Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways

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Most cervical carcinomas express high-risk human papillomaviruses (HPVs) E6 and E7 proteins, which neutralize cellular tumor suppressor function. To determine the consequences of removing the E6 and E7 proteins from cervical cancer cells, we infected HeLa cells, a cervical carcinoma cell line that contains HPV18 DNA, with a recombinant virus that expresses the bovine papillomavirus E2 protein. Expression of the E2 protein resulted in rapid repression of HPV E6 and E7 expression, followed ≈12 h later by profound inhibition of cellular DNA synthesis. Shortly after E6/E7 repression, there was dramatic posttranscriptional induction of p53. Two p53-responsive genes, mdm2 and p21, were induced with slightly slower kinetics than p53 and appeared to be functional, as assessed by inhibition of cyclin-dependent kinase activity and p53 destabilization. There was also dramatic posttranscriptional induction of $p105^{Rb}$ and p107 after E6/E7 repression, followed shortly thereafter by induction of p130. By 24 h after infection, only hypophosphorylated $p105^{Rb}$ was detectable and transcription of several Rb/E2F-regulated genes was dramatically repressed. Constitutive expression of the HPV16 E6/E7 genes alleviated E2-induced growth inhibition and impaired activation of the Rb pathway and repression of E2F-responsive genes. This dynamic response strongly suggests that the p53 and Rb tumor suppressor pathways are intact in HeLa cells and that repression of HPV E6 and E7 mobilizes these pathways in an orderly fashion to deliver growth inhibitory signals to the cells. Strikingly, the major alterations in the cell cycle machinery underlying cervical carcinogenesis can be reversed by repression of the endogenous HPV oncogenes.

High-risk human papillomaviruses (HPVs) such as HPV18 play a central role in the development of essentially all cases of cervical carcinoma (1). However, carcinoma develops infrequently even after infection by these HPV types, and it typically occurs years to decades after the initial infection. Two HPV oncogenes, E6 and E7, are expressed in cervical carcinomas and carcinoma-derived cell lines. The E6 and E7 proteins can immortalize cultured primary human keratinocytes, but these immortalized cells are not tumorigenic unless additional, undefined genetic events occur. These observations imply that the viral oncogenes do not directly induce tumor formation but rather set in motion a series of events that may ultimately result in tumorigenicity.

The high-risk HPV E6 and E7 proteins exert profound effects on the tumor suppressor proteins p53 and $p105^{Rb}$ (1). These tumor suppressor proteins normally control signaling pathways that regulate the cell cycle and monitor and protect the integrity of the genome. p53 is a transcription factor that activates transcription of a variety of genes including $p21^{Waf1/CIP1/SDI1}$ (p21) (reviewed in ref. 2). p21 directly inhibits the activity of cyclin-dependent kinase (cdk) complexes, which are required for cell cycle progression. Transcription of the mdm2 gene is also induced by p53. mdm2 in turn binds to p53 and stimulates its degradation in a negative feedback loop that controls p53 levels. $p105^{Rb}$ and the retinoblastoma (Rb) family members p107 and p130 regulate the activity of E2F transcription factors, which control transcription of a variety of genes required for cell cycle progression (reviewed in refs. 3 and 4). Hypophosphorylated Rb family members bind to E2F family members, thereby forming complexes that actively repress transcription of cell cycle genes (5–13). Phosphorylation of Rb proteins by cdks disrupts these complexes. Disruption of these complexes impairs repression and increases the concentration of unbound E2F family members, some of which, like E2F1, can stimulate transcription (3, 4).

The high-risk HPV E6 protein binds to p53 and targets it for accelerated ubiquitin-mediated degradation, and the high-risk HPV E7 protein binds to hypophosphorylated members of the retinoblastoma family, resulting in their destabilization and the disruption of Rb/E2F repressor complexes (14–21). Therefore, levels of p53 and hypophosphorylated Rb are typically low in cells expressing the E6 and E7 proteins. As a consequence of these interactions, expression of high-risk HPV E6 and E7 proteins in cultured cells disrupts cell cycle checkpoint control and results in increased rates of mutagenesis and genetic instability (18, 22–27). Thus, expression of the E6 and E7 proteins may facilitate acquisition of the additional genetic changes that drive carcinogenic progression. Similar processes appear to occur during cervical carcinogenesis *in vivo*. For example, HeLa cells, an aneuploid tumorigenic cell line derived from a malignant human cervical carcinoma, express E6 and E7 proteins from integrated HPV18 DNA and display aberrant checkpoint control (28, 29).

