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Inhibition of Cervical Carcinoma Cell Line Proliferation by the Introduction of a Bovine Papillomavirus Regulatory Gene

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Human papillomavirus (HPV) E6 and E7 oncogenes are expressed in the great majority of human cervical carcinomas, whereas the viral E2 regulatory gene is usually disrupted in these cancers. To investigate the roles of the papillomavirus E2 genes in the development and maintenance of cervical carcinoma, the bovine papillomavirus (BPV) E2 gene was acutely introduced into cervical carcinoma cell lines by infection with high-titer stocks of simian virus 40-based recombinant viruses. Expression of the BPV E2 protein in HeLa, C-4I, and MS751 cells results in specific inhibition of the expression of the resident HPV type 18 (HPV18) E6 and E7 genes and in inhibition of cell growth. HeLa cells, in which HPV gene expression is nearly completely abolished, undergo a dramatic and rapid inhibition of proliferation, which appears to be largely a consequence of a block in progression from the G_1 to the S phase of the cell cycle. Loss of HPV18 gene expression in HeLa cells is also accompanied by a marked increase in the level of the cellular p53 tumor suppressor protein, apparently as a consequence of abrogation of HPV18 E6-mediated destabilization of p53. The proliferation of HT-3 cells, a human cervical carcinoma cell line devoid of detectable HPV DNA, is also inhibited by E2 expression, whereas two other epithelial cell lines that do not contain HPV DNA are not inhibited. Thus, a number of cervical carcinoma cell lines are remarkably sensitive to growth inhibition by the E2 protein. Although BPV E2-mediated inhibition of HPV18 E6 and E7 expression may contribute to growth inhibition in some of the cervical carcinoma cell lines, the BPV E2 protein also appears to exert a growth-inhibitory effect that is independent of its effects on HPV gene expression.

Specific types of human papillomaviruses (HPV) are leading candidates for the infectious agents involved in the development of human cervical carcinoma (39, 71). The great majority of cervical carcinomas contain and express the E6 and E7 genes of HPV types 16 and 18 (HPV16 and HPV18; the high-risk virus types) (1, 2, 7, 18, 31, 40, 50, 52, 53, 57, 70). These same viral genes can immortalize or transform cultured epithelial cells, including human cervical epithelial cells (16, 41, 42, 67, 68). The HPV16 and HPV18 E6 and E7 proteins bind to the cellular tumor suppressor proteins p53 and $p105^{RB}$, respectively, and are thought to inactivate these growth-inhibitory proteins (19, 21, 30, 38, 49, 66). In contrast, the E6 and E7 proteins of HPV types that are infrequently detected in cervical carcinoma tissue display much lower ability to transform cells or bind these cellular proteins. Furthermore, the rare cervical carcinoma cell lines that are devoid of HPV DNA often contain mutant p53 and $p105^{RB}$ genes, whereas these genes appear wild type in carcinoma cell lines that contain viral DNA (11, 12, 48, 69). These findings strongly suggest that the interaction between HPV16 and HPV18 E6 and E7 proteins and cellular tumor suppressor proteins plays a causal role in the development of human cervical carcinoma.

Most benign HPV-induced cervical lesions do not progress to carcinoma, and the molecular mechanisms underlying progression are not well understood. Papillomavirus genomes replicate as extrachromosomal plasmids in premalignant lesions but are integrated in the great majority of cervical carcinomas and carcinoma-derived cell lines, resulting in disruption of the viral E1 and E2 genes (18, 33, 51, 56). The frequent loss of a functional E2 gene during malignant progression led to the suggestion that inactivation of this gene may play a role in this progression (10, 45, 51, 62). The E2 gene encodes site-specific DNA-binding proteins that stimulate or inhibit transcription, depending on the particular E2 protein synthesized and on the position of the E2 binding sites relative to other transcriptional control elements (26, 34). Cotransfection and in vitro transcription studies indicate that full-length bovine papillomavirus (BPV) E2 protein inhibits transcription of genes under the control of the HPV18 P_{105} and HPV16 P_{97} promoters, which normally direct transcription of the E6 and E7 genes, by binding to sites located within the promoters and interfering with the binding of essential transcription factors (17, 22, 46, 60–62). The HPV16 and HPV18 E2 proteins bind to the same consensus DNA recognition sequence as does the BPV E2 protein and exert a similar inhibitory effect on HPV transcription, although they appear to be less potent repressors than the BPV protein (5, 6). Moreover, introduction of the HPV18 E2 gene by transfection also appeared to inhibit expression of the E6 and E7 genes in HPV-immortalized human foreskin keratinocytes (47). On the basis of these results, it can be hypothesized that during the initial phases of HPV infection, expression of the E2 protein results in low levels of expression of the E6 and E7 proteins. Integration of the viral DNA disrupts the E2 gene, thereby allowing more efficient expression of the E6 and E7 genes, which in turn results in more complete inactivation of the cellular $p105^{RB}$ and p53 tumor suppressor proteins and further growth stimulation. It has also been suggested that papillomavirus E2 proteins are toxic or lethal to cervical carcinoma cells because attempts to establish stable derivatives of HeLa cells expressing BPV E2 were unsuccessful, and because

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microinjection of SiHa cells with a plasmid predicted to express a truncated form of the BPV or HPV16 E2 protein rapidly resulted in cell death (29, 62).

To examine the effect of the E2 protein on HPV gene expression and on growth of cervical carcinoma cells, we have used a BPV-simian virus 40 (SV40) recombinant virus (Pava1) to efficiently introduce the BPV E2 gene into a variety of cell lines. We show that BPV E2 expression results in specific inhibition of HPV gene expression in these cells and in rapid and dramatic growth inhibition. Surprisingly, BPV E2 expression also inhibits proliferation of a cervical carcinoma cell line apparently devoid of HPV DNA.

