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Accumulation of Human Papillomavirus Type 16 E7 Protein Bypasses G_1 Arrest Induced by Serum Deprivation and by the Cell Cycle Inhibitor p21

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The E7 oncoproteins encoded by the high-risk type of human papillomaviruses (HPVs) interact with the Rb family proteins Rb, p107, and p130. The Rb family proteins associate with the factors of the E2F family to form transcription repressor complexes, which control expression of several genes essential for S-phase entry and DNA replication. The E7 oncoproteins, by interacting with the Rb family proteins, dissociate the repressor complexes involving the factors of the E2F and Rb families, leading to a release of the E2F factors in their activator forms. In this study, we have addressed the mechanism by which the HPV type 16 (HPV16) E7 stimulates the cell cycle. Using a cell line that inducibly expresses the HPV16 E7 protein, we show that an accumulation of E7 induces quiescent cells to enter S phase and that this function of E7 depends on retention of the motif involved in binding to the Rb family proteins. To study the effects of E7 on normal human cells, we generated a recombinant adenovirus that expresses the HPV16 E7 protein. Infection of normal human fibroblasts, which were arrested in G_1 phase by serum deprivation, with the E7-expressing virus induced the cells to enter S phase. The E7-induced S phase entry was accompanied by an increase in the activator form of E2F, but no increase in the cyclin-dependent kinase (cdk) activity was detected. Infection of serum-stimulated fibroblasts with a recombinant adenovirus expressing the cdk inhibitor p21 inhibited progression into S phase. Coinfection with the E7-expressing virus abrogated the p21 inhibition of progression into S phase without increasing the cdk activity. These results are consistent with the notion that E7 stimulates entry into S phase through targets downstream of the cdks such as the proteins of the E2F and Rb families.

The products of the human papillomavirus (HPV) oncogenes E6 and E7 cooperate to immortalize and transform cells (17, 19, 28, 38, 51), and they inactivate the cellular tumor suppressor proteins p53 and Rb. The E6 gene product binds p53 and induces a proteolytic degradation of p53 through the ubiquitin pathway (7, 28, 30, 45). The ability to induce p53 degradation has been linked to the oncogenic function of E6. The E6 proteins encoded by HPV types 6 and 11 (HPV6 and -11), which are low risk for malignant transformation, are unable or much less efficient in inducing degradation of p53 compared to those encoded by the high-risk types of HPV such as HPV16 and HPV18 (7, 45). Primary human fibroblasts expressing the HPV16 E6 protein at a high frequency exhibit genomic instability, chromosomal rearrangements, and aneuploidy (54), which are hallmarks of cancer cells. These characteristics are consistent with an E6-mediated loss of p53 because p53 is critical for genomic integrity and stability (57).

The E7 oncoprotein exhibits both structural and functional similarities with other DNA virus oncoproteins such as adenovirus E1A and simian virus 40 T antigen (5, 14, 15, 35). These oncoproteins interact with the Rb family proteins Rb, p107, and p130. The N-terminal sequences of E7, E1A, and T antigen contain a motif (LXCXE) that is involved in binding to the Rb family proteins (5, 14). The growth suppression domains of the Rb family proteins are targeted by the LXCXE motifs of these viral oncoproteins (24, 39, 55). It is generally believed that E7, by binding to the growth suppression domains, eliminates the suppression functions of the Rb family proteins (21). The C-terminal half of E7 does not exhibit homology with E1A or T antigen; however, this region is also important for the transformation function (26, 32). The C-terminal region contains Cys-X-X-Cys zinc-binding motifs, which are crucial for dimerization and transformation (32). A mutation in one of the cysteine residues within a zinc-binding motif resulted in a loss of the immortalization function of E7 (26, 32). The biochemical function of the C-terminal region and the significance of dimerization of E7 are yet to be determined.