Most cervical carcinomas and cervical carcinoma cell lines, including HeLa cells, harbor wild-type p53 and $p105^{Rb}$ genes (30, 31). Thus, the growth regulatory machinery active in normal cells may be intact in these carcinoma cells but masked by expression of the HPV E6 and E7 proteins. To analyze the consequences of removing the HPV E6 and E7 proteins from cervical carcinoma cells, we and others have exploited the ability of the bovine papillomavirus (BPV) and HPV E2 proteins to repress E6/E7 transcription by binding directly to the HPV early promoter (32–41). Introduction of an ectopic E2 gene into a number of cervical carcinoma cells lines results in a great reduction in E6/E7 mRNA and in substantial growth inhibition (32–36, 38, 42). E2-mediated growth inhibition is observed only in cells containing HPV DNA, and E2-induced reduction in HeLa cell colony formation is prevented by constitutive expression of the HPV16 E6 and E7 genes (32, 38, 41, 42), indicting that repression of E6/E7 is required for the growth inhibitory effect.

Abbreviations: HPV, human papillomavirus; Rb, retinoblastoma; cdk, cyclin-dependent kinase; BPV, bovine papillomavirus.

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We used a recombinant SV40-based viral vector to express the E2 protein in cervical carcinoma cells (32). Two days after infection with this virus (but not after infection with viruses expressing inactive mutant E2 proteins), HeLa cells display profound growth inhibition, accumulating with a G_1/G_0 DNA content (32, 34, 42). At this time, HPV18 E6/E7 expression is repressed and p53, p21 and hypophosphorylated $p105^{Rb}$ are induced. However, these experiments examined a single time point after the imposition of severe growth inhibition and therefore provided a static snapshot of the cellular response to E2 expression. The sequence of biochemical events that occurs after E6/E7 repression is not known, nor is it known whether the cells arrested because of the orderly reestablishment of normal growth control mechanisms or to catastrophic derangement of cellular metabolism. Here, we examined the sequence of biochemical events induced by expression of the E2 protein in cervical carcinoma cells. This kinetic analysis revealed a dynamic and complex sequence of activating and repressing events in cell cycle regulatory components that strongly implied that the p53 and Rb tumor suppressor signaling pathways are intact in HeLa cells and able to transduce a growth inhibitory signal once HPV oncogene expression is extinguished. These findings have important implications for the pathogenesis and treatment of cervical carcinoma.

Materials and Methods

Cells and Virus Preparation. HeLa cells were maintained in standard media as described (42). The pPava-5′BΔS viral vector (42), which contains a wild-type BPV E2 gene but no SV40 T antigen gene and a disrupted BPV E5 gene, was further modified by replacing the AUG start codon for the internally initiated, E2 trans-repressor protein with an ATC codon, a mutation with no apparent effect on the E2 activities measured here (32). This repressor minus construct was renamed pPava-5′BΔS-RMC. Viral stocks were prepared and titered as described previously (38), and mock-infected cells were used as controls. Cellular DNA synthesis assays were performed in quadruplicate as described (42), with the modification that infections were at a multiplicity of infection of 20 and that [^{3}H]thymidine labeling was performed for only 2 h. Recombinant retroviruses expressing HPV16 E6/E7 and control retroviruses were obtained from Denise Galloway (Fred Hutchinson Cancer Research Institute) (43). After infection with these viruses and selection for G418-resistance, individual clones of drug-resistant HeLa cells were expanded into cell lines for analysis.

RNA Analysis. Cells were infected as described previously (42), and cell pellets were harvested and frozen at −80°C until fractionation. Total cellular RNA was purified by using Trizol reagent (Life Technologies), and 5 μg of RNA was subjected to formaldehyde-agarose gel electrophoresis, transferred to Nytran (Schleicher & Schuell) and crosslinked to the membrane by UV irradiation. The immobilized RNA was hybridized with the indicated random prime-labeled cDNA, and the signal was detected and quantified with a PhosphorImager (Molecular Dynamics). Sequential hybridizations were performed after stripping the previous probe from the membrane. RNA levels were normalized to the signal obtained with ubiquitin mRNA.

