

# Role of the Retinoblastoma Pathway in Senescence Triggered by Repression of the Human Papillomavirus E7 Protein in Cervical Carcinoma Cells

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## ABSTRACT

Repression of the endogenous human papillomavirus (HPV) type 18 E7 gene in HeLa cervical carcinoma cells by the bovine papillomavirus E2 transcription factor activates the retinoblastoma (Rb) pathway and induces cells to undergo senescence. To determine whether activation of the Rb pathway is responsible for senescence in response to HPV18 E7 repression, we tested the ability of wild-type and mutant E7 proteins to affect the activity of the Rb pathway and to modulate senescence in these cells. Enforced expression of the wild-type HPV16 E7 protein prevented Rb activation in response to E2 expression and impaired senescence. Importantly, there was an absolute correlation between the ability of mutant E7 proteins to inactivate the Rb pathway and to inhibit senescence in HeLa cells. Similar results were obtained in HT-3 cervical carcinoma cells. These results provide strong genetic evidence that activation of the Rb pathway is required for senescence in response to E7 repression. Hence, continuous neutralization of the Rb pathway by the E7 protein is required to maintain the proliferation of cervical carcinoma cells. Similarly, our results indicate that activation of the Rb pathway can prevent apoptosis induced by repression of the HPV18 E6 gene in HeLa cells.

## INTRODUCTION

Cervical carcinoma, a leading cause of cancer mortality in women worldwide, is initiated by infection with high-risk human papillomaviruses (HPV), usually HPV16 or HPV18. Integration of the viral DNA into the infected host cell genome often disrupts the gene that encodes the E2 protein, a viral transcription factor that can repress expression of the HPV E6 and E7 oncogenes (1). The high-risk HPV E6 protein binds to p53 and stimulates its ubiquitin-directed degradation (2–4). The E6 protein also stimulates the activity of telomerase, which maintains the ends of chromosomes (5).

An important target of the high-risk E7 protein is the retinoblastoma (Rb) family of tumor suppressor proteins (6). p105<sup>Rb</sup> and the Rb family members p107 and p130 regulate the activity of E2F transcription factors (7). Complexes consisting of E2F and hypophosphorylated p105<sup>Rb</sup> repress the transcription of genes such as cyclin A that are required for cell cycle progression, and repression is relieved by cdk-mediated phosphorylation of p105<sup>Rb</sup>. The high-risk HPV E7 protein binds to Rb family members and disrupts Rb/E2F complexes, resulting in increased expression of E2F-responsive genes (7–10). In addition, the E7 protein induces rapid degradation of hypophosphorylated Rb family members (11–15). Rb binding and degradation by the E7 protein are both required for full inactivation of Rb function. A conserved LXCXE motif in the E7 protein is absolutely required for E7/Rb binding and accelerated proteasome-mediated degradation of

Rb family members, and an NH<sub>2</sub>-terminal segment of the E7 protein is required for Rb degradation but not for Rb binding (11, 16–22). The E7 protein binds to numerous other cellular proteins, but the physiological consequences of these interactions are largely unknown (6).

The E6 and E7 proteins have profound effects on cells. Primary human cells cannot be passaged indefinitely in culture, rather they undergo replicative senescence, an irreversible growth-arrested state characterized by a constellation of features including flattened morphology, elevated autofluorescence, and increased senescence-associated  $\beta$ -galactosidase (SA $\beta$ -gal) activity (23). Genetic studies in primary keratinocytes revealed that activation of telomerase and inactivation of the Rb and p53 pathways are required to bypass senescence and immortalize cells (24–26). Expression of the high-risk E6 and E7 genes also allows primary keratinocytes to bypass senescence, but HPV-immortalized cells are not tumorigenic, implying that additional events are required for carcinogenic progression (2, 6).

Continuous expression of the high-risk HPV E6 and E7 proteins is required to maintain the proliferative state of cervical cancer cells. Antisense and RNA interference-mediated repression of HPV gene expression in cervical carcinoma cell lines typically results in a severalfold inhibition of proliferation (*e.g.*, Refs. 27 and 28). More dramatic effects are induced by the papillomavirus E2 proteins. Introduction of the bovine papillomavirus (BPV) E2 gene into cervical carcinoma cell lines or HPV-immortalized keratinocytes represses expression of the resident HPV genomes, resulting in activation of the p53 and Rb pathways and inhibition of telomerase activity (29–36). E2-mediated repression of E6 and E7 expression can cause close to 99% inhibition of cellular DNA synthesis within 2 days, loss of colony forming ability, and the rapid acquisition of a senescent phenotype by virtually every cell in the population, suggesting that the E6 and E7 proteins actively prevent the execution of a senescence program (31, 32, 34, 37). BPV E2-induced senescence requires repression of the HPV oncogenes and does not occur in cells devoid of HPV DNA (32, 38, 39).

Expression of the E2 protein in HeLa cells engineered to constitutively express the HPV16 E6 protein represses the endogenous HPV18 E7 protein, resulting in activation of the Rb pathway and senescence, even though continued expression of the HPV16 E6 protein prevents activation of the p53 pathway (39). Furthermore, E2-mediated repression of HPV30 expression induces senescence in HT-3 cervical carcinoma cells, even though these cells do not contain transactivation-competent p53 (31, 40). Taken together, these findings imply that repression of E7 is sufficient to induce a robust, p53-independent senescence response.

The cellular signaling pathways responsible for senescence induced by E7 repression have not been defined. In this report, we tested the hypothesis that Rb activation is required for induced senescence when the E7 gene is repressed. An exogenous HPV16 E7 gene inhibits senescence caused by E2-mediated repression of the endogenous HPV18 E7 gene in HeLa cells (39). Here, we tested the ability of a panel of E7 mutants to inhibit senescence triggered by E7 repression, with the rationale that senescence would not be inhibited by E7 mutants defective for Rb inactivation if senescence required Rb sig-

Received 12/1/03; revised 2/12/04; accepted 2/23/04.

**Grant support:** NIH Grants AI55403 and AR07016 (K. E. Yates and L. Manuelidis), NIH Grant CA16038 (D. DiMaio).

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naling. Our results provide strong evidence that activation of the Rb pathway is required for senescence in response to E7 repression.