One of the consequences of E7's binding to the Rb family of proteins is an increase in the activator form of the E2F family of transcription factors (39). The E2F factors exist in both activator and repressor forms, and they control expression of genes that are involved in DNA replication and cell division (3, 9, 10, 39). The repressor forms of the E2F factors involve the Rb family proteins. For example, Rb binds to E2F-1, E2F-2, E2F-3, and E2F-4 to generate complexes that are believed to be repressors of genes containing E2F-binding sites (22, 25, 31, 34, 44, 52, 53). p130 and p107 interact with E2F-4 and E2F-5 to generate similar repressor complexes (16, 34, 43, 49). E2F molecules that are not bound to the Rb family proteins are considered to be activators of transcription (13, 56). Highly purified E2F factors from HeLa nuclear extracts or recombinant E2F-1 could stimulate transcription in vitro (56). More-

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over, expression of E2F-1 has been shown to induce expression of several genes that are necessary for S-phase entry and DNA replication (13, 47). E7, by binding to the Rb family of proteins, disrupts their interactions with the E2F factors and releases the E2F factors in their activator forms. In addition, E7 has been shown to induce expression of B-*myb* (29) and of the cyclin E (58), cyclin A (58), and proliferating cell nuclear antigen (6) genes. These findings are also consistent with the effects of E7 on the activity of the E2F family factors because these genes have been shown to be regulated by the E2F factors (9).

It has been shown that differentiated human keratinocytes constitutively expressing the E7 protein remain replication competent (6). Moreover, keratinocytes expressing E7 could overcome growth arrest induced by DNA damage (11, 23). These findings are consistent with E7's effects on the activity of the E2F family of transcription factors as well as on the expression of the cell cycle genes such as those encoding cyclin A, cyclin E, and proliferating cell nuclear antigen. It is possible that E7, by stimulating expression of the cyclin genes, increases the activities of the cyclin-dependent kinases (cdks) to then induce cells to progress into S phase. Besides increasing the expression of cyclin genes, E7 has been shown to associate with cyclin E, cyclin A, and cdks (1, 8, 33, 48). These results imply a role of the cdks in the mechanism by which E7 stimulates DNA synthesis. We used a cell line that inducibly expresses the HPV16 E7 protein and an E7-expressing recombinant adenovirus to study the events that closely follow E7 expression. Here, we show that an accumulation of E7 in serum-deprived cells induces entry into S phase. This E7-mediated entry into S phase is accompanied by an increase in the activator form of the E2F family of transcription factors, but no significant increase in cdk activity is detectable. Moreover, E7 abrogates the cell cycle-inhibitory effects of p21 without increasing cdk activity.

MATERIALS AND METHODS

Cell culture. 3T3-E7 (wild type) and 3T3-E7 (mutant), NIH 3T3 cells expressing wild-type E7 and mutant E7, respectively, from the metallothionien promoter, were described before (36, 37). Normal human fibroblasts were a kind gift from G. R. Adami (University of Illinois at Chicago College of Dentistry). The cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum.

Construction of recombinant adenoviruses. Recombinant adenoviruses were generated by the overlap recombination method (2, 20). The wild-type E7 cDNA or a mutant E7 cDNA (harboring a deletion of amino acids DLYC in the Rb-binding region) was subcloned into the *Bam*HI/*Hin*dIII sites of plasmid pGEM CMV NEW (−) (a kind gift from the lab of J. R. Nevins [Duke University, Durham, N.C.]). This plasmid also contains adenovirus sequences for the overlap recombination. Purified adenovirus DNA (type 5 *in*340) was digested with *Xba*I and *Cla*I. The large piece of the viral DNA was purified by 1% agarose gel electrophoresis. Three micrograms of *Nar*I-linearized pGEM CMV NEW (−) containing E7 cDNA downstream of the cytomegalovirus promoter and 0.5 μg of the large fragment of adenovirus DNA obtained after *Cla*I/*Xba*I digestion were cotransfected into 293 cells grown in six-well cluster plates. Transfection was carried out by the calcium phosphate coprecipitation method (36). Precipitates were removed after 4 h, and cells were glycerol shocked with 1 ml of phosphate-buffered saline containing 10% glycerol for 1 min followed by two washes with DMEM containing 10% fetal bovine serum. Five days after transfection, lysates were prepared and potential recombinant viruses were isolated by plaque purification. The recombinants were confirmed by purification of the viral DNA, restriction digestion, and Southern blot hybridization. Stocks were prepared by infecting 293 cells. Crude lysates were subjected to a second round of plaque purification. The plaque-purified viruses were used to infect human normal fibroblasts.

Virus infection. Cells were infected at an average multiplicity of 30 PFU per cell. Infection was carried out in DMEM medium (2 ml/10-cm-diameter dish) containing no serum for 1 h at 37°C. After a 1-h infection, cells were replenished with DMEM (8 ml/10-cm-diameter dish); when necessary, 10% fetal bovine serum was added to the culture medium.

