmingham, 1918 University Blvd., Birmingham, AL 35294-0005. Phone: (205) 975-8300. Fax: (205) 975-6075.

† Present address: Molecular Hematology Branch, National Heart, Lung, and Blood Institute, Bethesda, MD 20892.

‡ Present address: Department of Biochemistry and Molecular Genetics, The University of Alabama at Birmingham, Birmingham, AL 35294-0005.

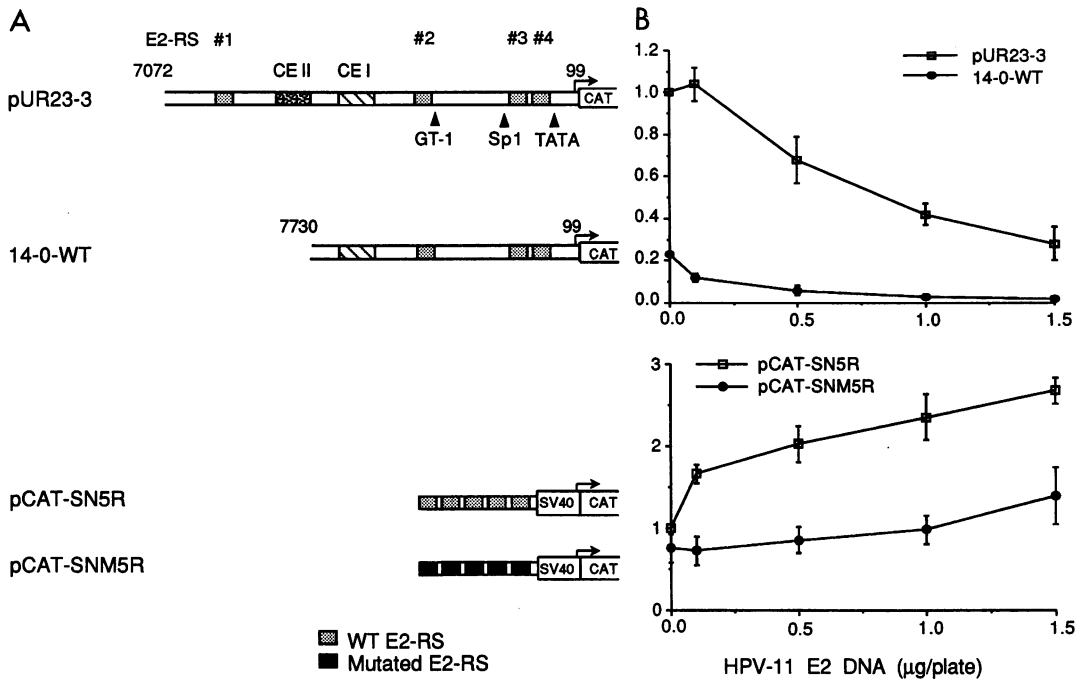


FIG. 1. Dual effect of HPV-11 E2 protein on the homologous E6 promoter. (A) Reporter plasmids. Simplified, schematic representations of the entire HPV-11 URR (clone 23-3) and 5′ deletion clone (14-0-WT) are presented in the top panel. *cis* elements depicted are CE II, nt 7677 to 7747; CE I, nt 7777 to 7821; four E2-RSs, each with the sequence $ACCGN_4CGGT$ (1, nt 7592 to 7603; 2, nt 7892 to 7903; 3, nt 35 to 46; and 4, nt 50 to 61); a putative GT-1 motif, 5′-CCACACCC-3′, 3 bp downstream of E2-RS 2 in the upper strand; Sp1, 5′-AGGAGG-3′, 1 bp upstream of E2-RS 3 in the upper strand; and the TATA motif of the E6 promoter 4 bp downstream of E2-RS 4 in the upper strand. The arrow represents the initiation site of the E6 promoter-derived transcripts at nt 99 (8). (B) Relative CAT activities of HPV-11 E6 promoter-CAT reporter clones, with and without CE II, when cotransfected with the increasing amounts of the HPV-11 E2 expression plasmid pPV020 (upper panel); and of the SV40 promoter-driven CAT gene placed downstream of five copies of either wild-type (-SN5R) or mutated E2-RS (-SNM5R) in the presence of increasing amounts of the E2 expression plasmid (lower panel). The total amount of DNA in each transfection was held constant by addition of the vector plasmid without an insert. The results are the average of three independent experiments, each performed in duplicate. Error bars are standard deviations.

the intact E2 protein. Insertion of multiple copies of the CE II sequences upstream of the minimal E6 promoter partially restores the basal activity and also partially abrogates the repression by the E2 protein. We have postulated that binding of active forms of CE II-cognate proteins stabilizes the preinitiation transcription complex assembled around the E6 promoter (8). However, the E2 or host factor binding sites that are involved in these interactions have not been localized. No CE II-like element has been defined in HPV-16 or HPV-18, and their homologous, full-length E2 proteins weakly repress the respective E6 promoters. The intact E2 protein of bovine papillomavirus type 1 (BPV-1) strongly represses the HPV-11 E6 promoter regardless of CE II (9), and it is also a stronger negative regulator of the E6 promoter of the other genital HPVs just described (17, 50, 59, 61). The basis for this difference between HPV and BPV E2 proteins is not understood.

Four copies of E2-RS are present in the URR of all HPV types sequenced to date that are trophic for mucosal epithelium, and their arrangement is highly conserved (Fig. 1). Copy 1 in HPV-11 is located well upstream of the enhancer region. Copy 2 precedes the E6 TATA motif by about 100 bp. A tandem pair of E2-RSs (3 and 4) is situated 1 bp downstream of a proven or putative variant Sp1 site and 3 to 4 bp upstream of the TATA motif. Repression of the E6 promoter of HPV-16 (P_{97}) and HPV-18 (P_{105}) by the BPV-1 E2 protein is mediated through E2 protein binding to one, two, or three copies of these E2-RSs proximal to the TATA motif (50, 59, 61). Repression is attributed to occlusion of the TATA-binding protein (TBP) or Sp1 protein from the flanking sequences (Fig. 1) by the BPV-1 E2 protein on the basis of in vitro binding experiments (17, 21, 59). Alternatively, repression may arise from DNA conformational changes upon binding by the E2 protein (21). The GGGCGT Sp1 motif in HPV-16 and HPV-18 is a variant of the consensus GGGCGG motif, and it has been speculated that other nonconsensus sequences in similar genomic regions of additional genital HPV types bind Sp1 (29, 59). The AGGAGG sequence in the comparable region of HPV-11 has not been tested directly, nor has Sp1 protein displacement been shown with homologous viral E2 proteins for any of the HPVs. In particular for HPV-11, the roles of each of the four E2-RSs have not been dissected until now. In this report, we describe detailed mutational analysis of the E2-responsive sites and nearby *cis* elements in the context of the HPV-11 enhancer-E6 promoter and in response to the homologous viral E2 and E2-C proteins. We show that copy 1 of the E2-RS distal to the promoter plays no apparent role in transcriptional regulation. Rather, promoter repression by the homologous E2 proteins is mediated through each of the three promoter-proximal E2-RSs, while CE II abrogated E2-mediated repression exerted at E2-RS 2. Our results also show that binding of Sp1 and Sp1-like proteins to the nonconsensus sequence AGGAGG upstream of E2-RS 3 is critical for high

basal activity of the E6 promoter and that E2 proteins preclude Sp1 from binding to this site, leading to promoter repression.

MATERIALS AND METHODS

Plasmids. pUR23-3 (nucleotides [nt] 7072-[7933/1]-99), 24-N (nt 7674-[7933/1]-99), and 14-0-WT (nt 7730-[7933/1]-99) containing various lengths of the HPV-11 URR contiguous with the E6 promoter were cloned upstream of the bacterial chloramphenicol acetyltransferase (CAT) reporter gene (8). Note that the revised nucleotide length for HPV-11, based on correction of the prototype sequence in the URR, is used (16a). pCAT-SN5R and pCAT-SNM5R contain five synthetic oligonucleotide copies of either wild-type or mutated E2-RS upstream of a minimal SV40 early promoter-driven CAT gene (28). pKV461 is a eucaryotic expression vector which contains a cytomegalovirus (CMV) immediate-early (IE) promoter (55). pPV020 expresses the HPV-11 E2 protein and was generated by transferring a blunt-ended *Bam*HI-*Hin*dIII fragment containing the HPV-11 E2 cDNA from clone pRS/$11E_{1-2}A^+$ (52) into pKV461 downstream of the CMV IE promoter. pPV021 expressing the HPV-11 E2-C protein was similarly generated by transferring the *Bam*HI fragment encoding the HPV-11 E2-C protein from pRSE2-C(3-11) (9). pPVE2AS contains an *Eco*RI fragment of the HPV-11 E2 cDNA from pMT2-E2 (7) cloned in the antisense orientation in pKV461. pSP72-Sp1 was prepared by transferring the *Xba*I-*Sma*I fragment of pSp1-778C containing the human Sp1 cDNA (34) to the compatible sites in pSP72 (Promega). pKV461, pPV020, pPV021, and pSP72-Sp1 were provided by Mark P. Sowden of our laboratory. p20-99 containing HPV-11 nt 20 to 99 cloned into the blunt-ended *Hin*dIII site of pUC19 and pUC-SN1 containing HPV-11 E2-RS 2 (nt 7891 to 7904) cloned into the *Hin*cII site of pUC-19 were provided by Jen-Sing Liu of our laboratory.