MATERIALS AND METHODS

Cells and viruses. HeLa cells were obtained from W. P. Summers (Yale University), and CaSki cells were obtained from M. Reiss (Yale University). The HeLa cells used here (HeLa-SHN6) were derived by single-cell cloning; the parental culture and all five additional clones tested showed similar responses to Pava1 infection. The other cervical carcinoma cell lines and A431 cells were obtained from the American Type Culture Collection (Rockville, Md.). Southern blotting was used to document the presence of the appropriate HPV DNA type in the HPV-positive cervical carcinoma cell lines and the absence of HPV sequences in C33a cells. HT-3 cells were shown to be HPV negative by using the polymerase chain reaction (PCR) using consensus primers for the L1 gene that amplify a large variety of HPV types infecting genital mucosa (the gift of K. Shah, Johns Hopkins University) (data not shown). In addition, PCR and DNA sequencing was used to confirm the presence of a G→T mutation in codon 245 of the HT-3 p53 gene (48) (data not shown). All cells were mycoplasma free and were propagated in Dulbecco's modified Eagle's medium (DMEM) or McCoy's 5a medium (for the HT-3 cells), both containing 10% fetal calf serum and antibiotics.

High-titer stocks of recombinant viruses were prepared essentially as described previously (54). Briefly, recombinant viral DNA was excised from the bacterial plasmid vector by *Eco*RI digestion, recircularized with DNA ligase in vitro, and transfected into CMT4 cells that were subsequently treated with heavy metals to induce expression of SV40 large T antigen (23). Stocks were serially passaged when significant cytopathic effect occurred. Wild-type Pava1 and mutant viruses containing the following previously described mutations were prepared: E2am1 (nonsense mutation in the 5′ portion of the E2 gene) (54), E5d29 (frameshift mutation in the E5 gene) (52), E4am1 (nonsense mutation in the E4 gene) (55), E2-RMC1 (mutation that eliminates the initiating methionine codon for the 3′ E2TR gene) (44), and E2-BMV (mutation that deletes the DNA binding domain common to the full-length E2 protein and to E2TR) (55). For viruses expressing an intact E2 gene, titers were estimated by measuring E2-mediated transactivation in NL3D cells as described previously (54, 55). For viruses containing E2 mutations, multiplicity of infection was judged to be roughly equivalent by comparing the expression of the E2TR protein or the BPV E5 protein (the E5 gene is intact in the E2 mutant viruses) in infected HeLa cells or by measuring the amount of viral DNA replication following infection of CMT4 cells. We estimate that the multiplicity of infection for all experiments reported here was greater than 100 infectious units per cell.

Expression of the BPV E2 protein and cellular p53. Approximately 5×10^5 cells were plated in a 6-cm-diameter dish and infected 2 days later with 40 μl of high-titer stocks of Pava1 or Pava-E2am1 (54). Two days after infection, the cells were washed twice in phosphate-buffered saline (PBS), incubated in cysteine-free, serum-free DMEM for 1 h, and labeled for 45 min in 1.0 ml of medium containing 200 μCi of [^{35}S]cysteine (Amersham) per ml. The cells were rinsed three times with PBS containing 1 mM phenylmethylsulfonyl fluoride, collected in a microcentrifuge tube, and lysed in 0.2 ml of radioimmunoprecipitation assay buffer (50 mM Tris Cl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 10 mM deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) on ice for 30 min. The lysate was vortex mixed and centrifuged for 5 min. Radioactivity incorporated into protein was determined by using ascending chromatography to separate incorporated from unincorporated label in a portion of the extract. Ninety microliters of B202 culture medium (containing an anti-E2 monoclonal antibody, supplied by Tim Nottoli, University of California, Berkeley, Calif.) (43) was added to each supernatant containing equivalent amounts of incorporated radioactivity; after rotation for 1 h at 4°C, 60 μl of a 1:1 suspension of protein A-Sepharose (Pharmacia) in 10% bovine serum albumin in Tris-buffered saline (10 mM Tris Cl [pH 7.4], 165 mM NaCl) was added, and the sample was further rotated for 1 h. The beads were pelleted, washed four times with cold radioimmunoprecipitation assay buffer, and resuspended in 45 μl of Laemmli SDS-polyacrylamide gel electrophoresis sample buffer. After boiling for 5 min, the samples were electrophoresed through a 17% polyacrylamide gel. The gel was dried and processed for fluorography using Amplify (Amersham). To detect the cellular p53 protein, cells were labeled for 3 or 4 h. Extracts were prepared and analyzed as described above except that 0.8 μl of a 1:3 dilution of anti-p53 antiserum CM-1 (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom) was used for immunoprecipitation per 10^6 cpm. The amount of p53 immunoprecipitated was assessed by using a PhosphorImager (Molecular Dynamics, Inc.) and Imagequant software.

RNA analysis. Two days after plating, cervical carcinoma cells in 6-cm-diameter plates were infected at high multiplicity with Pava1 or Pava-BMV. Approximately 70 h after infection, total cellular RNA was harvested and quantitated spectrophotometrically (9). Aliquots (10 μg) of total RNA were denatured, electrophoresed on an agarose gel containing formaldehyde, and transferred to nitrocellulose. HPV18 E6/E7 message was detected by hybridization to a ^{32}P-labeled probe extending from HPV18 nucleotides 7582 to 1128 (20). After autoradiography, blots were stripped by incubation at 65°C in 2 mM Tris HCl (pH 8.2)–1 mM EDTA–0.1% SDS and rehybridized to a radiolabeled human γ-actin gene (25).

Plating assays. HeLa cells (10^4) were plated in 1.5-cm-diameter wells 2 days prior to infection. Two days after high-multiplicity infection with virus, cells were trypsinized and 10^4 cells were plated in 6-cm-diameter plastic plates or in 0.3% agarose containing DMEM with 10% fetal calf serum. Cells were fed with fresh medium or agarose every 4 days. One week after plating, photographs were taken with an Olympus OM-2S camera and 451F550 yellow filter attached to an Olympus CK-2 phase-contrast inverted microscope.

Thymidine incorporation assay of DNA synthesis. Cells were plated in 1.5-cm-diameter wells at a density of 1×10^4 to 4×10^4 cells per well 3 days prior to infection with 4 μl of a high-titer stock of Pava1 or Pava-E2am1. Two to three days after infection, cells were incubated for 4 to 5 h in 0.5 ml of medium containing 1.5 μCi of [^{3}H]thymidine (Amersham) per ml. The cells were then rinsed three times with PBS, rinsed once with cold 10% trichloroacetic acid, and incubated in 10% trichloroacetic acid at 4°C for 20 min. DNA

synthesis in each well was determined by scintillation counting of radioactivity solubilized by incubation in 3% perchloric acid at 95°C for 40 min. All assays were performed in duplicate.

