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A Genomic Approach Reveals a Novel Mitotic Pathway in Papillomavirus Carcinogenesis

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ABSTRACT

More than 90% of cervical carcinomas are associated with human papillomavirus (HPV) infection. The two viral oncogenes E6 and E7 play a major role in transforming the cells by disrupting p53- and pRb-dependent cell cycle checkpoints. A hallmark of HPV-associated cervical carcinoma is loss of the expression of the viral E2 protein, often by disruption of E2-encoding gene. We showed previously that reintroduction of E2 in HPV18-associated cervical carcinoma cells induces cell cycle arrest in G_1 because of the transcriptional repression of the viral oncogenes E6 and E7 and concomitant reactivation of the p53 and pRb pathways. Here we describe global gene profiling of HeLa cells expressing different HPV18 E2 mutants to study the effects of repression of the viral oncogenes. We identified 128 genes transcriptionally regulated by the viral oncogenes in cervical carcinoma. Surprisingly, E2 repressed a subset of E2F-regulated mitotic genes in an E6/E7-dependent pathway. This was corroborated by the observation that E2 delayed mitotic progression, suggesting the involvement of a mitotic pathway in HPV carcinogenesis. These mitotic genes constitute an as yet unrecognized set of genes, which were also found deregulated in other HPV-associated cervical carcinoma cell lines and therefore represent new targets for both diagnosis and therapeutic approaches in cervical cancer.

INTRODUCTION

High-risk human papillomaviruses (HPV) are associated with the vast majority of cervical carcinomas. HPV18 is a prototype of these "high risk" viruses. It shows the most aggressive tumorigenicity, although it is not the most frequent HPV type associated with cervical carcinoma. Integration of the HPV18 genome into the host genome plays a crucial role in carcinogenic progression. Integration results in disruption of the genes coding for the two viral regulatory proteins, E1 and E2, and is a common feature of HPV18-associated carcinomas. E2 negatively regulates the transcription of the viral oncogenes E6 and E7 through specific binding to DNA recognition sequences located within the E6/E7 promoter. Therefore, loss of E2 expression leads to constitutive activation of the viral oncogenes. Reintroduction of E2 in HPV-associated cervical carcinoma cells was shown to suppress cellular growth, because of cell cycle arrest in G_1, senescence, and apoptosis (1–8). The proapoptotic function is an intrinsic property of the viral E2 protein that does not require its DNA binding and does not depend on repression of the viral oncogenes (2, 9). This conclusion is further reinforced by the observations that E2 can also induce apoptosis in HPV-negative cell lines. In contrast, cell cycle arrest and senescence are mainly phenotypic consequences of the E2-mediated down-regulation of the E6/E7 viral oncogenes. In particular, repression of E6 by E2 in HeLa cells stabilizes p53 leading to transcriptional activation of the cell cycle-negative regulator p21 and consequent G_1 cell cycle arrest (1, 3, 5, 8).

The E6 and E7 oncoproteins act mainly through protein/protein interactions to alter two major pathways regulating cell cycle progression. Through its interaction with the E6AP ubiquitin ligase, E6 targets p53 for degradation by the proteasome, consequently abrogating the p53 pathway (10, 11). On the other hand, E7 binds to the p105Rb protein leading to release of active E2F and activation of E2F target genes. Alteration of the p53 and pRb tumor suppressor pathways by the E6 and E7 proteins account for most of their transforming functions. Both pathways are often disrupted in cancers. They are independent but interconnected, explaining the difficulty to separate the phenotypic outcome of each of them in cervical carcinoma. To study the individual contributions of these two pathways, the E2 protein has been expressed in cervical carcinoma cell lines to down-regulate both endogenous E6 and E7 transcription, and either E6 or E7 were independently re-expressed from heterologous promoters. Unfortunately, these experiments did not permit clear discrimination between the p53 and pRb pathways (1, 8). For example, the senescence phenotype that occurs after long term expression of E2 and cell cycle arrest was found to be linked to both pRb and p21 proteins (8). Inhibition of E7 alone triggers senescence via the pRb pathway, whereas inhibition of E6 alone induces both senescence and apoptosis via the p53 pathway (1). Clearly, some phenotypic effects of E6 and E7 repression depend on the interconnection of p53 and pRb pathways. A global analysis of genes modulated after irreversible commitment to senescence was performed in HeLa cells infected with an E2 recombinant adenovirus, 4 days after infection (12). This study addressed alterations of the cellular transcriptome associated with the irreversible senescent phenotype, which was initiated by E2, but had become independent from E2 expression at the time of analysis (12). The genes identified are therefore not necessarily targets of E2. In contrast, our study depicts the direct consequences of the repression of E6/E7 transcription because of E2 expression in HeLa cells 40-h postinfection. In these conditions, the alterations of the cellular transcriptome reflect the transcriptional events that are responsible for cell cycle arrest by E2. Consequently, we describe a series of genes that are essentially different from the genes described in that work. Interestingly, however, a strong activation of p21 was found in the two systems, establishing this gene as one of the prominent actors in cell cycle arrest and senescence in HeLa cells.

At the transcriptional level, one of the main targets modulated by E6 repression via stabilization of p53 is the negative cell cycle regulator p21/CDKN1, which is highly activated in E2-expressing cells. p21 activation reflects stabilization of p53 by E2-mediated repression of E6. In the pRb pathway, three E2F target genes activated by E7 have already been described. These include the cdc25A tyrosine phosphatase involved in the G_1-S transition (13–16) and two S-phase genes, cyclin E and cyclin A (17, 18). Activation of these genes is due to reactivation of E2F by E7, rather than to reentry into S phase. With the dramatic increase in the number of E2F target genes identified recently by microarray analyses, it is expected that as many as 7% of

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mammalian promoters could be regulated by E2F (19). p53 microarray analyses have also revealed many highly heterogeneous p53 responsive genes (20, 21).

In the present study, different forms of the homologous HPV18 E2 protein were re-expressed by recombinant adenoviruses in HeLa cells, to identify cellular genes affected by the E6 and E7 viral oncogenes. Given the high number of transcriptional target genes involved in the p53 and pRb pathways, a comprehensive analysis of their modulation in cervical carcinoma was undertaken using microarrays containing 13,000 human cDNA sequences. Our experimental approach indicated that the main effect of E6/E7 transcriptional repression was to reactivate both the p53 and pRb pathways, confirming their essential roles in cervical carcinoma. Sixty cellular genes followed the expected expression pattern of p53-responsive genes, among which 10 were known p53 targets. Similarly, 67 cellular genes were repressed as expected for E2F-responsive genes, among which half were indeed known E2F targets. Unexpectedly, half of the repressed genes were strongly associated with mitosis, suggesting that the viral oncogenes can affect mitosis. Indeed, we show that E2 severely delayed the mitotic progression of HeLa cells, implicating a novel mitotic pathway in HPV-associated carcinogenesis, which could be mainly activated by the E7 oncoprotein.

