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# Suppression of Cellular Proliferation by the Papillomavirus E2 Protein

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**Carcinogenic progression of a human papillomavirus (HPV)-infected cell is often associated with integration of the viral genome in a manner which results in the loss of expression of the viral regulatory protein E2. One function of E2 is the regulation of expression of the viral oncogenes, E6 and E7. Introduction of the bovine papillomavirus type 1 (BPV-1) E2 transactivator (E2-TA) in HeLa cells, an HPV type 18 (HPV-18)-positive cervical carcinoma cell line results in growth arrest. In this study, we have found that the HPV-16 and HPV-18 E2 proteins share with BPV-1 E2-TA the ability to suppress HeLa cell growth. This property was not observed for the BPV-1 E2 transcriptional repressor (E2-TR). Analysis of various mutant E2 proteins for growth suppression revealed a requirement for the intact transactivation and DNA binding domains. A HeLa cell line (HeLa-*ts*E2) which expressed a conditional mutant E2 protein that was functional only at the permissive temperature (32°C) was established, permitting an analysis of the molecular and cellular consequences of E2 expression. Our data indicate that one mechanism by which E2 suppresses cellular growth is through repression of E6 and E7 expression, thereby enabling the cellular targets of E6 and E7 to resume regulation of the cell cycle.**

There are over 70 different human papillomaviruses (HPVs) now identified, of which at least 30 types have been associated with anogenital lesions (60). HPVs can be further classified as either high risk (HPV type 16 [HPV-16] and HPV-18) or low risk (HPV-6 and HPV-11) on the basis of the clinical lesions with which they are associated and the relative propensity for these lesions to progress to cancer. Approximately 85 to 90% of human cervical cancers harbor the DNA of a high-risk HPV.

In HPV-positive genital cancers, the viral genomes are transcriptionally active, and two viral genes, E6 and E7, are invariably expressed. The high-risk HPVs encode two oncoproteins, E6 and E7, whose expression can extend the life span of squamous epithelial cells, which are the normal host cells for the papillomavirus. E6 and E7 together can result in the efficient immortalization of primary human cells (21, 39, 55). E6 and E7 are expressed in HPV-positive cervical cancer-derived cell lines (42, 43, 45). Furthermore, although many genetic changes have occurred in cervical carcinoma cells, the continued expression of the viral oncoproteins is necessary since expression of antisense E6/E7 RNA results in decreased cell growth (53). Similar to the transforming proteins of the other small DNA tumor viruses, simian virus (SV40) and adenovirus, the transforming properties of the E6 and E7 oncoproteins appear to be due at least in part to their capacity to functionally inactivate the p53 and the retinoblastoma (pRB) tumor suppressor proteins. The E6 proteins of HPV-16 and HPV-18 can complex (56) and inactivate p53 (37). The high-risk HPV E7 proteins bind pRB more efficiently than the E7 proteins of low-risk HPVs (3, 14, 39a). It is believed that the functional inactivation of the p53 and pRB regulatory pathways by E6 and E7 are important steps in cervical carcinogenesis.

One characteristic of HPV-related carcinogenic progression is the frequent integration of the viral genome into the human chromosome in the cancer cells in a manner that results in the loss of expression of the viral E2 gene but maintains high levels of E6/E7 expression (13, 24). E2 encodes a regulatory protein, and for the genital HPVs, it is involved in the regulation of the viral promoter that directs expression of the E6 and E7 genes (10, 11, 20, 41, 48–50). Several observations suggest that the loss of E2 expression may be an important step in HPV-associated carcinogenic progression. First, E2 can repress activity from the HPV-16 and HPV-18 promoters which direct expression of the E6 and E7 gene products (5, 41, 49). Second, viral genomes with mutations in the HPV-16 E2 open reading frame (ORF) have an increased immortalization capacity in primary keratinocytes (40). Finally, the reintroduction of the BPV-1 E2 gene into cervical carcinoma cell lines leads to suppression of growth and cell cycle arrest (23, 50). This growth suppression correlates with inhibition of expression of the E6 and E7 transcripts, as demonstrated by infection of HPV-positive cervical carcinoma cell lines with a recombinant SV40 expressing the bovine papillomavirus type 1 (BPV-1) E2 protein (23).

The functions of the E2 proteins have been extensively characterized for BPV-1. The BPV-1 E2 ORF encodes three different proteins: an internally initiated E2 transcriptional repressor (E2-TR), a spliced product between E8 and E2 (E8-E2), and the E2 transactivator (E2-TA) (22, 27). The full-length E2 protein (E2-TA) can function as a transactivator or a repressor, depending on the location of E2 binding sites in a responsive promoter. It has two functional domains which are well conserved among the E2 proteins of different papillomaviruses (17, 35), a 200-amino-acid transactivation domain at the N terminus and a 90- to 100-amino-acid C-terminal domain that is essential for dimerization and DNA binding (30, 34, 36, 38). A flexible spacer region called "the hinge" separates the transactivation and DNA binding domains. Both E2-TR and E8-E2 can inhibit the transactivation function of the full-length protein, through mechanisms that may involve competition with E2-TA for DNA binding or by subunit mixing and heterodimer formation with the full-length protein. E2-TA can directly repress transcription of promoters in which the E2 binding sites are located near essential promoter elements

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such as the TATA box or SP1 binding sites (11, 18, 31, 41, 46, 49, 52). In these cases, E2-TA is believed to interfere with the assembly of the transcriptional initiation complex.

The studies presented here were designed to further examine the effect of E2 expression in cervical carcinoma cell lines. We were able to confirm earlier studies showing that expression of BPV-1 E2-TA results in growth suppression of HeLa cells. However, we found that E2-TR was unable to suppress cellular growth and, using a series of mutated E2 proteins, were able to determine which domains of the E2 protein were important for this activity. We were also able to demonstrate that HPV-16 and HPV-18 E2 proteins had similar growth suppression functions. Establishment of a HeLa cell line allowed further characterization of E2-mediated growth suppression. These studies suggest that at least one consequence of E2 expression in HPV-positive cell lines is decreased expression of the viral oncoproteins, resulting in cell cycle arrest.

## MATERIALS AND METHODS

**Recombinant plasmids.** The plasmids used for expression of the various BPV-1 E2 proteins were based on C59, which utilizes the SV40 early promoter to express the full-length E2 protein (46, 58). The C59-derived E2-TA plasmid used in this study has been modified to contain a Kozak initiation consensus sequence (CCACCATG [26]) as previously described (57). The plasmids used for expression of E2-TR and the E2-TR with the translation termination linker in E5 (p1175) were previously described (28). Plasmids which express the E2 mutant proteins $E2_{\Delta 1\text{-}15}$, $E2_{\Delta 157\text{-}282}$, $E2_{\Delta 220\text{-}309}$ (34, 57), and $E2_{1\text{-}218}$ have been previously described (58).