## MATERIALS AND METHODS

**Cells and Viruses.** All HeLa cell derivatives were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin and streptomycin, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. HeLa/sen2 cells, a cloned strain of HeLa cells that responds vigorously to E2 expression (31), were infected with the empty retrovirus vector LXS<sub>N</sub> and derivative retroviruses containing the wild-type HPV16 E7 gene or the following E7 mutants:  $\Delta 6-10$ ;  $\Delta 21-24$ ; S31G/S32G; and A50S (obtained from Denise Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA; Refs. 19 and 41). Stable cell lines were expanded from individual colonies after 2 weeks of selection in medium containing 1 mg/ml G418. Cells were maintained in medium containing 500  $\mu$ g/ml G418. HeLa/sen2 cells were also infected with RVY-HPV16/E6, a retrovirus expressing the HPV16 E6 gene and a gene conferring hygromycin resistance. Cell lines derived from individual colonies resistant to 250  $\mu$ g/ml hygromycin B were screened by Northern blotting for HPV16 E6 mRNA. One cell line, designated HeLa/16E6H cells, expressed similar levels of HPV16 E6 and HPV18 E6 mRNA and was chosen for additional experiments. HeLa/16E6H cells were infected with LXS<sub>N</sub> and derivative retroviruses expressing wild-type HPV16 E7, E7 $\Delta_{6-10}$ , or E7 $\Delta_{21-24}$ . Stable cell lines were expanded from individual colonies after 2 weeks of selection in medium containing 125  $\mu$ g/ml hygromycin and 1 mg/ml G418 and were maintained in medium containing 125  $\mu$ g/ml hygromycin and 500  $\mu$ g/ml G418. HT-3 cells maintained in McCoy's medium supplemented with 15% fetal bovine serum, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and penicillin and streptomycin were infected with LXS<sub>N</sub> and derivative viruses expressing wild-type HPV16 E7 or HPV16 E7 $\Delta_{21-24}$ . Polyclonal cell lines were established by pooling colonies on plates containing at least 100 colonies resistant to 200  $\mu$ g/ml G418.

High-titer stocks of the SV40/BPV type 1 (BPV-1) recombinant virus expressing the BPV-1 E2 protein prepared in CMT4 cells (hereafter referred to as the E2 virus) were used to infect cells at a multiplicity of infection of 20 as described previously (40). Cells were infected and maintained in the absence of drug selection with medium replaced every 3 days for the duration of the experiment.

**Western Blots.** To detect the HPV16 E7 protein,  $4 \times 10^5$  cells/100-mm dish were plated. The next day, cells were infected with E2 virus or mock-infected. Two days later the cells were harvested and resuspended in E7 lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% NP40, 0.5 mM DTT, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10  $\mu$ g/ml aprotinin and leupeptin (42)]. The cell lysates were incubated with rotation at 4°C for 30 min and spun at 15,000 rpm for 20 min at 4°C, and the supernatants were collected. Thirty or 50  $\mu$ g of total extract were resolved by electrophoresis in 15% SDS gels, transferred to Immobilon-P membranes (Millipore, Bedford, MA) in 12.5 mM Tris-0.1 M glycine-20% methanol transfer buffer, and blocked in 5% milk-TBST buffer [5% nonfat dry milk, 25 mM Tris-HCl (pH 8.0), 125 mM NaCl, and 0.5% Tween] for 1 h at 4°C. The membranes were then incubated in either 0.5  $\mu$ g/ml Zymed 8-C9 (which does not recognize E7 $\Delta_{6-10}$ ) or 1  $\mu$ g/ml SC-6981 (which does not recognize E7 $\Delta_{21-24}$ ) in 5% milk-TBST buffer for 1 h at room temperature. The membranes were then washed in TBST five times for 5 min at room temperature and then incubated in a 1:10,000 dilution of horseradish peroxidase-conjugated donkey antimouse antibody (Jackson ImmunoResearch, West Grove, PA) in 5% milk-TBST buffer for 1 h at room temperature. The membranes were then washed again in TBST five times for 5 min at room temperature and incubated with ECL+ (Amersham Biosciences, Little Chalfont, United Kingdom), and the signals were detected by Hyperfilm (Amersham Biosciences). Western blotting for p105<sup>Rb</sup>, cyclin A, and p53 was carried out as described previously (39).

**Phenotypic Analysis.** Cellular DNA synthesis was determined by incorporation of tritiated thymidine 2 (HeLa) or 3 (HT-3) days after mock infection or infection with E2 virus as described previously (32). Autofluorescence, annexin V binding, and SA $\beta$ -gal activity were measured as described previously (31, 39).

## RESULTS

**Expression of Mutant HPV16 E7 Proteins in HeLa Cells.** The E2 protein represses the endogenous HPV18 E6 and E7 genes in HeLa cells and induces greater than 95% of the cells to undergo growth arrest and senescence. However, when the wild-type HPV16 E7 protein is constitutively expressed in these cells, approximately one-third of the cells fail to undergo senescence in response to the E2 protein. These nonsenescent cells continue to proliferate and undergo a wave of p53-dependent apoptosis 5–7 days after E2 expression (39, 43). The ability of the E7 protein to provide partial protection against senescence and to induce apoptosis in response to E2 expression provides an assay for the activity of E7 mutants. We used recombinant retroviruses to introduce wild-type and mutant HPV16 E7 genes into HeLa cervical carcinoma cells. The following HPV16 E7 mutations were tested: deletion  $\Delta 6-10$ , located in the NH<sub>2</sub>-terminal segment of the E7 protein, which does not interfere with binding to Rb family members but does interfere with their degradation; deletion  $\Delta 21-24$ , which blocks Rb binding and degradation by removing the LXCXE motif; S31G/S32G, which is located near the LXCXE motif and eliminates E7 phosphorylation by casein kinase II; and A50S, a phenotypically silent mutation located near the middle of the E7 protein (44).

The empty retrovirus vector (LXS<sub>N</sub>) and vectors containing the wild-type and mutant HPV16 E7 genes were used to infect HeLa/sen2 cells, and individual G418-resistant colonies were expanded to generate clonal cell lines. Western blotting confirmed the absence of HPV16 E7 protein from the HeLa/LXS<sub>N</sub> cells and demonstrated that the other cell lines expressed similar levels of the wild-type or mutant HPV16 E7 protein (Fig. 1). The E2 protein was then acutely introduced into these cells by infection with a recombinant SV40-based viral vector expressing the BPV E2 protein, which acts as a transcriptional repressor of the endogenous HPV18 E6 and E7 genes in HeLa cells but not the introduced HPV16 E7 gene driven by the retroviral long terminal repeat. Thus, after E2 expression, the exogenous wild-type or mutant HPV16 E7 protein is the only HPV oncogene product expressed in the cells, providing an assay for the activity of the E7 protein.

**Biochemical Analysis of E7 Mutant Cell Lines after Repression of the Endogenous HPV18 E6 and E7 Oncogenes.** The biochemical and physiological responses of these cells to E2 expression were determined. As expected, 2 days after E2 infection, expression of the endogenous HPV18 E6-E7 oncogenes was markedly repressed in all of the cell lines (data not shown). The E2 protein caused a marked increase in the level of p53 in all of the cell lines due to repression of the endogenous HPV18 E6 gene and loss of E6-induced destabilization of p53 (Fig. 2, *bottom panel*). Furthermore, the E2 protein caused a decrease in the level of hyperphosphorylated p105<sup>Rb</sup> in all cell lines (Fig. 2, *top panel*), presumably at least in part as a consequence of p53-mediated induction of the cdk inhibitor, p21.