MATERIALS AND METHODS

Infection of HeLa Cells with Recombinant Adenoviruses. Recombinant adenoviruses expressing green fluorescent protein (gfp) fused to either the full-length E2 protein (gfp-E2), the protein deleted of its transactivation domain (gfp-del TAD) or the transactivation domain alone (gfp-TAD), were described previously (9). HeLa cells were infected with purified adenoviruses at a multiplicity of infection (m.o.i.) of 250 for adeno gfp-E2, gfp-delTAD, or gfp. An m.o.i. of 50 was used for adenovirus gfp-TAD, to avoid apoptotic induction that occurs at lower transactivation domain (TAD) expression than full-length E2 in HeLa cells (9). For each adenovirus, 6×10^6 cells were infected and plated on six P100 plates. The viral inocula were added to the cell cultures in a minimal volume of DMEM without serum, for 1 h at 37°C. After removal of the inoculum, 10 ml of fresh DMEM complemented with 7% serum was added to each plate. Forty-h postinfection, the cells were scraped and pooled. An aliquot of each cell population was taken to perform Western blot and flow cytometry analyses, the rest of the cells was processed for RNA preparation. Two independent sets of infections were done in the same conditions and processed separately for RNA preparations and microarray analysis.

Western Blot Analyses. Twenty-five μg of total proteins from each cell population were separated on a 12% denaturing polyacrylamide gel. Immunodetection was performed using a mouse monoclonal anti-gfp antibody (Chemicon International).

Immunofluorescence. HeLa cells grown on coverslips were infected with gfp-E2 and gfp-delTAD recombinant adenoviruses. Forty-h postinfection, cells were rinsed with PBS and fixed in 4% formaldehyde. After rehydration, cells were permeabilized with 0.1% Triton and incubated with the p53 monoclonal antibody (PAB1801; Santa Cruz Biotechnology) and a p21 polyclonal antibody followed by secondary antibodies coupled to Texas red. Nuclei were stained by 4′, 6′-diamidino-2-phenylindole (0.15 μg/ml).

Flow Cytometry. One-twenty-fifth of each cell population used for array analysis was fixed in ethanol. After a wash in PBS, cells were incubated in PBS, 10 μg/ml RNase A, and 20 μg/ml propidium iodide for at least 30 min. DNA content was analyzed by flow cytometry using an Epics XL fluorescence-activated cell sorter (Coulter Beckman). Cell cycle analysis was subsequently performed using the Mplus software (Coulter).

Time-Lapse Imaging and Analysis. HeLa cells were infected by gfp-E2 or gfp recombinant adenoviruses at m.o.i. 50. Twenty-four-h postinfection, the cells were installed in a thermostatic chamber maintained in 7% CO_2 at 37°, under the objective of the microscope. Images were captured every 5 min using a 20× objective on a Zeiss Axiovert microscope. Images were processed by Metaview and converted to Adobe Photoshop.

RNA Isolation. Total RNA was extracted with Trizol (Life Technologies, Inc.) according to the manufacturer's recommendations. RNA quality was assessed with Agilent 2100 BioAnalyzer.

Microarray Analysis. Five μg of total RNA were linearly amplified by *in vitro* transcription with T7 polymerase as described previously (22). Briefly, RNA was reverse transcribed using an anchored oligodeoxythymidylic acid + T7 promoter sequence. The resulting double-stranded cDNA served as a template for T7 RNA polymerase, which produced an average yield of 10–30 μg of amplified antisense RNA. Five μg of antisense RNA was reverse-transcribed into a single-stranded cDNA using random nonamer primers (Genset, Paris, France). The cDNA was labeled by incorporation of Cy3-dCTP or Cy5-dCTP (Amersham BioSciences, Buckinghamshire, United Kingdom). Labeled cDNAs from the various gfp-E2-expressing viruses (Cy5) and from gfp-expressing virus (Cy3) were resuspended in equi-molar concentrations in 30 μl of hybridization solution (50% formamide, 5× SSC, 0.1% SDS, 100 μg/ml salmon sperm DNA) and prehybridized with 1 μl of polydeoxythymidylic acid (1 mg/ml) at 42°C for 30 min to block hybridization on the polyA/T tails of the cDNA on the arrays. After addition of 1 mg/ml human COT DNA (Invitrogen, Belgium), the labeled cDNAs were hybridized at 45°C on type VIIstar silane-coated slides (Amersham BioSciences) that had been blocked previously in 3.5% SSC, 0.2% SDS, and 1% BSA for 10 min at 60°C. Microarrays consisted of three separate slides containing in total 13,800 human cDNA fragments (VIB MicroArray Facility, Flanders Interuniversity Institute for Biotechnology; www.microarrays.be). The clone set was composed from the 6K collection of Incyte (HumanGem I; Incyte) and from the 75K collection of the Resource Center and Primary Database (RZPD, Berlin, Germany). On each of the three slides, on average 4,300 unique cDNAs were spotted in duplicate, distant from each other. The cDNA inserts were PCR-amplified using M13 primers, purified with MultiScreen-PCR plate (Millipore, Belgium) and arrayed on the slides using a Molecular Dynamics Generation III printer (Amersham BioSciences). Approximately 10% of the genes are represented on the microarrays by >1 cDNA. Posthybridization washing was performed in 1 × SSC, 0.1% SDS, followed by 0.1 × SSC, 0.1% SDS, and 0.1 × SSC. Arrays were scanned at 532 nm and 635 nm using a Generation III scanner (Amersham BioSciences). Image analysis was performed with ArrayVision (Imaging Research Inc, Ontario, Canada). Spot intensities were measured as artifact removed total intensities subtracted with median intensity of the local background of each spot to represent the expression levels. For each gene, ratios of red (Cy-5) over green (Cy-3) intensities (I) were calculated and normalized via a running median of the $\log_{10}$ ratios [$\log_{10}$ ($I_{\text{Cy-5}}/I_{\text{Cy-3}}$)] over the $\log_{10}$ total intensity [$\log_{10}$ ($I_{\text{Cy-5}} \cdot I_{\text{Cy-3}}$)]. Mean ratios were calculated from the duplicate spots, and only values with covariance <0.5 were further taken into account. The genes for which any of the duplicate spot intensities were lower than 2-fold the background were considered as "genes not expressed" or expressed at a level too low to produce statistically significant results. Normalized ratios >1.7 and <0.4 were considered to represent overexpressed and repressed genes, respectively.