Oligonucleotide-directed mutagenesis. The *Hin*dIII fragment of 24-N that spans HPV-11 URR nt 7674-[7933/1]-99 was cloned into pBS+ (Stratagene) to generate pBS+24-N. Point mutations were introduced into E2-RS copies 3 and 4 or both according to the methods of Kunkel (37), as described previously (6). After identification of the mutated clones, the *Hin*dIII fragments from pBS+24-N-3M, pBS+24-N-4M, and pBS+24-N-34M were purified to replace their counterpart in 24-N in the same orientation, resulting in 24-N-3M, 24-N-4M, and 24-N-34M. The clone 24-N-2M with a mutated E2-RS 2 has been described by Chiang et al. (6). 24-N-23M, 24-N-24M, and 24-N-234M were then prepared by swapping fragments digested with restriction endonucleases *Sph*I and *Bst*EII that cut once in the 5′ polylinker and once downstream of E2-RS 2 in 24-N-3M, 24-N-4M, and 24-N-34M with fragments from 24-N-2M digested with the same two enzymes. 14-0-2M, 14-0-3M, 14-0-4M, 14-0-23M, 14-0-24M, 14-0-34M, and 14-0-234M were generated by swapping *Sph*I and *Bsu*36I fragments between clone 14-0 and each corresponding mutation in clone 24-N. 23-3-234M was similarly made by swapping between pUR23-3 and 24-N-234M. Each clone was confirmed by double-stranded DNA sequencing. E2-C protein expressed in bacteria was not able to bind to the single, double, or triple E2-RS mutations (but retained the ability to bind to any remaining wild-type copies), as assayed by DNase I footprinting (6) (data not shown).

14-0-Sp1M, 14-0-GT1M, 14-0-24MSp1M, and 14-0-34MGT 1M were similarly prepared by site-directed mutagenesis, as described, by using primer 5′ TTTCGGTTTCCCTCTCCCT AC 3′ for GT1M and 5′ AAAAGAGCTCAAACCGAAAAC 3′ for Sp1M. 23-3-Sp1M and 23-3-GT1M were constructed by swapping the corresponding *Sph*I-*Bsu*36I fragments from 14-0-Sp1M and 14-0-GT1M. 14-0-24MSp1+3 was constructed by first digesting 14-0-24M with *Ava*II and blunt-ending the fragments with the Klenow fragment of the *Escherichia coli* DNA polymerase I and then digesting the product with *Hin*dIII. The resulting two HPV-11 URR-containing fragments were purified by gel electrophoresis and then used to replace the URR in 14-0 contained in the *Hin*dIII fragment. Clones substituted with Sp1M were identified by the loss of the *Ava*II restriction site in the URR. 14-0-34MGT1+5 was constructed by digestion of 14-0-34M with *Bst*EII, followed by filling in the termini with DNA polymerase I Klenow fragment and sealing with T4 DNA ligase. 14-0-34MGT1+15 was constructed by the addition of a 10-bp *Bam*HI linker (Bethesda Research Laboratories) in the religation reaction of 14-0-34MGT1+5. All mutations were confirmed by double-stranded DNA sequence analysis.

Transient transfections and CAT assays. By using the calcium phosphate precipitation method (27), the wild type and each of the mutant URR-E6 promoter-driven CAT clones were transfected into C-33A cells, a human cervical carcinoma cell line that does not harbor endogenous HPV sequences, together with either the HPV-11 E2 expression vector pPV020 or the E2-C expression vector pPV021. Cotransfections with pKV461 or pPVE2AS were used as controls. Transfections were performed five or six times, each in duplicate, with several DNA preparations. Unless otherwise stated in the figure legends, 2 μg of each HPV-11 URR-E6 promoter-driven CAT plasmid and 1.5 μg of expression vector or control DNA were transfected into cells at 30% confluence in 60-mm-diameter plates. Cells were shocked for 1 min with 15% glycerol 4 to 6 h later and were then incubated in 5 mM sodium butyrate for 42 to 44 h and harvested. CAT assays with 40 to 50 μg of protein lysate for pUR23-3 and the 24-N series or 100 μg for the 14-0-WT series were carried out by the fluorodiffusion method for separation of substrate and products, as modified in our laboratory (5, 47). Protein concentrations were determined by using the Bradford assay (Bio-Rad). Initially, pCMV-β-galactosidase expression vector (equivalent to 1/10 of the total amount of DNA) was cotransfected as an internal control and the color reaction of β-galactosidase was performed as described earlier (42). We found that the CAT assays were reproducible and that this internal calibration was not necessary, and it was not included in later experiments.

Gel mobility shift assay. Human Sp1 messenger RNA was obtained by in vitro transcription of pSP72-Sp1 using the SP6 RNA polymerase. Sp1 protein was prepared by translation with a rabbit reticulocyte lysate (Promega) according to the manufacturer's recommendations. Small-scale preparations of C-33A cell nuclear extracts were made by the method of Lee et al. (38). The DNA probes for testing the Sp1 activity were made as follows: aliquots of gel-purified DNA *Dra*I-*Hin*dIII fragments (HPV-11 nt 20 to 99 from 14-0-4M and nt 20 to 99 from 14-0-4MSp1+3) were ^{32}P labelled at the 3′ end with the Klenow fragment of the *E. coli* DNA polymerase I. Unlabelled competitors used in the various experiments included similarly purified but unlabelled fragments as well as HPV-11 nt 20 to 99 from 14-0-4MSp1M, 14-0-Sp1M, and 14-0-34M, nt 7874-[7933/1]-20 from 14-0-WT, nt 7874-[7933/1]-20 from 14-0-GT1M, the SV40 early promoter region containing 6 tandem copies of the Sp1 binding site, or the 123-bp ladder (Life Technologies) as a nonspecific competitor. A probe spanning nt 7902-[7933/1]-15 and another containing the GT-1 motif and spanning nt 7874 to 7906 were separately prepared by 25 cycles of PCR amplification. Reactions were performed in a total volume of 50 μl containing, respectively, 50 ng of plasmid 23-0 (with HPV-11

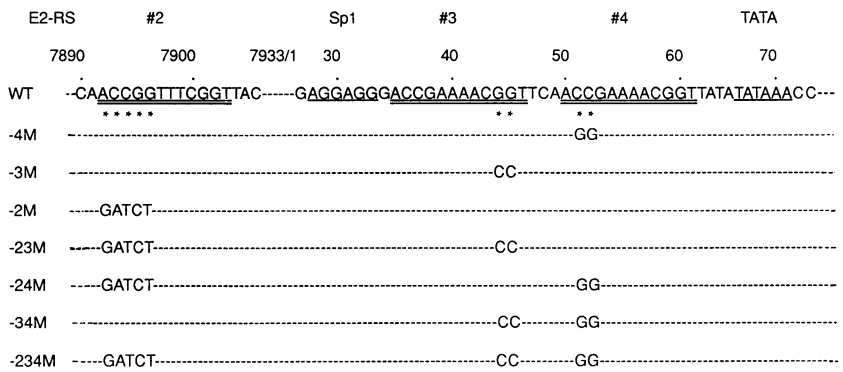


FIG. 2. E2-RS mutations in the HPV-11 URR. Only relevant sequences between nt 7890 and 73 are shown. E2-responsive sites are double underlined. Sp1 and TATA motifs have single underlines. Dashed lines in the mutants represent sequence identity, and substituted nucleotides in the mutations are indicated. WT, wild type. Asterisks, mutated bases; dots, nucleotides for which the positions are given.

nt 7902-[7933/1]-99) or plasmid p7874-7906 (6), 50 μCi of [α-^{32}P]dCTP (3,000 mCi/mM), 0.05 mM each of the deoxynucleotide triphosphates (dNTPs), 5 μl of 10× PCR buffer (10 mM Tris-HCl [pH 8.4], 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg of gelatin per ml), 0.5 μg of each of the flanking primers and 2.5 U of AmpliTaq polymerase (Perkin-Elmer Cetus). To make the unlabelled competitor DNA, [^{32}P]dCTP was omitted and the four dNTPs (0.1 mM each) were used.

For gel mobility shift assays (19), 3 μl of programmed reticulocyte lysates, control lysates without added Sp1 mRNA, or 0.5 μg of C-33A cell nuclear extract was preincubated in a total volume of 20 μl containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-HCl (pH 7.8), 10% glycerol, 5 mM $MgCl_2$, 60 mM KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, and 0.5 to 1.0 μg of poly(dI-dC)-poly(dI-dC) on ice for 10 min. ^{32}P-labelled DNA fragment (5,000 to 10,000 cpm [10 fmol]) was added, and incubation was continued on ice for 30 min. In competition experiments, 24- to 600-fold molar excesses of unlabelled DNA fragments or synthetic oligonucleotides were added to the tube prior to the labelled probe. When HPV-11 E2-C protein was included, 0.05 or 0.10 μg of the induced bacterial lysate was added either before or after the addition of Sp1 proteins (see the legend to Fig. 7). After a 30-min incubation on ice, the entire reaction mixture was loaded onto a 5% polyacrylamide gel (39.5:0.5) and run at 200 V in 0.25× Tris-borate-EDTA in the cold room (4°C). The gel was then dried and subjected to autoradiography at −70°C. The autoradiograms were either photographed (see Fig. 6 and 9) or scanned by densitometer (see the legend to Fig. 7).

RESULTS

Modulation of the E6 promoter by the homologous HPV-11 E2 proteins. To dissect the respective roles of the E2-responsive sequences in the regulation of the HPV-11 E6 promoter by the homologous HPV-11 E2 and E2-C proteins, we expressed the E2 proteins from the strong CMV IE promoter instead of from the relatively weaker Rous sarcoma virus long terminal repeat used in our previously described experiments (8). Consistent with the earlier results, clone 14-0-WT which lacks CE II was repressed when cotransfected into C-33A cells with as little as 0.1 to 0.5 μg of new expression vector (Fig. 1B, top panel). Unexpectedly, clone pUR23-3, which contains the entire URR, showed dose-dependent responses upon cotransfection with E2 protein expression vector (Fig. 1B, top panel). A small amount of the E2 expression vector had little or no effect, consistent with previous results (8). However, with greater amounts of E2 expression vector, the E6 promoter was increasingly repressed. We attribute the difference from the prior results to a greater amount of E2 protein generated by the more potent expression vector used in this study. However, the levels of E2 protein in the transfected C-33A cells remained below detectable limits by either Western blotting (immunoblotting) or immunoprecipitation (data not shown).

To substantiate that E2 protein was indeed expressed in the above experiments, we cotransfected the same amounts of the E2 expression plasmid with pCAT-SN5R, which contains five copies of consensus E2-RS inserted upstream of a minimal SV40 early promoter (28) (Fig. 1B, bottom panel). A reproducible activation was observed in a dose-dependent manner. The relatively weak fold stimulation even at a high concentration of the E2 expression vector can be attributed to the already high basal activity of the minimal SV40 promoter in C-33A cells. With pCAT-SNM5R, which contains five copies of mutated E2-RS (28), a weak activation was detected only with the greatest amount of E2 expression vector used (Fig. 1B, bottom panel). These results suggest that the effect of E2 proteins depends not only on the presence of other *cis* elements in the regulatory region but also on the amount of E2 protein expressed.

