Repression of the Integrated Papillomavirus E6/E7 Promoter Is Required for Growth Suppression of Cervical Cancer Cells

DELICIA A. FRANCIS, SUSANNE I. SCHMID, AND PETER M. HOWLEY*

Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115

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The human papillomavirus (HPV) E2 protein is an important regulator of viral E6 and E7 gene expression. E2 can repress the viral promoter for E6 and E7 expression as well as block progression of the cell cycle in cancer cells harboring the DNA of “high-risk” HPV types. Although the phenomenon of E2-mediated growth arrest of HeLa cells and other HPV-positive cancer cells has been well documented, the specific mechanism by which E2 affects cellular proliferation has not yet been elucidated. Here, we show that bovine papillomavirus (BPV) E2-induced growth arrest of HeLa cells requires the repression of the E6 and E7 promoter. This repression is specific for E2TA and not E2TR, a BPV E2 variant that lacks the N-terminal transactivation domain. We demonstrate that expression of HPV16 E6 and E7 from a heterologous promoter that is not regulated by E2 rescues HeLa cells from E2-mediated growth arrest. Our data indicate that the pathway of E2-mediated growth arrest of HeLa cells requires repression of E6 and E7 expression through an activity specified by the transactivation domain of E2TA.

The papillomaviruses are small DNA viruses that infect a variety of mammalian species, including humans. Human papillomavirus (HPV) infections are quite common and cause a variety of benign proliferations including cutaneous warts, venereal warts, genital squamous intraepithelial lesions, and orolaryngeal and -pharyngeal papillomas, as well as other types of hyperkeratoses (41). Certain HPV types have also been linked to specific malignant lesions, most notably cervical cancer and other anogenital cancers.

Of the over 80 HPV types identified, approximately 30 HPV types are associated with genital tract lesions. Subsets of the anogenital HPV types are classified as either “low risk” or “high risk” depending upon the potential of the associated lesions to progress to cancer. HPV6 and HPV11 are low-risk HPV types that are associated with low-grade squamous epithelial lesions (venereal warts or condyloma accuminata), which rarely progress to cancer. HPV16, HPV18, and others are high-risk HPV types that are associated with intraepithelial neoplasias or squamous intraepithelial lesions that can progress to cancer (54).

It is now evident that most cervical neoplasias, whether intraepithelial or invasive, can be attributed largely to HPV infection. The DNA of a high risk HPV type can be found in over 90% of human cervical cancers (54). In addition, transfection of high-risk HPV genomic DNA can extend the life span of primary human genital keratinocytes (the normal cellular host of HPVs) and can contribute to cellular immortalization (13, 39, 51). This ability of the high-risk HPV types to immortalize keratinocytes is not a property of low-risk HPV types (13, 39).

Oncogenic viruses often exploit the machinery of the cells they infect in order to promote their own survival and replication. As a part of this process, they interfere with normal cellular control mechanisms, leading to abnormal cell growth, genetic alterations and even malignant transformation. The high-risk HPV types encode two oncoproteins, E6 and E7,

both of which are involved in cellular immortalization (20, 22, 33). E6 and E7 function to stimulate cell proliferation and do so by interfering with the function of regulatory proteins in cells, including the p53 and pRb tumor suppressor gene products (15, 32, 34, 50). High-risk HPV E6 protein can complex with p53 and, as a result, promotes the ubiquitination and degradation of p53 (38). E7 complexes with members of the retinoblastoma family and inactivates their growth-suppressing functions (27). In addition, the papillomavirus E6 and E7 proteins have been shown to bind a variety of other cellular proteins; it is likely that some of these other interactions may also contribute to cellular transformation.

The papillomavirus E2 proteins play essential roles in transcriptional regulation and viral DNA replication. They contain well-conserved N-terminal and C-terminal domains. The conserved N-terminal 200-amino-acid domain is required for the transcriptional activation function, the DNA replication function, and the association with replication protein E1. The carboxy-terminal domain is responsible for dimerization and site-specific binding to DNA (21).

E2 has been best studied for bovine papillomavirus type 1 (BPV-1), which encodes full-length E2 protein (E2TA), as well as two truncated forms of E2, E2TR and E8/E2, an alternatively spliced (28 kDa) protein (21). Neither E2TR nor E8/E2 contains the N-terminal transactivation domain of E2TA, and both can act as dominant inhibitors of E2-mediated transcriptional activation (29).

