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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

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E2-C, a protein consisting mainly of the carboxy-terminal 45% of the human papillomavirus type 11 (HPV-11) E2 protein, was expressed from the Rous sarcoma virus long terminal repeat in mammalian cells. It competitively repressed the stimulatory action of the full-length E2 protein on the HPV-11 enhancer located in the upstream regulatory region, as assayed by the expression of a reporter gene from the simian virus 40 (SV40) early promoter in transiently transfected monkey CV-1 cells. A mutation in the initiation codon for E2-C protein eliminated repression. In the human cervical carcinoma cell line C-33A, which apparently lacks endogenous HPV DNA, the HPV-11 enhancer-SV40 promoter and the HPV-11 enhancer in its normal association with the E6 promoter had high constitutive activity. In these cells, E2 proteins had little or no stimulatory effect on the transcriptional activity of the HPV-11 enhancer-SV40 promoter. In contrast, the HPV-11 enhancer-E6 promoter was stimulated by the HPV-11 E2 protein but repressed by the bovine papillomavirus type 1 E2 protein, an effect due either to a quantitative difference in E2 expression levels or to a qualitative difference in the *trans*-activating abilities of the two E2 proteins. In this cell line, the HPV-11 E2-C protein suppressed both the constitutive activity and the HPV-11 E2 *trans* activation. The E2-C protein was also produced from an expression vector in *Escherichia coli*. The E2-C protein present in crude *E. coli* lysates or purified by DNA affinity chromatography associated in vitro with a specific sequence, $ACCN_6GGT$, in filter-binding assays. Moreover, the protein generated DNase I footprints spanning this motif identical to those of bacterially expressed full-length E2 proteins. This DNA sequence motif is necessary and sufficient for E2 binding in vitro and enhancer *trans* activation in vivo (H. Hirochika, R. Hirochika, T. R. Broker, and L. T. Chow, Genes Dev. 2:54–67, 1988). Mutations in this sequence that abolished interactions with E2 also precluded binding to the E2-C protein. These data strongly suggest that the full-length E2 protein consists of two functional domains: the amino-terminal half for *trans* activation and the carboxy-terminal half for DNA binding. The mechanism by which E2-C represses E2-dependent enhancer activity most likely involves competition with E2 for binding to a common transcriptional regulatory site. Steric hindrance with the binding of other transcription factors such as TFIID may be responsible for the suppression of E2-independent constitutive activity by either the E2 or the E2-C protein.

The papillomaviruses are a group of small DNA viruses that cause many different clinical manifestations of epithelial hyperplasia (for reviews, see references 6, 32, and 45). These viruses have a circular genome of about 7,900 base pairs and show strict species and tissue specificity. Viral gene expression, DNA replication, and vegetative propagation are all tied to the state of host keratinocyte differentiation (8), which has yet to be fully achieved in vitro. Because of the lack of a cell culture system for papillomavirus propagation, these processes are not particularly well characterized.

The E2 open reading frames (ORFs) of bovine papillomavirus (BPV) and several human papillomavirus (HPV) types encode analogous *trans*-acting proteins which stimulate papillomavirus enhancer-dependent gene transcription (18, 19, 33, 40). The enhancers are located in the upstream regulatory region (URR). The DNA sequence motif $ACCN_6GGT$, present in multiple copies in the URR of all papillomaviruses that have been sequenced (9, 12), has been suggested to mediate the effect of E2 on the enhancer. Consistent with this hypothesis, BPV type 1 (BPV-1) E2 protein expressed in *Escherichia coli* in an N-terminal-truncated form or fused to beta-galactosidase binds to restriction fragments containing this motif (2, 29). Hirochika et al. (20) demonstrated unequivocally that this motif is both necessary and sufficient for E2-dependent enhancer activation and that full-length, unmodified HPV-1 and HPV-11 E2 proteins and an N-terminal-truncated BPV-1 protein expressed in *E. coli* bind to DNA fragments containing one or more copies of this E2-responsive sequence (E2-RS) and leave discrete footprints spanning the motifs. When the motif is deleted or mutated, E2 binding in vitro and *trans*-activation in vivo are both lost. Within a 270-nucleotide (nt) enhancer region mapped in the HPV-11 URR, three $ACCN_6GGT$ motifs (copy numbers 2, 3, and 4) compose an E2-inducible domain, while adjacent segments containing sequences resembling the CCAAT transcription factor-binding site and enhancer elements in the simian virus 40 (SV40) and polyomavirus regulatory regions are apparently responsible for constitutive activity in monkey CV-1 cells (20).

The BPV-1 E2 ORF also encodes a transcriptional repressor which shares the same carboxy-terminal domain with the E2 protein and suppresses E2-dependent enhancer activity in monkey CV-1 cells and in mouse C127 cells (24). Cripe et al. (10) have expressed the carboxy-terminal 60% of BPV-1

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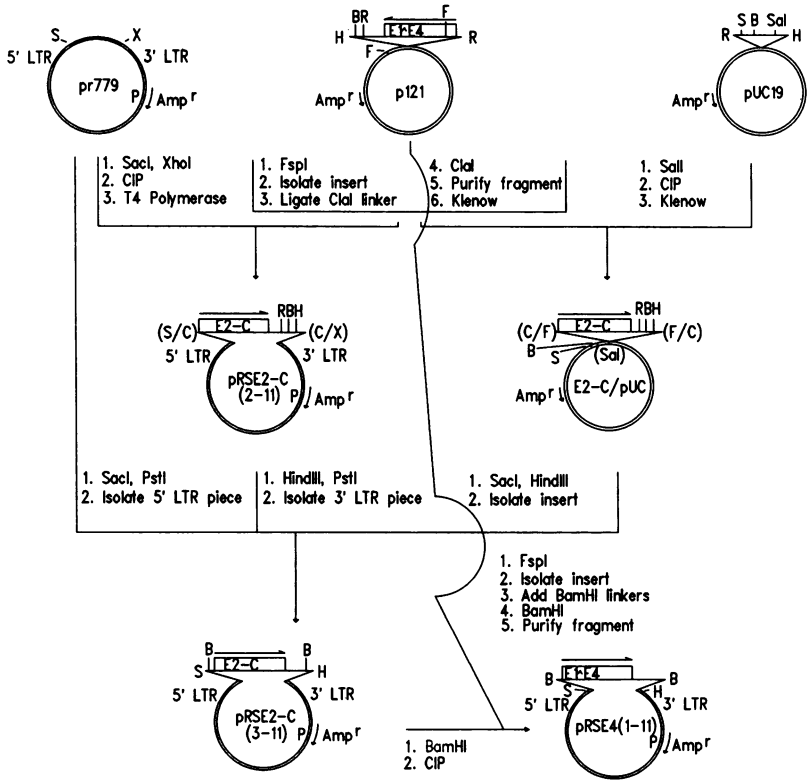

FIG. 1. Cloning of E2-C and E1^E4 into eucaryotic expression plasmids. The cDNA (p121) containing HPV-11 nt 716-847^3325-4388 encodes an E1^E4 protein (30). It also contains the carboxy-terminal 45% of the E2 ORF in an overlapping reading frame. The eucaryotic expression vector is pr779, which contains two Rous sarcoma virus LTRs. The plasmids were manipulated as shown and as described fully in Materials and Methods. The HPV-11 insertion is represented by open boxes. The gene orientations are indicated by the arrows. B, *Bam*HI; C, *Cla*I; F, *Fsp*I; P, *Pst*I; R, *Eco*RI; S, *Sac*I; Sa, *Sal*I; X, *Xho*I; CIP, calf intestine phosphatase. Restriction sites in parentheses indicate sites destroyed during manipulation.