Quantitative Real-Time Reverse Transcription-PCR Analysis. Five μg of total RNA were reverse transcribed into cDNA by MultiScribe reverse transcriptase, using the Reverse Transcription kit (Applied Biosystems) and the superscript II (Invitrogen) as recommended by the manufacturer. One-five-hundredths of the resulting synthesized single-stranded cDNA was used for each PCR reaction in the presence of 3 μM specific primers and Syber green PCR master mix (Applied Biosystems). Quantitative PCR was performed on a GeneAmp 5700 Sequence Detection System (Applied Biosystems), or ABI Prism 7000, with cycling conditions of 2′ at 50°C, 10′ at 95°C and 40× (15″ at 95°C, 1′ at 60°C). After the last cycle, the temperature was progressively raised to provide dissociation curves allowing for the assessment of the purity of the amplified product. Each PCR 96-well plate contained serial dilutions of β-actin, glyceraldehydes 3-phosphate dehydrogenase, or 18S ribosomal DNAs, which served to calibrate β-actin DNA concentration with the threshold cycle. Each PCR performed in triplicate and reported to a standard curve provided a mean threshold cycle value that was translated into an arbitrary DNA concentration. Relative expression values were expressed as the ratios between gfp-E2 and gfp-infected cell cDNA concentrations or thymidine-blocked gfp-infected cells.

RESULTS AND DISCUSSION

E2 Expression in HeLa Cells Represses Endogenous E6/E7 Transcription and Arrests Cells in G_1. The E2 protein dramatically affects cell proliferation by exerting direct transcriptional repression of endogenous viral E6/E7 oncogenes. Repression of E6/E7 transcription requires the specific binding of E2 to DNA, which is mediated by the COOH-terminal part of the protein. To discriminate between functions depending on the E6/E7 repression and independent functions of E2, we used two different truncated forms of E2 in addition to the full-length protein. DelTAD corresponds to the COOH-terminus of E2 and lacks the transactivation domain although it retains the ability to bind to E2 target DNA sequences. In contrast, the TAD construct only contains the amino terminus of the protein and retains its transactivation domain but does not bind to DNA. The three forms were fused to gfp and inserted into adenovirus expression vectors (Fig. 1*A*).

HeLa cells were infected with recombinant adenoviruses expressing the three different gfp-E2 isoforms or gfp alone, as described previously (9). Multiplicities of infection were such that 100% of infected cells became fluorescent 40 h after infection with no sign of apoptosis (9). Western blot analysis showed that all of the gfp fusion

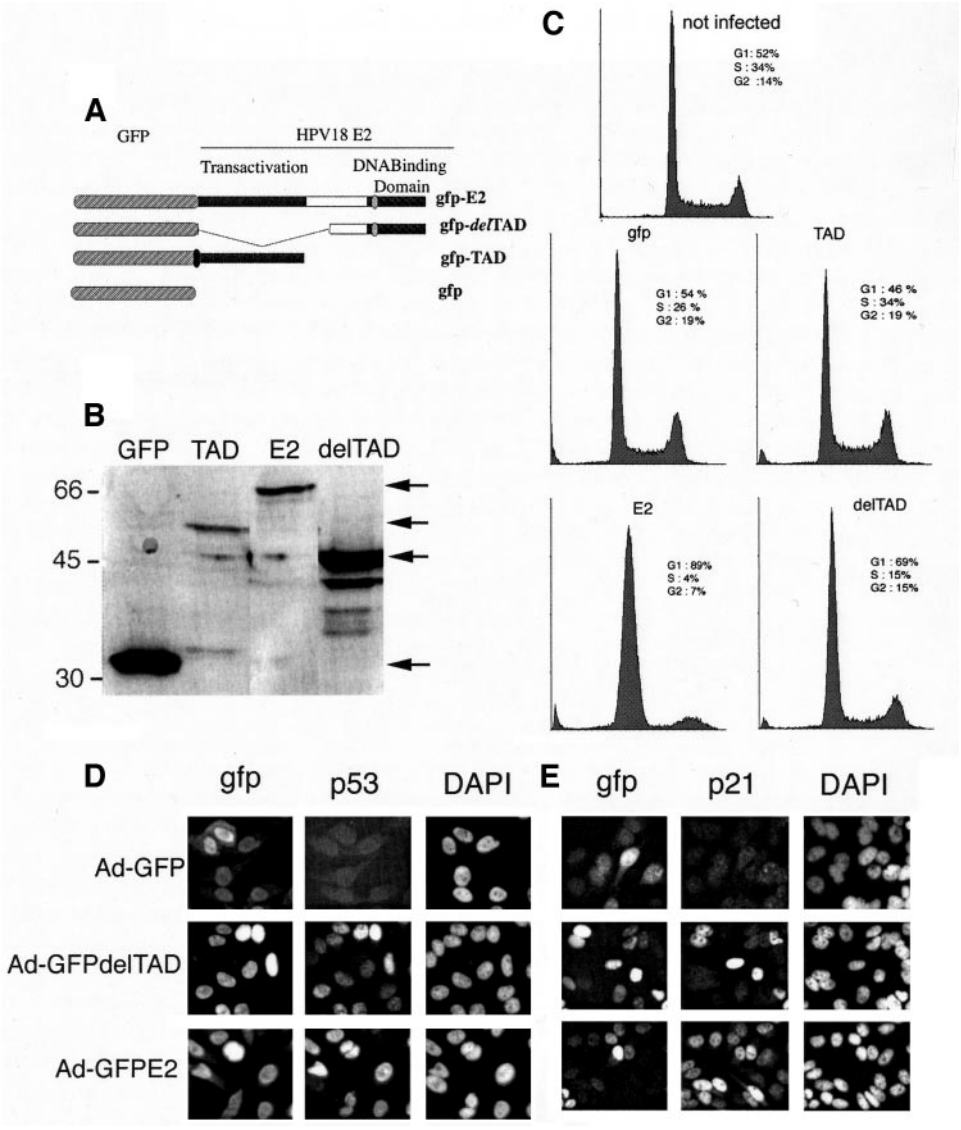

Fig. 1. HeLa cells infected by the four recombinant adenoviruses, 40-h postinfection. *A*, schematic representation of the green fluorescent protein (gfp)-fusion proteins is as follows; the gfp protein and the three domains of the E2 protein are shown as well as the natural nuclear localization signal (NLS) of E2 (▨) and an added NLS from the SV40 Tag (■). *B*, Western blot analysis with a GFP antibody showing expression of the four proteins in infected HeLa cells. *C*, cell cycle analysis by flow cytometry of the infected cells. *D*, immunofluorescence revealing stabilization of p53 in cells infected by adenoviruses expressing full-length E2 or delTAD but not GFP. *E*, immunofluorescence showing activation of p21 in cells infected by E2 and delTAD but not by GFP. *HPV*, human papillomavirus; *TAD*, transactivation domain; DAPI, 4′,6-diamidino-2-phenylindole.