The papillomavirus E2 proteins can repress transcription of the HPV16 (P₉₇) and HPV18 (P₁₀₅) viral promoters, which direct expression of the viral E6 and E7 oncoproteins (9, 19, 37, 48, 49). This repression occurs through E2 binding sites located in promoter regions of the viral genomes. Carcinogenic progression of HPV16- and HPV18-infected lesions has generally been associated with the integration of the viral genome into the host cell chromosome (14, 40). This integration usually occurs in a manner that results in the loss of viral E2 and E1 gene expression and a selection for the continued expression of E6 and E7 (1, 12, 40). Thus, the integration of the HPV genome and the concomitant loss of E2 expression may be an important step in the carcinogenic process that results in the deregulated expression of the viral E6 and E7 genes.

* Corresponding author. Mailing address: Department of Pathology, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115. Phone: (617) 432-2884. Fax: (617) 432-2882. E-mail: peter_howley@hms.harvard.edu.

Several experimental observations support the notion that the loss of E2 expression in cervical cancer cells is an important step in HPV-associated carcinogenic progression. First, as mentioned above, full-length E2 proteins can repress the HPV18 P₁₀₅ and HPV16 P₉₇ promoters (4, 37, 49). Second, a decrease in HPV18 E6 and E7 transcripts has been detected in HeLa cells following transfection of BPV-1 E2TA (7, 11, 16, 24). Third, a colony reduction assay first described for BPV-1 E2 by Thierry et al. showed that introduction of BPV-1 E2, HPV16 E2, or HPV18 E2 causes the growth arrest of the HPV-positive carcinoma cell lines HeLa and SiHa but not of the HPV-negative cell line C33A (7, 11, 16, 23, 49). The observed growth arrest correlates with the reduction of E6 and E7 transcript levels and is accompanied by an increase in the levels of p53 and of the cyclin-dependent kinase inhibitor p21/cip1/waf1 (11, 24).

Although reintroduction of E2 into HeLa cells represses E6 and E7 transcription, it is possible that this E2 repression function may be necessary, but not sufficient to account for the growth inhibitory effects observed. Several observations have suggested that other E2 functions are necessary to mediate the cellular growth arrest (7). A better understanding of the mechanism by which E2 mediates cell growth arrest in HPV-positive cells should provide further insight into the functions of E2 as a transcriptional regulator and possible evidence as to whether E2 has a direct effect on the cell cycle regulatory machinery. The simplest mechanism by which E2 could mediate cell growth arrest in HPV-positive cells is through repression of E6 and E7 expression. Inactivation of E6 and E7 would restore the normal functions of p53 and pRb, as well as that of any other possible targets of E6 and E7.

To further investigate the mechanism by which expression of E2 in HPV-positive cell lines induces a growth arrest, we tested whether repression of high-risk E6 and E7 expression is required for E2-mediated cellular growth arrest. Here we provide evidence that E2-mediated growth arrest can be rescued by E6 and E7 expressed from a heterologous promoter that is itself not regulated by E2 and that repression of the HPV18 E6/E7 promoter by E2 is therefore required for its cell growth-suppressive activity. Furthermore, we find that E2TR does not repress the endogenous HPV18 E6/E7 promoter in HeLa cells, accounting for the inability of E2TR to arrest their growth.

MATERIALS AND METHODS

Cells. Cervical carcinoma cell lines HeLa, SiHa, and C33a were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 1 mM glutamine, 10% fetal calf serum, and antibiotics.

Generation of stable cell lines. For the transduction of HeLa cells, retroviruses expressing HPV16 E6 and E7 were obtained from Denise Galloway (17). HeLa cells were plated on 10-cm-diameter plates and incubated for 8 h with 1 ml of the appropriate retroviral producer line supernatant (empty retroviral vector LXS or HPV16 E6 and E7), along with 4 ml of DMEM containing 4 μ g of Polybrene/ μ l. Twenty-four hours after infection, cultures were harvested and seeded at various dilutions onto 10-cm-diameter plates. Clones obtained were selected in culture for 2 to 3 weeks in DMEM containing 500 μ g of G418/ml. Several G418-resistant single-cell clones were randomly chosen and analyzed for HPV16 RNA and E7 protein expression.