In the absence of the E2 protein, all of the cell lines displayed

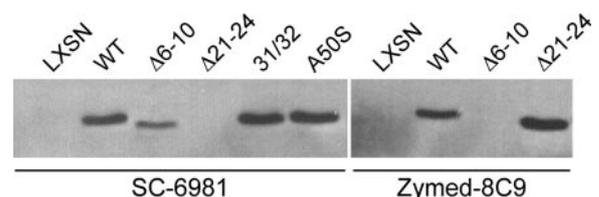


Fig. 1. Expression of exogenous HPV16E7 protein. HeLa/LXS<sub>N</sub> (LXS<sub>N</sub>), HeLa/16E7-3 (WT), HeLa/16E7 $\Delta_{6-10}$  clone 6 ( $\Delta 6-10$ ), HeLa/16E7 $\Delta_{21-24}$  clone 9 ( $\Delta 21-24$ ), HeLa/16E7<sub>G31S/G32S</sub> clone 4 (31/32), and HeLa/16E7<sub>A50S</sub> clone 5 (A50S) cells were harvested in E7 lysis buffer. Fifty  $\mu$ g of extracted protein were subjected to electrophoresis and immunoblotted with the indicated HPV16E7 antibodies.

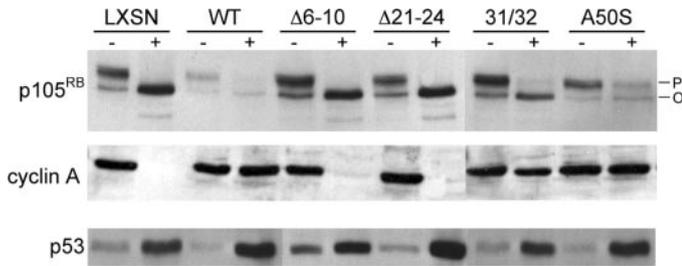


Fig. 2. Biochemical effects of HPV repression. Trizol protein extracts were made 2 days after mock infection (–) or infection with the E2 virus (+) from the cell lines listed in the legend to Fig. 1. Five  $\mu$ g of extracted protein of each sample were subjected to electrophoresis and immunoblotted with antibodies specific for p105<sup>Rb</sup>, cyclin A, and p53, as indicated. The hyperphosphorylated and hypophosphorylated species of p105<sup>Rb</sup> are indicated by P and O, respectively.

similar patterns of p105<sup>Rb</sup> expression and phosphorylation, in which the slowly migrating, hyperphosphorylated form is the most prominent species (Fig. 2, top panel). Less p105<sup>Rb</sup> was present in the cells transduced with the wild-type E7 gene than in the other cell lines. Expression of the E2 protein in HeLa/LXSN cells, which express no exogenous E7 protein, caused a marked increase in the level of the hypophosphorylated, active form of p105<sup>Rb</sup> due to repression of endogenous HPV18 E7 in these cells and loss of E7-mediated p105<sup>Rb</sup> destabilization. Constitutive expression of the wild-type HPV16 E7 protein or E7<sub>A50S</sub> prevented E2-mediated induction of hypophosphorylated p105<sup>Rb</sup>, because these E7 proteins bind this form of p105<sup>Rb</sup> and induce its accelerated degradation. In contrast, in cells expressing E7<sub>Δ6-10</sub> or E7<sub>Δ21-24</sub>, the E2 protein induced the expression of hypophosphorylated p105<sup>Rb</sup> to a level comparable with that in cells not expressing an exogenous E7 protein, consistent with the inability of these mutants to stimulate Rb degradation. Constitutive expression of E7<sub>S31G/S32G</sub> resulted in a slight increase in the level of the hypophos-

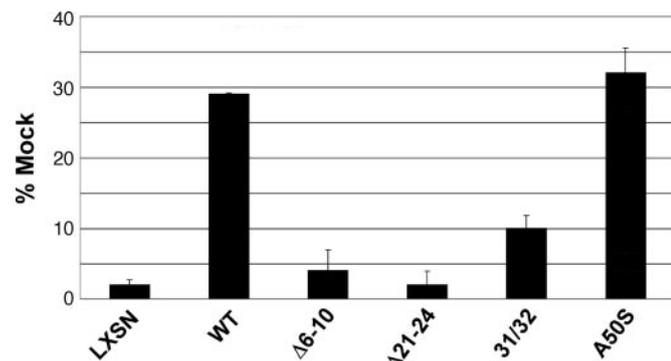
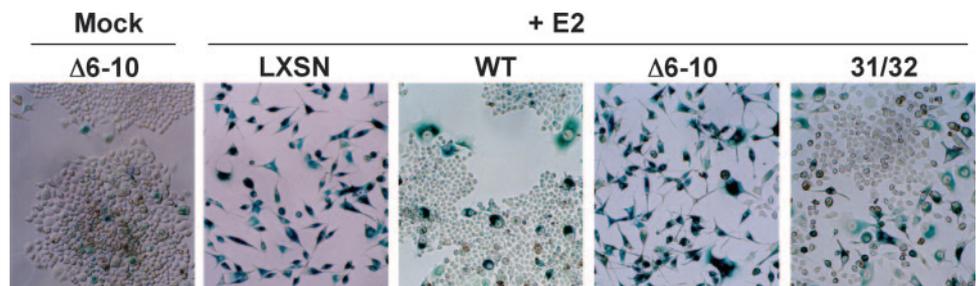


Fig. 3. Effect of HPV repression on DNA synthesis. Cell lines (designated as in the legend to Fig. 1) were infected with the E2 virus or mock-infected, and 2 days later, DNA synthesis was measured in triplicate by [<sup>3</sup>H]thymidine incorporation. DNA synthesis by the infected cells is expressed as percentage of synthesis by mock-infected cells (% Mock). The results of three independent experiments were averaged, and the error bars represent one SD.