and HPV-16 E2 ORFs in SiHa cells and have shown that both proteins can repress E2-dependent and E2-independent HPV-16 enhancer activities when reporter genes are linked to the SV40 early promoter. A comparative analysis of genomic organization, transcription patterns, and RNA splice sites suggests that the expression of this repressor is probably a general feature of many papillomaviruses (7). In this paper, we report the expression of HPV-11 E2-C, a protein consisting mainly of the carboxy-terminal 45% of the E2 protein, and the regulation of HPV-11 enhancer-SV40 early promoter and HPV-11 enhancer-E6 promoter activities by HPV-11 E2-C, HPV-11 E2, and BPV-1 E2 proteins in CV-1 cells and in the human cervical carcinoma cell line C-33A. We have also investigated the possible mechanism of E2-C regulation by expressing it in *E. coli* and studying its interaction with the E2-RS in vitro. Our results suggest that HPV-11 E2-C functions as a repressor by competing with E2 protein for direct binding to the E2-RS. Both E2 and E2-C proteins can also function as repressors for the HPV-11 E6 promoter, perhaps by interfering with the binding of other cellular transcriptional factors such as TFIID.

MATERIALS AND METHODS

Construction of eucaryotic expression clones and chloramphenicol acetyl transferase (CAT) vectors. The E4 cDNA completely overlaps the coding region of the carboxy-terminal 45% portion of the HPV-11 E2 protein. We therefore expressed E2-C protein from an HPV-11 E1^E4 cDNA (nt 716-847^3325-4388) in pUC8 (p121) (30) by introducing an AUG initiation codon contained in a *Cla*I linker, shifting translation into the E2 reading frame. p121 was digested with *Fsp*I which cuts at nt 811 in the cDNA and in the vector immediately downstream of the viral sequence. *Cla*I linkers were then added to the cDNA fragment. Expression plasmids with the Rous sarcoma virus long terminal repeat (LTR) promoter in vector pr779 were made as described below (Fig. 1). The *Cla*I cDNA fragment was treated with the Klenow fragment of *E. coli* DNA polymerase I and inserted between the *Sac*I and *Xho*I sites (made blunt with bacteriophage T4 DNA polymerase) of pr779 to create plasmid pRSE2-C(2-11). To place the AUG codon in a sequence context with strong initiation potential (23), the *Cla*I cDNA fragment was also temporarily cloned into the pUC19 *Sal*I site (filled in by treatment with the Klenow fragment of DNA polymerase I). The cDNA insertion in pUC19 was next excised with *Sac*I and *Hin*dIII and ligated to the *Hin*dIII-*Pst*I fragment of pRSE2-C(2-11) containing the 3' LTR and also to the *Pst*I-*Sac*I fragment of pr779 containing the 5' LTR to create pRSE2-C(3-11). To generate a very similar clone which does not express E2-C, pRSE2-C(3-11) was digested with *Bam*HI to remove the cDNA insertion. *Bam*HI linkers were added to the E1^E4 cDNA removed from p121 by *Fsp*I digestion (Fig. 1). The DNA fragment was then ligated to the *Bam*HI fragment of pRSE2-C(3-11). This clone, pRSE4(1-11), is identical to pRSE2-C(3-11), except that it no longer contains the AUG from which

E2-C translation can initiate; instead, it encodes in an overlapping translation frame the E1^E4 fusion protein by using the natural initiation codon at nt 832 to 834 in the cDNA (30).

The HPV-11 E2 expression clone pRSE2-11 is nearly identical to the previously described clone pRSE2-11 (19) except that the 3′ boundary of the HPV-11 insertion is nt 4388, identical to the E2-C expression plasmid, instead of nt 4988. The recombinant pUR23-3 containing the HPV-11 enhancer and E6 promoter from the URR (nt 7072-7931/1-99) was generated by first digesting cloned HPV-11 DNA with *Sfa*NI (nt 104) followed by treatment with S1 nuclease. The DNA was then digested with *Bam*HI, and the HPV-11 enhancer-E6 promoter fragment was isolated by gel electrophoresis and inserted at the *Bam*HI and blunted *Hin*dIII sites of pCAT-A upstream of the CAT expression cassette. The end point of the deletion was established by DNA sequencing. pCAT-A is an enhancer assay plasmid containing the CAT gene with an enhancerless SV40 early promoter (19). Other plasmids used include pUR27, in which the URR of HPV-11 (nt 7072-7931/1-64) is inserted in pCAT-A upstream of the SV40 promoter (19), and pSV2CAT, in which the CAT gene is expressed from the intact SV40 early promoter and enhancer (17).

Expression of HPV-11 E2-C protein in *E. coli*. For the expression of E2-C protein in bacteria, the *Fsp*I-truncated E1^E4 cDNA fragment (nt 811-847^3325-4388) with *Bam*HI linkers (Fig. 1) was transferred into the multiple cloning site of pEV-vrf1 (11) to generate the recombinant pEVE2-C. This vector contains a bacteriophage lambda P_L promoter, a synthetic (Shine-Dalgarno) ribosome-binding site and an initiation codon from which the correct translation frame for E2-C expression is achieved. This clone was introduced into the RR1 strain of *E. coli* containing pRK248*cI*ts, a low-copy-number plasmid which harbors a gene for a temperature-sensitive phage lambda repressor. Protein expression was induced by heat inactivation of the repressor at 42°C for 2 h.

Protein purification. Heat-induced bacteria were suspended in 50 mM Tris hydrochloride (pH 7.8)–2.5 mM EDTA–0.1 M NaCl, and lysozyme was added to 0.5 mg/ml. The digestion was allowed to proceed on ice for 30 min. NaCl and Nonidet P-40 were then added to 1 M and 2%, respectively. DNA and debris were removed by centrifugation. This crude lysate was stored at −20°C and used for filter binding or DNase I footprinting. To purify E2-C protein, heat-induced bacteria were sonicated in Z buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; K^+; pH 7.8], 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 20% [vol/vol] glycerol, 0.1% [vol/vol] Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) containing 0.1 M KCl (21). The lysates were then passed through a DNA-Sepharose affinity column containing the multimerized complementary oligonucleotide SN which contains an E2-RS (20; see Fig. 8) by the method of Kadonaga and Tjian (21). The E2-C protein was eluted with Z buffer containing 1 M KCl and stored at −70°C. Proteins were separated in 12.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate by electrophoresis and visualized by Coomassie blue staining.

DNase I footprinting. After binding reactions were performed in 100-μl volumes as described above, the DNA-protein complexes were subjected to mild pancreatic DNase I digestion. $MgCl_2$ was added to 10 mM, DNase I was added to 2 μg/ml, and the digestion was allowed to proceed for 1 min at room temperature. The reaction was stopped by the addition of 25 μl of 1.5 M sodium acetate–0.25 M EDTA–150 μg of pUC18 DNA per ml. The DNA was purified by phenol extraction and ethanol precipitation. Samples were then electrophoresed in 10% polyacrylamide sequencing gels, which were subsequently autoradiographed. A guanosine (G) or guanosine + adenosine (G+A) Maxam-Gilbert sequencing ladder of the same restriction fragment provided length markers.