Immunoblotting and cdk2 Kinase Activity. Protein for immunoblotting was prepared from the Trizol extracts after isolation of the RNA as described (38). Five micrograms of extracted protein was resolved by denaturing PAGE, transferred to an Immobilon-P membrane and probed with the antibodies specific for the following proteins: E2F1 (catalogue no. 05-379) from Upstate Biotechnology (Lake Placid, NY); p107 (sc-318), p130 (sc-317), mdm2 (sc-965), cdc25A (sc-7389), cdk2 (sc-748), and p21 (sc-397), all from Santa Cruz Biotechnology; p53 (15801A) and p105Rb (14001A), both from PharMingen; and cyclin A (from H. Zhang, Yale University). To measure cdk activity, HeLa extracts were immunoprecipitated as previously described (38), and histone H1 kinase activity was determined and quantitated with a PhosphorImager. After subtraction of the small signal resulting from kinase reactions after immunoprecipitation with nonimmune, species-matched antibodies, the signals were normalized to mock-infected controls.

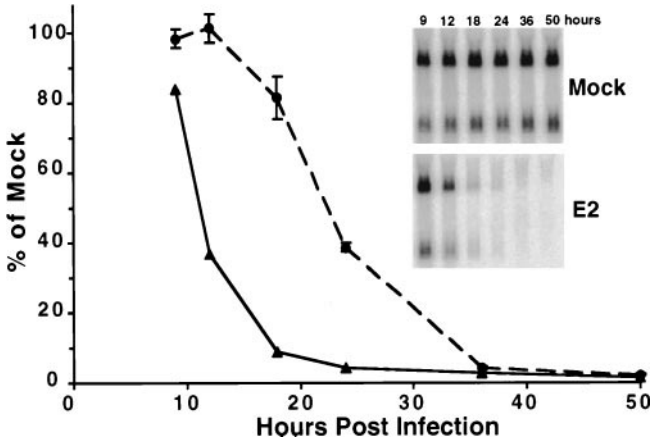

Fig. 1. Time course of HPV E6/E7 repression and growth inhibition. HeLa cells were infected or mock-infected, and, at the indicated time after infection, RNA was analyzed for HPV E6/E7 expression by Northern blotting (solid line), and cellular DNA synthesis was determined by incorporation of tritiated thymidine (dashed line). The error bars indicate two standard deviations of the mean. (*Inset*) Repression of HPV E6/E7 expression. Northern blot described above. RNA was isolated at the indicated hours after E2 infection (*Lower*) or mock-infection (*Upper*), electrophoresed, transferred, and probed with a radiolabeled HPV18 E6/E7 DNA fragment. The signal obtained was quantitated, normalized for the signal obtained with a ubiquitin probe, and expressed as the percentage of the normalized signal obtained with RNA from mock-infected cells.

Results and Discussion

E2-Mediated Inhibition of DNA Synthesis and Repression of HPV Gene Expression. We used a recombinant BPV/SV40 virus to introduce the BPV E2 gene into HeLa cells to determine the timing of events after expression of the full-length E2 protein. To measure cellular DNA synthesis, the cells were subjected to a 2-h pulse of [^{3}H]thymidine at various times after infection with the E2 virus at a multiplicity of 20 infectious units per cell or after mock infection, and acid-insoluble radioactivity was determined. As shown in Fig. 1, there was no difference in thymidine incorporation between infected and control cells at 12 h after infection. By 18 h after infection, infected cells showed a modest inhibition of DNA synthesis compared with mock-infected cells. The extent of inhibition increased with time, with DNA synthesis being approximately 40% of control levels by 24 h after infection and less than 5% by 36 h. These results demonstrated that the E2 protein exerted profound biological effects in the vast majority of cells in the population and established the time frame against which to measure biochemical changes in these cells.