Fig. 4. Effect of HPV repression on SA $\beta$ -gal activity. Mock-infected HeLa/16E7<sub>Δ6-10</sub> colonies (Δ6-10) and E2-infected HeLa/LXSN (LXSN), HeLa/16E7-3 (WT), HeLa/16E7<sub>Δ6-10</sub> (Δ6-10), and HeLa/16E7<sub>31/32</sub> (31/32) cells were stained at pH 6.0 with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside 10 days after infection with the E2 virus or mock infection and photographed by bright-field microscopy.



phorylated form of Rb after E2 infection. We also assessed the expression of cyclin A, the product of an E2F-responsive gene (Fig. 2, middle panel). The E2 protein repressed expression of cyclin A in HeLa/LXSN cells, indicating that the Rb pathway was activated, but not in HeLa/16E7 and HeLa/16E7<sub>A50S</sub> cells. Cyclin A expression was also inhibited by the E2 protein in the HeLa/16E7<sub>Δ6-10</sub> and HeLa/16E7<sub>Δ21-24</sub> cells, whereas HeLa/16E7<sub>S31G/S32G</sub> cells displayed slight repression of cyclin A expression. Thus, E7<sub>Δ6-10</sub> and E7<sub>Δ21-24</sub> are defective in their ability to inactivate the Rb pathway in cervical carcinoma cells, and repression of the endogenous E7 protein in cells expressing these mutants activates the Rb pathway.

**Effect of E7 Mutants on the Cellular Response to the E2 Protein.** To assess the effect of the E7 mutants on cell proliferation, we measured incorporation of tritiated thymidine into DNA 2 days after E2 infection or mock infection (Fig. 3). As reported previously, the E2 protein dramatically inhibited DNA synthesis in HeLa/LXSN cells due to repression of the endogenous HPV18 E6 and E7 genes (39). Constitutive expression of the wild-type HPV16 E7 protein provided substantial protection against E2-mediated growth inhibition, with E2-infected HeLa/16E7 cells displaying approximately 30% the DNA synthesis of uninfected cells. Notably, E2 expression caused dramatic inhibition of DNA synthesis in HeLa/16E7<sub>Δ6-10</sub> and HeLa/16E7<sub>Δ21-24</sub> cells, similar to the level of inhibition displayed by cells without constitutive E7 expression. In contrast, infected HeLa/16E7<sub>A50S</sub> cells displayed similar levels of DNA synthesis as cells expressing the wild-type E7 protein, and HeLa/16E7<sub>S31G/S32G</sub> cells displayed intermediate levels of DNA synthesis. Similar results were obtained with multiple independent isolates of each genotype. Thus, the wild-type E7 protein and E7<sub>A50S</sub>, both of which prevent activation of the Rb pathway, conferred substantial protection against E2-induced growth arrest, whereas the two mutants defective for Rb inactivation did not provide protection, and the mutant that allowed partial activation of the Rb pathway provided partial protection.

We also measured SA $\beta$ -gal expression (Fig. 4; data not shown). The colonies arising from mock-infected cells showed faint background staining (mock-infected HeLa/16E7<sub>Δ6-10</sub> cells are shown as a representative example). After infection with the E2 virus, virtually all HeLa/LXSN cells displayed intense blue staining indicative of SA $\beta$ -gal activity and cellular senescence. In contrast, as reported previously (39), the E2-infected HeLa/16E7 cultures contained cells displaying a flattened morphology and SA $\beta$ -gal activity interspersed with numerous proliferating colonies that did not stain, demonstrating partial protection from senescence by the wild-type HPV16 E7 protein. Notably, expression of the E2 protein in HeLa/16E7<sub>Δ6-10</sub> or HeLa/16E7<sub>Δ21-24</sub> (data not shown) cells resulted in uniform high-level SA $\beta$ -gal activity, demonstrating that these E7 mutants did not inhibit senescence. E2-infected HeLa/16E7<sub>A50S</sub> (data not shown) and HeLa/16E7<sub>S31G/S32G</sub> cells displayed a mixture of proliferating SA $\beta$ -gal-negative cells and flat, nonproliferating SA $\beta$ -gal-positive cells.

Because HeLa cells constitutively expressing the wild-type HPV16 E7 protein undergo apoptosis as well as senescence upon repression of