*ts*E2 was subcloned from plasmid pE2ts-1 (9) by PCR using oligonucleotides containing a *Hin*dIII site at the 5′ end and an *Xba*I site at the 3′ end of the E2 gene. *Pfu* polymerase (from Stratagene) was used to ensure high fidelity. The DNA fragment containing temperature-sensitive E2 (*ts*E2) was cloned into the vector pRC-CMV (Invitrogen) at the *Hin*dIII-*Xba*I sites of the polylinker. This vector also contains the gene for neomycin resistance. The sequence of this clone was verified by automated DNA sequence analysis (ABI model 373A sequencer). The mutant E2 gene contains an insertion of the four-amino-acid sequence Pro-Arg-Ser-Arg between amino acids 181 and 182 (9).

**Cell culture.** HeLa, HT-3, SiHa, Caski, and C33A are human cervical cancer cell lines obtained from the American Type Culture Collection that have been previously analyzed for the presence of HPV DNA and HPV RNA (59). Saos-2, a human osteosarcoma cell line, was obtained from Phil Hinds (Harvard Medical School). An immortalized human foreskin keratinocyte cell line (W16) was immortalized by using the full-length genome of HPV-16 linearized with *Bam*HI, as previously described (40).

HeLa, SiHa, Caski, C33A, and Saos-2 cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum. HT-3 cells were maintained in McCoy's 5A medium (Gibco/BRL) with 10% fetal bovine serum. The HPV-16-immortalized keratinocyte line (W16) was maintained in 3+1 medium (3 parts KGM plus 1 part Dulbecco modified Eagle medium).

**Growth suppression assay.** Cells were seeded at $1 \times 10^6$ to $2 \times 10^6$ cells per 10-cm-diameter dish the day before transfection. Cells were transfected by lipofection (Lipofectin; GIBCO BRL), using 8 to 10 μg of plasmid DNA purified through two CsCl gradient centrifugations per 10-cm-diameter plate. Sixteen hours after transfection, cells were refed; at 24 h posttransfection, the cells were split and placed under selection in medium containing 10% fetal bovine serum and G418 (concentration dependent on the cell line). Cells were maintained under selection for 2 to 3 weeks until the number of drug-resistant colonies could be determined. Cells were fixed in 10% formaldehyde for 15 min, washed, and stained with methylene blue for 15 min. Plates were washed, and G418-resistant colonies were counted.

**Chloramphenicol acetyltransferase assays.** Reporter plasmid was cotransfected into HeLa cells (6) with the indicated expression plasmids (empty vector was used to normalize the amount of total DNA transfected) by the calcium phosphate method. At 48 h after transfection, cells were harvested and lysed in 250 mM Tris (pH 8.0), and chloramphenicol acetyltransferase activity was determined (19).

**Generation of the HeLa-*ts*E2 cell line.** *ts*E2 was linearized with *Bgl*II and transfected into HeLa cells by lipofection. Transfected cells containing *ts*E2 were selected for in 450 μg of G418 per ml, and individual colonies were analyzed.

**RNA analysis.** The HeLa-*ts*E2 cell line and the control cell line containing the vector alone (HeLa-V) were split, and $10^6$ cells were plated. Cultures were at maintained at 32 or 38°C for 5 days. Total RNA was harvested by using a Biotex RNA isolation kit. Total RNA (14 μg) was separated by 1.2% agarose-formaldehyde gel electrophoresis for Northern (RNA) blot analysis. The RNA was transferred to a GeneScreen Plus filter and hybridized with a probe for HPV-18 E6/E7 mRNA levels and with the cyclophilin probe (7).

**Immunological procedures.** For immunoblotting, cell lysates were prepared in lysis buffer (0.1 M NaCl, 2 mM EDTA, 20 mM Tris [pH 8.0], 1% Nonidet P-40, 0.01% phenylmethylsulfonyl fluoride, 1 μg of aprotinin per ml, 1 μg of leupeptin per ml, 5 mM sodium fluoride, 1 mM sodium orthovanadate) on ice for 30 min. Lysates were cleared by centrifugation at 15,000 × *g* at 4°C for 5 min. A 100-μg aliquot of protein from the cell lysate of each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting (51). The protein concentrations were determined by the Bio-Rad protein assay. Monoclonal antibody 1801 (Ab-2 from Oncogene Science) was used for p53 detection. Monoclonal antibody 245 (PharMingen) was used for pRB detection. Mouse monoclonal antibody sdi-1/p21 (15091A; PharMingen) was used for p21/WAF1/CIP1 detection. Horseradish peroxidase-conjugated rabbit anti-mouse antibody (NA 931; Amersham) was used, followed by enhanced chemiluminescence detection (Renaissance system; NEN).

**Immunocomplex kinase assays.** Immunoprecipitations were carried out essentially as described previously (32), using 100 μg of cellular extract. Rabbit polyclonal antibody C-8 (Santa Cruz) was used to precipitate cyclin A. Mouse monoclonal antibody HE-111 (Santa Cruz) was used to precipitate cyclin E complexes.

Kinase assays were carried out as described previously (32), using histone H1 as a substrate. Proteins were separated by SDS-PAGE (11% polyacrylamide gel), Coomassie blue stained, and dried, and phosphorylated proteins were visualized by autoradiography.

## RESULTS

**Suppression of HeLa cell growth by BPV-1 E2-TA but not E2-TR.** Expression of the full-length BPV-1 E2-TA protein in HeLa cells results in suppression of cell growth as assayed by a reduction in colony formation (50). It is thought that the decreased expression of the viral oncoproteins may contribute to this growth suppression, since expression of the full-length E2 proteins of BPV-1, HPV-16, and HPV-18 as well as HPV-18C (an artificially truncated HPV-18 E2 repressor construct, analogous to BPV-1 E2-TR) is capable of down regulating transcription from the HPV-18 $P_{105}$ promoter (49). Furthermore, growth suppression of HeLa cells by BPV-1 E2-TA expressed from an SV40-based vector results in the down regulation of E6 and E7 expression from the viral $P_{105}$ promoter (23). To further investigate the mechanisms involved in the E2 regulation of cellular proliferation, we examined whether BPV-1 E2-TR could suppress HeLa cell growth like the full-length BPV-1 E2-TA. HeLa cells were cotransfected with a plasmid conferring neomycin resistance (pSV2neo) and a plasmid encoding either BPV-1 E2-TA or BPV-1 E2-TR. Cells were split after transfection and maintained in G418-containing medium for 14 to 21 days, at which time the number of G418-resistant colonies was determined. In agreement with previous reports, no growth suppression was observed with BPV-1 E2-TR (Fig. 1). Since the E2-TR plasmid used in this experiment contains an intact E5 gene which might mask or otherwise affect a growth-suppressive effect of E2-TR, an E2-TR construct (p1175) with a translation termination linker in E5 was tested and also found to be defective for HeLa cell growth suppression (data not shown). Similar results were obtained when puromycin selection rather than G418 selection was used, indicating that these results were not specific for neomycin resistance selection.