Transfections and CAT assays. CV-1 and C-33A cells (American Type Culture Collection) on 60-mm-diameter plates were cotransfected by the calcium phosphate procedure with combinations of plasmids pRSE2-BP or pRSE2-11 that express the E2 protein of BPV-1 or HPV-11, respectively (19), and pUR27 (containing HPV-11 enhancer-SV40 promoter-CAT) (19) together with pRSE2-C(3-11), pRSE4(1-11), or pr779 in the amounts indicated in Fig. 2 to 4. After 4 to 6 h, the CV-1 cells were shocked for 3 min with 15% glycerol and then treated with 1 mM sodium butyrate (16). C-33A cells were shocked for 1 min and treated with 5 mM sodium butyrate. Cell lysates were prepared 42 h after shock, and CAT assays were performed by the procedure of Gorman et al. (17), as modified by Hirochika et al. (19). Plasmid pSV2CAT (17) was used as a positive control, and pCAT-A lacking an enhancer (19) was the negative control in all transfections. Most transfection and CAT assays were carried out in duplicate. All experiments were performed several times.

RESULTS

The comparative analyses of genomic DNA sequences and the structures of mRNAs recovered from condylomata (7) suggest that HPV-6, HPV-11, and many other papillomaviruses may encode an E2-C protein which is translated from an mRNA species which shares the same splice acceptor site and downstream exon with the mRNA coding for the E1^E4 protein (30). These mRNAs originate from different putative promoters, as reflected by distinct short 5′ exons. The 5′ exons are thought to provide the initiation codon and a few additional amino acids before being spliced into different reading frames in the 3′ main bodies of the mRNAs. The existence of this HPV-11 E2-C mRNA in a human lesion has recently been confirmed by sequence analysis of cDNA (M. Rotenberg, T. R. Broker, and L. T. Chow, unpublished result). We took advantage of these features and expressed from vector pRSE2-C(3-11) or pRSE4(1-11) the E2-C or E1^E4 protein, respectively, by modifying the HPV-11 E1^E4 cDNA (30) (Fig. 1). The putative E2-C protein (8) differs from that expressed from pRSE2-C(3-11) only in that 9 amino acids (excluding the initiation methionine) at the amino terminus are replaced by 12 different amino acids in the recombinant. pRSE4(1-11) is expected to encode the same E1^E4 protein of 90 amino acids as deduced from the cDNA.

Repression of E2-dependent transcription in CV-1 cells by HPV-11 E2-C protein. To examine whether the HPV-11 E2-C protein is a transcriptional repressor, we cotransfected fixed amounts of the CAT enhancer assay plasmid pUR27 (HPV-11 enhancer-SV40 promoter) and the E2 expression plasmid together with various amounts of E2-C expression plasmid into CV-1 cells. Because the BPV-1 E2 expression clone (pRSE2-BP) stimulates the HPV-11 enhancer to a greater extent than either the HPV-11 or HPV-1 E2 expression clone does (19), it was used in most of the experiments discussed in this paper. pr779, the vector for E2 and E2-C expression, was used to normalize DNA quantities in each transfection. The results of competition between HPV-11 E2-C and

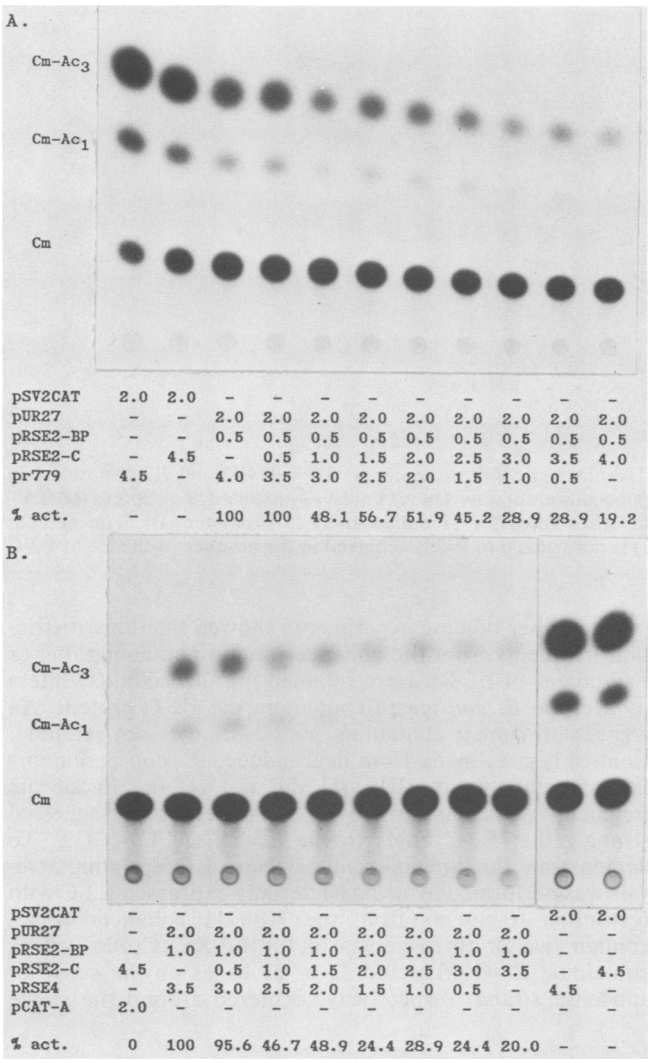

FIG. 2. Repression by E2-C of E2-dependent HPV-11 enhancer activity in CV-1 cells. Cells were cotransfected with various combinations of DNA plasmids in the amounts indicated, and the lysates were assayed as described in Materials and Methods. pSV2CAT containing the SV40 enhancer and promoter was the positive control. pCAT-A containing no enhancer was the negative control. pUR27 uses the HPV-11 enhancer-SV40 promoter for the expression of the CAT gene. BPV-1 E2 (pRSE2-BP), HPV-11 E2-C (pRSE2-C), and E1^E4 (pRSE4) were all expressed from the Rous sarcoma virus LTR in vector pr779. (A) pr779 was used as carrier; (B) pRSE4(1-11) was used as carrier. The percent conversion of chloramphenicol (Cm) to the acetylated forms (Cm-Ac_3 and Cm-Ac_1) is normalized to levels achieved in the presence of E2 expression plasmid only. act., Activity.

BPV-1 E2 are shown in Fig. 2A. Consistent with our previous reports, E2 stimulated the HPV-11 enhancer, as judged from the increased CAT activity. E2-C alone had no stimulatory effect on activity (data not shown), implying that the N terminus of the E2 protein was essential for enhancer activation. The E2-dependent CAT activity decreased with increasing amounts of cotransfected pRSE2-C DNA. At the highest amount of E2-C plasmid used, only 20% of the CAT activity remained. This repression was specific for the HPV-11 enhancer, because the E2-C protein had no effect on pSV2CAT, which relies on the SV40 enhancer. Control plasmid pr779 did not suppress enhancer activity. To demonstrate that the production of the E2-C protein was necessary for the observed suppression, the experiment was repeated with plasmid pRSE4(1-11) as a control and as the carrier DNA. pRSE4(1-11) differed from pRSE2-C(3-11) only in the linkers used for cloning and expressed the E1^E4 protein instead of E2-C. Again, the suppression of E2-dependent activity was only seen when E2-C plasmid was cotransfected (Fig. 2B). This result also ruled out the possibility that the repression of E2-dependent HPV-11 enhancer activity was due to competition by the cDNA sequence for positive transcriptional factors. These data showed that HPV-11 E2-C suppressed the HPV-11 enhancer stimulation by BPV-1 E2 protein but had no effect on pCAT-A or pSV2CAT. HPV-11 E2-C also suppressed the small stimulation of pUR27 by HPV-11 E2 protein (data not shown). Primer extension analysis of CAT mRNAs prepared from cotransfection experiments with E2 or E2 plus E2-C demonstrated that this phenomenon occurred at the level of mRNA transcription (data not shown), as was previously shown for the positive or negative effect on the papillomavirus enhancer by the intact or the amino-terminal-truncated E2 proteins (10, 18, 19, 24, 39).