Autoradiography. Cells plated on an eight-chamber glass slide (Nunc) were mock infected or infected with 2 μl of Pava1 or Pava-E2am1 per chamber; 48 h later, cells were labeled for 4 to 5 h with [^{3}H]thymidine, 1.5 μCi/ml in 0.2 ml per chamber. After rinsing in PBS, the cells were fixed in methanol-acetic acid (3:1) for 5 min and treated with 5% trichloroacetic acid at 4°C for 5 min. The slides were washed in 70% ethanol three times and in 95% ethanol three times, air dried, and then coated with autoradiography emulsion NTB2 (Kodak, Rochester, N.Y.). After exposure for 4 days at 4°C, the slides were developed in Kodak D-19 developer and fixed. Representative microscopic fields were photographed through phase-contrast microscopy, and the numbers of labeled and total nuclei were scored.

Cell cycle analysis. A total of 1.5×10^5 HeLa or HT-3 cells were plated in 35-mm-diameter plates 2 days prior to infection with 8 μl of Pava1 or mock infection. Three days after infection, cells were harvested by trypsinization, washed in RPMI 1640 medium, and collected by centrifugation. The cell pellet was stained by using a modification of the procedure of Bauer (4) by incubation at 4°C with 1 ml of a solution containing 0.005% propidium iodide (Sigma Co., St. Louis, Mo.), 2 mM sodium citrate, 0.002 RNase (Worthington Biochemical, Freehold, N.J.), 0.2% Triton X-100, and 0.5% Nonidet P-40. The nuclei were analyzed on a EPICS Profile flow cytometer (Coulter Electronics, Hialeah, Fla.). Cell cycle distribution was evaluated by using the software Verity Modfit (Verity Software House, Topsam, Maine). The fraction of cells in S phase was evaluated by using a second-degree polynomial.

RESULTS

For the experiments reported here, the following human cervical carcinoma cell lines were used: HeLa, MS751, and C-4I, all of which contain HPV18 DNA; SiHa and CaSki, which contain HPV16 DNA; and C33a and HT-3, which are devoid of HPV DNA (40, 70). We also analyzed CV-1 monkey kidney epithelial cells and A431 human epidermoid carcinoma cells. The BPV E2 gene was delivered into these cell lines by high-multiplicity infection with a replication-defective BPV-SV40 recombinant virus, Pava1, and its derivatives (54, 55). This recombinant virus also contains the BPV E4 and E5 genes, but these genes are not required for the growth-inhibitory effect in HeLa cells described below (see Fig. 7).

To determine whether viral infection introduces a functional E2 gene into cervical carcinoma cell lines, RNA was isolated 48 h after infection with Pava1 and analyzed by Northern (RNA) analysis using probes specific for the BPV E2 gene. Infected HeLa cells produced abundant BPV E2 RNA, C-4I and MS751 cells expressed moderate amounts of BPV E2 RNA, and CaSki, SiHa, and C33a cells expressed modest amounts of BPV E2 RNA (data not shown). Thus, there is considerable variability in the ability of cervical carcinoma cell lines to be infected by the recombinant virus or to express E2 RNA. To analyze the effect of the E2 protein on cervical carcinoma cells that express HPV gene products, we concentrated on HeLa cells because they expressed higher levels of BPV E2 RNA than do the other HPV-containing cell lines.

To document the expression of the BPV E2 protein following infection by BPV-SV40 recombinant viruses,

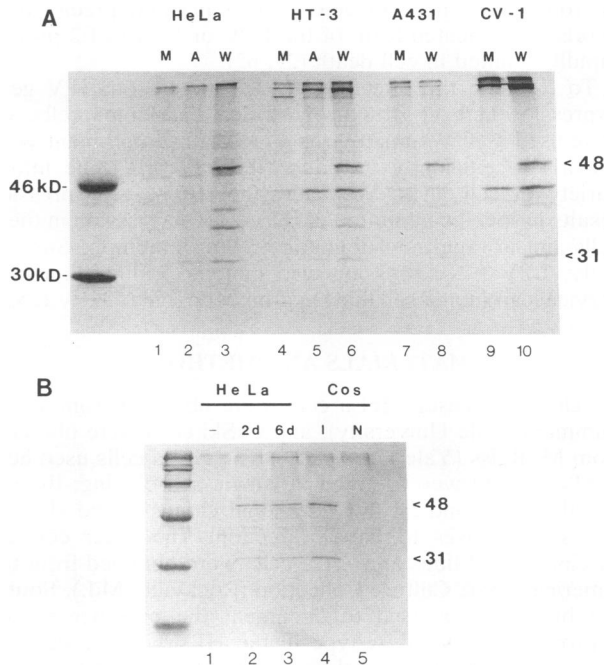

FIG. 1. BPV E2 protein expression in infected cell lines. Cells were metabolically labeled, and E2 protein was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis as described in Materials and Methods. The positions of the full-length 48-kDa E2 species and the 31-kDa E2TR are indicated, and the molecular weights of the markers are shown. (A) The indicated cell lines were mock infected (M) or infected with high-titer stocks of Pava1 (W), which expresses a wild-type E2 gene, or Pava-E2am1 (A), which contains an amber mutation preventing synthesis of the full-length E2 protein, and labeled 2 days later. (B) HeLa cells were labeled in the absence of infection (−) or 2 or 6 days after infection with wild-type Pava1, as indicated. As controls, extracts of Pava1-infected COS cells were immunoprecipitated with nonimmune (N) or anti-E2 (I) antibodies.

HeLa cells as well as HT-3, A431, and CV-1 cells, three cell lines devoid of HPV DNA, were metabolically labeled with [^{35}S]cysteine 48 h after infection, and extracts were immunoprecipitated with an E2-specific monoclonal antibody (Fig. 1A). The full-length 48-kDa E2 protein was absent from uninfected cells but readily detectable in cells infected with Pava1, as were two less abundant smaller species that appear to include the well-characterized 31-kDa internally initiated carboxyl-terminal E2TR protein (26, 34). HeLa, HT-3, and CV-1 cells express similar amounts of the 48-kDa E2 protein, whereas A431 cells express about threefold less. To confirm that the prominent species was the full-length E2 protein, cells were also infected with Pava-E2am1, a mutant virus containing a nonsense mutation in the 5′ half of the E2 gene (15), upstream of the site of translation initiation of the E2TR protein. As predicted, this mutation eliminated expression of the 48-kDa E2 protein while leaving expression of the 31-kDa species intact. Thus, the BPV-SV40 virus can be used to express the BPV E2 protein in a variety of human cell lines.