The lack of growth suppression by E2-TR was unexpected, since E2-TR has been shown to specifically repress E2-responsive promoters. To confirm that E2-TR was indeed expressed in HeLa cells, the E2-TR plasmid was tested for its ability to repress E2-TA transactivation in HeLa cells. HeLa cells were transfected with the E2-TA plasmid, the E2-responsive plasmid 6XE2TKCAT (47), and increasing amounts of the E2-TR plasmid. Figure 2 demonstrates that E2-TR was able to specifically repress E2-TA transactivation, indicating expression of functional E2-TR in HeLa cells.

**HPV-16 and HPV-18 E2 proteins suppress cell growth in HeLa cervical carcinoma cells.** The abilities of HPV-16 and HPV-18 E2 proteins to suppress HeLa cell growth were next

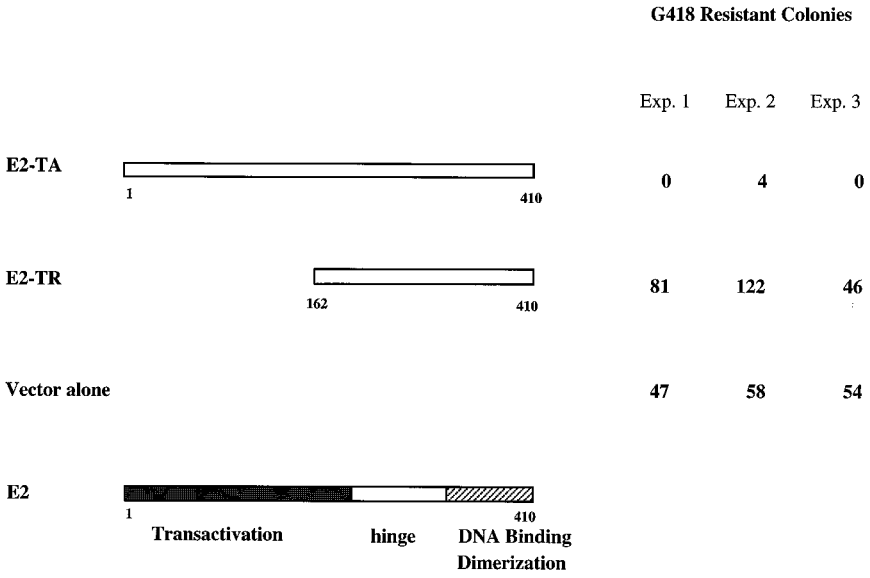


FIG. 1. Growth suppression of HeLa cells by the full-length BPV-1 E2-TA but not E2-TR. HeLa cells ($10^6$ per plate) were transfected with 1 μg of pSV2neo plus 10 μg of E2-TA, E2-TR, or vector alone, using lipofection. Cells were split 24 h after transfection into medium containing G418 (500 μg/ml). The number of drug-resistant colonies was determined at 2 to 3 weeks.

examined, since the expression of E2 is often lost as a consequence of the integration of the viral DNA in the course of carcinogenic progression of HPV-associated cancers. In this series of experiments, we tested whether the E2 proteins of the high-risk HPVs shared the growth-suppressive function shown for the BPV-1 E2-TA protein. The experiment was performed with HPV-16 E2 and HPV-18 E2 expression plasmids that had been previously shown to express functional E2 protein capable of supporting origin-dependent DNA replication and of activating E2-responsive promoters (8). Both HPV-16 E2 and HPV-18 E2 proteins suppressed HeLa cell growth in the colony formation assay (Table 1). This result differs from a previous report that expression of HPV-18 E2 was not able to suppress the growth of HeLa cells (50). We feel that the discrepancy between these two studies might be due to different levels of expression from the different HPV-18 expression vectors used in each study.

**Growth suppression mediated by E2 is observed in other HPV-positive cell lines.** To examine the specificity of E2 growth suppression, additional cell lines were assayed for their responses to E2 expression using the colony reduction assay. E2 growth suppression was observed in each of the three HPV-positive cell lines tested in which the viral genes are expressed from the viral long control region. These include two HPV-16-positive cervical carcinoma cell lines, SiHa and Caski, and W16, which is a human foreskin keratinocyte cell line immortalized by HPV-16 (Table 2). Growth suppression by BPV-1 E2-TA was observed in each of these cell lines, and no growth-suppressive effect was observed with the BPV-1 E2-TR protein. These data are in agreement with the results of Hwang et al. (23), who also reported growth suppression of other HPV-positive cervical cancer cell lines by BPV-1 E2-TA.

To determine whether E2 can act as a general growth suppressor, two cervical carcinoma cell lines which are HPV negative (C33A and HT-3) and a human osteosarcoma cell line (Saos-2) were tested for growth suppression by E2-TA. It was previously reported that E2-TA could suppress the growth of HT-3 cells when introduced by infection with an E2-expressing recombinant SV40 (23). In our assay, however, the growth of each of these HPV-negative cell lines, including HT-3, was unaffected by expression of the E2-TA protein (Table 3). Thus, the E2 growth suppression in the colony reduction assay is specific for HPV-positive cells.

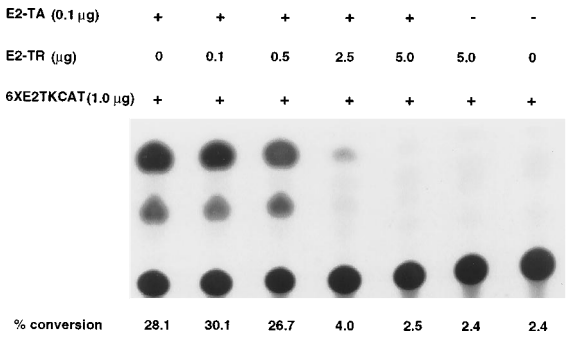


FIG. 2. E2-TR can specifically repress expression of CAT from an E2-responsive promoter. HeLa cells were transfected with 1 μg of the reporter construct 6XE2TKCAT and the indicated expression vectors. Cells were harvested 48 h after transfection, and the percentage conversion in each sample was determined with a Phosphoimager (Bio-Rad).

TABLE 1. HeLa cell growth suppression by HPV-16 and HPV-18 E2[a]

| Transfection | No. of G418-resistant colonies[b] | |
|---|---|---|
| | Expt 1 | Expt 2 |
| HPV-16 E2 | 0 | 0 |
| HPV-18 E2 | 0 | 0 |
| Vector alone | 34 | 41 |

[a] HeLa cells were transfected with 1 μg of pSV2neo and 10 μg of either HPV-16 E2- or HPV-18 E2-expressing plasmid or vector alone.

[b] Transfected cells were maintained under selection in medium containing 500 μg of G418 per ml for 2 to 3 weeks, and the number of G418-resistant colonies from each transfection was determined.