Plasmids. Plasmids used for the expression of BPV-1 E2TA and E2TR have been described previously (11, 30, 42, 52). Expression of E2 in each plasmid is under the control of the simian virus 40 promoter. The plasmid (p1321) contains the gene for HPV16 E6 and E7 under the control of the β -actin promoter (33). The puromycin selection plasmid, pBabe-puro, was obtained from Stratagene.

Transient transfections. Cells were cotransfected with a total of 10 μ g of DNA using Fugene (Boehringer Mannheim) following manufacturer recommendations.

Colony reduction assay. Cells (10^5 to 2×10^5) were seeded on 6-cm-diameter plates the day before transfection. Cells were transfected with 1 μ g of the pBabe-puro plasmid and 9 μ g of empty vector or the E2TA or E2TR plasmid using Fugene. At 24 h posttransfection, the cells were trypsinized and divided between two 10-cm-diameter plates. After cell attachment, the medium was

replaced with medium containing 10% fetal bovine serum and 0.7 μ g of the selection drug puromycin (Sigma)/ml. After 21 days, cells were washed with 1 $\times$ phosphate-buffered saline, fixed in 10% formalin, and stained with methylene blue. The total number of puromycin-resistant colonies was then determined.

Western blot analysis. Whole-cell lysates were prepared using lysis buffer (0.1 M NaCl, 2 mM EDTA, 20 mM Tris [pH 8.0], 1% Nonidet P-40, 0.01% phenylmethylsulfonyl fluoride, 5 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 μ g each of the protease inhibitors leupeptin, aprotinin, antipain, chymostatin, and pepstatin) (18). Lysed cells were scraped off the plates, transferred to centrifuge tubes, and incubated on ice for 30 min. Cell debris was then removed by centrifugation at 4°C for 10 min. Protein concentration of the supernatant was determined using the Bradford reagent (Bio-Rad). Protein extracts were then normalized for equal protein concentration and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P; NEN), and the membrane was blocked using 5% (wt/vol) nonfat milk in TBS-Tween (10 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1% Tween 20). The blot was then incubated for 1 h with HPV16 E7-specific mouse monoclonal antibody 8c9 (Zymed Laboratories Inc., San Francisco, Calif.). Proteins were detected using enhanced chemiluminescence (ECL; Amersham) and autoradiography. A β -actin monoclonal antibody was obtained from Calbiochem. Whole-cell extracts from rat kidney fibroblasts expressing HPV16 E7 (RKO-7.2) were obtained from K. Munger (26).

Northern blot analysis. Total RNA was isolated from confluent plates using Trizol reagent (BRL, Bethesda, Md.) according to manufacturer instructions. In some experiments, total RNA was also isolated from cytoplasmic extracts of cells using silica gel affinity purification columns (Qiagen) in order to minimize contamination by genomic DNA. RNA samples (10 μ g) were resolved on 1.2% agarose gels containing formaldehyde and transferred to a charged nylon membrane (N+; Amersham). Filters were hybridized at 42°C with randomly primed ³²P-labeled DNA probes specific for HPV16 E6/E7 (p1321) (33), HPV18 E6/E7 (39), or GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Specific bands were quantitated using a densitometer (Molecular Dynamics).

Luciferase assays. HeLa cells plated in six-well dishes were transfected with 1 μ g of 2x2x2E2BS-luciferase reporter plasmid (28) along with 20 ng of E2TA plasmid and increasing amounts of E2TR plasmid. Cell extracts were prepared 48 h after transfection. Cells were washed twice with 1 $\times$ phosphate-buffered saline and then incubated with 200 μ l of cell lysis buffer, consisting of 25 mM Tris-PO₄ (pH 7.8), 15% glycerol, 2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 1% lecithin, 1% bovine serum albumin, 4 mM EGTA (pH 8), 8 mM MgCl₂, and 0.4 mM phenylmethylsulfonyl fluoride, for 20 min at room temperature. Lysates were harvested and spun at 4°C for 5 min to pellet cell debris. For the luciferase assay, 20 μ l of cell lysate was incubated with 130 μ l of cell lysis buffer not containing EGTA. Tubes were placed in a luminometer (AutoLumat LB) automated to inject 300 μ l of luciferase assay buffer (15 mM KH₂PO₄ [pH 7.8], 25 mM glycylglycine, 15 mM MgSO₄, 1 mM dithiothreitol, and 2 mM ATP) along with 100 μ l of luciferin (Analytical Luminescence, Ann Arbor, Mich.) substrate as needed. The relative luciferase activity was calculated by subtracting the background luciferase value from the raw data.