Positive and negative regulation by HPV-11 E2 and E2-C and BPV-1 E2 proteins in C-33A cells. Because the E2-independent HPV-11 enhancer and enhancer-promoter activities in pUR27 (nt 7072-64) and pUR23-3 (nt 7072-99), respectively, were too low in CV-1 cells to detect possible negative effects of E2-C, we tested several human cervical carcinoma cell lines. We found that both pUR27 and pUR23-3 were active in HeLa (data not shown) and C-33A cells. HeLa cells contain integrated HPV-18 DNA and express the E6 and E7 regions rather actively (35). To avoid complications in interpreting E2-C effects, we concentrated on C-33A cells, which have been demonstrated not to contain HPV-6, HPV-11, HPV-16, or HPV-18 DNA (31a, 44). The cells were cotransfected with E2 and E2-C expression plasmids and control plasmid pRSE4(1-11) at a constant total DNA concentration (Fig. 3A). The constitutive activity of pUR23-3 was augmented by cotransfection with the HPV-11 E2 expression plasmid, and as anticipated, HPV-11 E2-C suppressed this stimulation. Unexpectedly, in the absence of the E2 expression plasmid, the constitutive activity from pUR23-3 was also repressed by 70% when 4 μg of pRSE2-C(3-11) was cotransfected. Unlike HPV-11 E2, BPV-1 E2 repressed pUR23-3 (Fig. 4A). The effect appeared to depend on the amount of plasmid pRSE2-BP transfected. When 1.0 or 0.2 μg of pRSE2-BP DNA was used, a small or no effect was observed. But when 4 μg of the BPV-1 E2 expression vector was introduced, 97% of the constitutive activity of pUR23-3 was abolished. Repression was not seen when control plasmid pRSE4(1-11) or pr779 was cotransfected. Very different results were obtained when pUR27 was used as test plasmid. Neither HPV-11 E2 nor E2-C had much effect on pUR27 (Fig. 3B). This lack of stimulation by HPV-11 E2 might be explained by the high E2-independent activity and the previous finding that HPV-11 E2 expression clones have rather weak positive effects (19). At both low and high concentrations, BPV-1 E2 stimulated pUR27 weakly. This small stimulation was eliminated by HPV-11 E2-C protein (Fig. 4B). All these experiments were performed several times, usually in duplicate, and the results were highly reproducible.

Binding of HPV-11 E2-C protein synthesized in *E. coli* to natural or synthetic E2-RS motif $ACCN_6GGT$. The E2 proteins almost certainly activate the papillomavirus enhancers by direct binding to the E2-RS $ACCN_6GGT$ (20). The

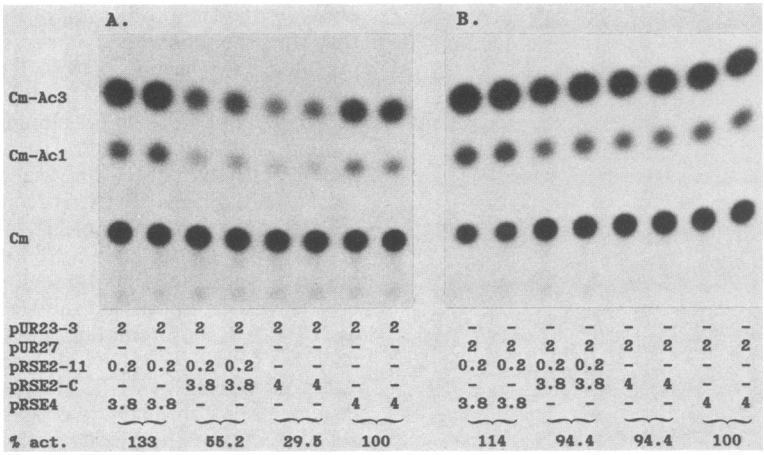

FIG. 3. Regulation of HPV-11 enhancer-E6 promoter and the HPV-11 enhancer-SV40 promoter by HPV-11 E2 and E2-C proteins in human cervical carcinoma cell line C-33A. Cells were cotransfected with combinations of different plasmids, as indicated. To express the CAT gene, pUR23-3 uses the HPV-11 enhancer-E6 promoter (A) and pUR27 uses the HPV-11 enhancer-SV40 promoter (B). The percent conversion of chloramphenicol to acetylated forms (Cm-Ac3 and Cm-Ac1) is normalized to levels achieved in the absence of the E2 or E2-C expression plasmid. act., Activity.

simplest mechanism by which E2-C might repress E2 activity is to bind to the same motif as the full-length E2 protein. We examined this hypothesis by studying the interaction between the E2-RS and the E2-C protein synthesized in *E. coli*. E2-C was expressed from the lambda P_L promoter under the control of a temperature-sensitive lambda repressor in the expression vector pEV-vrf1 (Fig. 5A), as described in Materials and Methods. This protein differs from the putative native protein in that 9 amino acids (excluding the initiation methionine) at the amino terminus encoded by the 5′ exon are replaced by 18 unrelated amino acids. E2-C was expressed at a high level in bacteria following induction at 42°C (Fig. 5A). After cell lysis, much of the E2-C protein remained insoluble and biologically inactive (data not shown).

Initial filter-binding experiments showed that only restriction fragments from the enhancer region containing one or two copies of E2-RS were retained on nitrocellulose filters by a crude *E. coli* lysate containing the E2-C protein. An adjacent fragment containing no E2-RS was not retained. Control lysates made from heat-induced *E. coli* containing the parental vector pEV-vrf1 did not retain any of the fragments (data not shown). These experiments suggested strongly that E2-C binds to the E2-RS, $ACCN_6GGT$. To demonstrate this unequivocally, DNase I footprinting analysis was performed by using bacterially expressed E2-C with restriction fragments from the HPV-11 enhancer which contain two or three copies of the E2-RS. Crude lysates containing E2-C protected 22 or 23 bases on the sense or antisense strand, respectively, centered around the single