Functional analysis of individual E2-responsive sites in the modulation of the HPV-11 E6 promoter by E2 proteins in the presence of CE II. To elucidate the functions of the four copies of E2-RS in E2-mediated regulation, point mutations were introduced alone or in combination into the three promoter-proximal copies of E2-RS (copies 2, 3, and 4) (Fig. 2) in reporter clones 23-3 and 24-N, which contain CE II, and in clone 14-0-WT, in which CE II is partially deleted and is nonfunctional (8). The single, double, or triple E2-RS mutations efficiently eliminated the binding of bacterially expressed HPV-11 E2-C protein (reference 6 and data not shown), which has previously been demonstrated to give identical DNase I footprinting as the intact E2 protein (9, 28). Only clone 23-3-234M still contains E2-RS 1. These CAT reporter plasmids thus contain 0, 1, 2, 3, or 4 wild-type copies of E2-RS.

To assess the role of E2-RS 1 in the presence of CE II, pUR23-3, 24-N, and their E2-RS mutation derivatives were transfected into C-33A cells. pPVE2AS, from which antisense E2 RNA was transcribed, or the vector without insertion was cotransfected as the control plasmid. As shown in Fig. 3, each of the E2-RS mutations resulted in only slight changes in the

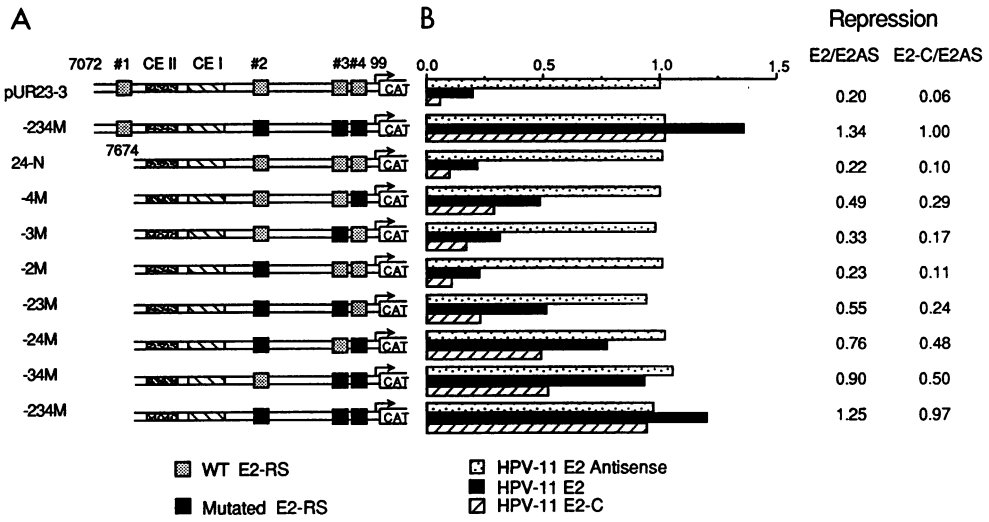


FIG. 3. Regulation of the HPV-11 E6 promoter and mutation derivatives by the homologous E2 proteins in the presence of CE II. (A) Schematic representation of E2-RS mutations in pUR23-3 and 24-N; and (B) their relative CAT activities. Transfections and CAT assays were carried out in C-33A cells as described in Materials and Methods. The relative CAT activities and fold repressions were obtained by comparison of the activities with that of pUR23-3 cotransfected with the plasmid expressing the antisense transcript of E2. Each transfection was performed in duplicate. The results shown were an average of two independent experiments that typically yielded results within 5 to 20% of each other, with the exception of E2-C repression of the wild-type (WT) clones, whose activities were very low (e.g., repression measured 0.04 and 0.07 for 23-3 and 0.07 and 0.12 for 24-N). The entire experiment was also repeated three times with the vector pKV461 as a negative control. The relative basal activities in the absence of E2 proteins were somewhat different from those obtained when antisense E2 clone was used as the control. Therefore, the data were not included. However, qualitatively similar results were obtained (data not shown).

basal activities, suggesting the mutations had little effect on the function of *cis* elements which interact with basic host transcription factors. Cotransfection with either the E2 or the E2-C expression vector led to repression by 5- to 10-fold, respectively. Whether this twofold difference in repression is due to the production, stability, or innate activity of the two forms of the E2 proteins expressed cannot be determined. Mutations of all three proximal E2-RSs in 23-3-234M eliminated the repression by E2-C and resulted in a slight stimulation by the intact E2 protein. We interpret this weak stimulation to be an E2-RS-independent, nonspecific effect because pCAT-SNM5R (Fig. 1), 24-N-234M (Fig. 3), 14-0-234M, and other mutation clones that contain no E2-RS (see Fig. 4, 5, and 8) were also stimulated to a similar degree. Deletion of E2-RS 1 (in clone 24-N) had no effect in either basal activity or response to either form of E2 protein. These results suggest that E2-RS 1 played little or no role in E2-mediated promoter regulation. Therefore, subsequent experiments were conducted in the sequence context of clone 24-N.

Mutations in any one of the three E2-RSs in reporter 24-N (i.e., 24-N-4M, 24-N-3M, and 24-N-2M) resulted in only a partial relief of repression by either E2 protein. Mutations 24-N-23M and 24-N-24M, eliminating two of the three E2-RSs, further reduced the degree of repression. Notably, 24-N-34M was not repressed by the full-length E2 protein but was still repressed by E2-C. No repression was observed even when twice the amount of the E2 expression vector was transfected (data not shown). Repression by E2-C was entirely alleviated only in 24-N-234M, in which all three E2-RSs proximal to the E6 promoter were mutated. As discussed above, this mutation was slightly stimulated by the intact E2 protein. On the basis of these results, we conclude that, in the presence of CE II, high amounts of E2 protein repress the E6 promoter through binding to E2-RS 3 or 4, whereas repression by the E2-C protein is mediated through binding to sites 2, 3, or 4. The more binding sites there are, the higher the repression is. The varied extent of residual repression observed with the particular mutations indicates that E2 proteins bind to the several sites with different affinities or that binding to the various copies of the E2-responsive site interferes with different stages of promoter activation or with specific components involved in transcription initiation.

Functional analysis of individual copies of E2-RS in HPV-11 E6 promoter regulation by the E2 proteins in the absence of CE II. The same set of E2-RS mutations was tested in the CE II-deleted reporter clone, 14-0-WT, in the presence or in the absence of the HPV-11 E2 or E2-C expression vector (Fig. 4). As was described previously (8), this clone exhibited fivefold reduced activity when compared to pUR23-3 or 24-N. The basal activity of each mutant derivative was similar to that of the parental clone with the exception of 14-0-4M and 14-0-34M, which consistently showed a 30% higher and 30% lower activity, respectively. Clone 14-0-WT was repressed by either form of HPV-11 E2 protein by 6- to 10-fold, as reported previously (8). Single and double mutations were all repressed to different degrees. 14-0-234M containing no wild-type E2-RS was no longer repressed by the E2-C protein and was consistently activated slightly by the intact HPV-11 E2 protein. These results indicated that, in the absence of CE II, either form of E2 protein represses the promoter by binding to E2-RS 2, 3, or 4.

This series of reporter clones as well as 23-3 and a derivative, 23-3-4M, were also cotransfected with BPV-1 E2 similarly expressed from the CMV IE promoter. All clones were repressed more severely, even with a lower dose (0.5 μg) of the expression plasmid, in comparison with the 1.5 μg used with the HPV-11 E2 or E2-C expression vector. Repression was reduced from 33- to 5-fold in clone 23-3-4M. Total relief of repression in clone 14-0 was observed only when all three E2-RSs were mutated (data not shown).

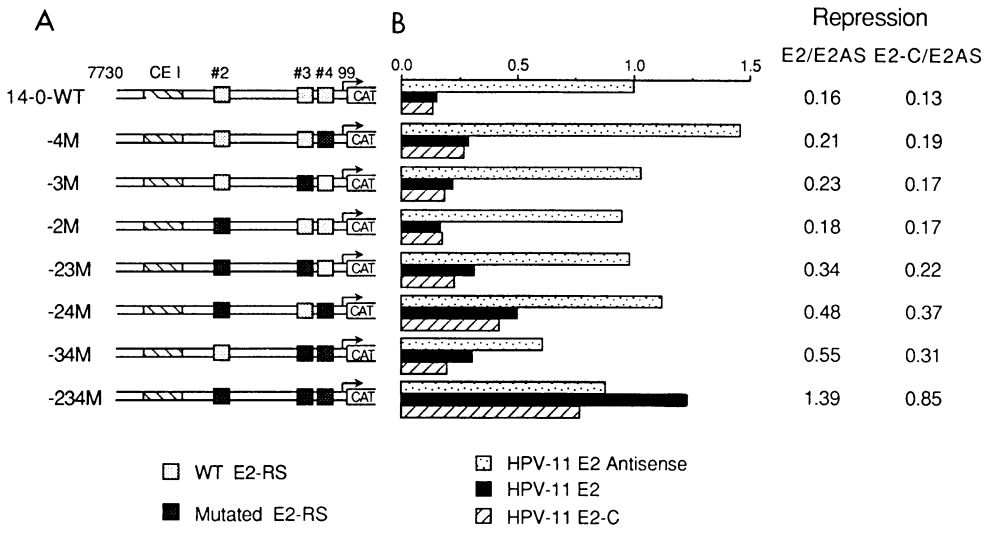


FIG. 4. Regulation of the HPV-11 E6 promoter and mutation derivatives by the homologous E2 proteins in the absence of CE II. (A) Schematic representation of E2-RS mutations in clone 14-0; and (B) their relative CAT activities. Transfections, CAT assays and data analyses were performed as described in Materials and Methods and in the legend to Fig. 3. E2 repression studies were also repeated twice with the vector pKV461 as a negative control. Selected clones were also similarly tested for E2-C repression. Qualitatively similar results were obtained (data not shown).