proteins were expressed at detectable levels, although E2 and TAD attained lower levels because of their reduced stability (Ref. 23; Fig. 1*B*). As expected, real-time-PCR analysis showed that both E2 and delTAD repressed transcription of the E6/E7 oncogenes, whereas TAD had no significant effect (Table 1). Repression by delTAD was less efficient than with the full-length protein (6-fold as compared with 12-fold for E2). These results were consistent with our previous findings showing that the full-length E2 protein represses E6/E7 transcription more efficiently than delTAD, although this latter protein is more efficiently expressed and can bind DNA *in vitro* as efficiently as the full-length protein (23). Repression of E6 transcription should result in the stabilization of the p53 protein and concomitant activation of p53 target genes. Indeed, both E2 and delTAD activated p53 at the protein level (Fig. 1*D*), but not at the RNA level (Table 1), confirming our previous findings (2). Consequently, the expression of p21 was increased both at the RNA and the protein levels (Table 1; Fig. 1*E*). Levels of p21 RNA activation achieved by E2 and delTAD (20- and 6-fold, respectively) correlated with the levels of E6/E7 repression (12- and 6-fold, respectively), indicating that activation of the cell cycle inhibitor p21 was proportional to the efficiency of E6 repression and consequent p53 stabilization. Activation of p21 leads to G_1 cell cycle arrest, which was more pronounced with E2 than with delTAD (Fig. 1*C*). As expected, TAD did not activate the p53 pathway (Table 1 and data not shown), and did not induce G_1 growth arrest (Fig. 1*C*). Overall, these results validate the use of the different E2 mutants to discriminate between a dependent (E2 and delTAD) or nondependent (TAD) effect of E6/E7 transcription.

Microarray Analysis of Cellular Genes Regulated by E2. Modulation of global gene expression by E2 in HeLa cells was investigated with microarrays containing about 13,000 human cDNA sequences spotted in duplicate. The expression of cellular genes in HeLa cells infected with recombinant adenoviruses expressing the three isoforms of gfp-E2 were compared with Ad-gfp-infected cells to generate relative ratio values for each cellular gene. This experimental approach allowed us to eliminate variations of cellular gene expression because of adenoviral infection. Strict criteria were applied to exclude genes with a level of expression similar to the background or with bad SDs among duplicates. The system of normalization used slightly compressed values of relative expression, which resulted in a systematic under-evaluation of modulations, as compared with quantitative reverse transcription-PCR (Table 1; Fig. 4). Therefore, the threshold to include significantly regulated genes was set up at 2-fold for both activation and repression. The values are from one experiment but were all confirmed by another independent experiment.

A total of 357 genes were modulated >2-fold by any form of E2.

Table 1 *Real-time PCR of genes modulated by HPV18 E2, the TAD domain deletant (delTAD) or the TAD domain alone in HeLa cells*

Control genes are in bold while genes not represented in the microarray are in italic. Values are the mean of at least two independent RT-PCR analyses done on the RNA preparations used for microarrays.

Genes	E2	delTAD	TAD
HPV-E6/E7	−12	−6	1,6
p53	−1,6	1	1,4
P21/CDKN1A	20	6	1
PLAB	21	6	2
GADD45A	3,3	2	ND
Bax	4	3	1
Bak	2,8	1,4	1
PIG3	3	2	−2
PA26	5	2,3	1
Bcl2	1	1	1
Bclx1	−1,3	−1,2	−1,4
B-actin	1,1	1,5	1,5
GADPH	1,5	1,3	−1,3

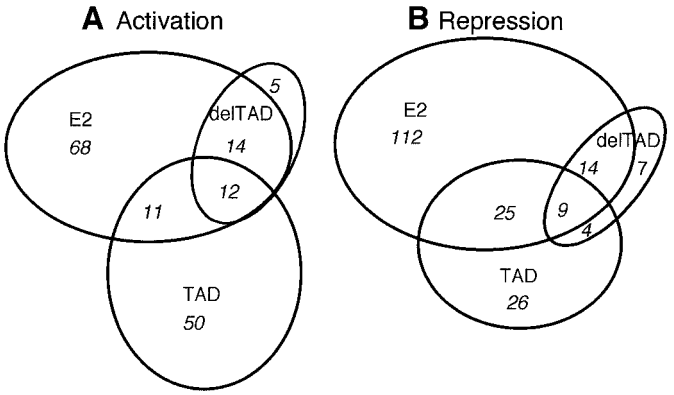

Fig. 2. Statistical analysis of genes modulated in the microarrays. *A*, number of genes found activated >2-fold when compared with green fluorescent protein (GFP) alone in microarray analysis using cells infected by the three types of adenoviruses expressing E2, delTAD, and TAD proteins. Total number of genes activated in each category as well as the overlaps between categories are shown. *B*, same as *A*, but for genes repressed >2-fold in the three conditions. *TAD*, transactivation domain.

Among them, 160 genes were activated and 197 were repressed >2-fold in at least one of the three conditions. The overlap between data sets common to full-length E2 and delTAD comprised 26 activated genes and 23 repressed genes (Fig. 2*A*). Among them, a fraction of genes were also modulated by TAD (12 for activation and 9 for repression). Because there is no conceivable functional overlap between the two deleted E2 proteins, delTAD and TAD, these genes were eliminated from further analysis. The use of two nonoverlapping deleted forms of E2 allowed an accurate assessment of the experimental limits of our approach and avoided the analysis of genes, the modulation of which is irrelevant to E2 expression.

In the present study, we focused the analysis on genes modulated both by E2 and delTAD, but not by TAD, to explore the cellular pathways mediated through transcriptional repression of the viral E6/E7 oncogenes. We confirmed that most of the cellular genes following this pattern of expression were not modulated by a direct effect of E2 by comparing them to microarrays done in Saos cells, which do not express the viral oncogenes, infected with the same adenoviruses expressing E2.[3] The overlapping data sets of genes regulated by both full-length E2 and delTAD comprised 14 activated genes and 14 repressed genes (Fig. 2). However, because delTAD was less efficient than E2 in modulating E6/E7 transcription (Table 1), it usually regulated cellular genes at a lower extent than the full-length E2 protein. Therefore, among the genes that were modulated >2-fold by E2, we lowered the level of modulation to 1.5-fold or delTAD. This extended the number of activated genes to 60 and the number of repressed genes to 67.