RESULTS

In order to further investigate the mechanism by which expression of E2TA in HPV-positive cell lines induces a growth arrest, we asked whether reexpression of E6 and E7 in the presence of E2TA could restore cellular growth of HeLa cells. We established HeLa cell clones in which expression of E6 and E7 was not repressed by E2TA. For these experiments we used a murine retrovirus expressing HPV16 E6 and E7 from the Moloney leukemia virus long control region (MLV-LTR), which is not regulated by E2 (data not shown). The HPV16 gene products expressed from this construct can be distinguished from the endogenous HPV18 E6 and E7 proteins normally expressed in HeLa cells. Since the HPV16 E6- and E7-expressing HeLa subclones required selection for neomycin resistance encoded by the retrovirus, an alternative antibiotic selection was necessary to conduct subsequent colony reduction assays. After evaluating the toxic effects of other selection antibiotics, puromycin was chosen for selection in the colony reduction assays.

Colony reduction assay. Previous experiments have demonstrated that HPV-positive cervical cancer cell lines (including HeLa, SiHa, and Caski) are susceptible to E2-mediated cellular growth arrest using neomycin resistance (Neo^r) as the cotransfection selection marker (11, 49). Before examining whether expression of E6 and E7 from the retroviral LTR

TABLE 1. Colony reduction assay^a

Cell line	HPV DNA status ^b	Status of gene for ^c :		No. of puromycin-resistant colonies after transfection with:		
		pRb	p53	Vector	E2TA plasmid	E2TR plasmid
HeLa	+	wt	wt	740	70	762
SiHa	+	wt	wt	111	10	117
C33A	—	mut	mut	1,424	1,486	1,392
U2OS	—	wt	wt	564	552	452

^a The indicated cell lines were transfected with vector alone or E2TA or E2TR plasmids, along with pBabe-puro. Twenty-four hours after transfection, cell cultures were divided between two 10-cm-diameter plates, allowed to attach, and placed under selection in media containing 0.7 μ g of the selection antibiotic/ml. The number of drug-resistant colonies was determined after 3 weeks.

^b +, positive; —, negative.

^c wt, wild type; mut, mutant.

could rescue HeLa cells from E2TA-mediated growth suppression, we investigated the effectiveness of puromycin selection in a colony reduction assay. HPV-positive cell lines HeLa (HPV18) and SiHa (HPV16), as well as the HPV-negative C33A cell line, were used. It has previously been reported that C33A cells, which contain mutant forms of p53 and pRb, are not growth inhibited by E2 (11, 24). We also assayed the effect of E2 expression in the human osteosarcoma cell line U2OS. Although U2OS cells do not harbor any HPV sequences, they do express wild-type p53 and pRb proteins as do HeLa and SiHa cells (8). The cell lines were cotransfected with 9 μ g of empty expression vector plasmid or the E2TA or E2TR plasmid and 1 μ g of the puromycin selection plasmid. The total number of puromycin-resistant colonies was determined after 21 days in culture.

The results from a representative experiment are shown in Table 1. Similar to the results of assays using neomycin selection, a significant reduction in the number of colonies was observed with puromycin selection in HeLa cells and SiHa cells following transfection with E2TA plasmid but not with E2TR plasmid. No reduction in colony number was observed for either of the HPV-negative cell lines regardless of the p53 or pRb status of the cell line. These results validated the use of puromycin selection for the colony reduction assay. Additionally, these results suggested that expression of mutated p53 or pRb in C33A cells was not directly responsible for the inability of E2TA to induce cellular growth arrest.

Selection of HeLa cell clones expressing HPV16 E6 and E7.