Flow cytometric DNA analysis. Nuclei from 5 million cells were isolated by a detergent-trypsin method followed by staining with propidium iodide (50). The flow cytometry analysis was performed in the Research Resources Center at the University of Illinois at Chicago with the help of K. Hagen. The flow cytometers used were Coulter EPICS 753 and Coulter EPICS ELITE ESP (Coulter Electronics Co., Hialeah, Fla.). The calculations of percentage of cells in the different phases of cell cycle were done by using the Multicycle AV program (Phoenix Flow Systems, San Diego, Calif.).

Northern blotting for E7 expression. Northern blot assays for E7 expression were carried out as previously described (36, 37).

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blot assays for E7 expression were described before (36). Western blot assays for p21, cdc2, cdk2, and cdk4 were carried out as described by Shiyanov et al. (46). cdc2, cdk2, and cdk4 antibodies were purchased from Santa Cruz Biotechnology.

Assays of cdks. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 0.1% Nonidet P-40, and 50 mM sodium fluoride. Cell lysates (250 μg) were incubated with 1 μg of antibodies against cdc2 (Santa Cruz), cdk2 (Pharmingen), cdk4 (Santa Cruz), cyclin A (Pharmingen), cyclin B (Pharmingen), cyclin E (Santa Cruz), or cyclin D1 (Santa Cruz) for 2 h at 4°C. The immune complexes were collected onto protein A-Sepharose beads. cdc2 and cdk2 were also purified from these extracts by binding to p13-Sepharose beads for 1 h on a nutator. The p13-Sepharose beads were prepared as previously described (42). The beads containing the immune complexes were washed three times with cell lysis buffer and once with kinase buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol). The washed beads were used to phosphorylate histone H1 in the presence of [γ-^{32}P]ATP and a protein kinase A inhibitor as described elsewhere (42). For cdk4 and kinases that associate with cyclin D1, glutathione *S*-transferase (GST)–Rb (0.3 μg) was used as the substrate.

Gel retardation assays for E2F. Gel retardation assays were performed as previously described (46).

RESULTS

Expression of the HPV16 E7 oncoprotein in quiescent NIH 3T3 cells induces entry into S phase. We have described the construction of cell lines that express wild-type or mutant HPV16 E7 oncoprotein in an inducible manner (36). These cell lines were constructed by transfecting NIH 3T3 cells with E7 expression plasmids under the control of a modified human metallothionein promoter (36). The mutant E7 construct harbors changes in the Rb-binding domain (LXCXE motif) and does not bind the Rb family proteins (41). Expression of E7 in these cells can be induced about 10-fold by adding 4 μM zinc chloride to the culture medium (Fig. 1A, lanes 1 and 2). The zinc chloride treatment was carried out typically for 3 h because these cells, irrespective of E7 expression, elicited a toxic response upon a longer incubation with zinc chloride (data not shown). The effect of E7 expression on cell cycle progression was studied by exposing these cells to zinc chloride for 3 h. To investigate the effect of E7 on quiescent cells, the cells were grown to 80% confluence and serum starved in DMEM containing 0.5% fetal bovine serum for 36 h. Zinc chloride was added to the serum-starved cells, and after a 3-h incubation, the medium was replaced by fresh medium. After an additional incubation for 15 h, cells were harvested, and nuclei were purified, stained with propidium iodide, and subjected to flow cytometry analysis. Results of three independent experiments are summarized in Table 1. Clearly, expression of the wild-type HPV16 E7 stimulated a significant proportion of the cells to enter S phase, whereas cells expressing a mutant E7 that is unable to bind the Rb family proteins behaved very much like the normal NIH 3T3 cells and remained arrested in the G_0/G_1 phase. The 3T3-E7 (wild type) and 3T3-E7 (mutant) cells expressed the wild-type and mutant E7 proteins at comparable levels (37). Interestingly, the E7-mediated entry into S phase required about 12 h, which is approximately the time required during serum-stimulated entry into S phase (not shown).