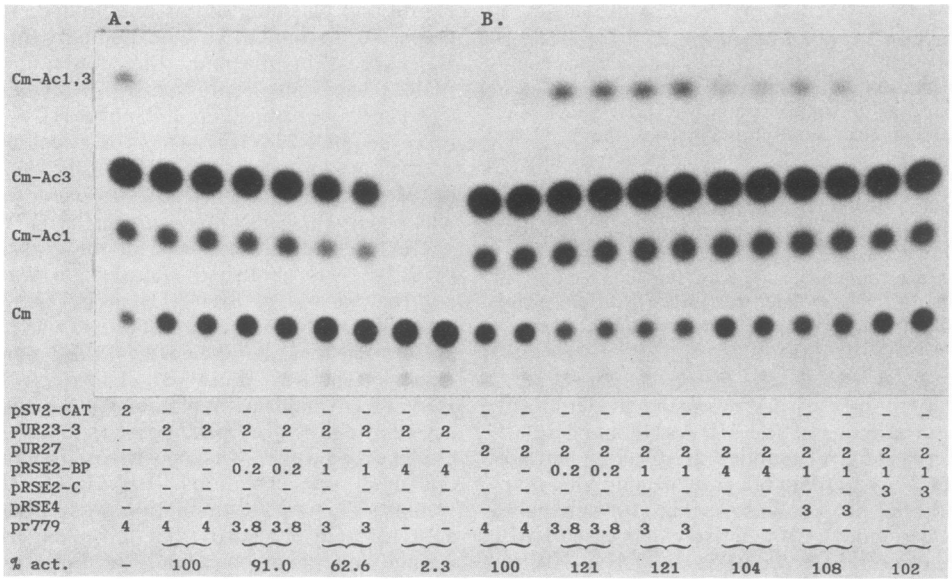

FIG. 4. Regulation of HPV-11 enhancer-E6 promoter (A) and HPV-11 enhancer-SV40 promoter (B) by BPV-1 E2 and HPV-11 E2-C proteins in human cervical carcinoma cell line C-33A. Cells were cotransfected with combinations of different plasmids as indicated. The CAT gene was expressed from the HPV-11-E6 promoter (pUR23-3) (A) or the HPV-11 enhancer-SV40 early promoter (pUR27) (B). The percent conversion of chloramphenicol (Cm) to acetylated forms (Cm-Ac1,3, Cm-Ac3, and Cm-Ac1) is normalized to levels achieved in the absence of the E2 or E2-C expression plasmid. act., Activity.

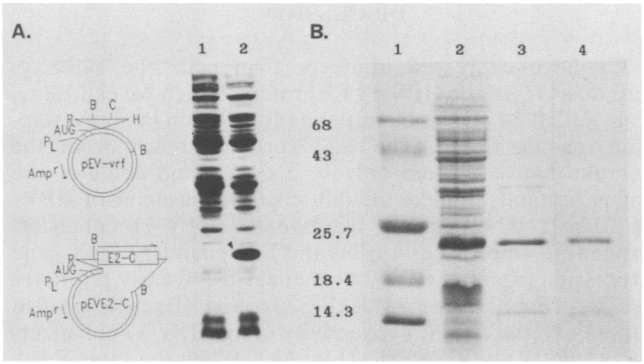

FIG. 5. Expression and purification of HPV-11 E2-C protein synthesized in *E. coli*. (A) Structures of procaryotic vector and expression plasmid described in Materials and Methods and expression levels of E2-C in uninduced (lane 1) and induced (lane 2, arrowhead) cells. (B) Whole lysate (lane 2) and E2-C purified by one (lane 3) and two (lane 4) passages over an E2-RS DNA affinity chromatography column, as described in Materials and Methods. Molecular weight markers are in lane 1. Proteins were visualized with Coomassie blue staining after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

number 2 motif (Fig. 6 and data not shown) and 37 or 38 nucleotides on the sense or antisense strand, respectively, centered around the tandem number 3 and number 4 copies of the E2-RS and overlapped the adjacent TATA motif for the E6 promoter (Fig. 6). All three motifs were equally protected at each protein concentration used. The DNA-protein complex created DNase I hypersensitivity in flanking nucleotides. Control lysates prepared from heat-induced bacteria containing pEV-vrf1 did not exhibit protection.

To test whether this interaction depends on sequences flanking the $ACCN_6GGT$ motif in the enhancer, footprinting analysis was also performed on a restriction fragment containing a single synthetic E2-RS, SN1 (Fig. 7). The size of the footprint was identical to that observed on the solitary number 2 copy of the E2-RS (Fig. 6). These results demonstrate unequivocally that the E2-C protein binds directly to the $ACCN_6GGT$ motif in the absence of specific flanking viral sequences.

Binding of E2-C protein to synthetic E2-RS but not to mutant motifs. The full-length HPV-11 E2 protein expressed in *E. coli* binds to synthetic E2-RS but not to its mutant versions (20). We next performed experiments to determine whether the HPV-11 E2-C protein has the same specificity. We purified bacterially expressed soluble E2-C protein by sequence-specific DNA affinity chromatography (21) with multimerized, synthetic complementary oligonucleotides containing the E2-RS as a ligand (20). Purity of near homogeneity was achieved by two passages through this column (Fig. 5B). Synthetic complementary oligonucleotides containing the E2-RS or its variants have been cloned into pCAT-A (20). Short restriction fragments containing one or two copies of the E2-RS motif were specifically retained on nitrocellulose filters by the purified E2-C protein (Fig. 8). On the contrary, a restriction fragment containing five copies of a synthetic oligonucleotide SNM in which the $ACCN_6GGT$

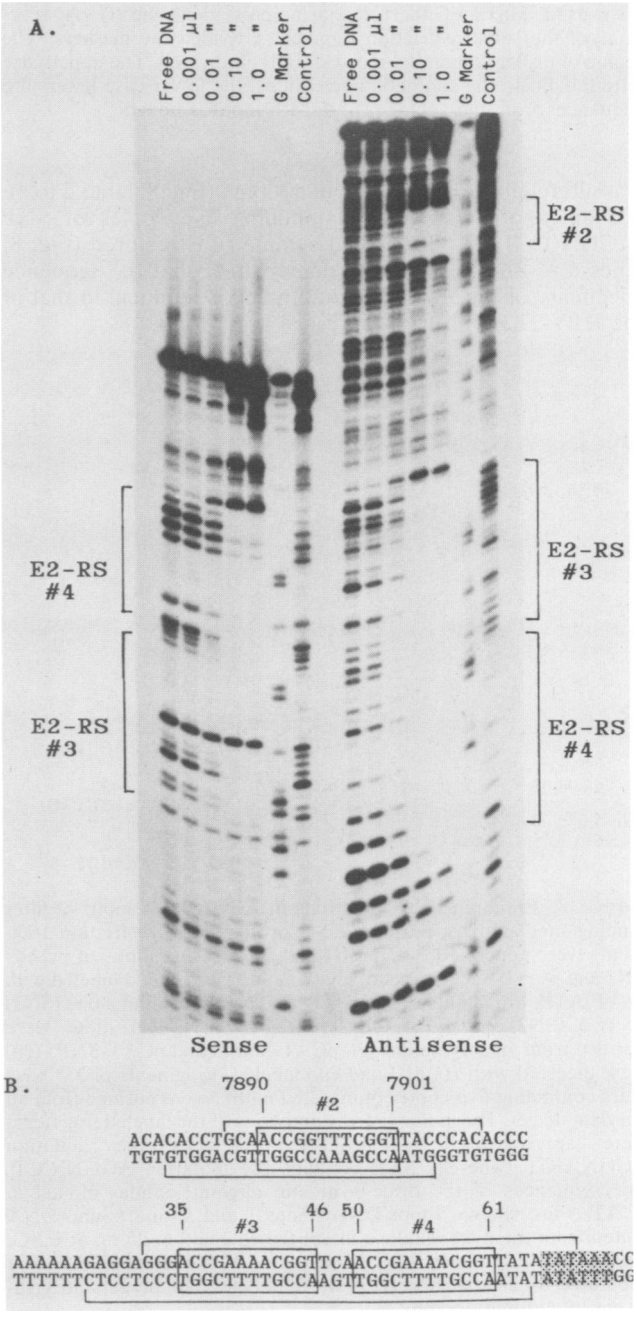

FIG. 6. DNase I footprinting of HPV-11 E2-C protein bound to HPV-11 enhancer DNA. For the sense-strand footprinting, pUR23-3 was digested with *Bst*EII and 5′ end labeled with [γ-^{32}P]ATP at nt 7900. The DNA was then recut with *Hin*dIII, and the fragment containing HPV-11 URR nucleotides 7900-7931/1-99 was used. For the antisense strand, pUR23-3 was cut with *Hin*dIII and 5′ end labeled. The approximately 1,000-base-pair fragment eluted from an agarose gel was recut with *Rsa*I. The fragment containing nt 7830-7931/1-99 (labeled at nt 99) was used. (A) DNase I footprints in the absence of E2-C or in the presence of various amounts (in microliters) of crude lysates of E2-C-expressing bacteria. Control lysate was prepared from induced bacteria that harbor vector pEV-vrf1. A Maxam-Gilbert guanosine (G) reaction of the same restriction fragment provided the markers. Copy numbers 2, 3, and 4 of the E2-RS in the DNA are identified by vertical brackets. The protection of both strands of motif number 2 was also obtained by using a different restriction fragment (data not shown). (B) The protected regions are shown schematically; they center on the $ACCN_6GGT$ motifs (located at nt 7890-7901, 35-46, and 50-61). The TATA motif of the putative E6 promoter is shaded.