Mutational analysis of the putative Sp1 motif. To examine whether the putative, nonconsensus Sp1 sequence motif AG GAGG located just 1 bp upstream of E2-RS 3 plays any role in the HPV-11 E6 promoter regulation mediated by the E2 proteins, mutations in this motif were introduced into either pUR23-3 or 14-0-WT to make 23-3-Sp1M and 14-0-Sp1M, respectively. 14-0-24MSp1M was also constructed to monitor this motif in the context of isolated wild-type E2-RS 3. In addition, a 3-bp insertion was placed between the putative Sp1 motif and E2-RS 3 in 14-0-24MSp1+3. All clones were tested in C-33A cells, and the results are presented in Fig. 5. In 23-3-Sp1M and in 14-0-Sp1M, as in HPV-16 and in HPV-18 (20, 29), the basal activities were compromised by at least 70%, indicating this motif is indeed important for the HPV-11 E6 promoter activity. Clone 14-0-24MSp1+3 retained over 80% of the activity exhibited by the parental clone 14-0-24M, suggesting that the 3-bp insertion did not significantly affect protein-DNA interaction. 14-0-Sp1M was still repressed by either of the homologous E2 proteins mediated through E2-RS 2 and 4. Consistent with the data shown in Fig. 4, 14-0-24M was repressed by either form of E2 protein but 14-0-234M was not repressed by the E2-C protein and was stimulated by the E2 protein. In contrast, clones 14-0-24MSp1M and 14-0-24MSp1+3 were no longer repressed by either E2 protein. These results clearly demonstrate that interference with the function of the putative Sp1 site by the E2 proteins bound to E2-RS 3 is responsible for the E6 promoter repression mediated via E2-RS 3 and that a 3-base insertion between the host and viral protein binding sites was sufficient to alleviate the repression.

Binding of recombinant Sp1 and Sp1-like proteins in C-33A cells to the variant binding site. To confirm that Sp1 binds to this nonconsensus AGGAGG motif, we performed electrophoretic mobility shift assays (EMSA) with in vitro-translated Sp1 protein. An 85-kDa protein was the most prominent translation product detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The probe used was an end-labelled DNA fragment spanning HPV-11 nt 20 to 99 containing the AGGAGG sequence, the wild-type E2-RS 3, and a mutated E2-RS 4 (Fig. 6A). A distinct retarded band was evident on the gel upon incubation with the in vitro-translated Sp1 protein in contrast to the unprogrammed reticulocyte lysate (Fig. 6B, lanes 1 and 2). To examine the specificity of the binding, complex formation was tested by the inclusion of excess unlabelled double-stranded DNA competitors. The unlabelled homologous fragment competed strongly (lane 3), as expected, whereas a comparable fragment containing a mutated Sp1 motif competed poorly (lane 4). The fragment containing the 3-bp insertion between the E2 and Sp1 binding sites competed as well as the wild type (lane 5), indicating that the Sp1 binding site is functional in this insertion mutation, consistent with the CAT assay (Fig. 5). A fragment of the SV40 early promoter and enhancer that contains six tandem copies of the SV40 Sp1 site (GGGCGG) also competed efficiently (lane 6). These results show that the AGGAGG motif does bind the Sp1 protein.

To demonstrate the presence of Sp1-like proteins in C-33A cells in which all our functional assays were performed, nuclear extracts were tested by EMSA. A fast minor band and a slower major band consisting of a doublet were observed in the same relative abundances in several preparations of nuclear extract. The faster complex of the doublet exhibited a mobility identical to that of the complex formed with the in vitro-translated Sp1 protein (compare Fig. 6, lanes 8 and 9). All three complexes exhibited the same competition patterns as the complex formed with the in vitro-translated Sp1 protein (compare lanes 10 to 13 with lanes 3 to 6). These results demonstrated that the sequence AGGAGG upstream of E2-RS 3 is recognized by Sp1 and Sp1-like proteins present in C-33A nuclear extracts, although binding of other factors in addition to Sp1 cannot be ruled out.

Interference with Sp1 binding by E2-C protein bound to E2-RS 3. As a direct physical test of whether E2 proteins associated with E2-RS 3 interfere with the binding of Sp1 or Sp1-like proteins to the variant motif, HPV-11 E2-C protein was expressed in *E. coli* and whole cell lysates were used in

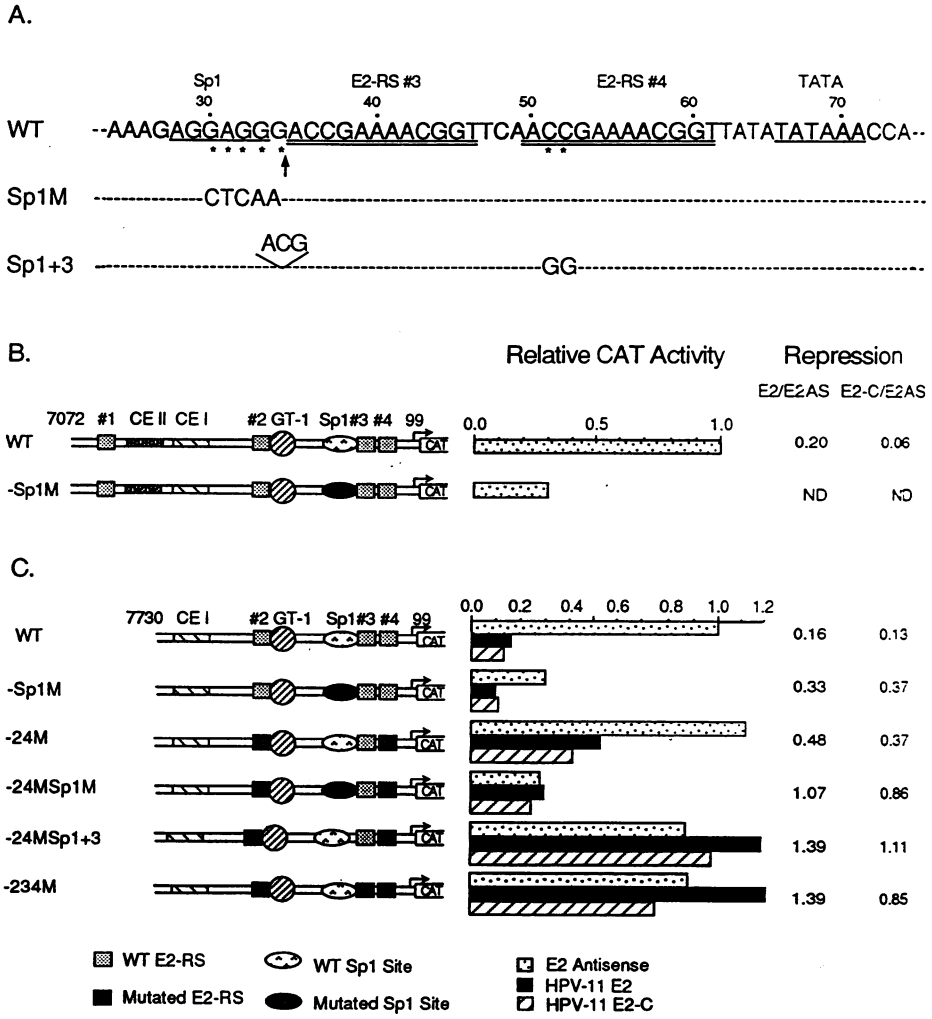


FIG. 5. Mutational analysis of the Sp1 motif in the HPV-11 URR and E2-mediated regulation. (A) DNA sequences of the URR mutations in or near the Sp1 motif. E2-RSs are double underlined and the Sp1 site and TATA motif have single underlines. *, mutation; ---, identical sequences; ↑, site of the insertion. The mutations in E2-RS 4 are also shown. (B) The relative CAT activity of the Sp1 mutation in pUR23-3 was compared with the basal activity of pUR23-3. The fold repression of pUR23-3 by E2 proteins was taken from Fig. 3. ND, not done. (C) Relative CAT activities of mutations and responses to cotransfected E2 or E2-C expression vector were compared with the basal activity of 14-0-WT. The experiment was also repeated one to three times for different clones with the vector pKV461 as a negative control. Qualitatively similar results were obtained (data not shown). WT, wild type.

EMSA with either in vitro-translated Sp1 protein or C-33A nuclear extracts. In this experiment (Fig. 7A) the DNA probe containing the Sp1 motif and a single wild-type E2-RS 3 was the same as that in Fig. 6B. Two different amounts of E2-C lysate formed a single complex as expected (lanes 9 and 10). This complex contained E2-C protein, as judged from the successful competition by a DNA fragment containing three copies of wild-type E2-RS versus one with mutated E2-RS. The complex was additionally supershifted by polyclonal antisera raised against the E2 protein (data not shown). Lanes 2 through 4 and 5 through 7 (Fig. 7A) each contained the same amounts of recombinant Sp1 or nuclear extract. In the presence of increasing amounts of E2-C protein (compare lanes 3 and 4 and lanes 6 and 7), the single Sp1 complex (compare lane 2 with lanes 3 and 4) and the three Sp1-like complexes (compare lane 5 with lanes 6 and 7) were correspondingly reduced or eliminated. These results demonstrated that the binding of E2-C protein to E2-RS 3 precluded Sp1 or Sp1-like proteins from binding to the adjacent Sp1 site.

Consistent with the functional assays (Fig. 5C), a 3-bp insertion between the Sp1 motif and E2-RS 3 allowed the simultaneous binding of both proteins, as shown in Fig. 7B. The DNA probe used (HPV-11 nt 20 to 99) was isolated from 14-0-24MSp1+3. Lanes 8 and 9 contained increasing amounts of E2-C protein alone, revealing the DNA:E2-C complex. Incubation of the probe in the presence of E2-C protein and in vitro-translated Sp1 protein (compare lanes 2, 3, and 4) or the C-33A nuclear extract (compare lanes 5, 6, and 7) resulted in slower migration bands at the expense of the original complexes.