Expression of E7 in serum-deprived primary human fibroblasts causes the cells to enter S phase. The effects of E7 on normal human fibroblasts and keratinocytes have been studied by comparing cells expressing no E7 with those constitutively expressing HPV16 E7 (6, 11, 54). These studies reported altered cell cycle behavior of cells expressing E7. For example, it

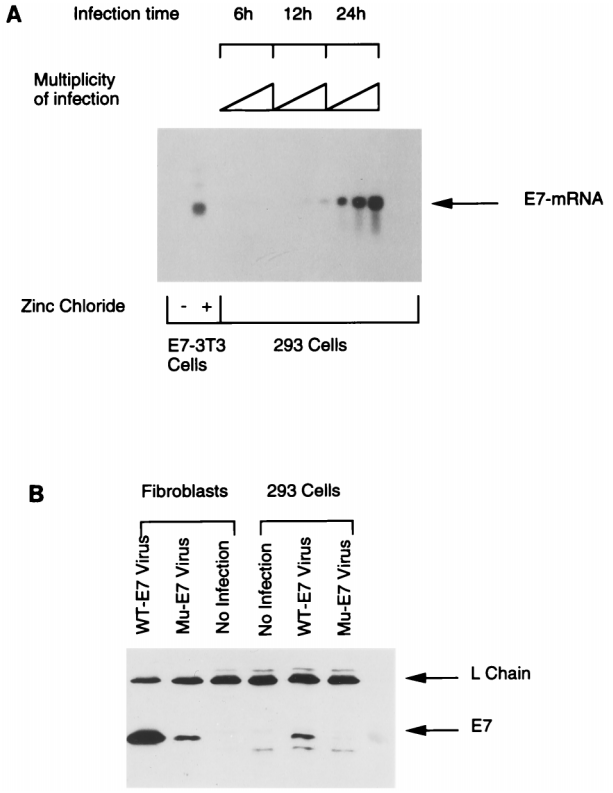

FIG. 1. Expression of E7 in cells infected with the E7 virus. (A) 293 cells were infected with the E7 virus at increasing multiplicity of infection for the indicated time periods. The 6-h infection was carried out at 100, 300, or 1,200 PFU/cell, whereas the 12- and 24-h infections were carried out at 30, 100, or 300 PFU/cell as described in Materials and Methods. The cells were maintained in DMEM containing 10% fetal bovine serum. A northern blot of 1.5 μg of total cellular RNA probed for E7 mRNA is shown. The two lanes at the left contained RNA from the inducible NIH 3T3 cells (37) before and after induction with zinc chloride. (B) 293 cells or primary human fibroblasts were infected with the wild-type or E7 (WT-E7) the mutant E7 (Mu-E7) virus at 30 PFU/cell for 20 h. Cell lysates (2 mg) were immunoprecipitated by a monoclonal antibody against E7. The immunoprecipitates were subjected to Western blot analysis. The blots were probed with the E7 antibody and developed with enhanced chemiluminescence.

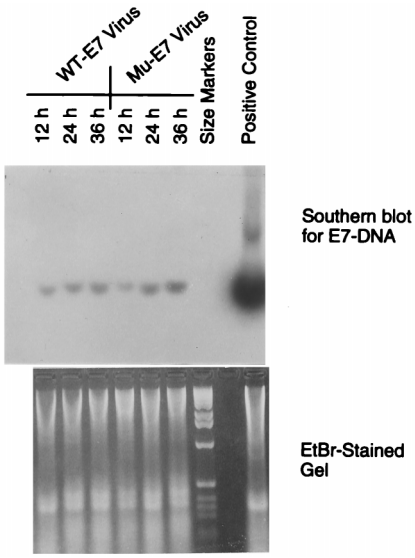

FIG. 2. The E7 virus does not replicate in primary human fibroblasts to any significant extent. Replication of the wild-type E7 (WT-E7) and mutant E7 (Mu-E7) virus was analyzed by assaying E7 DNA relative to total cellular DNA. Human fibroblasts were infected at 30 PFU/cell for the indicated time periods. The infected cells were then lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 0.1% sodium dodecyl sulfate, and 200 mM sodium chloride. The lysates were digested with proteinase K (0.2 mg/ml) and RNase A (0.1 mg/ml) for 1 h at 37°C. The DNA was isolated by phenol-chloroform and ethanol precipitation. To check for recovery, E7 virus was mixed with lysates from uninfected cells and subjected to a similar DNA isolation procedure (Positive Control). Ten micrograms of the isolated DNA was digested with *Eco*RI and *Bam*HI. The digests were subjected to a Southern blot analysis using E7-specific antisense RNA as a probe (upper panel). The lower panel shows an ethidium bromide (EtBr) stain of the DNA that was used in the Southern blot.

was shown that keratinocytes expressing the E7 protein do not exhibit G_1 arrest upon DNA damage, suggesting that E7 can bypass a G_1 block (11, 23). Moreover, differentiated keratinocytes expressing E7 remain replication competent (6). To investigate a more immediate effect of E7 on the cell cycle control mechanisms of mortal cells, we sought to construct a recombinant adenovirus that can be used to infect primary cells. Recombinant adenoviruses encoding wild-type or mutant E7 were constructed as described in Materials and Methods. The mutant E7-expressing virus (E7 virus) expressed the mutant protein at a much lower level than the wild-type virus (Fig. 1B); therefore, it was used only as an infection control and is referred to as control virus. Expression of the wild-type E7 at different multiplicities of infection is shown in Fig. 1A.