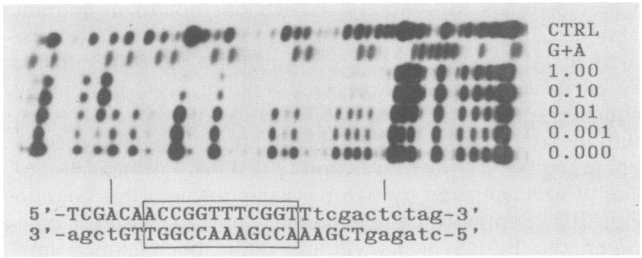

FIG. 7. DNase I footprinting of HPV-11 E2-C protein bound to synthetic E2-RS, SN1. Plasmid pCAT-SN1 (20) was digested with *Hin*dIII, 5′ end labeled with [γ-^{32}P]ATP and recut with *Bgl*I. The labeled fragment of 115 base pairs containing one copy of the synthetic $ACCN_6GGT$ motif was used. Various amounts (in microliters) of crude lysates containing E2-C and control (CTRL) lysate were used. Maxam-Gilbert guanosine-plus-adenosine (G+A) reactions of the same restriction fragment provided the markers. The region of protection on the sense strand is indicated. The upper-case letters indicate the synthetic nucleotides; the lower-case letters are sequences from the vector. The E2-RS motif is boxed.

was altered to $AGGN_6GGT$ did not bind (Fig. 8, lanes 2 to 4), nor did another fragment containing five copies of SNP ($AGGN_6CCT$) in which dyad symmetry is restored (Fig. 8, lanes 6 to 8). These results demonstrate that the sequence specificity of the E2-C protein binding is identical to that of the HPV-11 E2 protein expressed in *E. coli* (20).

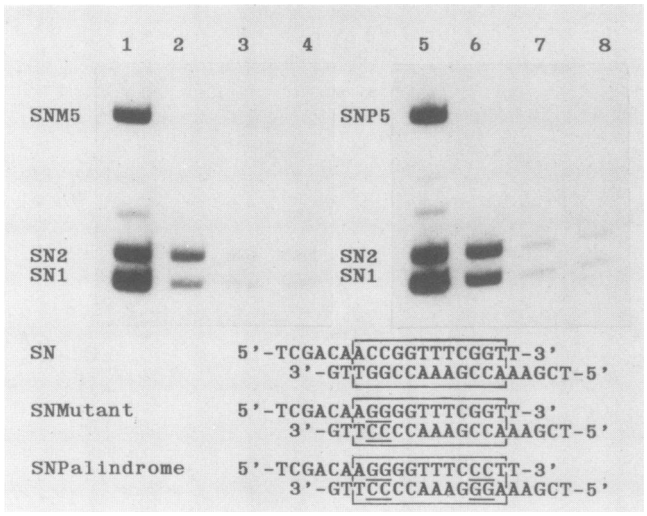

FIG. 8. Elimination by mutations in $ACCN_6GGT$ motif of filter binding mediated by purified E2-C protein. The restriction fragments were generated by *Hin*dIII and *Bam*HI digestions of pCAT-SN1 and pCAT-SN2, respectively (20), and were 5′ end labeled with [γ-^{32}P]ATP. Fragments of 55 and 70 base pairs containing one (SN1) or two (SN2) copies of the synthetic $ACCN_6GGT$ motif were purified from an acrylamide gel. pCAT-SNM5 and pCAT-SNP5 (20) were digested with *Hin*dIII and end labeled. Fragments of 315 base pairs containing five copies of mutated motifs were purified from an acrylamide gel. The molar specific activities of the labeled fragments were approximately equal. SNM contains the mutation $AGGN_6GGT$, whereas SNP contains the mutation $AGGN_6CCT$. The sequences of the three synthetic oligonucleotides cloned in pCAT-A are shown. Input DNA (lanes 1 and 5) and bound DNA eluted from the nitrocellulose filters (lanes 2 and 6, 25 ng of E2-C; lanes 3 and 7, 5 ng of E2-C; lanes 4 and 8, 2.5 ng of E2-C) were separated by electrophoresis in a 4% polyacrylamide gel and visualized by autoradiography.

DISCUSSION

We have expressed in mammalian cells the carboxy-terminal 45% of the HPV-11 E2 protein, which we call E2-C, from an E1^E4 cDNA by initiating translation in an overlapping reading frame. The E2-C protein differs from the putative native protein only in a few amino acids at the amino terminus. Unlike the full-length E2 proteins of HPVs or BPV-1, E2-C did not activate the HPV-11 enhancer. Instead, it suppressed in *trans* the E2-dependent CAT gene expression from the HPV-11 enhancer-SV40 early promoter in CV-1 (Fig. 2) and C-33A (Fig. 3A and 4B) cells. It also suppressed the constitutive activity of the HPV-11 enhancer-E6 promoter in C-33A cells (Fig. 3A). When the same DNA segment was expressed in a different reading frame into E1^E4 protein, inhibition was lost in both cell lines, indicating that the production of E2-C protein was required for repression (Fig. 2, 3A, and 4B). DNase I footprinting analysis showed that the E2-C protein synthesized in *E. coli* protected the E2-RS plus four or five flanking nucleotides on either side, and no other virus-specific sequences were required for this interaction (Fig. 6 and 7). These footprints were identical to those generated with full-length E2 proteins (20). Filter-binding assays with cloned synthetic oligonucleotides containing the E2 motif or mutant versions of it also demonstrated that this motif alone is both necessary and sufficient for the binding of E2-C protein expressed in bacteria (Fig. 8). All these DNA-binding properties of E2-C are identical to those observed for the full-length E2 proteins of HPV-1 and HPV-11 and the amino-terminus-truncated E2 protein of BPV-1 expressed in *E. coli* (20). Therefore, we conclude that the E2 protein consists of two domains, one near the amino terminus for transcriptional activation and the other near the carboxyl terminus for DNA binding. Reports of filter binding with the truncated BPV-1 E2 protein also suggest that the amino-terminal domain of BPV-1 E2 protein is not essential for DNA binding (2, 27, 29). Accordingly, the HPV-11 E2-C protein is a transcriptional repressor which acts by direct binding to the E2-RS. The mechanism by which this binding affects E2-dependent transcription most likely involves direct competition with the E2 protein for their common binding targets, or possibly, the binding occurs through the formation of a nonfunctional heterodimer with a full-length E2 protein.