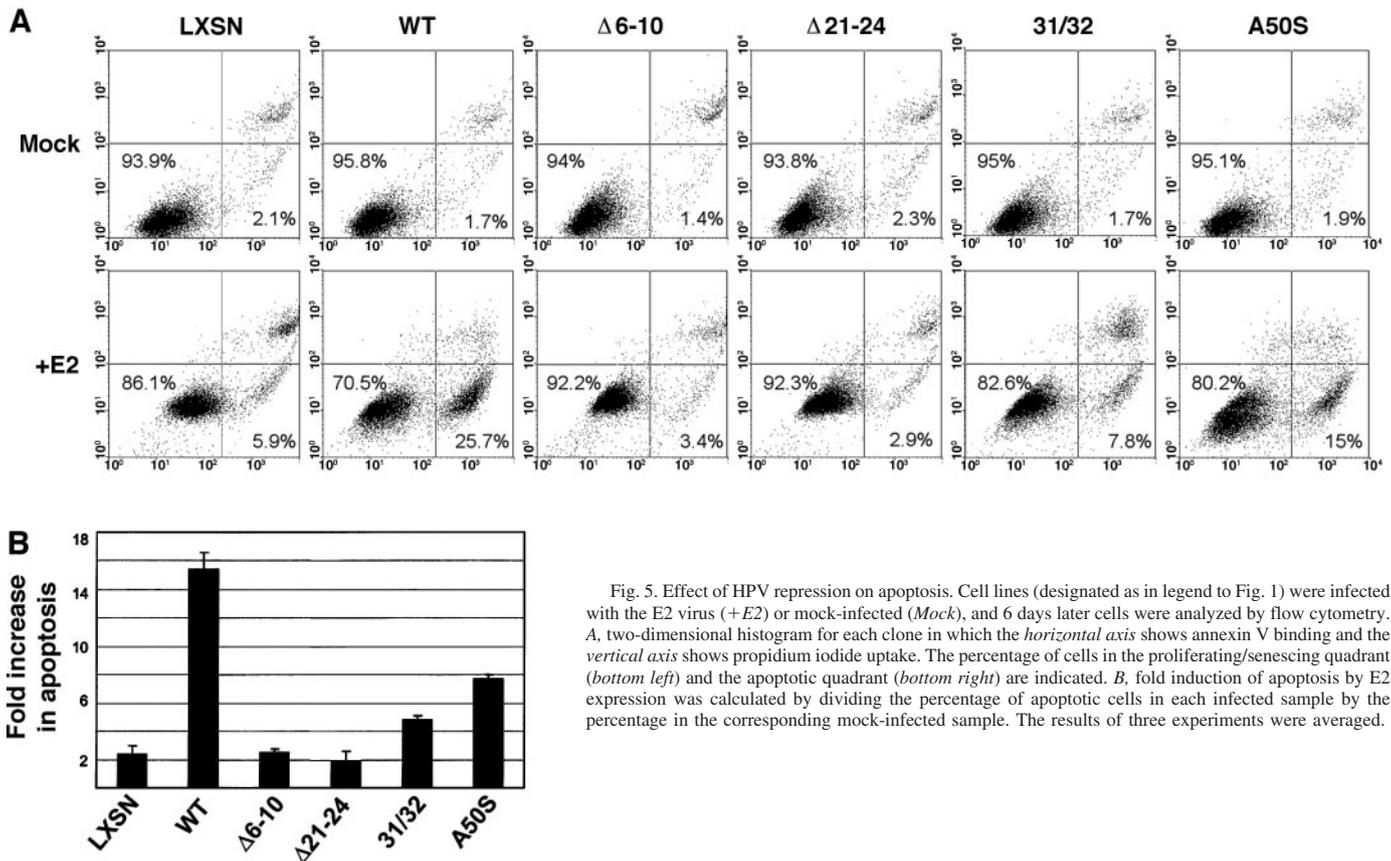


Fig. 5. Effect of HPV repression on apoptosis. Cell lines (designated as in legend to Fig. 1) were infected with the E2 virus (+E2) or mock-infected (Mock), and 6 days later cells were analyzed by flow cytometry. A, two-dimensional histogram for each clone in which the horizontal axis shows annexin V binding and the vertical axis shows propidium iodide uptake. The percentage of cells in the proliferating/senescent quadrant (bottom left) and the apoptotic quadrant (bottom right) are indicated. B, fold induction of apoptosis by E2 expression was calculated by dividing the percentage of apoptotic cells in each infected sample by the percentage in the corresponding mock-infected sample. The results of three experiments were averaged.

the endogenous HPV18 oncogenes (39), we also measured apoptosis. Nonpermeabilized cells were stained with annexin V and propidium iodide 6 days after E2 infection or mock infection and analyzed by flow cytometry (Fig. 5A). In this analysis, early apoptotic cells bind annexin but exclude propidium iodide and are visualized in the lower right quadrant of a two-dimensional plot. Few E2-infected HeLa/LXSN cells displayed annexin binding, although these cells showed increased fluorescence in the lower left quadrant, indicative of increased autofluorescence due to senescence. In contrast, E2-infected HeLa/16E7 cells displayed a greater than 10-fold increase in the number of apoptotic cells compared with mock-infected cells. Strikingly, E2 expression did not induce elevated apoptosis in cells expressing either of the Rb-defective mutants, E7 $_{\Delta 6-10}$  or E7 $_{\Delta 21-24}$ , but these cells displayed an autofluorescence shift characteristic of senescing cells (39). Thus, cells expressing E7 mutants unable to inactivate the Rb pathway underwent senescence but not apoptosis, as did cells without constitutive E7 expression. E2-infected HeLa/16E7 $_{\Delta 50S}$  and HeLa/16E7 $_{S31G/S32G}$  cells displayed intermediate levels of apoptosis. The results of multiple independent annexin-binding experiments are summarized in Fig. 5B.

The experiments described above indicated that E7 proteins able to inactivate the Rb pathway inhibited senescence and stimulated apoptosis in HeLa cells after repression of the endogenous HPV18 E6 and E7 genes. In contrast, cells expressing either of the E7 mutants defective for Rb inactivation efficiently senesced and failed to undergo apoptosis, whereas a mutant displaying partial Rb-inactivating activity was intermediate in blocking growth inhibition. Taken together, these results indicate that inactivation of the Rb pathway inhibits E2-mediated senescence and permits apoptosis. We infer that activation of the Rb pathway is required for senescence and inhibition of apoptosis after E7 repression.

**Biochemical Effect of Rb-Deficient E7 Mutants in Cells Constitutively Expressing the HPV16 E6 Protein.** The interpretation of the results described in the previous sections is complicated by the complex phenotype induced by the E2 protein in cells constitutively expressing the wild-type HPV16 E7 protein. HeLa cells engineered to constitutively express the HPV16 E6 protein display simpler responses to the E2 protein. In these cells, E2 expression efficiently induces senescence, but constitutive expression of the HPV16 E7 protein together with the HPV16 E6 protein prevents E2-induced senescence, and the cells continue to proliferate without undergoing apoptosis (39). Therefore, E7 mutants defective for Rb binding and degradation were introduced into cells constitutively expressing the HPV16 E6 protein, and the response of these cells to the E2 protein was determined.

HeLa/16E6H cells, which constitutively express the HPV16 E6 protein, were infected with the LXSN vector or retroviruses expressing the wild-type E7 protein, E7 $_{\Delta 6-10}$ , or E7 $_{\Delta 21-24}$ , and cell lines were established from individual colonies resistant to both G418 and hygromycin. The E2 protein repressed expression of the endogenous HPV18 E6 and E7 genes in these cells (data not shown). In the

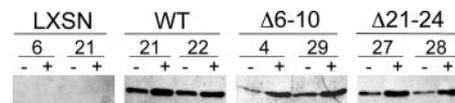


Fig. 6. Expression of exogenous HPV 16E7 in cells constitutively expressing the HPV16 E6 protein. Two clones of each of the HeLa/16E6H-LXSN (LXSN), HeLa/16E6H-16E7 (WT), HeLa/16E6H-16E7 $_{\Delta 6-10}$  ( $\Delta 6-10$ ), and HeLa/16E6H-16E7 $_{\Delta 21-24}$  ( $\Delta 21-24$ ) cell lines were mock-infected (-) or E2-infected (+) and then harvested 2 days later in E7 lysis buffer. Thirty  $\mu$ g of extracted protein of each sample were subjected to electrophoresis and immunoblotted with specific antibodies recognizing HPV16E7. The numbers are the clone designations.

presence of the E2 protein, these cell lines expressed similar levels of the wild-type and mutant HPV16 E7 proteins, which were higher than expression in the absence of the E2 protein (Fig. 6). Because high-risk HPV E6 proteins can act as transcriptional repressors of retroviral long terminal repeats (45), E2-mediated induction of the E7 protein may be a consequence of repression of the endogenous HPV18 E6 gene.