TABLE 2. BPV-1 E2 growth suppression of HPV-16-expressing human cell lines[a]

| Transfection | No. of G418-resistant colonies | | | | | |
|---|---|---|---|---|---|---|
| | SiHa | | Caski | | W16 | |
| | Expt 1 | Expt 2 | Expt 1 | Expt 2 | Expt 1 | Expt 2 |
| E2-TA | 6 | 4 | 2 | 0 | 4 | 8 |
| E2-TR | 52 | 42 | 59 | 67 | 18 | 19 |
| Vector alone | 46 | 57 | 51 | 55 | 24 | 23 |

[a] Indicated cell lines were transfected with 1 μg of pSV2neo and 10 μg of either BPV-1 E2-TA- or BPV E2-TR-expressing plasmid or vector alone.

**Intact DNA binding and transactivation functions of the E2 protein are required for inhibition of HeLa cell growth.** The domains of BPV-1 E2 necessary for growth suppression were mapped by using a series of truncation and deletion mutants of E2, which had been previously characterized for their transactivation and replication functions (57). The growth-suppressive phenotype conferred by the E2 deletion mutants was assayed in HeLa cells by using the colony reduction assay (Fig. 3A). Growth suppression was observed with E2-TA and with $E2_{\Delta 220\text{-}309}$, which is a mutant deleted of a large segment of the hinge region but containing an intact transactivation domain and an intact DNA binding/dimerization domain. No growth suppression was observed for either $E2_{\Delta 1\text{-}15}$ or $E2_{\Delta 157\text{-}220}$, both of which are deficient for the transcriptional transactivation and DNA replication functions in mammalian cells. Each of these E2 mutants can inhibit the transactivation properties of E2-TA in *trans*. An E2 mutant protein ($E2_{1\text{-}218}$) consisting of only the transactivation domain similarly had no negative effect on cell growth. The $E2_{\Delta 220\text{-}309}$ mutant, which has growth-suppressive activity, is able to transactivate an E2-responsive plasmid but is defective in origin-dependent DNA replication (57). We confirmed the transactivation or transrepression properties of each of these mutant E2 proteins in HeLa cells (data not shown). The expression of the E2 proteins was demonstrated in COS cells by immunoblotting, using expression from recombinant PAVA viruses. In these studies, it was noted that the $E2_{1\text{-}218}$ mutant was expressed at a somewhat lower level than the other E2 proteins (data not shown). These results indicate that the BPV-1 E2 growth-suppressive effect in HeLa cells requires a functional transactivation domain as well as an intact DNA binding domain but that an intact DNA replication function is apparently not necessary.

The genetic organization of BPV-1 is complex, with overlapping ORFs at the 3′ end of the early region (2), raising the possibility that another ORF contributes to the growth suppression phenotype. This, however, is quite unlikely. The intact E2-TA expression vector that was used contains additional ORFs, including E3 (3267 to 3551), E4 (3173 to 3526), and E5 (3714 to 4010). The $E2_{\Delta 220\text{-}309}$ deletion mutant plasmid, which expresses an E2 capable of growth suppression, is deleted of most of the coding regions of E3 and E4 (from 3265 to 3532 of the BPV-1 genome). Furthermore, E5 has been previously shown not to be associated with the growth-suppressive effect observed with the BPV-1 E2 expression vector (50). Thus, we conclude that the growth-suppressive phenotype can be attributed to E2.

We next examined the specificity of the E2 transactivation domain in suppressing HeLa cell proliferation by testing whether this function could be provided by other acidic activation domains. Chimeric constructs containing the E2 DNA binding/dimerization domain fused to either the acidic transactivation domain of the herpesvirus VP16 transcriptional activator (31) or the *spi* oncogene (16) were tested in the colony reduction assay (Fig. 3B). Although both chimeric proteins were able to efficiently transactivate E2-responsive reporter constructs in HeLa cells (data not shown), neither had the ability to suppress HeLa cell proliferation. This finding indicates that the ability of the E2 transactivation domain to suppress cellular proliferation is specific and not due to its general transactivation property.

The dimerization and DNA binding properties of BPV-1 E2 have been separated by point mutations in the DNA binding domain (4, 33). E2 mutants that can dimerize but not bind DNA and that can no longer dimerize or bind DNA have been described. Two of these E2 mutants were tested for the ability to suppress HeLa cell growth. Neither the $E2_{(I331R)}$ mutant, which is defective for both dimerization and DNA binding, nor the $E2_{(R344K)}$ mutant, which can dimerize but not bind DNA, was capable of suppressing HeLa cell proliferation (Fig. 3B). These results suggest that both the E2 transactivation and DNA binding functions are necessary for the E2 growth suppression phenotype.

**Molecular consequences of E2 expression.** To study the downstream effects of E2 expression in HPV-positive cells, we used a conditional BPV-1 E2 mutant that has normal replication and transactivation functions at 32°C which are inactivated at either 37 or 39°C (9). This mutant E2 gene was subcloned behind the cytomegalovirus promoter into an expression vector containing the neomycin resistance gene to generate plasmid pRC-tsE2. This plasmid was introduced into HeLa, SiHa, and C33A cells, and the number of G418-resistant colonies was determined after 21 days at 32 and 38°C (Table 4). Following transfection at the nonpermissive temperature (38°C), the cells were split and incubated at either 32 or 38°C under G418 selection. At the permissive temperature (32°C), *ts*E2 inhibited colony formation, similar to the effect of the wild-type E2-TA, in both the HeLa and SiHa cell lines. However, at the nonpermissive temperature (38°C), there was no growth inhibition in any of the cell lines tested. In agreement with the results obtained with wild-type E2, no growth suppression was observed with *ts*E2 in C33A cells at the permissive temperature. Control transfections performed with the pRC-neo vector alone confirmed that the growth suppression observed in HeLa and SiHa cells was not due to temperature, since G418-resistant colonies appeared for both cell lines at 32°C.

pRC-tsE2 was used to generate a G418-resistant HeLa cell line at 38°C which expressed the conditional E2 mutant protein (HeLa-*ts*E2). HeLa-*ts*E2 clonal lines were characterized for the ability to transactivate an E2-responsive plasmid and for their growth properties at 32 and 38°C. As a control, a G418-resistant HeLa cell line (HeLa-V) was also established by using the vector without insert. The HeLa-*ts*E2 cells were growth

TABLE 3. Lack of BPV-1 E2 growth suppression of HPV-negative human cell lines[a]

| Transfection | No. of G418-resistant colonies | | |
|---|---|---|---|
| | Saos-2 | | HT-3 |
| | Expt 1 | Expt 2 | |
| E2-TA | 67 | 80 | 24 |
| E2-TR | 81 | 102 | 28 |
| Vector alone | 62 | 99 | 19 |

[a] See the footnote to Table 2. For C33A cells, values were >500 in all assays in two experiments.