The repression by HPV-11 E2-C of E2-independent HPV-11 enhancer-E6 promoter activity from pUR23-3 observed in C-33A cells (Fig. 3A) could be due to interference with the binding of a TATA motif-binding factor such as TFIID (34) through steric exclusion. This hypothesis is supported by two observations. First, the E2-C repression was observed only when clone pUR23-3 which contains the HPV-11 enhancer-E6 promoter was used but not when pUR27 with the HPV-11 enhancer-SV40 promoter was used (Fig. 3). In pUR27, the TATA motif is 97 nucleotides from the E2-RS, compared with 4 nucleotides in pUR23-3. Second, the DNase I footprint of the E2-C protein on the tandem E2-RS elements overlapped the TATA motif of the putative E6 promoter (Fig. 6). An alternative explanation is that the SV40 promoter is not subject to the E2-C repression because the 21-base-pair repetitions adjacent to the TATA motif interact with additional transcriptional factors such as Sp1 and AP-2 (14, 28) that compensate for the negative effect of E2-C. Since E2 protein has the same footprint as E2-C, however, one must account for the stimulatory effect of the HPV-11 E2 protein on the E6 promoter. One possibility is that the amino-terminal domain of the E2 protein, which is

essential for activation, recruits and stabilizes the TATA-binding factor. A second possibility is that the E2-responsive sequences near the TATA motif are only occupied when E2 or E2-C protein is expressed at high levels. E2 would thus also be expected to decrease E6 promoter activity when it is highly expressed. The repression of pUR23-3 by BPV-1 E2 supports this notion (see below). Because HPV-11 E2 mRNA appears to initiate from the E6 promoter (8), E2 protein might then regulate its own expression and also that of E6 and E7 proteins. Thus we believe that the E2 protein should also be regarded as a repressor, as well as an activator. This hypothesis may explain the overexpression of HPV-16 and HPV-18 E6 and E7 ORFs in cervical carcinoma and in cell lines derived from them in which the E2 ORF is disrupted by integration into the host genome (3, 35–37).

Our results on the repression of the E2-dependent HPV-11 enhancer-SV40 promoter in pUR27 by HPV-11 E2-C in CV-1 and C-33A cells agree with those of other investigators who have reported that amino-terminal-truncated BPV-1 or HPV-16 E2 proteins repress E2-dependent BPV-1 and HPV-16 enhancer activities in CV-1, C127 (24), and SiHa cells (10). Our observation that the E2-independent HPV-11 enhancer-E6 promoter exhibits cell type specificity and is active in HeLa and C-33A cells (Fig. 3A) but not in CV-1 cells (data not shown) is also similar to observations with HPV-18 and HPV-16 (10, 15, 41, 42). The negative regulation of the HPV-11 constitutive enhancer-promoter activity by BPV-1 E2 protein in transfected cells (Fig. 4A) agrees with the BPV-1 E2 repression of the HPV-18 enhancer-E6 promoter (43) and the BPV-1 P1 promoter, which is adjacent to an E2-binding site (A. Stenlund and M. Botchan, personal communication). In addition, we have shown that this negative regulation depends on the amount of BPV-1 E2 protein (Fig. 4A). Repression was nearly complete when large amounts of BPV-1 E2 expression plasmid were introduced, whereas smaller effects were observed when less BPV-1 E2 DNA was cotransfected. The smaller quantity of BPV-1 E2 plasmid was, however, sufficient to stimulate the HPV-11 enhancer-SV40 promoter (pUR27) in the same cell line (Fig. 4B). Without a knowledge of the cellular factors with which these different E2 proteins interact or of the amount of E2 proteins present in the transfected cells, we do not know with certainty whether a difference in E2 properties or protein levels has resulted in the differential effects of HPV-11 and BPV-1 E2 proteins on the HPV-11 enhancer-E6 promoter. Our results demonstrating E2-C repression of the E2-independent HPV-11 enhancer-E6 promoter but not of the HPV-11 enhancer-SV40 promoter are at variance with those of Cripe et al. (10) who reported that a shortened BPV-1 or HPV-16 E2 molecule represses E2-independent HPV-16 URR-SV40 promoter activity in SiHa cells. The basis for this discrepancy is not clear, but we note a difference in the sizes of the E2-C expressed. It is also possible that different cellular factors are involved in the transcription of HPV-11 and HPV-16 in the different cell lines used.

The biological implications of repression by E2-C and E2 are a matter of conjecture, because no system for the in vitro propagation of papillomaviruses exists. Nevertheless, there are a number of interesting possibilities. One hypothesis is that E2-C, as well as E2 in high concentrations, acts as a switch, shutting off the expression of certain viral genes and activating others, in a manner analogous to SV40 T antigen (5, 22) and the herpes simplex virus immediate-early protein (ICP4) (13, 31). Another possibility is that E2-C attenuates viral gene expression so that the virus does not totally incapacitate the host cells but instead remains latent. E2-C might thus be analogous, in effect, to the 3′ ORF product of the human immunodeficiency virus, which decreases viral cytopathic effects (26).

In addition to the papillomavirus systems, another example of a single genetic region encoding both positive and negative factors is the adenovirus E1A region. However, the E1A products are thought to act through host proteins in a manner that does not involve their binding directly to responsive promoters (for a review, see reference 4). Interestingly, the E1A repressor and transforming activities map to the same protein domain, raising the possibility that the repression of certain cellular genes may be required for transformation (25). It is conceivable that E2 and E2-C also modulate host gene expression in addition to viral gene expression and that the perturbation in viral or host gene expression is necessary for oncogenic transformation. Thus, the counterbalancing regulatory mechanisms could play important roles in the life cycle of these species-, tissue-, and differentiation-specific pathogens.

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LITERATURE CITED

1. **Androphy, E. J., N. L. Hubbert, J. T. Schiller, and D. R. Lowy.** 1987. Identification of the HPV-16 E6 protein from transformed mouse cells and human cervical carcinoma cell lines. EMBO J. **6**:989–992.
2. **Androphy, E. J., D. R. Lowy, and J. T. Schiller.** 1987. Bovine papillomavirus E2 trans-activating gene product binds to specific sites in papillomavirus DNA. Nature (London) **325**:70–73.
3. **Baker, C. C., W. C. Phelps, V. Lindgren, M. J. Braun, M. A. Gonda, and P. M. Howley.** 1987. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J. Virol. **61**:962–971.
4. **Berk, A. J.** 1986. Adenovirus promoters and E1a transactivation. Annu. Rev. Genet. **20**:45–79.
5. **Brady, J., and G. Khoury.** 1985. *trans* activation of the simian virus 40 late transcription unit by T-antigen. Mol. Cell. Biol. **5**: 1391–1399.
6. **Broker, T. R., and M. Botchan.** 1986. Papilloma viruses: retrospectives and prospectives. Cancer Cells **4**:17–36.
7. **Chow, L. T., H. Hirochika, M. Nasseri, M. H. Stoler, S. M. Wolinsky, M. T. Chin, R. Hirochika, D. S. Arvan, and T. R. Broker.** 1987. Human papilloma virus gene expression. Cancer Cells **5**:55–72.
8. **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.
9. **Cole, S. T., and O. Danos.** 1987. Nucleotide sequence and comparative analysis of the human papillomavirus type 18 genome. Phylogeny of papillomaviruses and repeated structure of the E6 and E7 gene products. J. Mol. Biol. **193**:599–608.
10. **Cripe, T. P., T. H. Haugen, J. P. Turk, F. Tabatabai, P. G. Schmid III, M. Dürst, L. Gissmann, A. Roman, and L. P. Turek.** 1987. Transcriptional regulation of the human papillomavirus-16 E6-E7 promoter by a keratinocyte-dependent enhancer, and by viral E2 trans-activator and repressor gene products: implications for cervical carcinogenesis. EMBO J. **6**:3745–3753.
11. **Crowl, R., C. Seamans, P. Lomedico, and S. McAndrew.** 1985.