A series of HeLa clones expressing HPV16 E6 and E7 from a retroviral promoter was generated to test whether expression of E6 and E7 from an E2-nonresponsive promoter could rescue HeLa cells from E2-induced growth arrest. Total RNA was isolated from several independent clones, and HPV16 gene expression was examined by Northern blot analysis. A representative blot is shown in Fig. 1A, and the relative levels of HPV16 E6/E7 RNA, normalized to the GAPDH RNA levels, are shown in Fig. 1B. Various levels of HPV16 E6/E7 mRNA were detected among the clones tested. The highest levels of HPV16 E6/E7 mRNA expression were detected for clones 11 and 18; intermediate levels were detected for clones 2, 7, 16, and 17, and low levels of expression were detected for clones 5, 8, 9, 14, 15, and 20. As expected, no HPV16 E6/E7 mRNA was detected in HPV-negative C33A cells or in HeLa cells that were either not infected or that had been infected with the empty LXSN retroviral vector.

We also examined the levels of HPV16 E7 protein expression in a subset of the clones analyzed in Fig. 1. Whole-cell

extracts of protein were prepared, and 100 μ g of total protein was separated on a SDS-14% PAGE gel. The polyvinylidene difluoride membrane was then probed using monoclonal antibody 8c9 (Zymed Laboratories), which is specific for HPV16 E7 and which does not cross-react with HPV18 E7. As shown in Fig. 2, the levels of HPV16 E7 protein detected corresponded to the levels of E6/E7 mRNA detected by Northern blot analysis. For clones 1, 11, and 18, which expressed the largest amounts of HPV16 E6/E7 mRNA, the largest amounts of HPV16 E7 protein were detected. Clone 1 (not depicted in Fig. 1) was found to express levels of HPV16 E6/E7 mRNA comparable to those expressed by clone 11 (data not shown). Smaller amounts of E7 protein were detected for clones 2, 16, and 17. As a loading control, the membrane was stripped and reprobed with an antibody specific for β -actin (Fig. 2, bottom). We were unable to detect E6 expression due to the lack of a sensitive enough antibody against HPV16 E6. Nevertheless, since a single promoter (P₁₀₅) regulates the expression of both E6 and E7 in HeLa cells, the levels of HPV16 E6 protein likely parallel the levels of HPV16 E7 protein, which we could show to be expressed in the clones.

E6 and E7 can rescue HeLa cells from E2-mediated growth arrest. To investigate whether or not E6 and E7 expression could rescue HeLa cells from the E2-induced growth arrest and to determine whether P₁₀₅ promoter regulation was essential for this growth arrest, a subset of the HPV16 E6- and E7-expressing clones was used in a colony reduction assay. Four independent HPV16 E6- and E7-expressing clones expressing different E6/E7 mRNA levels were cotransfected with 9 μ g of either empty vector or BPV-1 E2TA or E2TR plasmid and 1 μ g of the selection plasmid, pBabe-puro. Transfected cells were then placed under puromycin selection. After 21 days, the puromycin-resistant colonies were fixed with formaldehyde, stained with methylene blue, and counted. The total numbers of colonies from three independent experiments were averaged (Fig. 3).

As expected, transfection with E2TA plasmid but not with E2TR plasmid resulted in a decrease in the number of puromycin-resistant colonies in HeLa cells or in HeLa cells that had been infected with the retroviral LXSN empty vector. In contrast, each of the HeLa cell clones expressing HPV16 E6 and E7 was resistant to the growth inhibition by E2TA. Interestingly, although individual HeLa cell clones analyzed in this experiment expressed different levels of HPV16 E7 protein (see above), all clones were resistant to E2TA-mediated growth inhibition. To ensure that E2TA was expressed and functional in each of the HeLa cell subclones, each clone was assessed for its ability to support E2TA-dependent activation of a luciferase reporter construct. As expected, the E2 activities observed in the HPV16 E6- and E7-expressing HeLa cell subclones were comparable (data not shown). Since the expression of HPV16 E6 and E7 in the HeLa clones is through a promoter not subject to E2TA repression, our results suggested that E2TA-mediated repression of E6 and E7 expression in HeLa cells was required for their growth arrest.