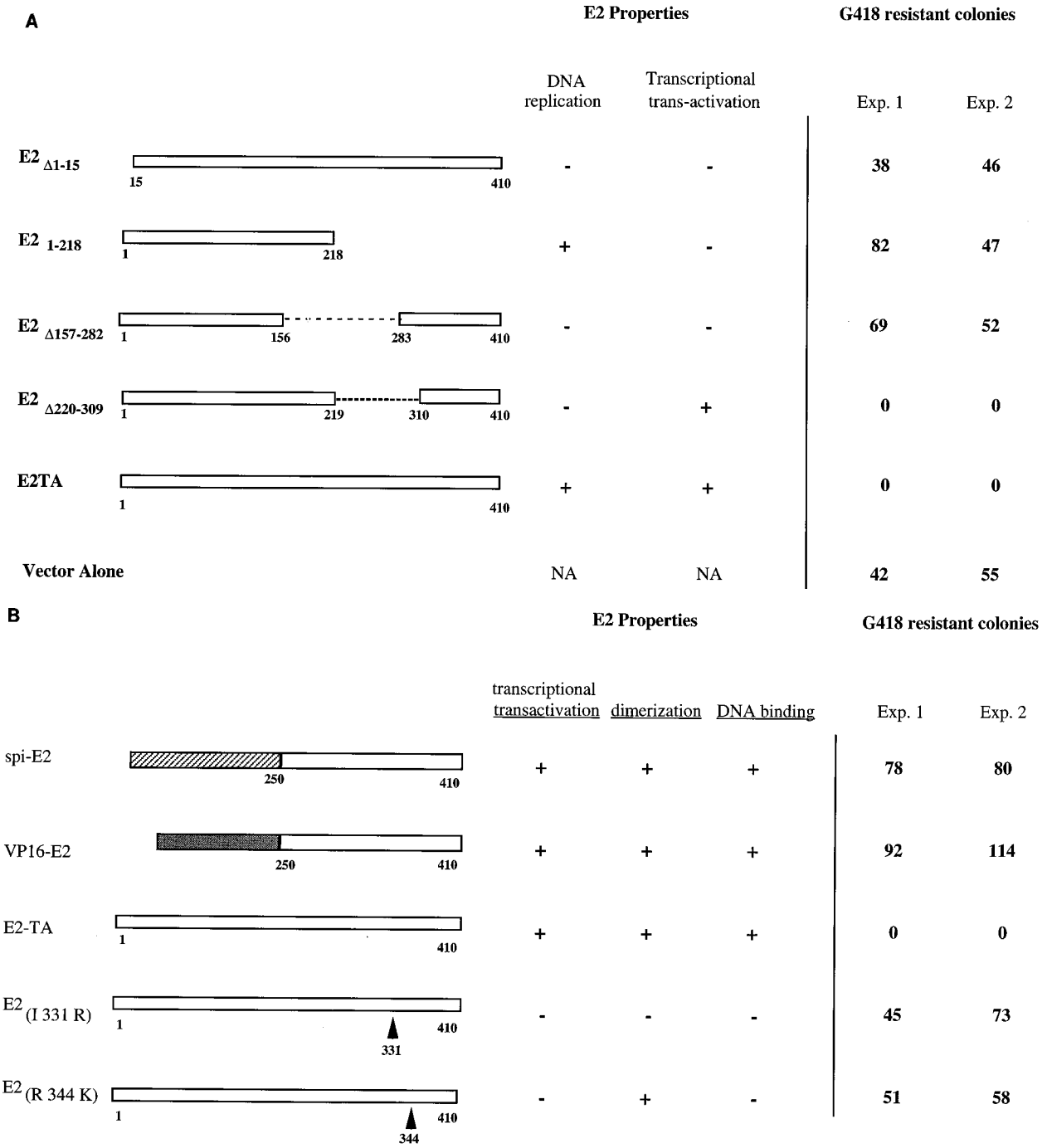


FIG. 3. The E2 transactivation domain and the E2 DNA binding domain are necessary for growth suppression. The number of G418-resistant colonies was determined at 2 weeks. (A) The BPV-1 E2 hinge region is not required for growth suppression. HeLa cells were transfected with E2 deletion mutants (57) as described in Materials and Methods. The properties of the various E2s are listed. (B) Chimeric E2s (VP16-E2 and Spi-E2) and point mutants in the DNA binding domain [$E2_{(I331R)}$ and $E2_{(R344K)}$] were transfected into HeLa cells as described in Materials and Methods. Open box, E2 sequence; hatched box, Spi sequence; shaded box, VP16 sequence.

arrested at 32°C, whereas the HeLa-V cells were not. Fluorescence-activated cell sorting analysis indicated that the HeLa-*ts*E2 cells were arrested in $G_1$ when grown at 32°C (data not shown), consistent with previously reported data indicating that BPV-1 E2 expression in HeLa cells from a recombinant SV40 leads to a $G_1$ cell cycle arrest (23). The HeLa-*ts*E2 cell line was characterized for HPV-18 RNA levels (Fig. 4). At the permissive temperature, the level of E6/E7 message was at least three- to fourfold lower than in HeLa-*ts*E2 cells at 38°C. There was a temperature-dependent decrease of 1.5-fold in the HeLa-V cell line at 32°C compared with HeLa-V cells grown at 38°C.

**Effects of E2 expression on cellular proteins in HeLa cells.** The E2-dependent loss of E6/E7 message and growth arrest

TABLE 4. Temperature-sensitive growth phenotype of cells containing the E2 mutant[a]

| Cell line | No. of G418-resistant colonies | | | |
|---|---|---|---|---|
| | pRC-tsE2 | | pRC-neo | |
| | 32°C | 38°C | 32°C | 38°C |
| HeLa | 0 | 73 | 41 | 61 |
| SiHa | 0 | 86 | 37 | 77 |
| C33A | >500 | >500 | >500 | >500 |

[a] Indicated cell lines were transfected with 1 μg of pSV2neo and 10 μg of either BPV-1 *ts*E2 or vector alone.

may be mediated through cellular targets of the E6 and E7 proteins. Since HPV-18 E6 promotes the degradation of p53, the decrease in expression of the endogenous viral mRNA would be predicted to result in lower levels of E6 and increased levels of p53. The levels of p53 were examined in the HeLa-*ts*E2 cell line following a shift to the permissive temperature. Immunoblot analysis of total cell extracts demonstrated a 20-fold increase (Fig. 5A) in the level of p53 in HeLa-*ts*E2 cells shifted to 32°C compared with normal HeLa cells or HeLa-*ts*E2 cells grown at 38°C. A decrease in p53 levels was noted in HeLa-V cells at 32°C. The reason for this lowered level of p53 is not known, but this finding suggests that the destabilizing effect of E6 might be even greater than the 20-fold change in the HeLa-*ts*E2 cell line.

Induction of the cyclin-dependent kinase inhibitor p21/WAF1/Cip1/Sdi1 occurs in response to increased levels of p53 (15). As shown in Fig. 5B, an increased level of p21 protein was observed in the HeLa-*ts*E2 cells after a shift to the permissive temperature (32°C). Notably, in the HeLa-V cell line at 32°C, which had very low levels of p53, no p21 was detected (Fig. 5B).