To determine whether the BPV E2 protein inhibited expression of HPV genes in cervical carcinoma cell lines, cells were infected with high-titer stocks of Pava1, which expresses the wild-type E2 gene, or with Pava-BMV, a virus containing a deletion in the E2 gene that prevents DNA binding by the E2 protein (55). RNA was isolated 48 h after

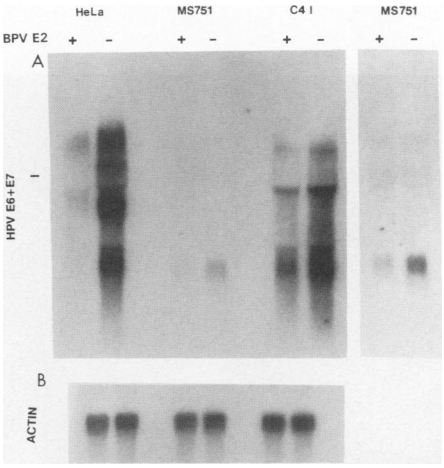

FIG. 2. Inhibition of HPV gene expression by the BPV E2 gene. (A) The indicated cervical carcinoma cell lines were infected at high multiplicity with wild-type Pava1 (+) or E2 mutant Pava-BMV (−) virus, and total cellular RNA was analyzed by Northern blotting for HPV18 E6 and E7 RNA as described in Materials and Methods. The panel at the right shows a longer exposure of the lanes containing MS751 RNA. (B) The filter shown in panel A after rehybridization to a γ-actin probe.

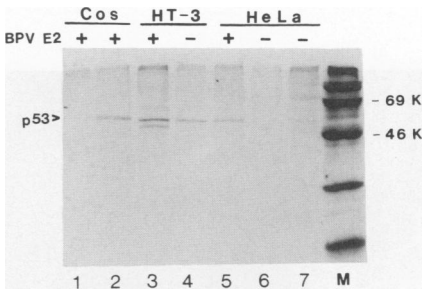

FIG. 3. Expression of the cellular p53 protein. Pava1-infected (+) and mock-infected (−) COS, HeLa, and HT-3 cells, as indicated, were labeled for 3 h. Equivalent amounts of radioactivity were immunoprecipitated with nonimmune rabbit serum (lane 1) or anti-p53 CM-1 antiserum (lanes 2 to 6). Threefold more of radioactivity was immunoprecipitated with CM-1 antiserum in lane 7. Proteins in the immunoprecipitate were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography. The arrowhead marks the position of p53. On some gels, the HeLa cell p53 resolves into two bands of similar electrophoretic mobility, as has been described for p53 synthesized in vitro from HeLa cell RNA (30).

infection and subjected to Northern analysis using probes specific for the HPV18 E6/E7 region. As shown in Fig. 2A, cervical carcinoma cells infected with the E2 mutant virus express readily detectable HPV18 E6/E7 RNA, which was indistinguishable in amount or mobility from that in uninfected cells (data not shown). In contrast, infection with the virus expressing the wild-type E2 gene caused a dramatic reduction in the amount of HPV18 E6/E7 RNA in HeLa cells. E2 expression also caused a moderate reduction in E6/E7 RNA in MS751 and C-4I cells, which express less BPV E2 RNA than do infected HeLa cells. Densitometric analysis demonstrated that HPV18 gene expression was reduced approximately 95% in HeLa cells, 75% in MS751 cells, and 50% in C-4I cells. Expression of the γ-actin gene was unaffected by infection (Fig. 2B), indicating that inhibition of HPV gene expression was not due to a generalized inhibition of transcription. Thus, expression of the BPV E2 gene efficiently represses expression of the resident HPV genomes in cervical carcinoma cell lines.

The E6 proteins of the high-risk HPV types target the cellular tumor suppressor protein p53 for rapid degradation in vitro, and the levels of p53 are low in HeLa cells and in E6-expressing keratinocyte cell lines, presumably as a consequence of this pathway (28, 30, 32, 47). To investigate whether BPV E2 expression and reduction in HPV18 E6 expression affect the level of p53 in HeLa cells, we measured p53 levels in uninfected HeLa cells, which express the HPV18 E6 protein, and in cells infected with Pava1, in which the BPV E2 protein represses expression of the HPV18 E6 gene (Fig. 3). As shown in lane 6, p53 is barely detectable in proliferating HeLa cells. Strikingly, inhibition of E6 expression by the BPV E2 protein is accompanied by a marked increase in the steady-state amount of p53 in HeLa cells (approximately ninefold; average of three experiments) (lane 5). In contrast, mutant p53 is readily detectable in HT-3 cells which do not express HPV E6, and the level of HT-3 p53 varies no more than approximately twofold upon BPV E2 expression (lanes 3 and 4). These results demonstrate a reciprocal correlation between HPV18 E6 and p53 expression in HeLa cells and suggest that E6-mediated p53 destabilization is responsible for the low amount of cellular p53 in these cells.

To determine the effect of BPV1 E2 expression on cell proliferation, HeLa cell growth was assessed in plating assays. Two days after mock infection or infection with Pava1, HeLa cells were passaged, and their ability to form colonies on plastic or in agarose was determined. Uninfected HeLa cells efficiently formed large colonies on plastic or in agarose (Fig. 4A and C). In contrast, colony formation by HeLa cells was markedly inhibited by infection with the virus that expresses the wild-type E2 gene (Fig. 4B and D). The growth-inhibited HeLa cells assumed a more extended appearance and remained metabolically active as assessed by trypan blue exclusion. In these and similar experiments, infection with the wild-type virus caused an approximately 25-fold reduction in the number of macroscopic colonies that developed. The small number of Pava1-infected HeLa cells that formed colonies on plastic were not genetically resistant to infection or growth inhibition; cell lines derived from individual colonies that developed after infection are growth inhibited upon reinfection with E2-expressing virus. Evidently, at the time of initial infection, these cells did not express sufficient levels of E2 protein for growth inhibition or were transiently refractory to its effects.