The effect of the E2 protein was determined in two independent cell lines expressing each of these retroviruses. Because all of these cell lines constitutively expressed the HPV16 E6 protein, p53 was not induced by E2 expression (Fig. 7, bottom panel). In control HeLa/16E6H-LXSN cells, the E2 protein caused a marked induction of the hypophosphorylated form of p105<sup>Rb</sup>, due to repression of the endogenous HPV18 E7 protein. In addition, the level of the hyperphosphorylated form was decreased, due to Rb-mediated repression of E2F-responsive genes required for maximal cdk activity, such as cyclin A and cdc25A. The E2 protein had no effect on the expression or phosphorylation of p105<sup>Rb</sup> in cells expressing the wild-type HPV16 E7 protein. Strikingly, hypophosphorylated p105<sup>Rb</sup> was induced and hyperphosphorylated p105<sup>Rb</sup> was eliminated in cells expressing the E7 mutants defective for Rb binding and degradation. Induction of hypophosphorylated p105<sup>Rb</sup> was more dramatic in cells expressing E7<sub>Δ21-24</sub>, which is unable to bind or degrade Rb family members, than in cells expressing E7<sub>Δ6-10</sub>, which can bind but not degrade p105<sup>Rb</sup>. Similarly, the E2 protein repressed cyclin A expression in cells with no added E7 protein or expressing the E7 mutants unable to bind or degrade Rb, whereas cyclin A expression persisted in cells expressing the wild-type HPV16 E7 protein. Thus, as expected, the E2 protein did not activate the Rb pathway in cells constitutively expressing the wild-type HPV16 E7 protein, but it did activate the pathway in cells expressing E7 mutants defective in their interaction with Rb family members.

**Phenotypic Effect of Rb-Deficient E7 Mutants in HeLa Cells Constitutively Expressing the HPV16 E6 Protein.** To assess the effect of the E7 mutants on cell proliferation after expression of the E2 protein in cells constitutively expressing the E6 protein, incorporation of tritiated thymidine into DNA was measured in several cell lines of each genotype (Fig. 8). The E2 protein dramatically inhibited DNA synthesis in cells constitutively expressing HPV16 E6, whereas constitutive expression of the wild-type E7 protein in these cells largely abrogated E2-induced growth inhibition. E2 expression caused dramatic inhibition of DNA synthesis in multiple clones of HeLa/16E6-16E7<sub>Δ6-10</sub> and HeLa/16E6-16E7<sub>Δ21-24</sub> cells also. Thus, unlike the wild-type E7 protein, the two E7 mutants defective for Rb inactivation were unable to prevent growth arrest induced by repression of the endogenous HPV18 E7 protein in these cells.

SAβ-gal expression was also examined in cell lines that were

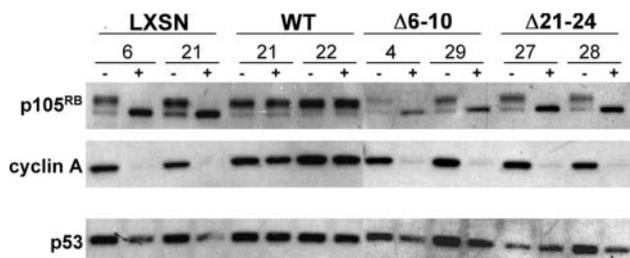


Fig. 7. Biochemical effects of HPV repression in cell constitutively expressing the HPV16 E6 protein. Trizol protein extracts were made from two clones of each of the cell lines (designated as in the legend to Fig. 6) 2 days after mock infection (-) or infection with the E2 virus (+). Five  $\mu$ g of extracted protein were subjected to electrophoresis and immunoblotted with antibodies specific for p105<sup>Rb</sup>, cyclin A, and p53, as indicated. The hyperphosphorylated and hypophosphorylated species of p105<sup>Rb</sup> are indicated by P and O, respectively.

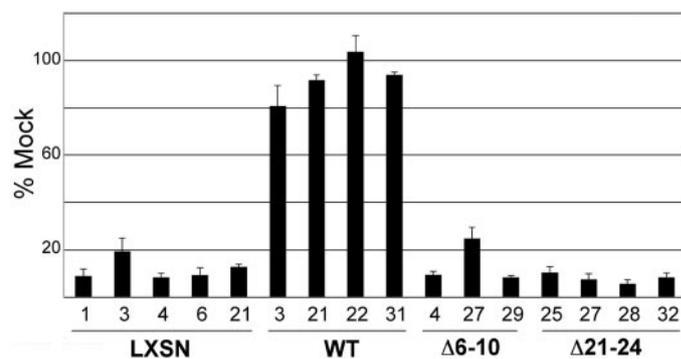


Fig. 8. Effects of HPV repression on DNA synthesis in cells constitutively expressing the HPV16 E6 protein. Cellular DNA synthesis after infection with the E2 virus was measured in several independent cloned cell lines of each genotype, as indicated. The results of a typical experiment are shown, and the error bars show SD of triplicate samples.

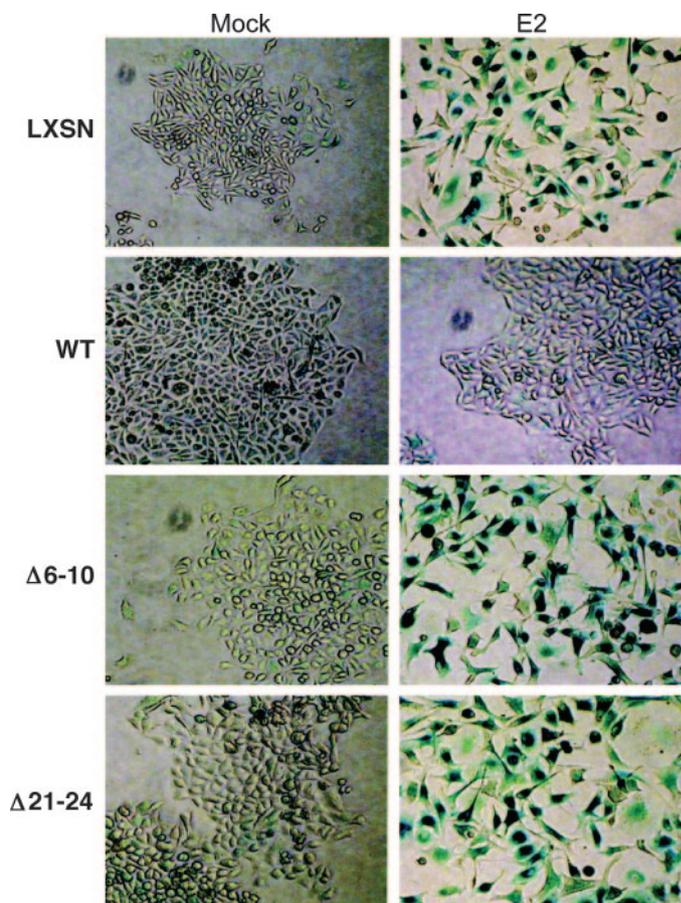


Fig. 9. SAβ-gal activity in cells constitutively expressing the HPV16 E6 protein. HeLa/16E6H-LXSN clone 21 (LXSN), HeLa/16E6H-16E7 clone 21 (WT), HeLa/16E6H-16E7<sub>Δ6-10</sub> clone 29 (Δ6-10), and HeLa/16E6H-16E7<sub>Δ21-24</sub> clone 27 (Δ21-24) cells were mock-infected (left panels) or infected with the E2 virus (right panels), and after 10 days, cells were stained at pH 6.0 with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and photographed by bright-field microscopy.

mock-infected or infected with the E2 virus (Fig. 9). In all cases, mock-infected cell lines generated colonies that showed only faint background staining, as did E2-infected cells constitutively expressing the wild-type E7 protein. After infection with the E2 virus, virtually all of the HeLa/16E6H-LXSN cells displayed a flat, loosely packed morphology and blue staining, demonstrating SAβ-gal activity and senescence. Expression of the E2 protein also induced SAβ-gal activity and senescent morphology in cells coexpressing HPV16 E6 and

either E7 $_{\Delta 6-10}$  or E7 $_{\Delta 21-24}$ , demonstrating that these mutants did not protect against senescence.