Genes Activated by E2 and DelTAD in the E6/E7 Pathways. We reasoned that transcriptional repression of E6, which is known to induce a strong stabilization of the p53 protein, should reactivate p53 target genes in HeLa cells as schematically illustrated in Fig. 3. At least 10 of the 60 activated genes were known p53 targets. Their activation ranged from 2-fold for the *TRAIL* receptor *TNFRSF10B* to 13-fold for the *p21/CDKN1* cell cycle regulator (Table 2). This level of activation was confirmed at the RNA level by real-time PCR (Table 1) and at the protein level by immunofluorescence (Fig. 1*E*). Among the other activated p53 targets, some are DNA damage-induced proteins such as *DDB2* or *GADD45A*. Others, such as transforming growth factor β or major vault proteins (20, 21), are not linked to the cell cycle but could play an important role in cell proliferation.

Of the 50 activated genes that were unknown p53 targets, very few

[3] Manuscript in preparation.

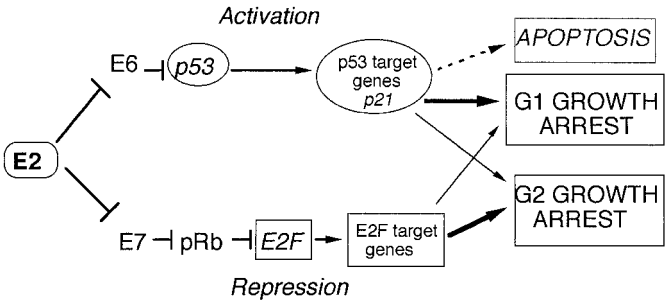

Fig. 3. Model for transcriptional modulation of cellular genes mediated by E2 repression of human papillomavirus (HPV)18 oncogenes in HeLa cells.

appeared directly linked to cell cycle regulation, and they do not form recognizable clusters (Table 4 in supplementary data). Because of their specific pattern of activation by E2 and delTAD, at least a subset of these genes might represent unknown p53 targets. Additional studies are in progress to explore this hypothesis further.

Although p53 can induce proapoptotic genes, only three genes potentially linked to apoptosis were activated about 2-fold by E2. These corresponded to the p53 target tumor necrosis factor-related apoptosis-inducing ligand receptor, TNFRSF10B gelsolin (Table 2) and a *Bcl*II-binding protein (BNIP3) (Table 4 in supplementary data). Other known proapoptotic p53 targets, such as *bax* and *bak*, were absent from the array. We therefore tested E2 modulation of their transcription by reverse transcription-PCR. We found that E2 activated transcription of *bax* and *bak* 4-fold and 2.8-fold, respectively (Table 1). However, these levels of activation were relatively modest compared with the levels of *p21*, for instance, 5 and 7 times <p21 activation in parallel experiments (Table 1). In contrast, antiapoptotic genes of the *Bcl*II family, such as *bcl2* or *bclxl* were not significantly affected by E2 (Table 1). Only the antiapoptotic gene *BAG4*, a silencer of death domain protein, was repressed 3-fold by E2 (Table 5 in supplementary data). Altogether, these results provide a global picture of the transcriptional effect of p53 stabilization because of E2 expression and provide a rationale for cell cycle arrest rather than apoptosis.

Many Genes Repressed by E2 in the E6/E7 Pathway Are Known E2F Targets. Repression of E7 transcription reactivates pRb with the concomitant repression of E2F target genes (Fig. 3). Of a total of 67 genes, repressed by full-length E2 and delTAD, 33 were known E2F targets although 34 were not known E2F targets (Table 3 and supplementary data). Most of the E2F target genes are linked to the cell cycle; eight are active in S phase, although 16 are active in mitosis (Table 3 and supplementary data). Our study indicates that expression of E2 preferentially affects E2F target genes linked to mitosis. These findings corroborate recent microarray studies showing a major role of E2F in the regulation of mitotic genes (24–30). Our study indicates that many of these mitotic genes are also targeted by E7 in cervical carcinoma. Of the repressed genes, which are not recognized E2F target genes, many were mitotic genes (Table 3), and we propose that they may represent new E2F targets. Experiments are in progress to validate this hypothesis.

Given the relatively high number of E2F target genes, only a subset of these are expected to be regulated in any particular condition, as illustrated in a study involving transcriptional modulations by pRb (26). Not surprisingly, many of these pRb targets were also found modulated by E2 in our study such as *cdc2*, *cyclin B2*, *topoisomerase II*, *RRM2*, *thymidine kinase*, or *cyclin A*. However, given the fact that the main effect described for E2 is a cell cycle arrest in G_1 (3, 4, 6), with a strong reduction in S phase (31), we expected mainly E2F-responsive S-phase genes to be repressed by E2. Although a number of S-phase E2F targets were found repressed by E2, the majority of E2F target genes affected were mitotic genes. This was unexpected and strongly suggested that the E7 viral oncogene, through its interaction with pRb, reactivates a mitotic pathway regulated by E2F in addition to G_1-S-dependent genes. Of particular interest, genes related to serine threonine kinases, important for centrosomal functions, such as aurora B (*STK12*), polo (*STK18*), polo-like (*PLK*), highly expressed in cancer (*HEK*), or the *NIMA* family (*NEK* 2, *NEK* 3 and *NEK* 6) are repressed by E2 (Table 3; Refs. 32 and 33). Aurora A and B, for instance, has been found overexpressed in various human solid tumors such as primary colorectal tumors, gastric cancers, or mammary carcinomas. Aurora A overexpression correlated with aneuploidy in gastric cancers (33), and its overexpression has been shown to over-

Table 2 *Genes activated by E2 that are known p53 targets*

Genes activated by E2 and delTAD, but not by TAD, which are known p53 target genes. Levels of transcriptional activation are given for E2 and delTAD as well as Unigene numbers. Some genes were represented more than once in the array and values are given for each representation. References of works where the genes are described are given as follows: Z, 21; K1, 42; K2, 20; L, 43. Note that for MVP, although a p53 motif was found in its promoter, no regulation by p53 has yet been shown.

Genes	E2	delTAD	Unigene Hs.[a] number	Short description	(Ref.)
TNFRSF10	2,2	1,6	51233	Dead receptor 5, Trail[b] R2, tumor necrosis factor receptor superfamily.	(Z)
GSN	2,5	3,3	446537	Gelsolin. Capping protein, actin regulatory protein.	(K)
MVP	3	1,7	80680	Major vault protein: nucleo-cytoplasmic transport	p53 motif (L)
″	2,8	1,5		(drug resistance to chemotherapy)	
DDB2	3	2	446564	DNA damage binding protein p48 subunit (*Xeroderma*	
″	3,2	2,2		*pigmentosum*)	
GADD45A	3,3	1,8	80409	DNA damage inducible gene, proapoptotic?	(Z)
				(*ataxia-telangiectasia*)	(K)
RRMB2	3,5	2	94262	Activated by p53 wt. Role in genomic instability	
″	9	3			
CYFIP2	4	2	211201	Interacts with FMR1 fragile X mental retardation protein.	
″	4	2			
PA26	4,4	3	14125	Member of the GADD family?	
″	7	3,5			
PLAB	9,6	2,6	296638	TGFB superfamily	(Z)
					(K)
P21/CDKN1A	8	3	370771	Cell cycle regulator, G_1 growth arrest.	
″	11	6			
″	13	4			

[a] Hs. refers to the Unigene cluster identification.