The effect of BPV E2 expression was also determined in short-term DNA synthesis assays in which incorporation of [^{3}H]thymidine into cellular DNA was assessed by measuring acid-insoluble radioactivity 2 to 3 days after infection. As shown in Table 1, infection with the virus expressing the wild-type E2 gene, but not the mutant E2 gene, caused a marked (>95%) decrease in HeLa cell DNA synthesis, in accord with the reduction in colony formation described above. E2-dependent inhibition of cellular DNA synthesis was observed in many experiments using independently derived stocks of recombinant virus, and at low multiplicities of infection, the inhibition of DNA synthesis was proportional to the dose of virus. Growth inhibition was still substantial 6 days after infection, although the amount of DNA synthesis increased between 2 and 6 days, presumably because the rare cells that escaped inhibition continued to proliferate and became a significant proportion of the population of cells. In a typical experiment, 2 days after infection,

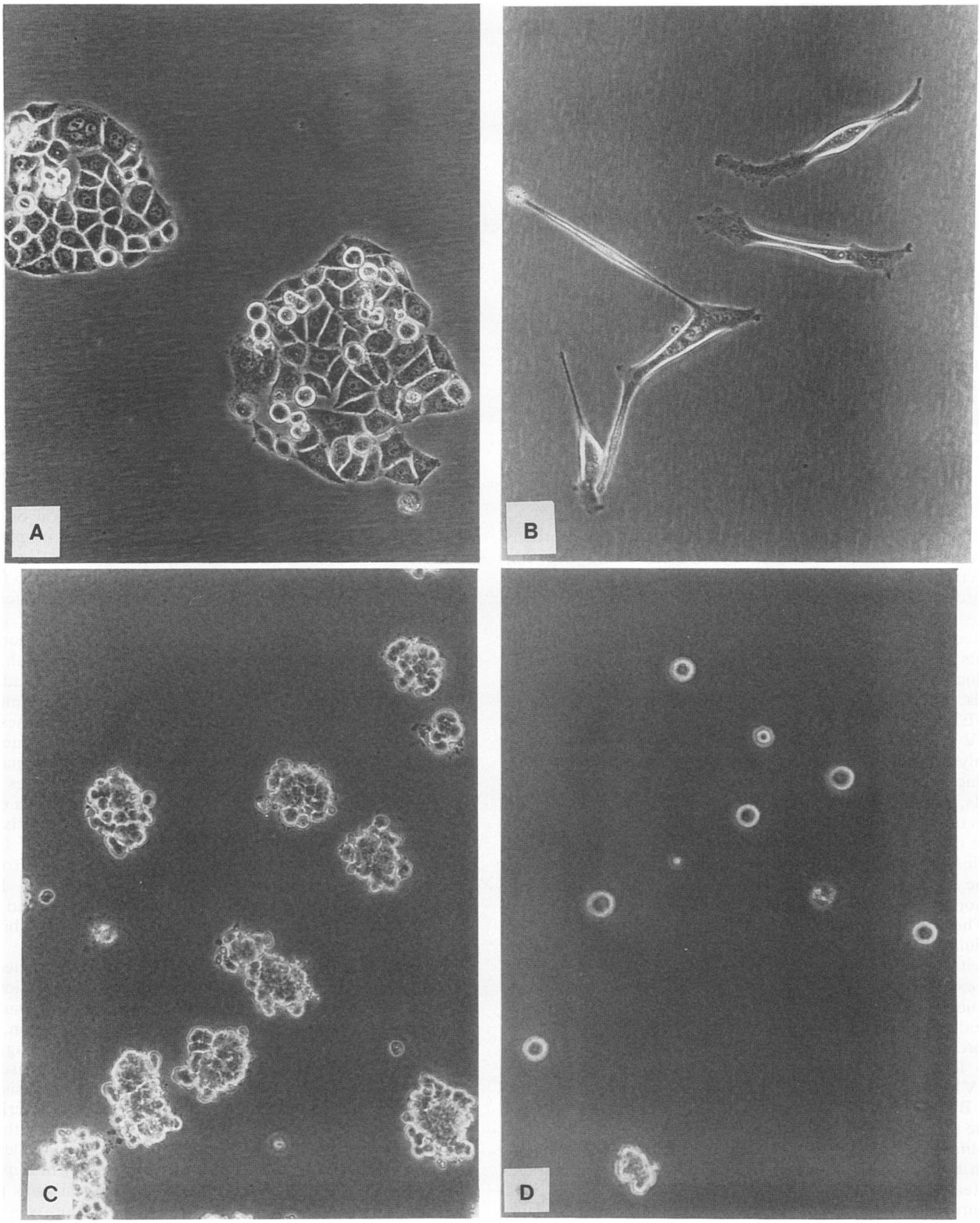

FIG. 4. Inhibition of HeLa cell colony formation by the BPV E2 gene. HeLa cells were mock infected (A and C) or infected with Pava1 (B and D). After being passaged, the cells were plated on plastic (A and B) or in agarose (C and D).

TABLE 1. Cellular DNA synthesis assay

E2 gene	DNA synthesis[a]			
	HeLa	HT-3	A431	CV-1
None	100	100	100	100
Wild type	2.5 ± 0.4	13.8 ± 5.8	93.3 ± 3.9	163.4 ± 22.5
Nonsense mutant	84.9 ± 13.6	87.1 ± 18.2	89.5 ± 9.7	137.1 ± 51.3

[a] Incorporation of [^{3}H]thymidine into acid-insoluble form by the infected samples, expressed as the percentage (± standard deviation) of synthesis by mock-infected cells. The results shown are averages of two (A431 and CV-1 cells) or more (HeLa and HT-3 cells) independent experiments (each determination performed in duplicate). HeLa and HT-3 cells were tested with at least two independent stocks of wild-type and mutant viruses. In a typical experiment, uninfected cells incorporated the following approximate amounts of radioactivity: HeLa cells, 3.63×10^5 cpm/2×10^4 cells plated; HT-3 cells, 1.02×10^5 cpm/4×10^4 cells plated; A431 cells, 1.65×10^5 cpm/4×10^4 cells plated; and CV-1 cells, 1.8×10^4 cpm/2×10^4 cells plated.