TABLE 1. Expression of HPV16 E7 in quiescent cells induces entry into S phase

Cells	% of cells[a]					
	−Zinc chloride			+Zinc chloride (4 μM)		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
NIH 3T3	85	9	6	83	11	6
3T3-E7 (wild type)	81	12	7	49	39	12
3T3-E7 (mutant)	85	7	8	77	15	8

[a] Average of three experiments. The variation did not exceed 5%.

The wild-type E7 virus did not replicate to any significant extent in normal human fibroblasts during a 36-h infection period. To detect viral DNA replication, the amount of E7 DNA relative to total cellular DNA was measured at different time points after infection. For a positive control, viral DNA was added to an equivalent lysate of uninfected cells. Ten micrograms of RNase-treated total DNA was digested with *Eco*RI and *Bam*HI, which releases E7 DNA from the viral genome. The E7 DNA was analyzed by a Southern blot assay using E7-specific RNA probes. As can be seen in Fig. 2, we did not detect any significant increase of the E7 DNA relative to total cellular DNA after 12, 24, or 36 h of infection, which would be consistent with a lack of viral DNA replication. Also, we did not detect any cytopathic effect within the 36-h time period, which is routinely seen in 293 cells. In addition, we did not detect any difference in levels of the adenovirus E4 gene expression (which is important for the viral DNA replication) between the wild-type E7 and the control virus (not shown).

The normal human fibroblasts were used to determine the effects of E7 expression on the cell cycle control of primary human cells because these cells can be arrested in G_1 phase by serum starvation. Fibroblasts were grown to 40% confluence in DMEM medium containing 10% fetal bovine serum. The cells were then starved for 48 h in serum-free DMEM medium. The serum-starved cells were infected with 30 PFU of the wild-type E7 virus or the control virus. The cells were harvested after 15 h of infection. Nuclei were purified, stained with propidium

TABLE 2. Accumulation of HPV16 E7 protein in serum-deprived normal human fibroblasts induces entry into S phase

Virus infection	% of cells in[a]:		
	G_0/G_1	S	G_2/M
None	87.5	6	6.5
Wild-type E7 virus	16	75	10
Control virus	89	3	8

[a] Average of three independent experiments. The variation did not exceed 10%.

iodide, and subjected to flow cytometry analysis. The results of the flow cytometry analysis are summarized in Table 2. After serum starvation, the majority of cells were in G_0/G_1 phase of the cell cycle. The expression of wild-type E7 stimulated entry into S phase. The control virus-infected cells remained arrested in G_0/G_1 phase. These results are consistent with the notion that E7 overrides a G_1 block induced by serum starvation and stimulates cells to enter S phase.

Recent studies on E2F-1 indicated that the expression of E2F-1 in quiescent NIH 3T3 cells or in primary rodent cells stimulates entry into S phase. HPV16 E7 has been shown to be a regulator of the E2F activity (1, 5, 40). E7 disrupts the interaction between E2F and the Rb family proteins, leading to an increase in the level of the activator form of E2F (5, 40). Moreover, E7 also associates with a cyclin A-containing complex of E2F (1). To investigate whether the cell cycle-stimulatory effect of E7 correlates with its effect on the E2F complexes, the extracts from control virus- and E7 virus-infected cells were compared by E2F-specific gel retardation assays. Consistent with previous results, the extract of the wild-type E7 virus-infected cells contained significantly more free E2F (which is considered to be the activator form of E2F) than the extract of the control virus-infected cells (Fig. 3). Moreover, one of the complexes in the extract of E7 virus-infected cells remained bound to E7, which would be consistent with a previous observation that E7 binds to a complex of E2F containing cyclin A and p107 (1).