To examine whether the increased levels of p21 in the HeLa-*ts*E2 cell line at the permissive temperature resulted in an inhibition of the cyclin-dependent kinases, cell lysates were prepared and immunoprecipitated with cyclin-specific antibodies, and kinase assays were performed with histone H1 as a substrate (32). Cyclin E is associated with Cdk2 and is active in late $G_1$ (12, 25). Inhibition of the cyclin E-associated kinase activity was observed in the HeLa-*ts*E2 cells at the permissive temperature (Fig. 5C). The cyclin E-associated kinase was active in the HeLa-*ts*E2 cells at 38°C and in the HeLa-V cells at both 32 and 38°C. Cyclin A-associated kinase activity was also

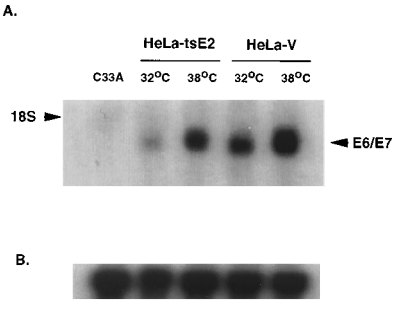


FIG. 4. Down regulation of E6/E7 mRNA in cells expressing BPV-1 E2. Cultures of HeLa-*ts*E2 and HeLa-V cells were incubated at 32 or 38°C for 5 days. Fourteen micrograms of total cellular RNA was electrophoresed through a 1.2% formaldehyde gel, transferred to GeneScreen Plus, and hybridized with a probe for HPV-18 E6/E7 (A). The position of the 18S rRNA is indicated. Hybridization with a cyclophilin radioactive probe was used as a control for the quantity and integrity of the RNAs (B).

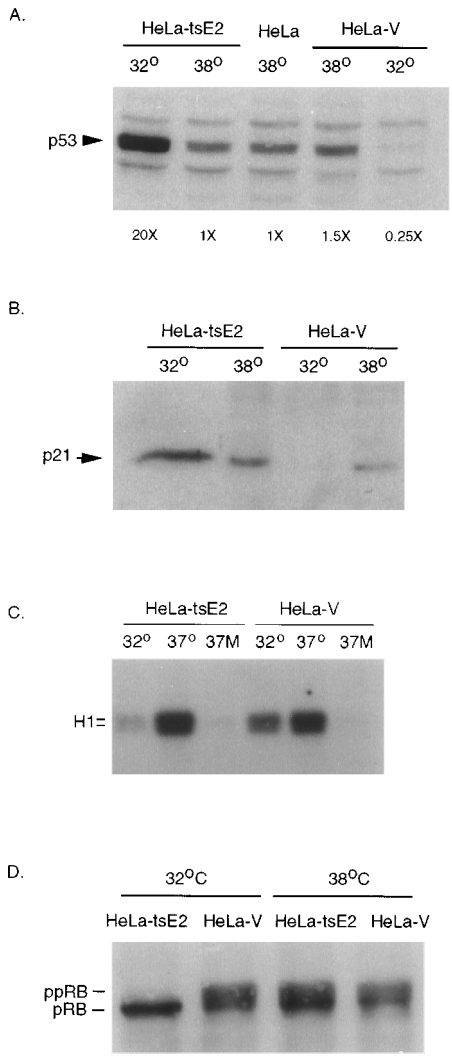


FIG. 5. HeLa-*ts*E2 and vector control HeLa-V cells were incubated at 32 or 38°C for 6 days. (A) p53 levels in the HeLa-*ts*E2 cell line. Cells were lysed, 100 μg of total protein was separated by SDS-PAGE (10% polyacrylamide gel), and the levels of p53 were determined. The relative levels of p53 in each sample were determined with a Phosphoimager (Bio-Rad). (B) p21 levels in the HeLa-*ts*E2 cell line. Cell lines were prepared as described for p53, and 100 μg of total cellular protein was separated by SDS-PAGE (14% polyacrylamide gel). (C) Cyclin E-dependent kinase activity. Total cell extracts (100 μg) were immunoprecipitated by either cyclin E antibody or normal mouse serum (M). Kinase assays were performed with histone H1 as a substrate as described in Materials and Methods. The position of histone H1 is indicated. (D) Accumulation of the hypophosphorylated form of pRB in E2 growth-arrested cells. HeLa-*ts*E2 and control HeLa-V cells were incubated at 32 or 38°C for 3 days. Equal amounts of protein (100 μg) extracted from the indicated cell lines were separated by SDS-PAGE (7% polyacrylamide gel). The positions of hyperphosphorylated (ppRB) and hypophosphorylated (pRB) pRB are indicated.

inhibited in the HeLa-*ts*E2 cells at the permissive temperature but not at the nonpermissive temperature or in the HeLa-V cell line (data not shown).

An immunoblot analysis of pRB was performed on extracts from the HeLa-*ts*E2 and HeLa-V cell lines grown at 32 or 38°C. Both the hyperphosphorylated and hypophosphorylated forms of pRB were detected in each case; however, the pRB forms in the HeLa-*ts*E2 cell line shifted to 32°C were predominantly hypophosphorylated (Fig. 5D). There was little difference in the phosphorylation state of pRB in the HeLa-*ts*E2

cells grown at 38°C and pRB in the HeLa-V control cell line at either temperature.

## DISCUSSION

Previous studies have demonstrated that expression of BPV-1 E2-TA in HeLa cells results in growth inhibition (23, 50). In this study, we have confirmed these results and have shown that the capacity of the full-length E2 proteins to inhibit cell growth is shared by the E2 proteins of HPV-16 and HPV-18. Furthermore, HeLa cell growth was not inhibited by E2-TR. This result was somewhat surprising because both forms of E2 have been reported to repress the HPV E6/E7 promoter and because the ability of E2-TA to suppress HeLa cell growth correlates well with its ability to inhibit the expression of the endogenous HPV-18 genome in HeLa cells. Previous studies found that N-terminally deleted forms of HPV-18 E2, analogous to BPV-1 E2-TR, were able to transcriptionally repress the HPV-18 $P_{105}$ promoter (49). These data taken together suggest that the mechanism for E2-mediated growth suppression may involve more complex mechanisms or that E2-TR is not an effective repressor of the HPV promoters integrated into the host chromosome. Progression of high-risk HPV lesions to cervical cancer is often associated with integration of the viral genome with disruption of the E1/E2 region (1, 43). This integration leads to the deregulation of the expression of the viral E6 and E7 transforming genes. This may be due in part to the release of the E6/E7 promoter from the repressor effects of E2. We have shown that HPV-positive cervical carcinoma cells are sensitive to the reintroduction of HPV E2 proteins, consistent with this model. The growth-suppressive effect of E2 is presumably mediated by transcriptional repression of E6 and E7 expression from the HPV-16 $P_{97}$ promoter. However, since mutations in the long control region that eliminate E2 binding in the HPV-16 long control region only partially relieve E2 repression of immortalization (40), it appears that E2 may affect viral transcription and cellular growth regulation through additional mechanisms.