HeLa cells synthesized 6% as much DNA as did uninfected cells, whereas by 6 days this value had increased to 26%. The BPV E2 protein was still readily detectable by immunoprecipitation of extracts prepared from cells metabolically labeled 6 days after infection (Fig. 1B). In contrast to the inhibitory effect on DNA synthesis, infection with Paval did not cause a significant inhibition of the incorporation of ^{35}S-labeled amino acids into protein or cause a reduction in cell number relative to the number of cells plated (data not shown), indicating that the cells remained viable. DNA synthesis in MS751 and C-4I cells was inhibited approximately 80 and 50%, respectively, by introduction of the wild-type E2 gene, mirroring the degree of BPV E2 expression, whereas SiHa cells, which express the E2 gene poorly, were not significantly growth inhibited (data not shown). These results indicate that there was a good correlation between the level of BPV E2 expression and the inhibition of cell growth in cervical carcinoma cell lines that contain HPV DNA.

The effect of BPV E2 expression was also assessed in cells devoid of HPV DNA (Table 1). Surprisingly, expression of the wild-type, but not mutant, E2 gene caused substantial growth inhibition of HT-3 cells, one of the prototype HPV-negative cervical carcinoma cell lines. In general, E2 expression in HT-3 cells resulted in approximately 80 to 90% inhibition of DNA synthesis. The absence of HPV DNA in HT-3 cells and the presence of a characteristic p53 mutation in these cells were confirmed as outlined in Materials and Methods. Infection of C33a cells, the other well-characterized HPV-negative cervical carcinoma cell line, had no effect on DNA synthesis or plating efficiency, but these cells did not express detectable E2 protein (data not shown). In contrast, Paval infection caused no specific growth inhibition of two noncervical cell lines, CV-1 and A431, both of which express readily detectable E2 protein following infection. Thus, the E2 protein does not exert a nonspecific growth-inhibitory effect in all epithelial cells. These results demonstrate that the growth-inhibitory effect of the BPV E2 protein is not restricted to cells expressing the HPV E6 and E7 genes but also can occur in an HPV-negative cervical carcinoma cell line.

DNA synthesis at the individual cell level was measured by autoradiography (Fig. 5 and Table 2). During asynchronous growth, approximately 37% of the HeLa cells and 25% of HT-3 cells incorporated detectable label during a 5-h pulse with [^{3}H]thymidine. These values were virtually unchanged 2 days after infection with a virus carrying the E2 nonsense mutation, whereas introduction of a wild-type E2 gene eliminated DNA synthesis in virtually all HeLa cells and in the great majority of HT-3 cells. In contrast, introduction of the E2 gene did not inhibit A431 cell DNA synthesis as measured in this assay. This autoradiographic analysis indicates that the BPV-SV40 virus induces a very large fraction of susceptible cervical carcinoma cells to undergo growth arrest and implies that biologically active E2 genes were delivered into the vast majority of cells exposed to this virus.

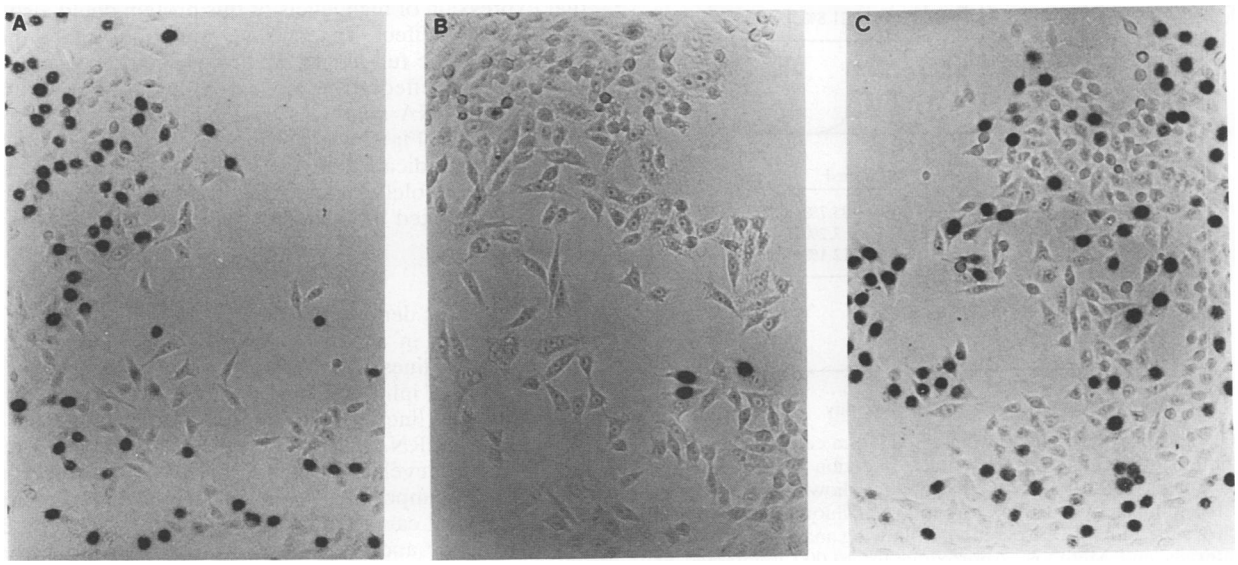

FIG. 5. Autoradiographic analysis of HeLa cell DNA synthesis. DNA synthesis was measured in HeLa cells by autoradiography as described in Materials and Methods, with silver grains accumulated over the nuclei that synthesized DNA during the pulse label. (A) HeLa cells 2 days after mock infection; (B) HeLa cells 2 days after infection with Paval expressing the wild-type E2 gene; (C) HeLa cells 2 days after infection with Pava-E2am1 expressing the E2 gene containing a nonsense mutation. The small cluster of replicating HeLa cells in panel B are probably derived from a single cell that escaped inhibition and underwent division following infection.

TABLE 2. Autoradiographic analysis of cellular DNA synthesis

E2 gene	DNA synthesis[a]		
	HeLa	HT-3	A431
None	37.2	25.0	62.4
Wild type	≤0.5	5.7	61.3
Nonsense mutant	33.3	24.3	65.9

[a] The fraction of nuclei that had undergone DNA synthesis, expressed as a percentage of total nuclei. The minimum numbers of cells counted for each treatment were as follows: HeLa, 650; HT-3, 810; and A431, 146.