To determine the effects of E7 expression on the activities of cdks, the extracts from infected cells were compared for cdk activities. These extracts contained comparable levels of the cdc2, cdk2, and cdk4 proteins (Fig. 4B). We were unable to detect cdk6 in these extracts (not shown), which is consistent with a poor expression of cdk6 in fibroblasts (33a). Antibodies against cdc2, cdk2, and cdk4 were used to immunoprecipitate the kinases from extracts of cells that were infected with the control virus or E7 virus for 10 or 15 h. The immunoprecipitates were washed and subjected to phosphorylation assays using either histone H1 (for cdc2 and cdk2) or GST-Rb (for cdk4) as substrates. Antibodies against cyclin A, cyclin B1, cyclin E, and cyclin D1 were also used to purify the associated kinases. The activities of these kinases were compared for their abilities to phosphorylate histone H1 (cyclins, A, B, and E) or GST-Rb (cyclin D1). The phosphorylation experiments were performed as described in Materials and Methods. These experiments allowed us to assay specifically the cdks because the phosphorylating activities could be eliminated by the cdk inhibitor p21 and immunoprecipitates obtained with normal immunoglobulin G did not exhibit any histone H1 phosphorylation activity (not shown). As can be seen in Fig. 4A, we did not detect any significant increase of these kinase activities by the expression of E7. Moreover, we used p13-Sepharose beads to collect the cdks from cell extracts. The p13-bound kinases were assayed for histone H1 phosphorylation activity as described in Materials and Methods. Again, no significant difference was observed between the extracts from E7 virus-infected cells and control virus-infected cells (Fig. 4C).

Expression of the E7 gene overrides the cell cycle inhibition by p21. Results presented above suggested that the HPV16 E7 protein could stimulate entry into S phase without requiring an up-regulation of the activity of the cdks. To further investigate the role of the cdks in the E7-mediated entry into S phase, we used the cdk inhibitor p21 (12, 18). A recombinant adenovirus expressing p21 (p21 virus) was used for this purpose. Construction of the p21 virus has been described elsewhere (4). Primary human fibroblasts were grown to 30% confluence and then starved in serum-free medium for 48 h. The cells were then infected with viruses. Five hours after virus infection, the culture medium was replaced by medium containing 10% fetal bovine serum; the incubation was continued for another 15 h, and cells were then harvested and subjected to flow cytometry analysis. Results of three independent experiments are summarized in Table 3.

Infection of cells with the p21 virus resulted in an inhibition of the serum-induced entry into S phase (Table 3). This inhibition correlated with an inhibition of the cdks in the extracts (Fig. 5). The cdk activities were measured by collecting the kinases on p13-Sepharose beads, which were subsequently assayed by the ability to phosphorylate histone H1. These results are consistent with the notion that p21 inhibits cell cycle pro-

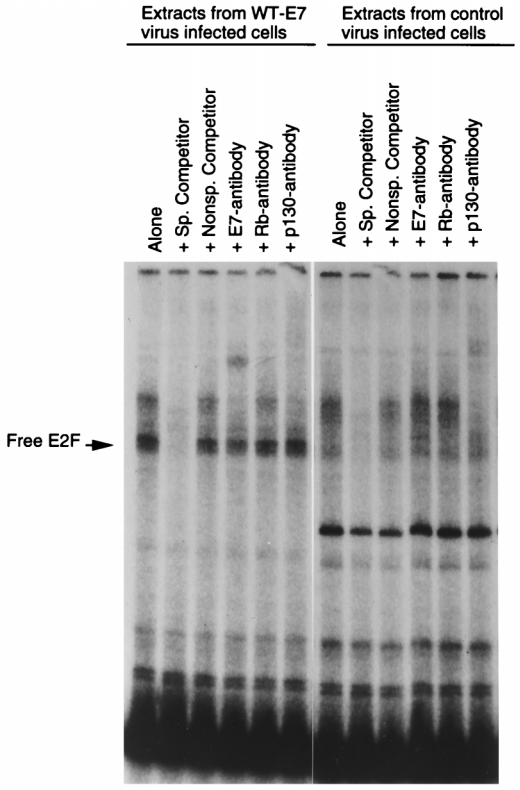

FIG. 3. Extracts from wild-type E7 virus-infected cells contain high levels of free E2F. Primary human fibroblasts were grown to 40% confluence and then maintained in DMEM containing no serum for 48 h. The starved cells were infected with the wild-type E7 (WT-E7) virus or the control virus for 15 h. Whole-cell extracts were prepared as described before (46). The extracts (10 μg) were subjected to the E2F-specific DNA-binding assay in the presence of specific (Sp.) or nonspecific (Nonsp.) competitor DNA (46). Where indicated, the reaction mixtures were also incubated with E7 antibody (0.1 μg), Rb antibody (0.1 μg), or p130 antibody (1 μg). The p130 antibody at this level of assay recognizes both p130- and p107-containing complexes.