[b] Trail, tumor necrosis factor-related apoptosis-inducing ligand; TAD, transactivation domain; TGFB, transforming growth factor B; GADD, growth arrest and DNA damage-inducible; wt, wild type.

Table 3 *Mitotic genes repressed by E2*

Mitotic genes repressed by E2 and delTAD that are either known E2F targets or not, as indicated. References of the microarray analysis, where the E2F target genes are described, are given as follows: I, 23; R, 28; P, 27; M, 26; Ma, 24; and W, 29. Functions are given as well as the Unigene numbers.

E2F targets	E2 Rep[a]	Unigene Hs.[b] number	Short description	Ref.
ODC1	−3 −2,7	443409	Ornithine decarboxylase. Peaks twice in the G_1-S and G_2-M boundaries. Biosynthesis of polyamine involved in spindle formation and chromosome condensation.	Ma
KNSL5	−3,6	270845	Kinesin-like protein 5.	M
RECQLA	−3	31442	DNA helicase: unwind DS DNA to SS DNA (Rothman-Thomson syndrom) premature aging and cancer predisposition.	W
NEK2	−3,3	153704	Ser/Thr kinase: NIMA-related kinase 2.	R
UBE2C	−4	93002	Ubiquitin-conjugating enzyme E2C. Destruction of cyclins A and B at the end of mitosis.	W
MAD2L1	−3,6 −3	79078	Execution of mitotic checkpoint. Mad2 monitors the spindle kinetochore attachment.	I,R
Cdc20	−4	82906	Subunit of APC, active in mitosis. Decrease drastically in early G_1. Directs degradation of substrates with D box.	I,R
STK12	−4,3 −4	442658	Ser/Thr kinase: related to aurora B. Involved in cytokenesis during mitosis. Highly expressed in transformed cell lines.	R,P
PTTG1	−5	350966	Securin, APC substrate that associates with separin. Interacts with p53 and inhibits its functions (oncogenic potential).	R
STK18	−5	172052	Ser/Thr kinase: related to *Drosophila polo*. Specific G_2-M.	I,R
TOP2A	−5	156346	Topoisomerase: alters the topological state of the DNA. Chromosome condensation.	R,P
RRM2	−5	226390	Ribonucleotide reductase M2: catalysis the formation of deoxyribonucleotides from corresponding ribonucleotides.	I
CCNB2	−5,2	194698	Cyclin B2: less essential than B1. Binds to and activates p34 cdc2 to form MPF.	I
CNNA2	−5,2	85137	Cyclin A: complex with an activated Ser/Thr kinase during G_2-M. Repressed during G_1.	I,R
MYBL2	−6	179718	Vmyb: cell cycle progression (activates cdc2, cyclin D1); broader functions than the other members of the family.	I
Ki67	−6	80976	Antigen of proliferating cells.	I
Unknown E2F targets				
YWHAG	−2	25001	14.3.3 γ. Signal transduction leading to mitosis and cell proliferation.	
ZWINT	−3	42650	ZW10 interactor. ZW10 is a kinetochore protein.	
KNSL2	−3	20830	Kinesin-like 2.	
MPP1	−3	240	M phase phosphorylated protein. Kinesin, interacting with PIN1 and KRMP1.	
″	−3,2			
″	−3,4			
NEK6	−3,2	387222	Ser/Thr kinase: NIMA-related kinase 6.	
NEK3	−2,7	2236	Ser/Thr kinase: NIMA-related kinase 3.	
CNAP1	−3,2	5719	Related SMC-associated protein. Chromosome condensation.	
HEC	−3,6	414407	Highly expressed in cancer. Spindle checkpoint signaling.	
FoxM1	−3,8	239	Fork head: transcription factor with a PEST sequence. Cell cycle specific expression. Activation of cyclins B1 and D1. Reduced expression in aging cells.	
ESPL1	−4,5	153479	Separin. Spindle pole-like 1.	
PLK	−5,5	329989	Ser/Thr kinase: *Drosophila polo*-like kinase.	
BRRN1	−5,6	308045	Barren is necessary for sister chromatid separation and modulates topoisomerase II activity.	

[a] Rep, repression; DS, double stranded; SS, single stranded; APC, adenomatous polyposis coli; MPF, mitosis-promoting factor; SMC, structural maintenance of chromosomes; PEST sequence, sequences rich in proline, glutamic acid, serine, and threonine.
[b] Hs. refers to the Unigene cluster identification.

ride the mitotic spindle checkpoint (34). In contrast, specific depletion of aurora B in HeLa cells has been shown recently to affect mitotic progression (35). Our results indicate that aurora B is also activated in cervical carcinoma and could represent a novel marker for carcinogenic progression. These data provide a framework for understanding the role of E7 in centrosomal duplication leading to abnormal mitosis and aneuploidy (36).

Interestingly, not all E2F target genes present in the arrays were modulated by E2. For example, several S-phase genes involved in DNA replication, such as *MCM*, *ORC*, *RFC*, primase, *PCNA*, and *RPA* are not modulated by E2. We studied modulation of *RPA*, *PCNA*, and *MCM5* genes (present three times in the arrays) by real-time PCR from independent infection experiments and confirmed that E2 did not notably repress transcription of these genes (data not shown). Another group of E2F target genes that were unaffected by E2 are linked to chromatin assembly such as histone genes (*H2A* and *H2B*). The few E2F target genes, reported previously to be repressed in cervical carcinoma cells by E2, were S-phase genes, such as *cdc25A* or *cyclins A* and *E* (13–18). Besides these specific examples, very little is known about the E7/E2F pathway in cervical carcinomas. Our study provides a comprehensive group of genes, which are likely to be modulated during carcinogenic progression in cervical carcinoma by the E7 oncoprotein.