Northern blotting was used to analyze expression of HPV18 E6/E7 mRNA at various times after infection or after mock-infection (Fig. 1 *Inset*). By 9 h after infection with the E2 virus, there was a reduction in HPV E6/E7 expression in infected cells compared with control cells. Quantitation in comparison to ubiquitin mRNA revealed that the level of E6/E7 RNA in E2-expressing cells was less than 40% the level in control cells by 12 h after infection and less than 10% by 18 h (Fig. 1). Expression of the E7 protein was repressed with similar kinetics

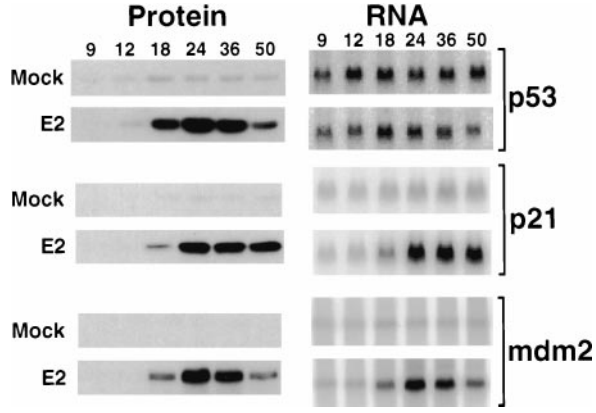

Fig. 2. (*Left*) Western analysis of p53 pathway. HeLa cell proteins were harvested at the indicated hours after E2 infection or mock-infection, electrophoresed, transferred, and probed with antibodies specific for p53, p21, or human mdm2, as indicated. (*Right*) Northern analysis of p53 pathway. HeLa cell RNA was isolated at the indicated hours after E2 infection or mock-infection. After electrophoresis and transfer, p53, p21, and mdm2 mRNA was detected by hybridization to the appropriate radiolabeled cDNA probe.

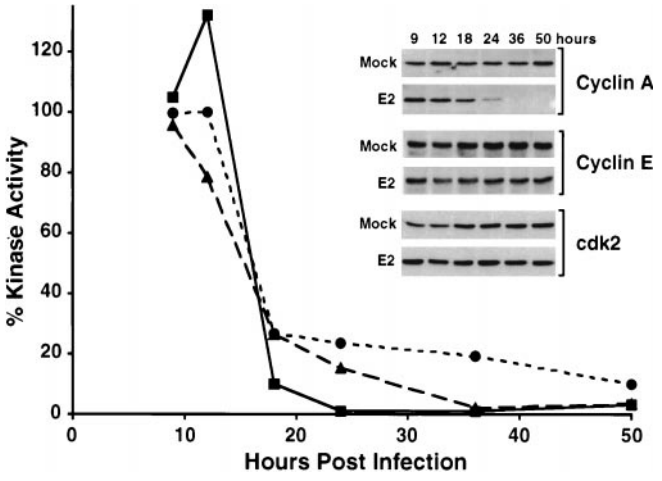

Fig. 3. Cyclin-dependent kinase activity. HeLa cell extracts were prepared at the indicated hours after E2 infection or mock-infection. After immunoprecipitation with control antibodies or antibodies specific for cyclin E (dotted line), cdk2 (dashed line), or cyclin A (solid line), kinase activity toward histone H1 was measured. The signal obtained was quantitated and expressed as the percentage of the corrected signal obtained with mock-infected samples. (*Inset*) Western analysis of cdk components. HeLa cell proteins were harvested at the indicated times from mock-infected or E2-infected cells. After electrophoresis and transfer, samples were probed with antibodies specific for cyclin A, cyclin E, and cdk2, as indicated.

(see Fig. 4). Therefore, HPV repression was a relatively early event after infection of HeLa cells with an E2-expressing virus and in fact was the earliest biochemical change we have detected. Importantly, HPV E6/E7 repression clearly preceded inhibition of DNA synthesis by about 12 h, a result consistent with repression playing a causal role in growth arrest.

Activation of the p53 Tumor Suppressor Pathway. The stability of p53 is increased in growth-arrested HeLa cells (32), an effect presumably due to the loss of the HPV18 E6 protein, which otherwise promotes accelerated, ubiquitin-mediated degradation of p53. Furthermore, in these growth-arrested cells, expression of the p53-responsive p21 gene is induced at the transcriptional level. Here, we used immunoblotting to determine the steady state level of p53 and two of its transcriptional targets, p21 and mdm2, at various times after infection (Fig. 2 *Left*). There was little change in the abundance of these proteins in mock-infected cells. In response to E2 expression, p53 displayed a complex kinetic profile, showing a dramatic induction by 18 h, followed by a drop after 24 h, so that by 50 h after infection the steady state level of p53 was only modestly higher than that observed in mock-infected cells. The mdm2 protein also showed a complex profile, with maximum levels attained at 24 h, after which there was a significant decline from the peak levels. p21 was induced with kinetics similar to mdm2, but its expression persisted for at least 50 h. p53 induction was approximately half-maximal by 18 h, whereas mdm2 and p21 induction was much less pronounced at this time point.