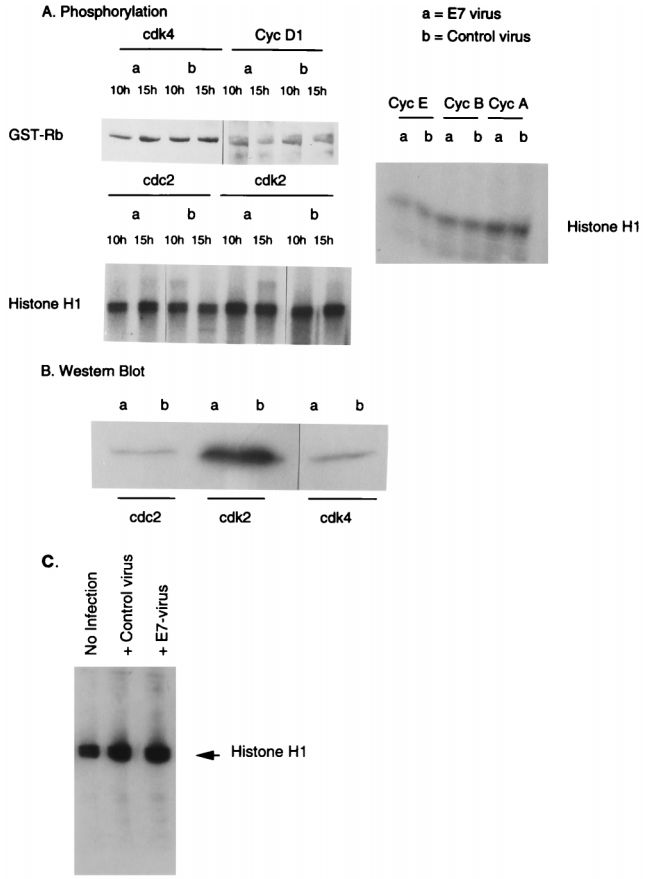

FIG. 4. Extracts from wild-type E7 virus- and control virus-infected cells contain similar levels of cdk activities. Human fibroblasts were grown to 30% confluence and then maintained in DMEM for 48 h. The serum-starved cells were infected with the wild-type or control virus for 10 or 15 h. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM sodium chloride, 0.1% Nonidet P-40, and 50 mM sodium fluoride. The cell lysates were used to perform the following experiments. (A) The indicated antibodies against the various cdks and cyclins were used to immunoprecipitate the kinases from the cell lysates (250 μg) as described in Materials and Methods. After extensive washing, the immunoprecipitates were subjected to phosphorylation assays as previously described (42) (see Materials and Methods). (B) Cell lysates (100 μg) were analyzed in Western blot assays for the levels of cdc2, cdk2, and cdk4 proteins. Antibodies (Santa Cruz) were used at 1:200 dilution. The blots were developed by enhanced chemiluminescence (Amersham) as instructed by the manufacturer. (C) Infection was carried out for 20 h. Cell lysates (150 μg) were incubated with 10 μl of p13 beads (about 2.5 mg of p13 per ml of beads). After an extensive washing, the beads were used to assay for histone H1 kinase activity as previously described (42).

gression by inhibiting cdks (12, 18). Coinfection of cells with the wild-type E7 virus, but not the control virus, overcame the inhibition conferred by the p21 virus. However, the extracts did not exhibit a return of the cdk activities (Fig. 5). Cells infected with the E7 virus alone entered S phase with greater efficiency than cells that were coinfected with p21 virus, most likely because of a decrease in the level of E7 expression in p21 virus-coinfected cells. In the coinfection experiments, E7 did not alter the level of p21 expression, but p21 reduced the level of E7 (Fig. 6). On the basis of these results, we conclude that E7 stimulates entry into S phase without requiring an increase of the cdk activities.