To demonstrate the slower-migrating (supershifted) bands indeed contained both E2-C and Sp1 or Sp1-like proteins, various competitors (illustrated in Fig. 7C) were included during the formation of complexes. The results were totally

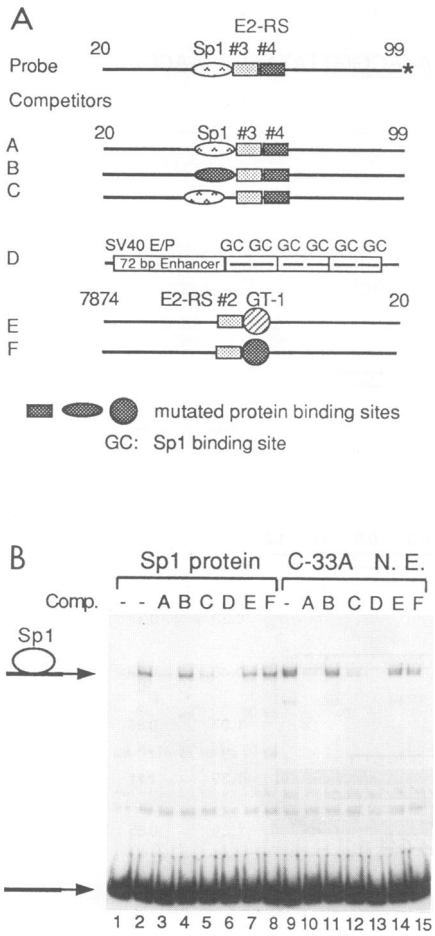
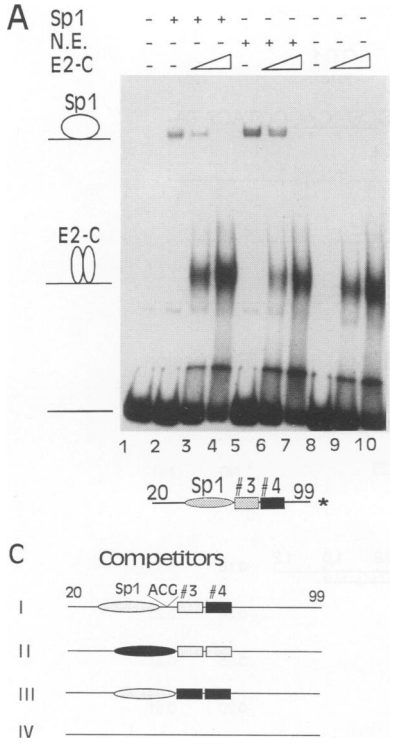


FIG. 6. Binding of human Sp1 protein and Sp1-like proteins in C-33A cells to the sequence AGGAGG located 1 bp upstream of HPV-11 E2-RS 3. (A) Schematic presentation of the labelled DNA probe, which spans HPV-11 nt 20 to 99 containing a wild-type putative Sp1 motif and E2-RS 3 but a mutated E2-RS 4, and unlabelled competitors used in the EMSAs. Numbers above the clones indicate the nucleotide positions in HPV-11. (B) Autoradiogram of EMSA. Aliquots of 10 fmol of 3′ end-labelled DNA fragment plus 0.5 to 1 μg of poly(dI-dC) were incubated on ice with either in vitro-translated human Sp1 protein (Sp1) or C-33A cell nuclear extract (N.E.) in the presence or absence of the indicated diagnostic competitor DNA (Comp.). Lane 1, 3 μl of unprogrammed rabbit reticulocyte lysate; lanes 2 to 8, 3 μl each of in vitro-translated Sp1 protein; lanes 9 to 15, 0.5 μg each of C-33A nuclear extract. Lanes 3 to 8 and 10 to 15, 100-fold molar excesses of unlabelled competitor DNA as follows: competitor A, homologous DNA, nt 20 to 99 (lanes 3 and 10); competitor B, nt 20 to 99 with mutated Sp1 (lanes 4 and 11); competitor C, nt 20 to 99 with a 3-bp insertion between the Sp1 motif and E2-RS 3 (Fig. 5A) (lanes 5 and 12); competitor D, SV40 enhancer-promoter fragment from pSV2CAT containing six copies of the Sp1 motif (GGGCGG) (lanes 6 and 13); competitor E, HPV-11 nt 7874-[7933/1]-20 spanning E2-RS 2 and the adjacent GT-1 motif (lanes 7 and 14); HPV-11 nt 7874-[7933/1]-20 with mutated GT-1 (GT1M), as described in Fig. 8 (lanes 8 and 15). The positions of free probe (lower arrow) or Sp1:DNA complexes observed are indicated on the left. In addition, a faster-migrating Sp1-like complex exhibiting an identical competition pattern was observed in lanes 9, 11, 14, and 15 where nuclear extracts were used.

consistent with this interpretation, as shown in Fig. 7B. Specifically, unlabelled homologous competitor I completely eliminated the E2-C:DNA complex as well as the supershifted ternary complex formed with the nuclear extracts, and only a trace amount of the Sp1-like DNA binary complex was still visible (lane 10). This competitor also completely eliminated all the complexes formed with in vitro-translated Sp1 and the E2-C protein (lane 14). Competitor II contained a mutated Sp1 motif but retained wild-type E2 binding sites. It completely eliminated any E2-C DNA complex as well as the supershifted bands formed with nuclear extracts or with genuine Sp1; however, the binary complex formed with Sp1 or Sp1-like proteins remained (lanes 11 and 15). Competitor III contained the wild-type Sp1 binding site but mutated E2-RSs. It eliminated the binary and ternary complexes formed with either the nuclear extract or the in vitro-translated Sp1, but the E2-C:DNA complex remained (lanes 12 and 16). Nonspecific competitor IV did not compete for the supershifted band or for the E2-C:DNA complex (lanes 13 and 17). These results firmly established that both E2-C and Sp1 or Sp1-like proteins could bind simultaneously when a 3-bp insertion was introduced between the binding sites, in complete agreement with the functional assays.

***cis* elements downstream of E2-RS copy 2.** We noted that the sequence 5′ CCACACCC 3′ (i.e., 5′ GGGTGTGG 3′) located 3 bases downstream of E2-RS 2 (Fig. 8A) is identical to the GT-1 motif in the SV40 enhancer, the CACCC motif in the β-globin gene promoter and the GT-1 motif downstream of the BPV-1 P1 promoter (13, 45, 56, 62). Sp1, transcriptional enhancer factor II, and a factor(s) expressed only in erythrocytes have been shown to recognize this motif (13, 18, 32, 39, 62, 63). To test its function, mutations in this GT-1 motif were introduced into pUR23-3 and 14-0-WT (Fig. 8A). As shown in Fig. 8B and C, 23-3-GT1M- and 14-0-GT1M-based expression vectors were reduced to 80 and 60% of the CAT activities of their respective parental clones. In clone 14-0-34MGT1M, much of the repression by E2 was relieved but repression by the E2-C protein persisted, whereas 14-0-34M was repressed by either form of E2 protein, as is shown in Fig. 4. Furthermore, a 5-bp insertion between the GT-1 motif and E2-RS 2 (clone 14-0-34MGT1+5) did not alleviate the repression by the intact E2 protein but a 15-bp insertion (clone 14-0-34MGT1+15) did. However, both were still repressed by the E2-C protein to an extent similar to that of the parental clone. These results suggest that the GT-1 motif played a role in the E6 promoter function when CE II was absent and also implied that there were some interactions among the intact E2 protein and host proteins recognizing the CE II element, the GT-1 motif, and perhaps additional motifs nearby.

Protein binding activities to sequences flanking E2-RS copy 2. To test whether the GT-1 motif binds Sp1 protein as has been demonstrated in other systems, a fragment (nt 7902-[7933/1]-15) spanning the GT-1 motif was labelled and used as the probe and tested by EMSA. To our surprise, the in vitro-translated Sp1 protein failed to bind the probe (Fig. 9, left panel, lanes 2 and 3). Consistent with this result, the unlabelled fragment spanning nt 7874-[7933/1]-20 containing either wild-type or mutated GT-1 motif competed poorly, if at all, for the complex formation between the variant Sp1 motif AGGAGG and the recombinant Sp1 protein or Sp1-like proteins in the C-33A nuclear extracts (Fig. 6B, lanes 7, 8, 14, and 15). Nuclear extracts from C-33A cells, however, generated four complexes with the wild-type GT-1 containing probe (Fig. 9, left panel, lane 4, A to D). Only complex C was eliminated by competition with a 21-bp-long double-stranded oligonucleotide containing the wild-type GT-1 motif of



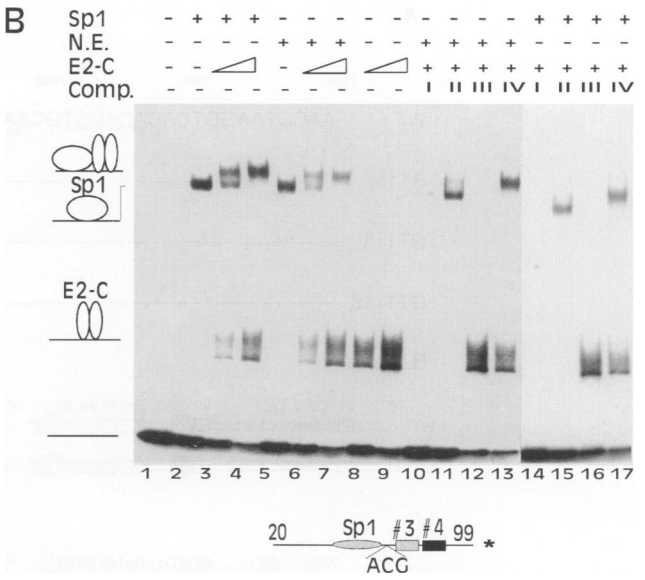


FIG. 7. Preclusion of Sp1 binding to AGGAGG by HPV-11 E2-C protein binding to E2-RS 3, and relief of preclusion by a 3-bp insertion between the binding sites. (A) EMSA with the same ^{32}P-labelled DNA probe described in the Fig. 6 legend. (B) The comparable DNA fragment with a 3-bp insertion between the Sp1 site and E2-RS 3 was used as a probe. (C) Specific and nonspecific competitors (comp.) used in the EMSA shown in B. Solid symbols, mutated sites as described for Fig. 5. Competitor DNAs were added in 600-fold molar (I, II, and III) or weight (IV) excesses of the labelled probe. In vitro-translated Sp1 protein (Sp1) (3 μl) or of C-33A cell nuclear extract (N.E.) (0.5 μg) was used in lanes as indicated: 0.05 μg of induced bacterial lysate containing HPV-11 E2-C protein (E2-C) (lanes 3, 6, and 9 [A and B]); 0.1 μg of the same E2-C lysate (lanes 4, 7, 10 [A and B] and 11 through 17 [B]). The proteins were added to the probes at the same time, and the total volume of each reaction was kept constant by adding protein dilution buffer. Lanes 14 to 17 of B were from a separate gel which was run for a slightly different length of time, accounting for the minor difference in mobility. The positions of schematically represented protein-DNA complexes and the free probe (solid line) are indicated on the left. The Sp1–DNA–E2-C complex is also indicated. *, ^{32}P label at the *Hin*dIII site at nt 99; wedge, increasing amounts of E2-C protein. The autoradiograms were scanned into a Personal Densitometer (Molecular Dynamics, Inc.). The image was then generated by ImageQuant (version 3.3; Molecular Dynamics, Inc.) and printed on Tekcolor Phaser IISD (Tektronix, Inc.).