Flow cytometry was used to measure autofluorescence in cells expressing the HPV16 E6 protein and either the wild-type E7 protein or the E7 mutants defective for Rb inactivation (Fig. 10). As expected, the entire population of E2-infected HeLa/16E6H-LXSN cells displayed a dramatic increase in autofluorescence, consistent with the efficient induction of senescence, whereas constitutive expression of the wild-type E7 protein blocked this increase. E2 expression also caused markedly increased autofluorescence in cells coexpressing HPV16 E6 and either E7 mutant. Similar results were obtained with multiple independent cell lines of each genotype. Thus, cells expressing Rb-deficient E7 mutants, like cells not expressing an exogenous E7 protein, efficiently underwent senescence in response to E2 expression.

**Modulation of Senescence by Adenovirus E1A Protein.** We also isolated clonal HeLa cell lines coexpressing HPV16 E6 and similar levels of the adenovirus 5 13S E1A protein or a mutant E1A protein containing a point mutation that disrupted the Rb-binding motif. The wild-type E1A protein markedly inhibited E2-mediated senescence, but the mutant did not (data not shown). Thus, as was the case for the HPV16 E7 protein, the E1A protein inhibited E2-induced senescence in HeLa cells in an Rb-dependent fashion, confirming that Rb activation was required for senescence induced by E7 repression in HeLa cells.

**Role of the Rb Pathway in HT-3 Cells.** We also tested the requirement for the Rb pathway in HT-3 cervical carcinoma cells, which express HPV30 E6 and E7 genes. E2 expression activates the Rb pathway and induces senescence in these cells (31, 35, 40), although this process is less efficient than in HeLa cells. Because HT-3 cells harbor only transactivation-defective p53, the E2 protein does not activate the p53 pathway in these cells. HT-3 cells were infected with the empty retrovirus vector or retroviruses expressing wild-type HPV16 E7 or E7 $_{\Delta 21-24}$ , and G418-resistant colonies were pooled to establish polyclonal cell lines. DNA synthesis was measured after mock infection or E2 infection. As shown in Fig. 11, the E2 protein inhibited DNA synthesis in HT-3/LXSN cells to a significant

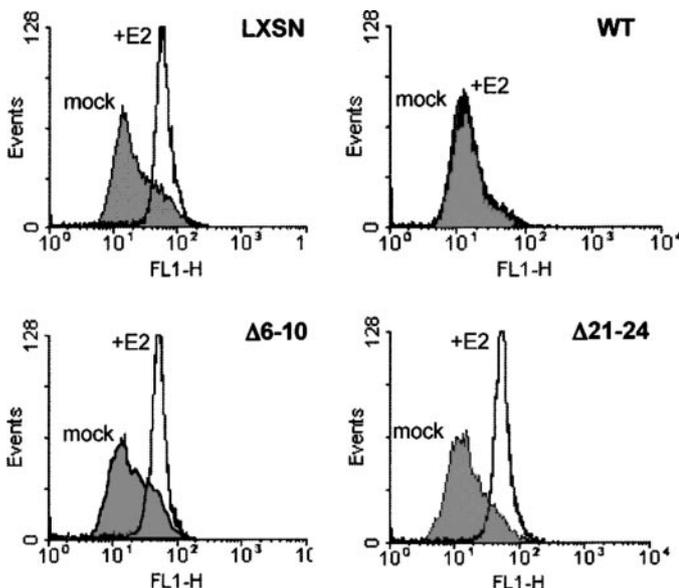


Fig. 10. Autofluorescence in cells constitutively expressing the HPV16 E6 protein. HeLa/16E6H-LXSN clone 21 (LXSN), HeLa/16E6H-16E7 clone 22 (WT), HeLa/16E6H-16E7 $_{\Delta 6-10}$  clone 29 ( $\Delta 6-10$ ), and HeLa/16E6H-16E7 $_{\Delta 21-24}$  clone 28 ( $\Delta 21-24$ ) cells were assayed by flow cytometry for autofluorescence 6 days after E2 infection (open histograms) or mock infection (shaded histograms).

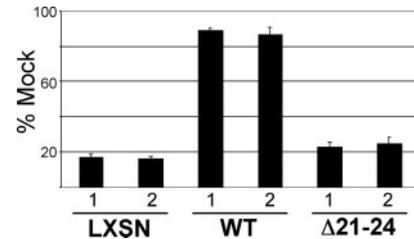


Fig. 11. Effect of Rb activity on HT-3 cell DNA synthesis. Polyclonal HT-3 cell lines harboring an empty retrovirus vector or vectors expressing the wild-type HPV16 E7 protein or E7 $_{\Delta 21-24}$  were infected with the E2 virus or mock-infected. Three days later, DNA synthesis was measured in quadruplicate by [ $^3$ H]thymidine incorporation. DNA synthesis by the infected cells is expressed as percentage of synthesis by mock-infected cells (% Mock). The results of a typical experiment involving two independent cell lines of each genotype are shown, and the error bars show one SD of the quadruplicate samples.

extent, consistent with the induction of senescence in most of the cells. Constitutive expression of the wild-type HPV16 E7 protein largely eliminated the E2-induced reduction in DNA synthesis in HT-3/16E7 cells, demonstrating that senescence required E7 repression in HT-3 cells. Strikingly, the E7 $_{\Delta 21-24}$  mutant did not prevent E2-induced senescence in HT-3/16E7 $_{\Delta 21-24}$  cells. Similarly, other measures of senescence, *i.e.*, cell enlargement and flattening, SA $\beta$ -gal activity and increased autofluorescence were inhibited by the wild-type E7 protein but not by E7 $_{\Delta 21-24}$  (data not shown). Thus, senescence induced by E7 repression in HT-3 cells required activation of the Rb pathway, as it did in HeLa cells. This result also implies that E6 repression did not activate a p53-independent senescence pathway in HT-3 cells.