To determine whether expression of the BPV E2 gene induced arrest at a particular stage of the cell cycle, infected HeLa and HT-3 cells were harvested, stained with propidium iodide, and subjected to flow cytometry to determine the DNA content of individual nuclei. Uninfected HeLa cells (or cells infected with the E2 mutant virus [data not shown]) consisted of discrete populations of cells in the G_1 and G_2/M phases of the cell cycle, as well as a clear fraction of cells in S phase (Fig. 6). Infection with Paval resulted in a marked reduction in the proportion of cells in S phase, with a concomitant increase in the proportion of cells in the G_0/G_1 phase. In these experiments, a substantial percentage of cells remained in the G_2/M phase, despite the depletion of S-phase cells. Similar effects were also seen in HT-3 cells expressing the E2 protein, although the reduction in S-phase cells and the increase in G_0/G_1-phase cells were not as marked (data not shown). These results indicate that E2 expression inhibits the progression of cells from the G_0/G_1 phase of the cell cycle into S phase and that there may be a block in progression from the G_2/M phase as well. The blockage at specific points in the cell cycle also supports our conclusion that E2 expression in sensitive cells causes specific growth arrest and not nonspecific toxicity.

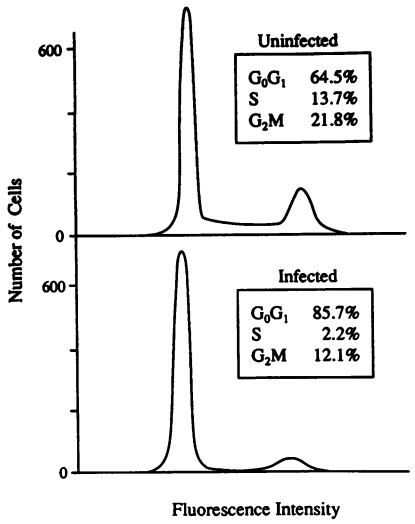

FIG. 6. Cell cycle analysis of infected HeLa cells. The cell cycle distribution of HeLa cells after mock infection or infection with Paval expressing the wild-type E2 protein is shown. The histograms show the number of nuclei as a function of fluorescence intensity (which is a measure of DNA content) following analysis as described in Materials and Methods. Approximately 10,000 uninfected and 7,000 infected cells were analyzed. The major peak at the left represents G_0/G_1-phase cells, the peak at the right represents G_2/M-phase cells, and cells in S phase are distributed between these peaks. The insets show the fraction of analyzed nuclei present in each phase.

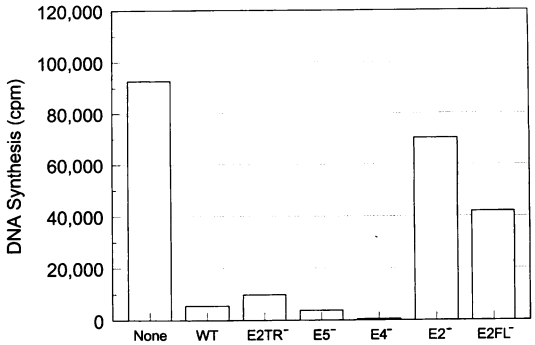

FIG. 7. Genetic requirements for inhibition of HeLa cell proliferation. HeLa cells were infected with the indicated viruses, and DNA synthesis was measured by incorporation of [^{3}H]thymidine into acid-insoluble form. The averages of duplicate determinations are shown. Viruses used for infection (described in Materials and Methods): WT, wild-type Paval; E2TR$^-$, Pava-RMC1 (defective for expression of the E2TR protein); E5$^-$, Pava-Ed29 (defective for expression of the E5 protein); E4$^-$, Pava-E4aml (defective for expression of the E4 protein); E2$^-$, Pava-BMV (defective for expression of both the full-length E2 protein and the E2TR protein); E2FL$^-$, Pava-E2aml (defective for expression of the full-length E2 protein).

The Paval genome has the potential to express the BPV E4 and E5 proteins in addition to the proteins encoded by the E2 gene. To confirm that the BPV E2 gene was responsible for growth inhibition following Paval infection, HeLa cells were infected with a panel of viral mutants, and the effects of these mutants on DNA synthesis were determined (Fig. 7). Substantial growth inhibition was still observed with mutants unable to express the E4, E5, or E2TR protein, demonstrating that these BPV proteins are not required for inhibition. However, the BPV-SV40 recombinant viruses express relatively low levels of the 31-kDa E2TR protein (Fig. 1), so these experiments do not rule out the possibility that expression of high levels of this protein could also exert an inhibitory effect. In contrast, mutations that inhibited synthesis of the full-length E2 protein markedly attenuated the inhibitory effect. The modest inhibition (approximately twofold) of DNA synthesis by Pava-E2aml in the experiment shown in Fig. 7 was not reproducibly observed. These experiments indicate that the full-length E2 protein is primarily, if not solely, responsible for the significant growth inhibition induced by infection of HeLa cells with Paval.

DISCUSSION

These results demonstrate that expression of the BPV E2 protein results in significant growth inhibition of cervical carcinoma cell lines regardless of whether they contain HPV DNA. Growth inhibition was evident in all four cervical carcinoma cell lines tested that expressed significant levels of the BPV E2 RNA, and the extent of inhibition correlated well with the levels of E2 expression. These experiments establish two important points. First and most important, human cervical carcinoma cells, even after they progress to full malignancy and after in vitro propagation for extended periods, remain remarkably sensitive to growth-inhibitory signals. Introduction of a single viral regulatory gene can cause relatively rapid and near complete cessation of replication of cells thought to proliferate in an uncontrolled manner. This growth inhibition does not appear due to a

toxic effect because of the continued viability of the cells and the blockage at specific sites in the cell cycle. The ability of the E2 protein to induce growth arrest in HeLa cells may open new avenues of study involving these well-characterized cells, and the uniform response of these cells to E2-mediated inhibition makes this system amenable to biochemical analysis. Second, the growth-inhibitory effect of the E2 protein is not restricted to cells expressing HPV DNA, indicating that the BPV E2 protein has a growth-inhibitory activity that is not mediated by transcriptional repression of HPV gene expression. Analysis of additional cell lines that efficiently express the BPV E2 protein is required to determine the spectrum of cell types sensitive to E2-mediated growth inhibition.