HPV-11 (nt 7893 to 7914) but not with a mutated GT-1 oligonucleotide (Fig. 9, right panel, lanes 3 to 5 and 6 to 8, respectively) or by a fragment which contained the multiple cloning site of pUC19 flanking an E2-RS (lanes 9 to 11). These results suggested that complex C was formed specifically between a host protein and the GT-1 motif. The fragment of 14-0-34MGT1+15 (nt 7883-[7933/1]-15) which has a 15-bp insertion between the two binding sites also successfully competed with this complex (data not shown). Thus, the insertional mutation retains the binding motifs, consistent with the CAT assay results. Taken together, these results suggest that the GT-1 motifs bind a host protein other than Sp1 or an Sp1-like protein under the conditions used. However, it cannot be ruled out that binding of the Sp1 proteins was much weaker but was stabilized by interactions with other host proteins bound to other motifs that are not present in the probes used.

DISCUSSION

By testing a large number of mutations in the E2-RSs and flanking *cis* elements in the HPV-11 URR, we investigated the mechanisms of HPV-11 E6 promoter regulation by the homologous viral E2 proteins and host transcription factors. We demonstrated that promoter repression by the E2 proteins was mediated via each of the three promoter-proximal copies of the E2-RS, and CE II-cognate proteins abrogated the repression via E2-RS 2 by the intact E2 but not by the E2-C protein (compare Fig. 3 and 4). Repression was relieved when the two (for E2) or three (for E2-C) promoter-proximal copies of E2-RS were mutated (Fig. 3 and 4) and were no longer able to bind the E2 proteins (6). These results are consistent with our previous hypothesis that CE II-cognate proteins interact with the host proteins bound to site(s) near the promoter located downstream and, in so doing, may stabilize the transcription initiation complex and contribute to the differential effects of E2 versus E2-C protein (8). Moreover, we showed that the expression vector used also affected the outcome, implying that the amount of the E2 protein expressed is an important factor. When cotransfected with a relatively high amount of a strong expression vector of HPV-11 E2, the wild-type HPV-11 URR E6 promoter is repressed by 5- to 10-fold regardless of the presence or absence of CE II (Fig. 1). In contrast, repression by the E2-C protein can occur in the presence of CE II (Fig. 1, 3, and 4), although CE II seemed to reduce the extent of repression (compare Fig. 3 and 4).

There are similarities as well as differences between our conclusions and those of Romanczuk et al. (50) and Thierry and Howley (60) who studied, respectively, the regulation of the HPV-16 E6 promoter by the BPV-1 E2 protein and of the HPV-18 E6 promoter by the HPV-18 or BPV-1 E2 proteins. Only the two promoter-proximal copies of E2-RS mediated repression in HPV-16, whereas, for HPV-18, all three proximal copies of E2-RS mediated repression. The distal E2 binding site was thought to respond positively to the intact E2 protein,

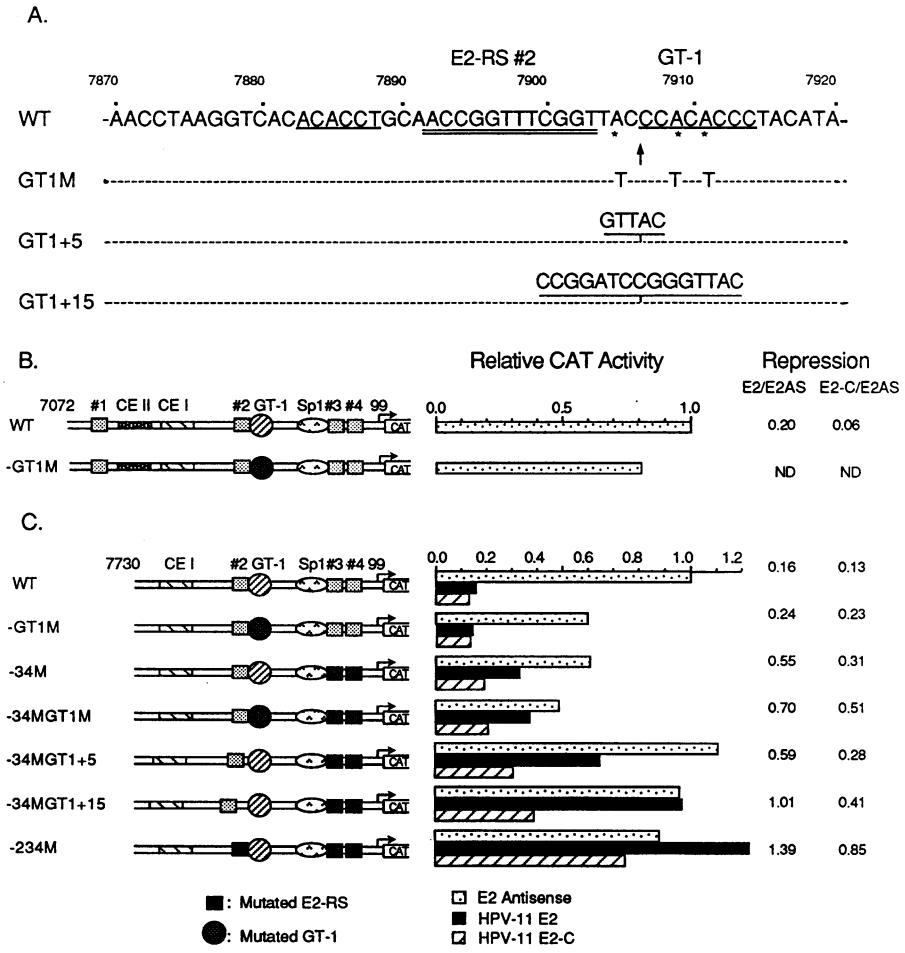


FIG. 8. Mutational analysis of the GT-1 motif in the HPV-11 URR and E2-mediated regulation. (A) DNA sequences flanking E2-RS 2 (doubly underlined) with a wild-type (WT) or mutated GT-1 motif (underlined). A GT-1-like motif 5′ of E2-RS 2 is also underlined. The arrow points to the position of insertions. (B) Relative CAT activity of the GT-1 mutation in pUR23-3 compared with the basal activity of pUR23-3. The fold repressions of pUR23-3 by E2 proteins were taken from Fig. 3 data. (C) Relative CAT activities of 14-0 mutation clones compared with the basal activity of 14-0-WT. The experiment was also repeated one to three times for different clones, with the vector pKV461 as a negative control. Qualitatively similar results were obtained (data not shown).

but no HPV-16 or HPV-18 clone tested did not contain this site. However, this E2 stimulation could, in part or in whole, correspond to the E2-RS-independent activation by either intact E2 or by the amino-terminal portion of E2 protein (data not shown) which we observed with the homologous viral promoter (Fig. 1, 3, 4, 5, and 8). A similar phenomenon of E2-RS-independent transactivation has previously been observed with the BPV-1 E2 protein (24, 26, 28, 41). Whether this nonspecific stimulatory effect is mediated through interactions with the Sp1 protein (41) cannot be deduced from our results. Our data clearly showed that the promoter-distal E2-RS 1 played little or no role in regulating the HPV-11 E6 promoter in vitro (Fig. 3; compare pUR23-3-234M and 24N-234M).

Repression of the HPV-11 E6 promoter in C-33A cells (this study) or in primary human keratinocytes (16) by the homologous viral E2 proteins was much more effective than that reported for HPV-16 and HPV-18 (3, 61). The heterologous BPV-1 E2 protein behaved similarly to HPV-11 E2-C and repressed the HPV-11 E6 promoter via all three promoter-proximal copies of the E2-RS except that it was a more potent repressor (data not shown). In contrast, BPV-1 E2 protein repressed the HPV-16 or HPV-18 E6 promoter mainly by binding to one or both of the TATA-proximal copies of E2-RS (50, 59, 60). These variations in URR regulation among HPV-16, HPV-18, and HPV-11 may be due to a genuine distinction by their engaging different host transcription factors, consistent with the absence of CE II-equivalent enhancer elements in the URR sequences of the high-risk HPV types. Moreover, Oct-1 and AP-1 binding proteins regulate the HPV-16 and HPV-18 URR (10, 12, 15, 30, 61), whereas HPV-11 does not contain an Oct-1 site and the AP-1 sites are not important for activity in primary human keratinocytes (16). Other explanations are also possible, such as variations in the levels of the E2 protein expressed from various vectors or the use of different cell types in some of the studies.