Mitotic Genes Are Specifically Repressed by E2 in Different Cervical Carcinoma Cell Lines. We speculate that the repression of many mitotic genes observed in the present study is because of E2-mediated repression of E7 transcription. Alternatively, it could be the consequence of the G_1 cell cycle arrest following E2 expression and p21 induction. To distinguish between these two possibilities, we compared the regulation of gene expression by E2 in infected HeLa cells to that achieved by synchronization in G_1-S with a thymidine block. Quantitative real-time PCR was performed on five mitotic and two S-phase-specific genes that were repressed by E2 in the arrays (Fig. 4*A*) and on two control genes, E7 and p21, that were highly repressed (E7) or activated (p21) by E2 (Fig. 4*B*). Among the seven repressed cellular genes, five are known E2F targets whereas *BRRN1*

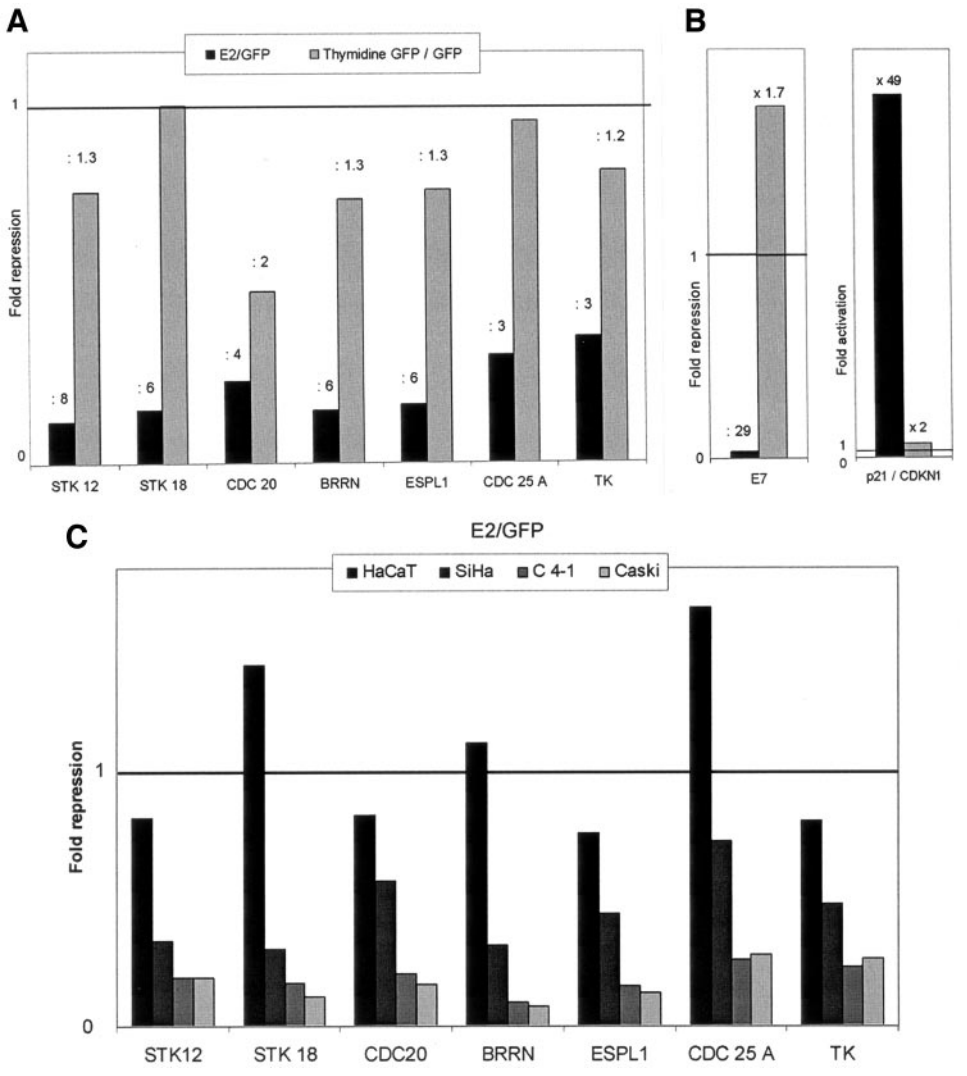

Fig. 4. Control real-time-PCR analysis of cellular genes in cervical carcinoma cell lines infected by E2. *A*, transcription of seven genes repressed in microarrays was studied by reverse transcription-PCR in green fluorescent protein (GFP)-infected HeLa cells arrested by thymidine treatment (▤) and E2-infected cells (■). Values given were the activities in each condition compared with asynchronous adeno-GFP-infected cells from which transcription was set up to a basal level of one for each gene. *B*, real-time-PCR done on the E7 and p21 transcription in HeLa cells. *C*, real-time-PCR analysis of the same set of genes in cervical carcinoma cell lines C4-1 [human papillomavirus (HPV)18], SiHa, Caski (HPV16), and human keratinocytes HaCaT. Values given are levels of gene expression in adeno-E2-infected cells when compared with adeno-GFP-infected cells.

and *Espl1* are not. Repression by E2 varied from 8-fold (for the *Stk12* gene) to about 3-fold (for the two S-phase E2F targets, *cdc25A*, and *thymidine kinase*) when compared with adenovirus GFP-infected cells. In contrast, synchronizing cells in G_1-S did not significantly alter the expression of these genes, with the exception of *cdc20*, a subunit of the anaphase-promoting complex, which was repressed 2-fold (Fig. 4). These experiments were also done with cells released at different time-points after thymidine block. They showed that variations of gene transcription were always less dramatic during cell phases than with E2 (Fig. 6 in supplementary data). These results indicated that the strong repression of the majority of these genes by E2 was a consequence of a direct repressive function of the viral protein.

To further analyze the impact of E2 expression in cervical carcinoma cells, we infected other cell types with the E2 recombinant adenovirus in conditions comparable with HeLa cells. We could show that E2 efficiently arrests the cell cycle of cervical carcinoma cells associated with HPV type 18 (C4–1) or type 16 (SiHa and Caski) but not of human keratinocytes (HaCaT) and that this cell cycle arrest was accompanied by induction of the p21 gene expression (data not shown). Real-time PCR analysis of a set of E2F target genes, repressed by E2 in HeLa cells, indicated a comparable transcriptional repression of these genes in all cervical carcinoma cells, in contrast to HaCaT cells where they were not markedly modulated (Fig. 4*C*). These experiments indicated that the cellular genes modulated by E2 in HeLa cells may constitute a general transcriptional signature of cervical cancer. Analysis of these genes in cervical lesions should allow for the validation of this hypothesis. A recent report identified markers of cervical carcinoma by cDNA and tissue microarrays (37). A small subset of these markers, linked to cellular proliferation, is common with our study (about five).

Our data were further compared with those from microarrays studies of HeLa cells in which the expression of genes in G_2 was compared with G_1 or S phases of the cell cycle (38, 39). A subset of genes repressed by E2 corresponds to genes specifically activated in G_2-synchronized HeLa cells as expected. However, not all G_2-M genes were repressed by E2 in our experiments and, on the other hand, many of the mitotic genes repressed by E2 (Table 3) were not modulated in these cell cycle studies. These comparative studies confirm that many genes are modulated by E2 independently of cell cycle modification.