The E2 transactivation and DNA binding/dimerization domains were each necessary for E2-mediated growth suppression of HeLa cells. $E2_{\Delta 220\text{-}309}$, which is defective in the replication function but retains the ability to transactivate an E2-responsive plasmid (57), was able to suppress growth of HeLa cells, indicating that the growth-suppressive properties of E2 can be unlinked from its DNA replication properties. The fusion of two other transactivation domains to the E2 DNA binding domain did not suppress the growth of HeLa cells, even though each of these chimeric proteins could function as E2-dependent transactivators in these cells. Therefore, some characteristic of the E2 transactivation domain is specific for cell growth suppression and is not shared with other transactivation domains. It is possible that the E2 transactivation domain recruits specific cellular factors to the promoter which may play a role in the transcriptional repression and growth suppression activities of E2.

The dependence of a functional transactivation domain may be in part due to its ability to relieve nucleosome-mediated repression. The E2 transactivation domain has been shown to counteract the nucleosome repression of DNA replication (29). The transcriptional repression properties of the E2-TR have been characterized on transiently transfected naked DNA. This, however, cannot be the only explanation for E2-mediated growth suppression, since the VP16 transactivation domain also has the ability to relieve nucleosome repression, but in our experiments, expression of the chimeric protein does not result in growth suppression.

The growth-suppressive effect of E2 was demonstrated in other HPV-positive cell lines but not in HPV-negative cell lines by the cotransfection assay used in this study. This result contrasts with the finding of Hwang et al. (23), who observed a growth-suppressive effect with BPV-1 E2-TA in the HPV-negative cervical carcinoma cell line HT-3. One explanation for the difference between these two studies may involve the different assays used and the different levels of expression of E2 achieved in the two experimental approaches. Our studies were done with DNA transfection, whereas the studies by Hwang et al. used the recombinant SV40 virus PAVA-E2, which resulted in much higher levels of protein expression. We have not been able to detect E2 protein by immunoblot analysis in HeLa cell lines transfected with the E2 plasmid, even though we can readily detect functional levels of E2 in transactivation or transcriptional repression assays. However, E2 is readily detectable by immunoblotting in HeLa cells infected with the PAVA-E2 virus (23), indicating a significantly higher level of expression. The two studies also differed in the experimental conditions to assess the biologic effects of E2 expression. Hwang et al. examined the transient effects of E2 by analyzing the cells 2 days postinfection. In the colony growth suppression assay used in our study, selection was maintained for 2 weeks, at which time the number of drug-resistant colonies was counted. Thus, the different effects of E2 observed on the growth of the HPV-negative HT-3 cell line may be due to differences in the assays, to the levels of E2 expression achieved by the different vectors, or to both.

Repression of HPV-18 E6/E7 transcription was observed in the HeLa-*ts*E2 cells after a shift to the permissive temperature, resulting in a growth arrest. Our data indicate that in HeLa cells, the mechanism of E2-mediated growth arrest involves a decrease in E6 and E7 mRNA and reactivation of the p53/p21 and pRB pathways. The fact that the p21/Cdk inhibitory pathway is still intact and can be activated by disruption of E6 function suggests that therapeutic strategies targeted at interfering with E6 function could be effective in inhibition of cellular proliferation in HPV-positive cancers. We also observe an accumulation of hypophosphorylated pRB, which may be a consequence of a p53-mediated $G_1$ growth arrest (44). Cells blocked in $G_1$ prior to activation of the cyclin D/Cdk complexes do not phosphorylate pRB. The shift in the phosphorylation state of RB to its hypophosphorylated form suggests that the cell cycle block occurs prior to the point of pRB phosphorylation. Our studies are in agreement with the observation that expression of antisense E6/E7 in C4-1, an HPV-18-derived cell line, was able to inhibit the growth rate of these cells (53, 54).

The studies presented here indicate that the transactivation domain of E2 is involved in the growth suppression and that the characteristic of the E2 transactivation domain in this suppression is not a general property shared with the VP16 and Spi transactivation domains. Future studies will be designed to see whether the transactivation function of E2-TA can be unlinked from its growth-suppressive effects and to identify cellular factors that may be involved in mediating the E2-dependent growth response.

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## REFERENCES


1. **Baker, C. C.** 1993. The genomes of the papillomaviruses, p. 1.134–1.146. *In* S. O'Brien (ed.), Genetic maps: locus maps of complex genomes. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
2. **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.
3. **Barbosa, M. S., C. Edmonds, C. Fisher, J. T. Schiller, D. R. Lowy, and K. H. Vousden.** 1990. The region of the HPV E7 oncoprotein homologous to adenovirus E1a and SV40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. EMBO J. **9:**153–160.
4. **Barsoum, J., S. S. Prakash, P. Han, and E. J. Androphy.** 1992. Mechanism of action of the papillomavirus E2 repressor: repression in the absence of DNA binding. J. Virol. **66:**3941–3945.
5. **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.
6. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
7. **Danielson, P. E., S. Forss-Petter, M. A. Brow, L. Calavetta, J. Douglas, R. J. Milner, and J. G. Sutcliffe.** 1988. p1b15: a cDNA clone of the rat mRNA encoding cyclophilin. DNA **7:**261–267.
8. **Del Vecchio, A. M., H. Romanczuk, P. M. Howley, and C. C. Baker.** 1992. Transient replication of human papillomavirus DNAs. J. Virol. **66:**5949–5958.
9. **DiMaio, D., and J. Settleman.** 1988. Bovine papillomavirus mutant temperature sensitive for transformation, replication and transactivation. EMBO J. **7:**1197–1204.
10. **Dong, G., T. R. Broker, and L. T. Chow.** 1994. Human papillomavirus type 11 E2 proteins repress the homologous E6 promoter by interfering with the binding of host transcription factors to adjacent elements. J. Virol. **68:**1115–1127.
11. **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.
12. **Dulic, V., L. F. Drullinger, E. Lees, S. I. Reed, and G. H. Stein.** 1993. Altered regulation of G(1)-cyclins in senescent human diploid fibroblasts—accumulation of inactive cyclin-E-cdk2 and cyclin-D1-cdk2 complexes. Proc. Natl. Acad. Sci. USA **90:**11034–11038.
13. **Dürst, M., A. Kleinheinz, M. Hotz, and L. Gissmann.** 1985. The physical state of human papillomavirus type 16 in benign and malignant genital tumors. J. Gen. Virol. **66:**1515–1522.
14. **Dyson, N., P. M. Howley, K. Münger, and E. Harlow.** 1989. The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934–937.
15. **Eldeiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler, and B. Vogelstein.** 1993. WAF1, a potential mediator of p53 tumor suppression. Cell **75:**817–825.
16. **Gauthier, J.-M., and B. V. Bourachot Doucas, M. Yaniv, and F. Moreau-Gachelin.** 1993. Functional interference between the spi-1/pu.1 oncoprotein and steroid hormone or vitamin receptors. EMBO J. **12:**5089–5096.
17. **Giri, I., and M. Yaniv.** 1988. Structural and mutational analysis of E2 trans-activating proteins of papillomaviruses reveals three distinct functional domains. EMBO J. **7:**2823–2829.
18. **Gloss, B., and H. U. Bernard.** 1990. The E6/E7 promoter of human papillomavirus type 16 in the absence of E2 proteins by a sequence-aberrant Sp1 distal element. J. Virol. **64:**5577–5584.
19. **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.
20. **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.
21. **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.
22. **Hubbert, N. L., J. T. Schiller, D. R. Lowy, and E. J. Androphy.** 1988. Bovine papillomavirus transformed cells contain multiple E2 proteins. Proc. Natl. Acad. Sci. USA **85:**5864–5868.
23. **Hwang, E. S., D. J. Riese, J. Settleman, L. A. Nilson, J. Honig, S. Flynn, and D. Dimaio.** 1993. Inhibition of cervical carcinoma cell line proliferation by the introduction of a bovine papillomavirus regulatory gene. J. Virol. **67:** 3720–3729.
24. **Jeon, S., and P. F. Lambert.** 1995. Integration of HPV-16 DNA into the human genome leads to increased stability of E6/E7 mRNAs: implications for cervical carcinogenesis. Proc. Natl. Acad. Sci. USA **92:**1654–1658.
25. **Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza, and J. M. Roberts.** 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science **257:**1689–1694.
26. **Kozak, M.** 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. **266:**19867–19870.
27. **Lambert, P. F., N. L. Hubbert, and P. M. Howley.** 1989. Genetic assignment of the multiple E2 gene products in bovine papillomavirus-transformed cells. J. Virol. **63:**3151–3154.
28. **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:**68–78.
29. **Li, R., and M. R. Botchan.** 1994. Acidic transcription factors alleviate nucleosome-mediated repression of DNA replication of bovine papillomavirus type 1. Proc. Natl. Acad. Sci. USA **91:**7051–7055.
30. **Li, R., J. Knight, G. Bream, A. Stenlund, and M. Botchan.** 1989. Specific recognition nucleotides and their context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. Genes Dev. **3:**510–526.
31. **Li, R., J. D. Knight, S. P. Jackson, R. Tjian, and M. R. Botchan.** 1991. Direct interaction between Sp1 and the BPV enhancer protein mediates synergistic activation of transcription. Cell **65:**493–505.
32. **Matsushime, H., D. E. Quelle, S. A. Shurtleff, M. Shibuya, C. J. Sherr, and J. Y. Kato.** 1994. D-type cyclin-dependent kinase activity in mammalian cells. Mol. Cell. Biol. **14:**2066–2076.