Because p53 and mdm2 displayed a similar expression pattern, namely an initial induction by the E2 protein followed by a decline, it seemed likely that these changes in the levels of the mdm2 protein were due to p53-mediated regulation of mdm2 transcription. Northern blotting demonstrated that mdm2 RNA levels did in fact rise and fall in parallel with mdm2 protein levels (Fig. 2 *Right*). Similarly, p21 was induced at the mRNA level. Consistent with the model that p21 and mdm2 induction was mediated by p53-transcriptional activation, neither gene was induced by E2 expression in HT-3 cells, an HPV30-containing cervical carcinoma cell line that expresses a transactivation-defective p53 protein (refs. 30, 31, and 38; data not shown). If the loss of HPV E6-directed degradation of p53 is responsible for the initial increase in p53 levels in HeLa cells, and the imposition of mdm2-directed degradation is responsible for the later decline, then the level of p53 mRNA should not change after expression of the E2 protein. In accord with this prediction, p53 mRNA levels changed little during the course of infection (Fig. 2 *Right*), despite the dramatic fluctuations in the level of p53 itself.

Because p21-mediated inhibition of cdk activity is an important consequence of p53 activation, we measured the kinase activity of cdk complexes *in vitro.* HeLa cell extracts prepared at various times after infection were immunoprecipitated with control antibodies or antibodies recognizing cdk2, cyclin A, and cyclin E, and the kinase activity of the immunoprecipitates toward histone H1 was measured. As shown in Fig. 3, E2 expression reduced the activity of all three types of cdk complexes, compared with complexes isolated from mock-infected cells. Inhibition of kinase activity was substantial by 18 h after infection and persisted for the duration of the experiment. To determine whether the components of cdk complexes were expressed at a reduced level in HeLa cells, we used immunoblotting to measure the expression of cdk2, cyclin A, and cyclin E. As shown in Fig. 3 *Inset*, the levels of cdk2 and cyclin E in cells expressing the E2 protein did not differ from those in mock-infected cells at any time during the course of the experiment. In contrast, cyclin A levels were essentially unchanged during the first 18 h after E2 infection and then declined to undetectable levels by 36 h after infection.

Our results suggest that the following sequence of events occurred in the p53 pathway. Binding of the E2 protein to the HPV18 early promoter caused transcriptional repression of E6/E7 expression. The resulting decay in the E6 protein reduced the amount of p53 targeted to the ubiquitin degradation system, leading to increased levels of p53. p53 induction caused increased transcription of the p21 and mdm2 genes and the accumulation of p21 and mdm2 proteins. As the mdm2 protein accumulated, it targeted p53 for accelerated degradation, leading to a posttranscriptional drop in p53 levels. As p53 levels dropped, it no longer induced mdm2 transcription, and levels of mdm2 RNA and protein dropped. This kinetic analysis provided evidence that the p53/mdm2 negative feedback loop is intact in

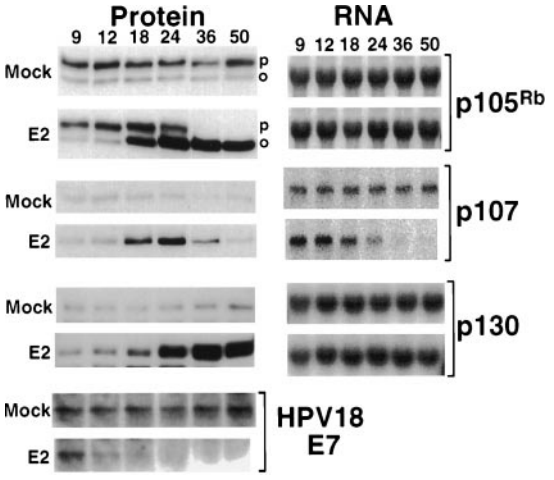

Fig. 4. (*Left*) Western analysis of retinoblastoma family members. HeLa cell protein was prepared at the indicated hours after E2 infection or mock-infection. After electrophoresis and transfer, specific antibodies were used to detect HPV18 E7, $p105^{Rb}$, p107, and p130. The hyperphosphorylated (p) and hypophosphorylated (o) form of $p105^{Rb}$ are indicated. (*Right*) Northern analysis of retinoblastoma family members. HeLa cell RNA was prepared at the indicated hours after mock-infection or E2 infection. After electrophoresis and transfer, $p105^{Rb}$, p107, and p130 mRNA were detected by hybridization to the appropriate radiolabeled cDNA probe.