Rescue of HeLa cells by transient coexpression of HPV16 E6 and E7 and E2TA. To further confirm the ability of E6 and E7 to rescue E2-mediated growth arrest in HeLa cells, we also performed colony reduction assays following cotransfection of HeLa cells with E2TA plasmid along with a plasmid for expression of HPV16 E6 and E7. The expression of HPV16 E6 and E7 was under the control of the human β -actin promoter, another promoter that is not repressed by E2TA. Increasing amounts of the HPV16 E6/E7 expression plasmid were able to rescue HeLa cells from the E2-mediated growth arrest (Fig. 4). At the largest amount of the HPV16 E6/E7 plasmid (4 μ g), we

FIG. 1. HPV16 and E6 and E7 expression levels in HeLa cell subclones. (A) Total RNA was isolated from confluent plates of each indicated HeLa clone. Ten micrograms of total RNA was subjected to electrophoresis in a 1.2% formaldehyde gel, transferred to a positively charged nylon membrane (Amersham), and hybridized with a 32 P-radiolabeled probe specific for HPV16 E6/E7 mRNA (Top). Hybridization with a GAPDH probe was used to normalize the data for loading variability (bottom). (B) The relative levels of HPV16 E6/E7 mRNA were quantitated using a densitometer (Molecular Dynamics) and normalized against the GAPDH RNA levels. The relative levels of normalized mRNA relative to SiHa HPV16 mRNA levels were graphed. RNA from HPV18-positive HeLa cells was used as the negative control. RNA from the HPV16-positive SiHa cell line was used as a positive control. HPV16 and HPV18 probes do not cross-hybridize under the stringent annealing and washing conditions used.

observed approximately 60% rescue of the E2-mediated colony reduction. Considering that a full rescue was observed in HeLa cells constitutively expressing HPV16 E6 and E7, the partial rescue detected by plasmid cotransfection may be reflective of the percentages of cells that were successfully co-transfected with both the E2TA and the HPV16 E6/E7 plasmid. These results, however, do confirm that expression of HPV16 E6 and E7 can rescue HeLa cells from E2-mediated growth arrest.

E2TR does not repress HPV18 E6 and E7 expression in HeLa cells or clonal lines. We next examined the effect of E2 on the integrated HPV18 P₁₀₅ promoter. Although it has been reported that full-length E2 represses expression of E6 and E7 in HPV-positive cervical cancer cells, conflicting evidence exists in the literature regarding the regulation of the E6/E7 promoter (P₁₀₅) by E2TR. Although E2TR contains the DNA-binding domain and dimerization domain and can inhibit the transactivation activity of full-length E2 (2, 31), it is unable to induce growth arrest in HeLa cells (11, 16). Also, Goodwin et al. found that neither transactivation-defective BPV-1 E2 mutants nor forms of E2 lacking the transactivation domain could repress the HPV18 E6/E7 promoter in Northern analysis of E6

and E7 transcripts in HeLa cells (16). In contrast, Desaintes et al. reported that E2TR was capable of repressing the HPV18 E6/E7 promoter in RNA primer extension assays (7). Since our data indicated that E6/E7 promoter regulation by E2TA is required for growth arrest, we investigated whether E2TA still repressed the integrated HPV18 P₁₀₅ promoter in the HeLa cell clones expressing HPV16 E6 and E7 from the MLV-LTR. We also examined whether E2TR could repress the integrated HPV18 P₁₀₅ promoter in HeLa cells as well as in a representative HeLa clone (clone 16) stably expressing HPV16 E6 and E7. Cells were transfected with empty vector or the E2TA or E2TR plasmid and selected with puromycin for 5 days. Following selection, cytoplasmic RNA was isolated, electrophoresed on a 1.2% formaldehyde gel, transferred to a membrane,

FIG. 2. HPV16 E7 protein levels in HeLa cell subclones. Whole-cell lysates from HPV16 E6- and E7-expressing clones were resolved by SDS-PAGE and then analyzed by immunoblotting with monoclonal antibody 8c9, specific for HPV16 E7, as indicated (top). Extracts from normal HeLa cells were used as a negative control. As positive controls, whole-cell extracts from rat kidney fibroblasts cells constitutively expressing HPV16 E7 (RKO-7.2) (27), as well as from the HPV16-positive cell line SiHa, were used. To control for protein loading, the blot was also probed with an antibody specific for actin (bottom).

FIG. 3. Colony reduction assay. Normal HeLa cells, retroviral LXSN vector cells, and HPV16 E6- and E7-expressing HeLa cell clones 1, 2, 16, and 17 were maintained in DMEM supplemented with 10% fetal bovine serum. Cells were seeded into six-well dishes at densities that resulted in approximately 50% confluence after 24 h. As described in Materials and Methods, cells were transfected with 1 μ g of pBabe-puro along with 9 μ g of vector or the E2TA or E2TR plasmid using Fugene (Boehringer Mannheim). After 21 days, the remaining cells were fixed and stained with methylene blue and the colonies were counted. The averaged total numbers of colonies from three independent experiments are shown. E2TA, full-length BPV-1 E2 protein; E2TR, BPV-1 E2 variant lacking the N terminus transactivation domain; vector, empty expression plasmid.