E2 Affects HeLa Cells Both in G_1 and in Mitosis. We deduced that many genes, specifically repressed through E2-mediated repression of E7 could result in E2-induced growth arrest in G_2-M phase. This putative mitotic arrest would not have been visible in previous experiments (3, 9) because we showed previously that the expression of the transgene is closely linked to the cell cycle in transfected cells, inducing a dominant G_1 growth arrest (23). Indeed, fluorescence-

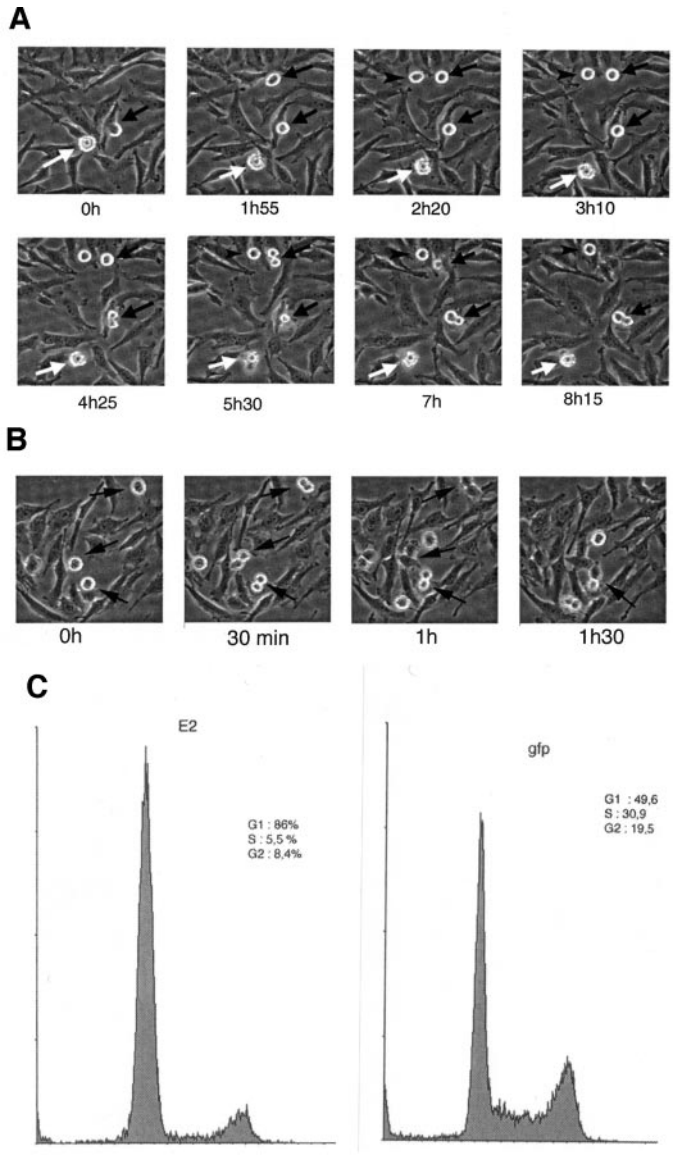

Fig. 5. Time-lapse microscopy of E2-infected HeLa cells. *A,* HeLa cells were infected by the recombinant adenovirus expressing E2 at an m.o.i. of 50, and cells were recorded between 16- and 26-h postinfection with pictures taken every 5 min. Pictures containing four mitotic cells in the same field are shown at different time-points. *Black arrows* show delayed mitoses, the cells staying round for more than 3 h. *Black arrowhead* shows a cell that remained round for more than 6 h. The *white arrow* shows a dying cell. Division times were calculated on a total of 70 mitoses giving a mean value of 110 min. *B,* HeLa cells were infected at an m.o.i. of 50 by the adenovirus expressing green fluorescent protein (gfp). *Black arrows* show mitotic cells. As for the E2-infected cells, time of division of 70 mitoses were calculated giving a mean time of 45 min. *C,* Cell cycle analysis of the E2- or gfp-expressing cells at the end of the time-lapse experiments, 27-h postinfection.

activated cell sorter analysis of HeLa cells infected by E2 recombinant adenoviruses revealed the existence of a G_2-M population, representing 30 to 60% of the equivalent peak in control cells, although the S phase was almost totally repressed (Fig. 5*C*). These analyses indicate that the G_1 growth arrest does not occur at the expense of the two other phases of the cell cycle equally, the G_2-M phase being less affected than S.

To further explore the role of E2 in mitosis, cells were followed by time-lapse microscopy from 16 to 26 h after infection with gfp-E2 or gfp recombinant adenoviruses. As visualized by time-lapse microscopy, mitoses occurred throughout the 10 h-recording time. However, most of the mitoses were slowed down by a mean value of about 2.5-fold (110 min, compared with about 45 min in control cells). Most of the observed mitoses slowed down but resolved normally and about 5% of them stopped before division, remaining round for the total length of the time-lapse experiment and eventually died. The delay in mitotic progression could be explained by E2-mediated repression of mitotic genes and was reflected by the fluorescence-activated cell sorter analysis that showed a noticeable remaining G_2-M peak representing 50% of the same population in control cells, despite a strong G_1 growth arrest. More work is needed to decipher how and which are the mitotic genes involved in this E2-induced phenotype.

Other Direct Targets of E6 and E7. The two viral oncogenes E6 and E7 have been shown to interact with other proteins in addition to the cell cycle regulators p53 and pRb (reviewed in Ref. 40). We looked for potential gene modulation because of these interactions and could not find any relevant correlation, contrary to our search for genes of the p53 and E2F pathways. The E6 targets for protein/protein interactions are from various functional backgrounds and disruption of the interaction of E6 with many of them such as paxillin, E6BP, or telomerase may not perturb transcriptional regulation of cellular genes. E6 can also interact with at least two different transcriptional regulators, p300 and interferon regulatory factor 3, and disruption of these interactions might be involved in transcriptional modulation of some of the cellular genes found in our study. In addition, E6 itself has been shown to behave as a transcriptional regulator (41), so that some of the genes repressed by E2 could be direct target genes for E6.

Regarding the interactions of E7 with proteins other than the pocket proteins, the picture is even more complex than with E6 (40). There are 17 known targets for protein interaction with E7, besides the pocket proteins, of which seven are transcription factors. In most studies, interaction with E7 repressed the transcriptional activity of the interacting factors, implying that disruption of these interactions by E2 should activate transcription of the genes controlled by these factors. Some of the E7-interacting factors belong to the general transcriptional machinery, such as TATA box binding protein or TAF 110, although others are more specific such as activator protein-1 or Oct4. Does this imply that some of the genes activated by E2 could be targets of the transcription factors repressed by E7? More work is needed to answer this question.

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