Versatile expression vectors for high-level synthesis of cloned gene products in Escherichia coli. Gene **38**:31–38.
12. **Dartmann, K., E. Schwarz, L. Gissmann, and H. zur Hausen.** 1986. The nucleotide sequence and genome organization of human papilloma virus type 11. Virology **151**:124–130.
13. **Gelman, I. H., and S. Silverstein.** 1987. Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the virus transcriptional activators. J. Virol. **61**: 3167–3172.
14. **Gidoni, D., W. S. Dynan, and R. Tjian.** 1984. Multiple specific contacts between a mammalian transcription factor and its cognate promoters. Nature (London) **312**:409–413.
15. **Gloss, B., H. U. Bernard, K. Seedorf, and G. Klock.** 1987. The upstream regulatory region of the human papilloma virus-16 contains an E2 protein-independent enhancer which is specific for cervical carcinoma cells and regulated by glucocorticoid hormones. EMBO J. **6**:3735–3743.
16. **Gorman, C. M., B. Howard, and R. Reeves.** 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. Nucleic Acids Res. **11**:7631–7648.
17. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
18. **Haugen, T. H., T. P. Cripe, G. D. Ginder, M. Karin, and L. P. Turek.** 1987. Trans-activation of an upstream early gene promoter of bovine papilloma virus-1 by a product of the viral E2 gene. EMBO J. **6**:145–152.
19. **Hirochika, H., T. R. Broker, and L. T. Chow.** 1987. Enhancers and *trans*-acting E2 transcriptional factors of papillomaviruses. J. Virol. **61**:2599–2606.
20. **Hirochika, H., R. Hirochika, T. R. Broker, and L. T. Chow.** 1988. Functional mapping of the human papillomavirus type 11 transcriptional enhancer and its interaction with the trans-acting E2 proteins. Genes Dev. **2**:54–67.
21. **Kadonaga, J. T., and R. Tjian.** 1986. Affinity purification of sequence-specific DNA binding proteins. Proc. Natl. Acad. Sci. USA **83**:5889–5893.
22. **Keller, L. M., and J. C. Alwine.** 1984. Activation of the SV40 late promoter: direct effects in the absence of viral DNA replication. Cell **36**:381–389.
23. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
24. **Lambert, P. F., B. A. Spalholz, and P. M. Howley.** 1987. A transcriptional repressor encoded by BPV-1 shares a common carboxy-terminal domain with the E2 transactivator. Cell **50**:69–78.
25. **Lillie, J. W., M. Green, and M. R. Green.** 1986. An adenovirus E1a protein region required for transformation and transcriptional repression. Cell **46**:1043–1051.
26. **Luciw, P. A., C. Cheng-Mayer, and J. A. Levy.** 1987. Mutational analysis of the human immunodeficiency virus: the orf-B region down-regulates virus replication. Proc. Natl. Acad. Sci. USA **84**:1434–1438.
27. **McBride, A., R. Schlegel, and P. M. Howley.** 1988. The carboxy-terminal domain shared by the bovine papillomavirus transactivator and repressor proteins contains a specific DNA binding activity. EMBO J. **7**:533–539.
28. **Mitchell, P. J., C. Wang, and R. Tjian.** 1987. Positive and negative regulation of transcription in vitro: enhancer-binding protein AP-2 is inhibited by SV40 T antigen. Cell **50**:847–861.
29. **Moskaluk, C., and D. Bastia.** 1988. DNA bending is induced in an enhancer by the DNA-binding domain of the bovine papillomavirus E2 protein. Proc. Natl. Acad. Sci. USA **85**:1826–1830.
30. **Nasseri, M., R. Hirochika, T. R. Broker, and L. T. Chow.** 1987. A human papilloma virus type 11 transcript encoding an E1^E4 protein. Virology **159**:433–439.
31. **O'Hare, P., and G. S. Hayward.** 1985. Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. J. Virol. **53**:751–760.

31a. **Pater, M. M., and A. Pater.** 1985. Human papillomavirus types 16 and 18 sequences in carcinoma cell lines of the cervix. Virology **145**:313–318.

32. **Pfister, H.** 1987. Papillomaviruses: general description, taxonomy, and classification, p. 1–38. *In* N. P. Salzman and P. M. Howley (ed.), The papillomaviruses. Plenum Publishing Corp., New York.
33. **Phelps, W. C., and P. M. Howley.** 1987. Transcriptional *trans*-activation by the human papillomavirus type 16 E2 gene product. J. Virol. **61**:1630–1638.
34. **Sawadogo, M., and R. G. Roeder.** 1985. Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. Cell **43**:165–175.
35. **Schneider-Gädicke, A., and E. Schwarz.** 1986. Different human cervical carcinoma cell lines show similar transcription patterns of human papillomavirus type 18 early genes. EMBO J. **5**:2285–2292.
36. **Schwarz, E., U. K. Freese, L. Gissmann, W. Mayer, B. Roggenbuck, A. Stremlau, and H. zur Hausen.** 1985. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. Nature (London) **314**:111–114.
37. **Shirasawa, H., Y. Tomita, S. Sekiya, H. Takamizawa, and B. Simizu.** 1987. Integration and transcription of human papillomavirus type 16 and 18 sequences in cell lines derived from cervical carcinomas. J. Gen. Virol. **68**:583–591.
38. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in a cervical cancer and a cancer-derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83**:4680–4684.
39. **Spalholz, B. A., P. F. Lambert, C. L. Yee, and P. M. Howley.** 1987. Bovine papillomavirus transcriptional regulation: localization of the E2-responsive elements of the long control region. J. Virol. **61**:2128–2137.
40. **Spalholz, B. A., Y. C. Yang, and P. M. Howley.** 1985. Transactivation of a bovine papilloma virus transcriptional regulatory element by the E2 gene product. Cell **42**:183–191.
41. **Swift, F. V., K. Bhat, H. B. Younghusband, and H. Hamada.** 1987. Characterization of a cell type-specific enhancer found in the human papilloma virus type 18 genome. EMBO J. **6**:1339–1344.
42. **Thierry, F., J. M. Heard, K. Dartmann, and M. Yaniv.** 1987. Characterization of a transcriptional promoter of human papillomavirus 18 and modulation of its expression by simian virus 40 and adenovirus early antigens. J. Virol. **61**:134–142.
43. **Thierry, F., and M. Yaniv.** 1987. The BPV1-E2 trans-acting protein can be either an activator or a repressor of the HPV 18 regulatory region. EMBO J. **6**:3391–3397.
44. **Yee, C., I. Krishnan-Hewlett, C. C. Baker, R. Schlegel, and P. M. Howley.** 1985. Presence and expression of human papillomavirus sequences in human cervical carcinoma cell lines. Am. J. Pathol. **119**:361–366.
45. **zur Hausen, H., and A. Schneider.** 1987. The role of papillomaviruses in human anogenital cancer, p. 245–264. *In* N. P. Salzman and P. M. Howley (ed.), The papillomaviruses, vol. 2. The papovaviridae. Plenum Publishing Corp., New York.