HeLa cells. p21 mRNA levels also dropped at times later than the 50-h time point analyzed here (data not shown). The induced p21 bound to cdks and inhibited their activities, an effect that was reinforced by the absence of cyclin A in the case of total cdk2 and cyclin A-associated cdk activity.

Activation of the Retinoblastoma Tumor Suppressor Pathway. We previously reported that there is a marked increase in the levels of the hypophosphorylated form of $p105^{Rb}$ in E2-arrested cervical carcinoma cells, presumably due to reduction in proteosome-mediated degradation, as well as a reduction in the amount of the hyperphosphorylated form (34, 38, 44). Here, as shown in Fig. 4 *Left*, we used immunoblotting to examine the E7 protein and the Rb family members $p105^{Rb}$, p107 and p130 at various times after infection. A reduction in E7 protein expression was clearly evident by 12 h after infection, in parallel with the decrease in HPV E6/E7 RNA level. By 18 h after infection, there was an abrupt and dramatic induction of the level of hypophosphorylated $p105^{Rb}$ that increased until 24 h and persisted throughout the course of the experiment. In addition, at later times, hyperphosphorylated $p105^{Rb}$ disappeared, an effect likely due to the decline in cdk activity. Induction of hypophosphorylated $p105^{Rb}$ before the reduction in the hyperphosphorylated form was observed in multiple independent experiments. p107 was induced with similar kinetics as that observed for hypophosphorylated $p105^{Rb}$, but at later times the level of p107 dropped to that found in proliferating HeLa cells. p130 was induced more gradually than $p105^{Rb}$ or p107, with a significant increase only evident by 24 h, and the level of p130 remained elevated. The reciprocal expression of p107 and p130 correlates with a shift from cellular proliferation to a nonproliferative state in other systems as well (e.g., see citations in ref. 44).

If the increase in the abundance of the Rb proteins as infection proceeded was due to posttranslational stabilization as a consequence of the disappearance of the E7 protein, then the amounts of Rb family member mRNA are predicted not to increase. In support of this model, there was little increase in $p105^{Rb}$, p107, or p130 mRNA at any time after E2 expression (Fig. 4 *Right*). In contrast, the decline in p107 levels at later

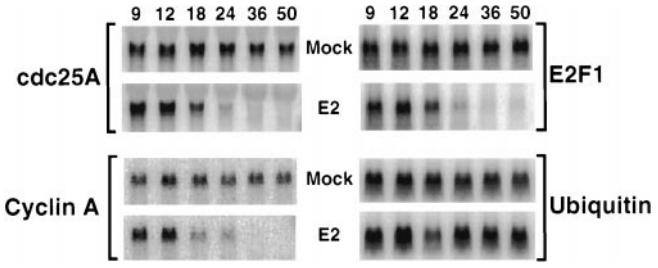

Fig. 5. Northern analysis of E2F-responsive genes. HeLa cell RNA was prepared at the indicated hours after mock-infection or E2 infection. After electrophoresis and transfer, E2F1, cyclin A, cdc25A, and ubiquitin mRNA were detected by hybridization to the appropriate radiolabeled cDNA probe.

times was due to a reduction in the amount of p107 mRNA as infection proceeded.

Rb family members exert their effects in large part by regulating the activity of E2F transcription factors, which themselves regulate the expression of genes involved in cell cycle progression. Here, we examined the time course of mRNA expression for four E2F-regulated genes, p107, cyclin A, E2F1, and cdc25A (3, 4, 45). In response to E2 expression, the level of these four mRNAs underwent a dramatic reduction, first evident at 18 h after infection with the E2 virus [Fig. 4 (p107) and Fig. 5], and the corresponding proteins underwent a similar reduction in amount [Fig. 3 *Inset* (cyclin A), and Fig. 4 (p107); data not shown for cdc25A and E2F1]. The repression of these E2F responsive genes in the same time frame as the induction of hypophosphorylated $p105^{Rb}$ strongly suggest that repression was due to a common, Rb-mediated mechanism. Elsewhere, we show that E2-induced repression of cdc25A expression in cervical carcinoma cells was mediated by increased formation of E2F4/Rb complexes that bound to an E2F site in the cdc25A promoter (44). Taken together, these results demonstrated that the induced $p105^{Rb}$ and p130 proteins formed complexes with E2F family members and repressed a panel of E2F-regulated cell cycle regulatory genes.