FIG. 4. Dose response of transient HPV16 E6 and E7 expression on E2-mediated growth arrest. HeLa cells were cotransfected with 6 μ g of empty vector or the E2TA plasmid along with increasing concentrations of HPV16 E6/E7 plasmid as indicated. After 21 days, the total number of puromycin-resistant colonies was determined as described in the legend to Fig. 3. Results shown are representative of a single experiment.

and probed for levels of HPV18 E6/E7 (Fig. 5A), HPV16 E6 and E7 (Fig. 5B), and GAPDH (as a loading control) (Fig. 5C) mRNA. As demonstrated in Fig. 5, decreased levels of HPV18 E6/E7 mRNA were observed only with E2TA-transfected cells. No decrease was detected in HeLa cells transfected with either the E2TR plasmid or the empty vector. These results confirm the observation by Goodwin et al. that E2TA, but not E2TR, represses the integrated HPV18 E6/E7 promoter in HeLa cells. Additionally, this repression does not appear to be affected by exogenously expressed E6 and E7.

In order to ensure that the E2TR expressed in the HeLa cells was functional in these experiments, we tested its ability to interfere with E2TA-mediated transcriptional activation of a reporter plasmid (30). E2TA transcriptional activation requires the binding of full-length E2TA homodimers to E2-binding sites in E2-responsive promoters. E2TR can competitively inhibit the ability of E2TA to activate the transcription

of genes with E2-binding sites in the promoter (30). Increasing amounts of the E2TR plasmid were cotransfected into HeLa cells with a constant amount of E2TA plasmid and an E2TA-responsive luciferase reporter gene (2x2xE2BS-luc) (28). E2TR did repress E2TA-mediated transcriptional activation of the luciferase gene in a dose-dependent manner (Fig. 6), confirming that the transfected E2TR was functional in our experiments. We therefore conclude that E2TR is unable to repress transcription of the integrated HPV18 P₁₀₅ promoter in HeLa cells.

DISCUSSION

Understanding the mechanism by which E2 induces cellular growth arrest is complicated by the multifunctional roles of this transcription factor. E2 can function as either an activator or repressor of viral gene transcription depending upon the location of the E2-binding sites within the promoter (4, 19). The papillomavirus E2 proteins can strongly activate heterologous promoters through E2-responsive elements in a position- and orientation-independent manner (43). This activation has been shown in transient transfection assays (as depicted in our experiments to assess E2TR function [Fig. 6]), as well as in *in vitro* transcription with purified E2 protein (46).

For the mucosal HPVs that infect the genital tract however, E2 proteins primarily down-regulate transcription of the viral promoters that control expression of E6 and E7 (4, 37, 44, 47). The HPV18 and HPV16 E6/E7 promoters (P₁₀₅ and P₉₇, respectively) each contain four E2-binding sites located upstream of the TATA box. Two of these E2-binding sites are in close proximity to the TATA box and overlap with an SP1 site. Binding of E2 to these two sites is thought to interfere with SP1 binding and with formation of the preinitiation complex, resulting in promoter repression (6, 10, 45).

The expression of E2 in HeLa cells induces cellular growth arrest (11, 24) and an apoptotic response in a subset of the cells (7). Through repression of E6 and E7, there is an increase in p53 levels resulting from the loss of E6 protein targeting of p53

FIG. 5. Northern blot analysis of HPV18 E6/E7 mRNA levels. Cytoplasmic total RNA was isolated from the indicated cell lines transfected with empty vector (vector) or the E2TA or E2TR plasmid and electrophoresed on a 1.2% agarose gel containing formaldehyde. The membrane was hybridized with a ³²P-radiolabeled probe specific for HPV18 E6/E7 (A), HPV16 E6/E7 (B), or GAPDH (C) mRNA.