The mechanism through which the BPV E2 protein inhibits cell growth is not known. Our results demonstrate that expression of the BPV E2 protein in human cervical carcinoma cell lines that contain HPV 18 DNA results in substantial inhibition of the expression of the HPV E6 and E7 oncogenes, presumably via the well-documented repressing effect of the BPV E2 protein on HPV transcription (6, 17, 22, 46, 60–62). In these cells, decreased levels of these HPV gene products may contribute to growth inhibition. According to this scenario, depletion of the HPV E6 and E7 proteins by expression of the BPV E2 protein increases the amount of the p53 and $p105^{RB}$ cellular tumor suppressor proteins in their growth-inhibitory state and is functionally equivalent to introducing the wild-type versions of the p53 and RB genes or proteins into cells, a treatment that inhibits cells from entering S phase (3, 8, 13, 14, 24, 27, 35, 36, 59). Our results demonstrate that expression of the BPV E2 protein results in a marked increase in the steady-state level of the cellular p53 tumor suppressor protein in HeLa cells. Furthermore, the increased amount of p53 in infected HeLa cells does not appear to be due to an increased rate of p53 synthesis (28a). We have not demonstrated that reduced E6 levels are responsible for the increase in p53 in HeLa cells expressing BPV E2. Nevertheless, our ability to modulate HPV18 RNA levels in HeLa cells and the demonstration that reduced HPV gene expression is accompanied by increased p53 levels provides strong support for the contention that the HPV18 E6 protein targets p53 for rapid degradation in human cancer cells, as well as in vitro and in transfected cell lines (28, 30, 49).

In accord with the view that HPV oncogene expression is required to maintain the transformed state of human cervical cancer cells, studies in other laboratories have demonstrated that induction of E6/E7 antisense RNA in one of three C-4I derivatives tested caused a moderate (approximately 40 to 60%) decrease in cell proliferation and reduced tumorigenicity, implying that HPV gene expression may play a role in maintaining these growth properties (63–65). Although Storey et al. showed that some HPV16 E6- and E7-specific antisense oligonucleotides inhibited proliferation of CaSki cells, the mechanism of this effect is unknown because oligonucleotide treatment did not detectably affect HPV gene expression (58).

The hypothesis that E2-mediated inhibition of E6 and E7 expression is responsible for growth inhibition of cervical carcinoma cell lines is challenged by the finding that HT-3 cells are also inhibited even though they appear devoid of HPV DNA. Either these cells contain a divergent, as yet undetected HPV DNA type or the BPV E2 protein can inhibit proliferation via a mechanism independent of its effects on HPV gene expression. It seems unlikely that HT-3 cells harbor HPV DNA because its absence has been confirmed in several laboratories by using sensitive PCR-based methods and because, like other HPV-negative cell lines, they harbor mutations in the cellular RB and p53 genes. Thus, the growth inhibition of HT-3 cells is not mediated by these cellular tumor suppressor proteins. If HT-3 cells are truly HPV DNA negative, we can envision three possible mechanisms of E2-mediated growth inhibition of these cells. First, the E2 protein may bind DNA adjacent to cellular growth-regulatory genes and either stimulate or inhibit their expression. Second, the E2 protein may bind to and sequester cellular transcription proteins, a phenomenon known as squelching, thereby inhibiting expression of genes essential for cell proliferation. Finally, growth inhibition may be a consequence of activities of the E2 protein distinct from its role in transcription regulation, such as its ability to bind proteins directly involved in DNA replication (37).

It is possible that both an HPV-independent growth-inhibitory effect and transcriptional repression of the HPV18 E6 and E7 genes contribute to E2-mediated growth inhibition of cervical carcinoma cell lines containing HPV DNA, but further experiments are required to determine the relative importance of these two effects. Comparison of the effects of various E2 mutants in HT-3 and HeLa cells may reveal whether the same activities of the E2 protein are responsible for growth inhibition of these two cell types and may provide information concerning the mechanism(s) responsible for this effect. Elucidation of the mechanism responsible for growth inhibition of cervical carcinoma cells by the BPV E2 protein may provide new insights into growth control of human tumor cells and may suggest new strategies for cancer therapy, including the delivery of viral negative regulatory genes or gene products into carcinoma cells.

Integration of HPV16 and HPV18 DNA and disruption of the viral E2 gene frequently occur during cervical carcinoma progression, leading to the suggestion that inactivation of the HPV E2 gene is important to allow efficient expression of the E6 and E7 genes, which in turn provide cervical epithelial cells with a growth stimulus (10, 41, 42, 62, 67, 68). The results described here demonstrate that BPV E2 can inhibit expression of HPV18 E6 and E7 genes stably integrated during natural infection and that this inhibitory effect can be imposed even after the cells progress to carcinoma. In addition, our results in HT-3 cells indicate that the BPV E2 protein can exert HPV-independent growth-inhibitory effects in cervical epithelial cells. The HPV and BPV E2 proteins share a number of structural and functional features, including overall genetic organization, DNA binding specificity, and role in transcriptional regulation and viral DNA replication (26, 34). Therefore, our results raise the possibility that growth inhibition of cervical carcinoma cells by BPV E2 reflects activities expressed by the HPV E2 proteins as well. If this is the case, disruption of the HPV E2 gene during carcinoma progression confers a growth advantage by increasing E6 and E7 expression and by removing HPV-independent growth-inhibitory effects. To test this hypothesis, it will be necessary to examine the consequence of expressing HPV E2 proteins in cervical carcinoma cells. The notion that the effects of the E2 protein on cell growth are not mediated exclusively via transcriptional regulation of HPV E6 and E7 genes is also supported by the results of experiments which determined the ability of transfected HPV16 DNA to immortalize primary human foreskin keratinocytes (45). These experiments showed that mutations that prevented synthesis of the HPV16 E2 protein stimulated immortalization efficiency to a greater extent than did mutations that uncoupled E6 and E7 expression from E2-mediated repression. Thus, it appears likely that papillomavirus E2 proteins play a complex role in the pathogenesis of

cervical carcinoma. Further studies promise to provide new insights into the role of these proteins in the pathogenesis of papillomavirus-associated diseases and in the maintenance of the malignant phenotype.

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