These results suggest that the following sequence of events occurred in the Rb pathway (Fig. 6). Reduction in HPV18 E7 expression caused the stabilization of Rb family members, resulting in a posttranscriptional increase in their intracellular concentration. The increased concentration of $p105^{Rb}$ and p130 caused the assembly of E2F/Rb transcriptional repressor complexes, which bound to E2F sites located in the promoters of E2F-responsive genes required for cell cycle progression (45), resulting in their transcriptional repression. The repression of E2F-regulated genes also instituted positive feedback loops that

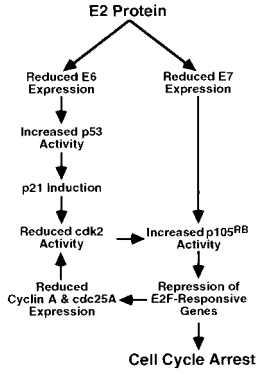

Fig. 6. Model for the growth regulatory pathway activated by the E2 protein. See text for details.

reinforced the growth inhibitory signal and ensured that growth inhibition was maintained. First, E2F1-mediated activation of genes required for S phase progression was reduced. Second, because E2F1 and p107 were absent from the cell, $p105^{Rb}$/E2F4 and p130/E2F4 complexes were more likely to form. Complexes containing these Rb and E2F family members have potent transcriptional repressor activity in nonproliferating cells (46–50). Finally, because both cyclin A and cdc25A stimulate cdk activity (51), their absence, together with the p53-mediated induction of p21, is predicted to impair cdk-mediated phosphorylation of $p105^{Rb}$ at the G_1/S boundary. The sequential induction of $p105^{Rb}$ followed by Rb-mediated repression of E2F-responsive genes that encode cdk activators may provide an explanation for the finding that levels of hypophosphorylated $p105^{Rb}$ rose before the reduction in the level of the hyperphosphorylated form.

Role of HPV E6/E7 Repression in Activation of the Rb Pathway. Francis *et al.* (41) previously reported that the E2-induced reduction in HeLa cell colony formation was impaired by constitutive expression of the HPV16 E6 and E7 genes, but cellular regulatory components were not examined in these experiments. Here, we assessed the effect of constitutively expressed HPV16 E6/E7 on the acute cellular and biochemical response to the E2 protein. All seven cell clones generated by infection with the empty retrovirus vector showed high level inhibition of DNA synthesis after introduction of the E2 gene, whereas the clones generated by infection with the HPV16 E6/E7 retrovirus displayed varying amounts of E2-resistant DNA synthesis (Fig. 7 *Top*; and R. DeFilippis & D.D., unpublished results). The incomplete protection of HeLa cells from the E2 protein may reflect suboptimal expression of HPV16 E6 and E7 from the heterologous promoter. A representative control cell clone and two clones generated by the HPV16 E6/E7 retrovirus were selected for biochemical analysis. As expected, expression of the E2 protein caused ≥99% reduction in the level of endogenous HPV18 mRNA (Table 1). In contrast, the transduced HPV16 E6/E7 genes were not repressed. E2-mediated induction of hypophosphorylated $p105^{Rb}$ and loss of hyperphosphorylated $p105^{Rb}$ were severely impaired in cells constitutively expressing HPV16 E6/7 compared with control cells (Fig. 7 *Middle*). In addition, E2-induced repression of cyclin A (Fig. 7 *Bottom*), and cdc25A (data not shown) was largely eliminated. We conclude that repression of HPV E6/E7 expression is required for E2-mediated induction of hypophosphorylated $p105^{Rb}$ and repression of E2F-responsive genes in HeLa cells.