FIG. 6. E2TR inhibition of reporter gene transcription in HeLa cells. HeLa cells were cotransfected with 20 ng of the E2TA plasmid, 1 µg of a luciferase reporter plasmid containing four E2-binding sites, and increasing amounts of the E2TR plasmid. Cellular extracts were prepared at 48 h posttransfection, and luciferase activity was measured. The basal activity of the luciferase reporter plasmid in the absence of E2TA was 391. The graphed results are the averages of three independent experiments.

ubiquitination (38, 50) and an accumulation of hypophosphorylated Rb (11, 23). The increase in p53 levels induces the transcription of its downstream effectors including the cyclin-dependent kinase (cdk) inhibitor p21/cip1/waf1 (11, 23). The accumulation of the hypophosphorylated Rb is presumably a direct consequence of E7 no longer triggering its degradation. There may be other cell cycle targets affected. Naeger et al. have reported that E2 causes a repression of the *cdc25A* and *cdc25B* genes, which may result in a reduction in cdk2 activity, contributing to an increase in pRb activity (35).

Although the effects of E2 on the cell cycle through key regulators including p53 and pRb can be explained by turning off HPV18 E6 and E7 expression in HeLa cells, it had not been formally established that repression of E6 and E7 expression was required for E2-mediated growth arrest. The rescue of HeLa cells from E2-mediated growth arrest by ectopic expression of E6 and E7 establishes the necessity of E6/E7 promoter repression for this effect. Taken together, our results are consistent with a simple model for the E2-mediated growth arrest of HPV-positive cervical cancer cells, based on E6/E7 promoter repression triggering cellular growth arrest through lowering the levels of E6 and E7, leading to the reestablishment of the p53 and pRb pathways (11, 24). We are currently examining the individual contributions of the p53 and the pRb pathways to the establishment of the growth inhibition in HeLa cells.

There have been conflicting reports in the literature regarding the ability of E2TR to repress the P_{105} promoter. According to results by Desaintes et al. (7), E2TR is able to repress the E6/E7 promoter in HeLa cells. In experiments reported by Goodwin et al. (16), neither E2TR nor a panel of transactivation-deficient E2 mutants was able to repress this promoter. Recently, it was demonstrated through use of a series of transactivation-defective mutants of HPV16 E2 that an intact transactivation function is required for repression of the E6/E7 promoter (35a). This is also in agreement with the findings of Goodwin et al. We also did not observe repression of the

HPV18 E6/E7 promoter in HeLa cells transfected with E2TR (Fig. 5). Our results therefore confirm the findings by Goodwin et al. but not those of Desaintes et al. Presumably, the differences among these various studies are due to different experimental conditions or the particular assays employed in the respective studies.

The critical function conferred by the E2 transactivation domain in suppressing cell growth and in repressing the E6/E7 promoter appears to be a unique characteristic of the papillomavirus E2TA domain that is not shared by other transactivation domains. Chimeric E2 proteins composed of the E2 DNA-binding domain and other cellular or viral transactivation domains do not arrest HeLa cell growth (11, 16). One critical question that still remains is why E2TR is unable to repress the endogenous HPV18 promoter for E6 and E7 expression although it has an intact DNA-binding/dimerization domain. Further studies will be necessary to address this question. Other questions still to be answered include, What other relevant targets of E6 and E7 contribute to the E2-mediated growth arrest? and Are there other functions of E2, independent of its effects on the E6/E7 promoter, that are involved in mediating the cellular growth inhibition?

Nishimura et al. (35a) have found that an intact E2 transactivation function is required for binding of E2 to the E6/E7 promoter of the integrated HPV18 genome in HeLa cells. Their observations suggest that the intact transactivation domain of E2 is required to enhance the sequence specific binding of E2 to chromatin. The identification of the mechanism by which the transactivation domain of E2 facilitates this binding will likely involve an interaction of E2 with specific cellular factors. E2 has been shown to associate with a number of cellular proteins, including components of the basal transcriptional machinery such as TFIIB (3, 36, 53) and TFIID (36). In addition, using a yeast two-hybrid screen, Breiding et al. (5) have shown that the transactivation domain of BPV-1 E2 interacts with the transcription factor AMF-1 when E2 is bound to DNA. This interaction with E2 appears to be important for

E2 to stimulate transcription (5). A clearer understanding of the mechanisms and cellular factors involved in the transactivation and repression functions of E2 should help to elucidate whether or not E2 functions in addition to the repression of the integrated E6/E7 promoter are required for growth suppression of cervical cancer cells.

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