Previous investigations of HPV-18 and HPV-16 E6 promoter regulation demonstrated that the BPV-1 E2 protein can preclude the binding of recombinant TBP (17) or Sp1 protein in mouse cell C127 nuclear extracts (59), resulting in promoter repression. Sp1 proteins are a family of highly glycosylated and phosphorylated sequence-specific transcription factors (34, 36). Our mutational analyses and EMSAs have provided direct

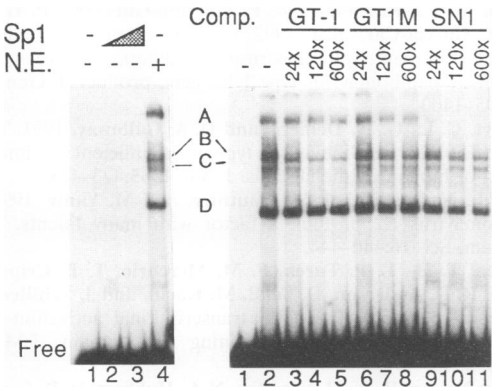


FIG. 9. Specific binding of a host protein to the GT-1 motif in the HPV-11 URR. The probe was a PCR-amplified, ^{32}P-labelled DNA fragment spanning HPV-11 nt 7902-[7933/1]-15 (see Fig. 5 and 8 for sequences). Aliquots of 10 fmol were used in each lane. Left panel: lane 1, 4 μl of unprogrammed rabbit reticulocyte lysate; lanes 2 and 3, 2 and 4 μl, respectively, of in vitro-translated Sp1 protein; lane 4, 0.1 μl of C-33A nuclear extract (N.E.). Right panel: lane 1, no protein; lanes 2 to 11, 0.1 μg of C-33A cell nuclear extract. Competitor DNAs in lanes 3 to 5 were 24-, 120-, and 600-fold molar excesses of double-stranded 21-mer spanning nt 7897 to 7917. Competitor DNAs in lanes 6 to 8 were 24-, 120-, and 600-fold molar excesses of the comparable fragment containing the GT-1 mutations (GT1M) described in the Fig. 8 legend. Competitor DNAs in lanes 9 to 11 were the same fold molar excesses of a fragment consisting of a single E2-RS flanked by pUC19 multiple cloning sites. Wedge, increasing amounts of Sp1 protein. Free, unbound probe; A to D, protein-probe complexes.

evidence that the nonconsensus sequence AGGAGG in HPV-11 binds Sp1 and Sp1-like proteins and functions as a positive *cis* element for the basal activity of the HPV-11 E6 promoter (Fig. 5B and C). The displacement of Sp1 or Sp1-like proteins from this site by the homologous viral E2 proteins bound to E2-RS 3 is responsible in part for the promoter repression. This conclusion is supported by the relief of repression (Fig. 5C) and simultaneous binding of both viral and host proteins when a 3-bp insertion is placed between E2-RS 3 and the Sp1 motif (Fig. 7).

Host proteins bound to the GT-1 motif downstream of E2-RS 2 appear to be partially responsible for interactions with CE II-cognate proteins. This interaction contributed to the E6 promoter activity and overcame E2 repression mediated through E2-RS 2. Although reported to bind Sp1 protein previously (62, 63), in the HPV-11 sequence context and under the conditions employed, the GT-1 motif bound neither recombinant Sp1 protein nor Sp1-like proteins in C-33A cells (Fig. 6 and 9) but it did form a specific complex with other components in the nuclear extracts (Fig. 9). That the triple mutation in 14-0-34MGT1M or in 14-0-34MGT1+15 largely or totally relieved the repression by the intact E2 protein but not that by the E2-C protein (Fig. 8C) suggested that there may be additional *cis* elements nearby and that the bound host proteins interacted with the E2, but not the E2-C, protein. We note that there is a perfect transcriptional enhancer factor 1 motif (ACATATTT) (31) which is 1 bp downstream of the GT-1 motif. This motif is not important when the URR is in the enhancer configuration linked to a surrogate promoter (28). Whether it is important for the contiguous URR E6 promoter activity remains to be determined. We also point out that the sequence ACACACCT located 3 bp upstream of E2-RS 2 has a 75% homology to GT-1. Mutations of this motif to AGAATTCT in parental 14-0-WT or 14-0-GT1M reduced the basal activities slightly relative to those of the parental clone but failed to relieve the repression by either E2 protein (data not shown). At this juncture, the host proteins interacting with the E2-RS copy 2 flanking sequences and with CE II remain to be identified. As to copy 4 of the E2-RS proximal to the TATA motif, the results of our functional assays (Fig. 3 and 4) are consistent with the hypothesis that interference with binding or function of TBP leads to the E6 promoter repression, as has been demonstrated for HPV-18 (17, 21). Taken together, these results strongly argue that the homologous E2 proteins can repress the E6 promoter through binding to any and all of the three promoter-proximal E2-RSs, interfering with the binding of host proteins.

HPV E1 and E2 proteins are essential for viral episomal DNA replication, and the E2-responsive sequences are absolutely required (6, 7, 14, 48). Furthermore, replication efficiency increases with the copy number of E2-RS. In contrast, the HPV-11 E1M^E2C and E2-C proteins repress replication (6). Since the URRs of all genital and oral tract-specific HPV genotypes have conserved the arrangement of four copies of the E2-RS and because all three E2-RSs involved in E6 promoter modulation overlap the strong origin of replication (6), the E2 proteins play a key role in coregulating transcription and replication of the viral genome. We suspect that in vivo the primary regulatory mediator is the intact E2 protein, whereas E2-C proteins are much rarer, judging from electron microscopic heteroduplex analysis of mRNAs isolated from patient specimens (11). The E2 protein is translated from a polycistronic mRNA initiated from the E6 promoter in both the low-risk and high-risk viruses (11, 46, 51–53). Therefore, the hypothesis that the E2 protein autoregulates the E6 promoter is central to the pathogenesis of all genital HPVs. We have previously proposed that the function of the CE II-cognate proteins is epithelial cell differentiation dependent (4, 57). As the keratinocytes ascend and differentiate in a stratified squamous epithelium, the promoter repression by the E2 protein is abrogated and the viral infection then proceeds into the productive phase. In rare cases, the loss of the negative regulation by the homologous viral E2 proteins due to the destruction of the E2 transcriptional unit as a result of viral DNA integration in the E1/E2 region can derepress transcription of the E6 and E7 oncogenes from the E6 promoter, initiating a degenerative course of neoplastic progression (57). Recent immortalization studies carried out with primary human keratinocytes in vitro are entirely consistent with this latter hypothesis (49). In summary, the differential interactions between the viral and host factors lead to an intricate and precise regulation of viral genetic activities that are tightly linked to epithelial differentiation.

ACKNOWLEDGMENTS

This work was supported by Public Health Service award CA36200 and by a Fellowship from The James P. Wilmot Cancer Research Foundation to Gang Dong.

We thank Mark P. Sowden and Jen-Sing Liu for providing some of plasmids used in this study.

REFERENCES

1. **Androphy, E. J., D. R. Lowy, and J. T. Schiller.** 1987. Bovine papillomavirus E2 trans-acting gene product binds to specific sites in papillomavirus DNA. Nature (London) **325**:70–73.
2. **Barbosa, M. S., W. C. Vass, and D. R. Lowy.** 1991. In vitro biological activities of the E6 and E7 genes vary among human papillomaviruses of different oncogenic potential. J. Virol. **65**:292–298.
3. **Bernard, B. A., C. Bailly, M.-C. Lenoir, M. Darmon, F. Thierry,**

and M. Yaniv. 1989. The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. J. Virol. **63:**4317–4324.

4. **Broker, T. R., L. T. Chow, M. T. Chin, C. R. Rhodes, S. M. Wolinsky, A. Whitbeck, and M. H. Stoler.** 1989. A molecular portrait of human papillomavirus carcinogenesis. Cancer Cells **7:**197–208.
5. **Chiang, C.-M., T. R. Broker, and L. T. Chow.** 1991. An E1M/\E2C fusion protein encoded by human papillomavirus type 11 is a sequence-specific transcription repressor. J. Virol. **65:**3317–3329.
6. **Chiang, C.-M., G. Dong, T. R. Broker, and L. T. Chow.** 1992. Control of human papillomavirus type 11 origin replication by the E2 family of transcriptional regulators. J. Virol. **66:**5224–5231.
7. **Chiang, C.-M., M. Ustav, A. Stenlund, T. F. Ho, T. R. Broker, and L. T. Chow.** 1992. Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. Proc. Natl. Acad. Sci. USA **89:**5799–5803.
8. **Chin, M. T., T. R. Broker, and L. T. Chow.** 1989. Identification of a novel constitutive enhancer element and its associated binding protein: implications for human papillomavirus type 11 enhancer regulation. J. Virol. **63:**2967–2976.
9. **Chin, M. T., R. Hirochika, H. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Regulation of human papillomavirus type 11 enhancer and E6 promoter by activating and repressing proteins from the E2 open reading frame: functional and biochemical studies. J. Virol. **62:**2994–3002.
10. **Chong, T., D. Apt, B. Gloss, M. Isa, and H.-U. Bernard.** 1991. The enhancer of human papillomavirus type 16: binding sites for the ubiquitous transcription factors oct-1, NFA, TEF-2, NF1, and AP-1 participate in epithelial cell-specific transcription. J. Virol. **65:**5933–5943.
11. **Chow, L. T., M. Nasseri, S. M. Wolinsky, and T. R. Broker.** 1987. Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata. J. Virol. **61:**2581–2588.
12. **Cripe, T. P., A. Alderborn, R. D. Anderson, S. Parkkinen, P. Bergman, T. H. Haugen, U. Pettersson, and L. P. Turek.** 1990. Transcriptional activation of the human papillomavirus 16 P97 promoter by an 88-nucleotide enhancer containing distinct cell-dependent and AP-1 responsive modules. New Biol. **2:**450–463.
13. **Davidson, I., J.-H. Xiao, R. Rosales, A. Staub, and P. Chambon.** 1988. The HeLa cell protein TEF-1 binds specifically and cooperatively to two SV40 enhancer motifs of unrelated sequence. Cell **54:**931–942.
14. **Del Vecchio, A. M., H. Romanczuk, P. M. Howley, and C. C. Baker.** 1992. Transient replication of human papillomavirus DNA. J. Virol. **66:**5949–5958.
15. **Dent, C. L., G. A. McIndoe, and D. S. Latchman.** 1993. The constitutively expressed octamer binding protein OTF-1 and a novel octamer binding protein expressed specifically in cervical cells binds to an octamer-related sequence in the human papillomavirus 16 enhancer. Nucleic Acids Res. **19:**4531–4535.
16. **Dollard, S. C., T. R. Broker, and L. T. Chow.** 1993. Regulation of the human papillomavirus type-11 E6 promoter by viral and host transcription factors in primary human keratinocytes. J. Virol. **67:**1721–1726.