Implications. These results have several important implications. First, E6/E7 repression clearly preceded growth inhibition and was required for efficient E2-induced growth inhibition and for acute activation of the Rb pathway and repression of E2F-responsive genes. In addition, the earliest biochemical changes we have detected in cell cycle components, the posttranscriptional increase in p53, $p105^{Rb}$, and p107 levels, can be simply explained by the loss of the E6 and E7 proteins, which otherwise target these tumor suppressor proteins for accelerated proteosome-mediated degradation. Thus, although numerous events drive the malignant conversion of cervical carcinoma cells, E6/E7 expression appears to be continuously required to maintain their proliferative state. Second, the response of the cell cycle machinery to E6/E7 repression is complex and dynamic, demonstrating that examination of cellular physiology at a single time point can be misleading. Third, the cellular response can be explained by the known regulatory circuits comprising the p53 and Rb tumor suppressor pathways, strongly suggesting that these two major tumor suppressor pathways are functionally intact in

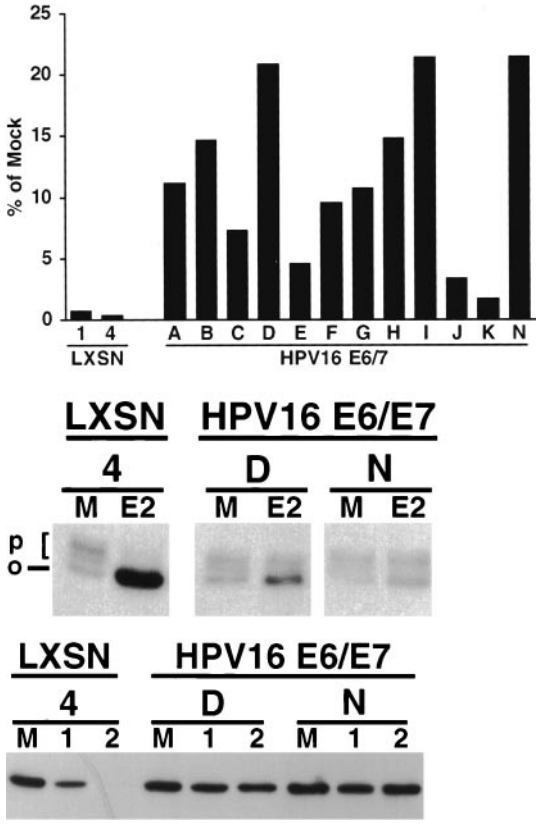

Fig. 7. Effect of HPV16 E6/E7 expression on the acute response to the E2 protein. Cell lines derived from individual clones of cells infected with control retrovirus (LXSN) or HPV16 E6/E7 retrovirus were analyzed. (*Top*) DNA synthesis by the indicated cell lines was measured by incorporation of tritiated thymidine 48 h after infection with the E2 virus, expressed as the percentage of DNA synthesis by each clone after mock-infection. (*Middle*) Expression of $p105^{Rb}$ in the indicated cell lines after mock-infection or 1 day after infection with the E2 virus. (*Bottom*) Expression of cyclin A in the indicated cell lines after mock-infection or 1 or 2 days after infection with the E2 virus.

HeLa cells. Although these aneuploid cells express viral proteins that induce genetic instability, and they have accumulated numerous genetic aberrations during their progression to a malignant carcinoma, this underlying regulatory machinery is intact. Finally, the E2 protein reactivates these dormant tumor suppressor pathways in an orderly fashion, resulting in the transmission of multiple reinforcing signals that converge on the repression of E2F-responsive genes required for entry into S phase. Thus, a surprisingly simple

Table 1. Repression of HPV18 mRNA in cells constitutively expressing HPV16 E6/7 mRNA

	% of mock*	
	HPV18 E6/E7	HPV16 E6/E7
LXSN-4	0.4	NA†
HPV16 E6/7-D	1.0	98.2
HPV16 E6/7-N	0.8	220

*HPV E6/7 mRNA prepared 24 h after infection with the E2 virus was measured by Northern blotting, normalized, and expressed as the percentage of the signal from mock-infected cells. The average of two different experiments is shown.

†NA, not applicable.

genetic manipulation is sufficient to mobilize this regulatory machinery and impose a cell cycle block.

Unlike most cancers, in which the brakes on cell growth are broken, in HeLa cells the driver is asleep. Expression of the E2 protein is sufficient to wake up the driver and impose growth control. Our results suggest that other manipulations that inhibit the expression or activity of the HPV E6 and E7 proteins will have a similar effect. Thus, the integrity of tumor suppressor pathways in cervical cancer cells may provide a unique target for therapy.

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