32a. **McBride, A. A.** Unpublished data.

33. **McBride, A. A., J. B. Bolen, and P. M. Howley.** 1989. Phosphorylation sites of the E2 transcriptional regulatory proteins of bovine papillomavirus type 1. J. Virol. **63:**5076–5085.
34. **McBride, A. A., J. C. Byrne, and P. M. Howley.** 1989. E2 polypeptides encoded by bovine papillomavirus type 1 form dimers through the common carboxy-terminal domain: transactivation is mediated by the conserved amino-terminal domain. Proc. Natl. Acad. Sci. USA **86:**510–514.
35. **McBride, A. A., H. Romanczuk, and P. M. Howley.** 1991. The papillomavirus E2 regulatory proteins. J. Biol. Chem. **266:**18411–18414.
36. **McBride, A. A., R. Schlegal, and P. M. Howley.** 1988. The carboxy-terminal domain shared by the bovine papillomavirus E2 transactivation and repressor proteins contains a specific DNA binding activity. EMBO J. **7:**533.
37. **Mietz, J. A., T. Unger, J. M. Huibregtse, and P. M. Howley.** 1992. The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. EMBO J. **11:**5013–5020.
38. **Moskaluk, C. A., and D. Bastia.** 1988. Interaction of the bovine papillomavirus type 1 E2 transcriptional control protein with the viral enhancer: purification of the DNA-binding domain and analysis of its contact with DNA. J. Virol. **62:**1925–1931.

39a. **Münger, K., B. A. Werness, N. Dyson, W. C. Phelps, E. Harlow, and P. M. Howley.** 1989. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. EMBO J. **8:**4099–4105.

39. **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.
40. **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.
41. **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.
42. **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.
43. **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.
44. **Slebos, R. J., M. H. Lee, B. S. Plunkett, T. D. Kessis, B. O. Williams, T. Jacks, L. Hendrick, M. B. Kastan, and K. R. Cho.** 1994. p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. Proc. Natl. Acad. Sci. USA **91:**5320–5324.
45. **Smotkin, D., and F. O. Wettstein.** 1986. Transcription of human papillomavirus type 16 early genes in cervical cancer and a cervical cancer derived cell line and identification of the E7 protein. Proc. Natl. Acad. Sci. USA **83:** 4680–4684.
46. **Spalholz, B. A., S. B. Vande Pol, and P. M. Howley.** 1991. Characterization of the *cis* elements involved in basal and E2-*trans*-activated expression of the bovine papillomavirus $P_{2443}$ promoter. J. Virol. **65:**743–753.
47. **Thierry, F., N. Dostantni, F. Arnos, and M. Yaniv.** 1990. Cooperative activation of transcription by bovine papillomavirus type 1 E2 can occur over a large distance. Mol. Cell. Biol. **10:**4431–4437.
48. **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.

49. **Thierry, F., and P. M. Howley.** 1991. Functional analysis of E2-mediated repression of the HPV18 P105 promoter. New Biol. **3:**90–100.
50. **Thierry, F., and M. Yaniv.** 1987. The BPV-1 E2 trans-acting protein can be either an activator or repressor of the HPV-18 regulatory region. EMBO J. **6:**3391–3397.
51. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **86:**4350–4354.
52. **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.
53. **von Knebel-Doeberitz, M., T. Oltersdorf, E. Schwarz, and L. Gissmann.** 1988. Correlation of modified human papillomavirus early gene expression with altered growth properties in C4-I cervical carcinoma cells. Cancer Res. **48:**3780–3785.
54. **von Knebel-Doeberitz, M., C. Rittmüller, H. zur Hausen, and M. Dürst.** 1992. Inhibition of tumorigenicity of cervical cancer cells in nude mice by HPV E6-E7 anti-sense RNA. Int. J. Cancer **51:**831–834.
55. **Watanabe, S., T. Kanda, and K. Yoshiike.** 1989. Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J. Virol. **63:**965–969.
56. **Werness, B. A., A. J. Levine, and P. M. Howley.** 1990. Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science **248:**76–79.
57. **Winokur, P. L., and A. A. McBride.** 1992. Separation of the transcriptional activation and replication functions of the bovine papillomavirus-1 E2 protein. EMBO J. **11:**4111–4118.
58. **Yang, Y.-C., B. A. Spalholz, M. S. Rabson, and P. M. Howley.** 1985. Dissociation of transforming and trans-activation functions for bovine papillomavirus type-1. Nature (London) **318:**575–577.
59. **Yee, C. L., 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.
60. **zur Hausen, H.** 1991. Human papillomaviruses in the pathogenesis of anogenital cancer. Virology **184:**9–13.