## DISCUSSION

Repression of the endogenous HPV E7 oncogene triggers senescence in cervical carcinoma cell lines. However, because the high-risk E7 protein binds to numerous cellular proteins, the biochemical pathways responsible for this senescence response have not been established. To determine whether activation of the Rb pathway was required for senescence in response to E7 repression, we tested the ability of the wild-type and mutant E7 proteins to affect the activity of the Rb pathway and modulate induced senescence.

Expression of the wild-type HPV16 E7 protein inhibited senescence caused by E2-mediated repression of the endogenous HPV18 E7 gene in two HeLa cell genetic backgrounds. When the endogenous HPV18 E6 and E7 genes were both repressed in HeLa cells, the exogenous E7 protein provided partial protection against senescence, and some of the cells underwent apoptosis. When the HPV18 E7 gene was repressed but HPV16 E6 expression was maintained in HeLa/16E6H cells, the exogenous E7 protein prevented senescence. In both cell types, two independent E7 mutants unable to inactivate the Rb pathway failed to inhibit senescence. Similarly, HeLa cell senescence was inhibited by the wild-type E1A protein but not an E1A mutant unable to bind Rb. An E7 mutant that retained the ability to inactivate the Rb pathway provided the same level of protection against senescence in HeLa cells as did the wild-type E7 protein, and an E7 mutant with an intermediate effect on Rb activity partially blocked growth inhibition. Senescence induced by E7 repression in HT-3 cells was also blocked by the wild-type HPV16 E7 protein but not by an Rb-binding-defective mutant. These experiments provide compelling genetic evidence that senescence initiated by E7 repression in HeLa cervical carcinoma cells requires activation of the Rb pathway. Rb signaling is also required for *ras*-induced senescence in human fibroblasts (46) and for replicative senescence (24–26, 47, 48) upon serial

cell passage. Furthermore, introduction of an exogenous Rb gene can induce senescence in human cancer cells (49, 50).

Wells *et al.* (37) also found that senescence triggered by combined repression of the HPV18 E6 and E7 genes in HeLa cells was partially inhibited by the wild-type HPV16 E7 gene but not by an E7 mutant unable to bind Rb. However, there are important differences between these earlier experiments and those reported here. Importantly, we conducted biochemical analysis to establish the activity of the Rb pathway in HeLa cells expressing the wild-type and mutant exogenous E7 proteins. In addition, as well as examining senescence when both E6 and E7 were repressed, we also examined the requirement for Rb signaling in senescence triggered by repression of E7 in the absence of E6 repression and in HT-3 cells in the absence of p53 signaling. Because E6 repression initiates p53-dependent signaling in HeLa cells, which can also induce senescence in the absence of Rb activation (39, 43), interpretation of experiments involving repression of both E6 and E7 is difficult. Thus, it is important to separately analyze the activities of the E6 and E7 proteins to dissect their influence on cellular physiology.

We also explored the role of the Rb pathway in apoptosis induced by E6 repression. When the endogenous HPV18 E6 protein is repressed in cells expressing the wild-type HPV16 E7 protein, a fraction of the cells undergo apoptosis after a delay, a response that requires p53 activity (39, 43). We found here that E6 repression induced apoptosis only in cells expressing E7 proteins that inactivated the Rb pathway. Thus, active Rb signaling inhibited p53-dependent apoptosis in cervical carcinoma cells. In several additional cell systems, Rb activity inhibits E7-mediated apoptosis (17, 51, 52).

Previous mutational and biochemical studies identified two features of the wild-type E7 protein required for full inactivation of Rb, the LXCXE Rb-binding motif and an NH<sub>2</sub>-terminal degradation motif. The inability of the E7<sub>Δ6-10</sub> mutant to prevent senescence initiated by repression of the HPV18 E7 gene indicates that Rb binding is not sufficient to inactivate the Rb senescence pathway in HeLa cells and that Rb degradation is also required. The S31G/S32G double mutant, which lacks the casein kinase II phosphorylation sites, partially protects against both Rb activation and the senescence response, implying that this mutant interferes with senescence by impairing the Rb pathway. Other investigators showed that phosphorylation of the CKII sites does not modulate Rb binding *in vitro*, but that mutations at these sites can impair Rb degradation (17, 44, 53). Similarly, our experiments show that mutations at these sites can result in inefficient Rb degradation and impaired repression of E2F-responsive S-phase genes such as cyclin A.

The available genetic analysis implies that E7 is a modular protein and that it is possible to assign individual activities to specific, fairly well-defined segments of the protein. For example, biochemical and gene transfer experiments demonstrated that NH<sub>2</sub>-terminal mutations that interfere with Rb degradation do not impair Rb binding, stimulation of S phase or apoptosis, E2F displacement *in vitro*, and transactivation of E2F-responsive genes; mutations at the CKII phosphorylation sites do not affect Rb binding at the adjacent LXCXE site but do affect Rb degradation and repression of p53 transcriptional activity; and p21 binding is separable from Rb inactivation (22, 44, 52–58). In addition, a COOH-terminal activity of the E7 protein is required for abrogation of exogenous growth inhibitory stimuli by neutralizing p21 activity (42, 55). Therefore, the inability of E7<sub>Δ6-10</sub> or E7<sub>Δ21-24</sub> to prevent senescence is unlikely to be due to E7 activities other than loss of Rb inactivation, although we note that the regions of the E7 protein required for Rb binding and degradation are also required for binding IRF-1 (59). Analysis of additional E7 mutants may reveal the existence of other E7 activities in addition to Rb inactivation that are required to prevent senescence.

In summary, both binding and degradation of the Rb proteins by the HPV E7 protein are essential for sustained proliferation of HeLa cervical carcinoma cells, and E7 repression triggers senescence at least in part by activating the Rb pathway in both HeLa and HT-3 cells. We previously showed that senescence initiated by E6 repression in HeLa cells required activation of the p53 pathway (43). Thus, HPV repression activates at least two pathways that can result in senescence, the Rb pathway when E7 is repressed and the p53 pathway when E6 is repressed. Our results also imply that Rb signaling can prevent the delayed, p53-dependent apoptosis elicited by E6 repression. Future strategies to inhibit the growth of cervical cancer cells should include approaches that manipulate these dormant growth inhibitory pathways.

## ACKNOWLEDGMENTS

We thank Denise Galloway for retroviruses expressing the wild-type and mutant E7 genes and Kathi Fries and Lara Ely for preliminary experiments with the E1A gene. We thank other members of the DiMaio laboratory for helpful comments on this manuscript and Jan Zulkeski for assistance in preparation of the manuscript.

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## Role of the Retinoblastoma Pathway in Senescence Triggered by Repression of the Human Papillomavirus E7 Protein in Cervical Carcinoma Cells

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*Cancer Res* 2004;64:3079-3086.

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