16a. **Dollard, S. C., L. T. Chow, J. W. Kreider, T. R. Broker, N. L. Lill, and M. K. Howett.** 1989. Characterization of an HPV type 11 isolate propagated in human foreskin implants in nude mice. Virology **171:**294–297.

17. **Dostatni, N., P. F. Lambert, R. Sousa, J. Ham, P. M. Howley, and M. Yaniv.** 1991. The functional BPV-1 E2 trans-activating protein can act as a repressor by preventing formation of the initiation complex. Genes Dev. **5:**1657–1671.
18. **Emerson, B. M., J. M. Nickol, and T. C. Fong.** 1989. Erythroid specific activation and derepression of the chick beta-globin promoter *in vitro*. Cell **57:**1189–1200.
19. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**6505–6525.
20. **Gloss, B., and H.-U. Bernard.** 1990. The E6/E7 promoter of human papillomavirus type 16 is activated in the absence of E2 proteins by a sequence-aberrant Sp1 distal element. J. Virol. **64:**5577–5584.
21. **Guido, M. C., R. Zamorano, E. Garrido-Guerrero, P. Gariglio, and A. Garcia-Carranca.** 1992. Early promoters of genital and cutaneous human papillomaviruses are differentially regulated by the bovine papillomavirus type 1 E2 gene product. J. Gen. Virol. **73:**1395–1400.
22. **Halbert, C. L., G. W. Demers, and D. A. Galloway.** 1991. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J. Virol. **65:**473–478.
23. **Ham, J., N. Dostatni, J.-M. Gauthier, and M. Yaniv.** 1991. The papillomavirus E2 protein: a factor with many talents. Trends Biochem. Sci. **16:**440–444.
24. **Haugen, T. H., L. P. Turek, F. M. Mercurio, T. P. Cripe, B. J. Olson, R. D. Anderson, D. Seidl, M. Karin, and J. Schiller.** 1988. Sequence-specific and general transcriptional activation by the bovine amphipathic helix-containing E2 domain. EMBO J. **7:**4245–4253.
25. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. EMBO J. **8:**3905–3910.
26. **Heike, T., S. Miyatake, M. Yoshida, K.-I. Arai, and N. Arai.** 1989. Bovine papillomavirus encoded E2 protein activates lymphokine genes through DNA elements, distinct from the consensus motif, in the long control region of its own genome. EMBO J. **8:**1411–1417.
27. **Hirochika, H., T. R. Broker, and L. T. Chow.** 1987. Enhancers and *trans*-acting E2 transcriptional factors of papillomaviruses. J. Virol. **61:**2599–2606.
28. **Hirochika, H., R. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Functional mapping of the human papillomavirus transcriptional enhancer and its complexes with the trans-acting E2 protein. Genes Dev. **2:**54–67.
29. **Hoppe-Seyler, F., and K. Butz.** 1992. Activation of human papillomavirus type 18 E6-E7 oncogene expression by transcription factor Sp1. Nucleic Acids Res. **20:**6701–6706.
30. **Hoppe-Seyler, F., K. Butz, and H. zur Hausen.** 1991. Repression of the human papillomavirus type 18 enhancer by the cellular transcription factor Oct-1. J. Virol. **65:**5613–5618.
31. **Ishiji, T., M. J. Lace, S. Parkkinen, R. D. Anderson, T. H. Haugen, T. P. Cripe, J.-H. Xiao, I. Davidson, P. Chambon, and L. P. Turek.** 1992. Transcriptional enhancer factor (TEF)-1 and its cell-specific co-activator activate human papillomavirus 16 E6 and E7 oncogene transcription in keratinocytes and cervical carcinoma cells. EMBO J. **11:**2271–2281.
32. **Jackson, D. P., T. Evans, J. M. Nickol, and G. Felsenfeld.** 1989. Developmental modulation of protein binding to beta-globin gene regulatory sites within chicken erythrocyte nuclei. Genes Dev. **3:**1860–1873.
33. **Johnson, P. F., and S. L. McKnight.** 1989. Eukaryotic transcriptional regulatory proteins. Annu. Rev. Biochem. **58:**799–839.
34. **Kadonaga, J. T., A. J. Courey, J. Ladika, and R. Tjian.** 1988. Distinct regions of Sp1 modulate DNA binding and transcriptional activation. Science **242:**1566–1570.
35. **Kaur, P., and J. K. McDougall.** 1988. Characterization of primary human keratinocytes transformed by human papillomavirus type 18. J. Virol. **62:**1917–1924.
36. **Kingsley, C., and A. Winoto.** 1992. Cloning of GT box-binding proteins: a novel Sp1 multigene family regulating T-cell receptor gene expression. Mol. Cell. Biol. **12:**4251–4261.
37. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
38. **Lee, K. A. W., A. Bindereif, and M. R. Green.** 1988. A small scale procedure for preparation of nuclear extracts that support efficient transcription and pre-mRNA splicing. Gene Anal. Tech. **5:**22–31.
39. **Lewis, C. D., S. P. Clark, G. Felsenfeld, and H. Gould.** 1988. An erythrocyte-specific protein that binds to the poly(dG) region of the chicken beta-globin gene promoter. Genes Dev. **2:**863–873.
40. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3:**510–526.
41. **Li, R., J. D. Knight, S. P. Jackson, R. Tjian, and M. R. Botchan.** 1991. Direct interaction between Sp1 and BPV enhancer E2

protein mediates synergistic activation of transcription. Cell **65:** 493–505.
42. **Lin, W.-C., and L. A. Culp.** 1991. Selectable plasmid vectors with alternative and ultrasensitive histochemical marker genes. BioTechniques **11:**344–351.
43. **McBride, A. A., H. Romanczuk, and P. M. Howley.** 1991. The papillomavirus E2 regulatory proteins. J. Biol. Chem. **266:**18411–18414.
44. **Münger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63:**4417–4421.
45. **Myers, R. M., K. Tilly, and T. Maniatis.** 1986. Fine structure analysis of a beta-globin promoter. Science **232:**613–618.
46. **Nasseri, M., J. R. Gage, A. Lörincz, and F. O. Wettstein.** 1991. Human papillomavirus type 16 immortalized cervical keratinocytes contain transcripts encoding E6, E7, and E2 initiated at the P_{97} promoter and express high levels of E7. Virology **184:**131–140.
47. **Newmann, J. R., C. A. Morency, and K. O. Russian.** 1987. A novel rapid assay for chloramphenicol acetyltransferase gene expression. BioTechniques **5:**444–447.
48. **Remm, M., R. Brain, and J. R. Jenkins.** 1992. The E2 binding sites determine the efficiency of replication for the origin of human papillomavirus type 18. Nucleic Acids Res. **20:**6015–6021.
49. **Romanczuk, H., and P. M. Howley.** 1992. Disruption of either the E1 or the E2 regulatory gene of human papillomavirus type 16 increases viral immortalization capacity. Proc. Natl. Acad. Sci. USA **89:**3159–3163.
50. **Romanczuk, H., F. Thierry, and P. M. Howley.** 1990. Mutational analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P_{97} and type 18 P_{105} promoters. J. Virol. **64:**2849–2859.
51. **Rotenberg, M. O., T. R. Broker, and L. T. Chow.** 1989. Characterization of rare human papillomavirus type 11 mRNAs coding for regulatory and structural proteins by the polymerase chain reaction. Virology **172:**489–497.
52. **Rotenberg, M. O., C.-M. Chiang, M. S. Ho, T. R. Broker, and L. T. Chow.** 1989. Characterization of cDNAs of spliced HPV-11 E2 mRNA and other HPV mRNAs recovered via retrovirus-mediated gene transfer. Virology **172:**468–477.
53. **Smotkin, D., H. Prokoph, and F. O. Wettstein.** 1989. Oncogenic and nononcogenic human genital papillomaviruses generate the E7 mRNA by different mechanisms. J. Virol. **63:**1441–1447.
54. **Sousa, R., N. Dostatni, and M. Yaniv.** 1990. Control of papillomavirus gene expression. Biochim. Biophys. Acta **1032:**19–37.
55. **Sowden, M., S. Harrison, R. Ashfield, A. J. Kingsman, and S. M. Kingsman.** 1989. Multiple cooperative interactions constrain BPV-1 E2 dependent activation of transcription. Nucleic Acids Res. **17:**2959–2972.
56. **Stenlund, A., and M. Botchan.** 1990. The E2 trans-activator can act as a repressor by interfering with a cellular transcription factor. Genes Dev. **4:**123–136.
57. **Stoler, M. H., C. R. Rhodes, A. Whitbeck, S. M. Wolinsky, L. T. Chow, and T. R. Broker.** 1992. Human papillomavirus type 16 and 18 gene expression in cervical neoplasias. Hum. Pathol. **23:**117–128.
58. **Storey, A., D. Pim, A. Murray, K. Osborn, L. Banks, and L. Crawford.** 1988. Comparison of the *in vitro* transforming activities of human papillomavirus types. EMBO J. **7:**1815–1820.
59. **Tan, S.-H., B. Gloss, and H.-U. Bernard.** 1992. During negative regulation of the human papillomavirus-16 E6 promoter, the viral E2 protein can displace Sp1 from a proximal promoter element. Nucleic Acids Res. **20:**251–256.
60. **Thierry, F., and P. M. Howley.** 1991. Functional analysis of E2-mediated repression of the HPV18 P_{105} promoter. New Biol. **3:**90–100.
61. **Thierry, F., G. Spyrou, M. Yaniv, and P. Howley.** 1992. Two AP1 sites binding JunB are essential for human papillomavirus type 18 transcription in keratinocytes. J. Virol. **66:**3740–3748.
62. **Vande Pol, S. B., and P. M. Howley.** 1990. A bovine papillomavirus constitutive enhancer is negatively regulated by the E2 repressor through competitive binding for a cellular factor. J. Virol. **64:** 5420–5429.
63. **Xiao, J.-H., I. Davidson, M. Macchi, R. Rosales, M. Vigneron, A. Staub, and P. Chambon.** 1987. *In vitro* binding of several cell-specific and ubiquitous nuclear proteins to the GT-1 motif of the SV40 enhancer. Genes Dev. **1:**794–807.
64. **zur Hausen, H.** 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184:**9–13.