DISCUSSION

Previous studies on the effects of E7 on cell cycle progression were carried out with cells that constitutively express the E7 protein (6, 11, 23). While these studies generated valuable insights into the function of E7, the mechanism by which E7 modifies the cell cycle remains poorly understood. In this study, we used an inducible system and a recombinant adenovirus to express E7 in an on/off manner, which allowed us to analyze the events that closely follow E7 expression. Using NIH 3T3 cells that express HPV16 E7 under the control of a metal-inducible promoter (36), we showed that expression of E7 stimulates serum-starved NIH 3T3 cells to enter S phase. Expression of a mutant E7 which does not bind the Rb family protein had no effect on serum-starved NIH 3T3 cells. These results suggest that the Rb-binding function of E7 is important for its cell cycle-stimulatory activity. We also observed that expression of the wild-type but not mutant E7 generated a higher level of free E2F factors in the cell extracts (not shown). Therefore, it is possible that E7 stimulates cell cycle progression by increasing the levels of free E2F, which would be

TABLE 3. HPV16 E7 overrides the p21-mediated inhibition of S-phase entry

Addition of serum and virus infection[a]	% of cells in[b]:	
	G_0/G_1	S
No addition	87	11
10% FBS	63	35
10% FBS + p21 virus	80	18
10% FBS + p21 virus + WT E7 virus	51	42
10% FBS + p21 virus + control virus	82	15
10% FBS + control virus	52	37
10% FBS + WT E7 virus	39	57

[a] FBS, fetal bovine serum; WT, wild-type.
[b] Average of three independent experiments. The variation did not exceed 5%.

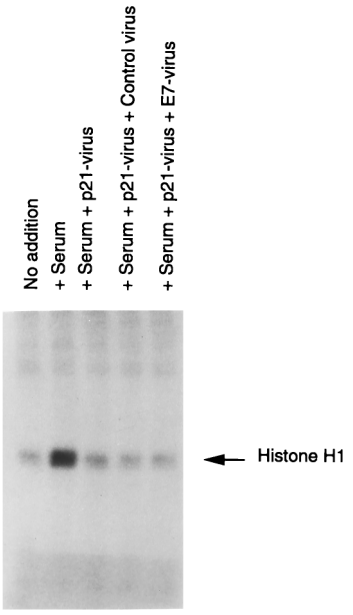

FIG. 5. E7 does not reverse the p21 inhibition of histone H1 kinase activities in primary human fibroblasts. Primary human fibroblasts were grown to 30% confluence and then incubated in DMEM without serum for 48 h. The starved cells were infected with the indicated viruses. Five hours following infection, cells were stimulated by adding 10% fetal bovine serum to the culture medium. Cell lysates were prepared 15 h after serum stimulation. Histone H1 kinases activities were purified by p13-Sepharose beads and subjected to histone H1 kinase assays as described before (42).

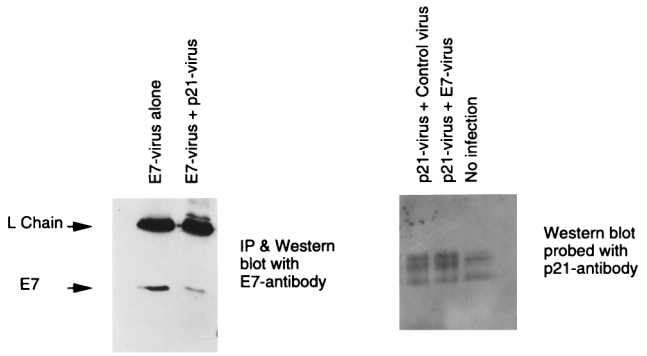

FIG. 6. Coinfection with the p21 virus reduces expression of E7 in E7 virus-infected cells. Human fibroblasts were infected with the indicated viruses as described in the legend to Fig. 5. The cell lysates were analyzed for E7 and p21 expression. For E7 expression, 2 mg of the cell lysates was subjected to immunoprecipitation (IP) with an E7 antibody (37). The immunoprecipitates were analyzed for E7 by a Western blot assay (37). For p21 expression, 200 μg of the cell lysates was directly analyzed in a Western blot assay using a p21-specific antibody (46) as a probe.

consistent with the observation of Johnson et al. (27) that expression of E2F-1 in quiescent NIH 3T3 cells stimulates the cells to enter S phase.

To determine the effects of E7 on primary human cells, we constructed recombinant adenoviruses that express wild-type or mutant (defective in Rb binding) E7. The mutant E7 virus produced much less protein in 293 cells and in primary human fibroblasts compared to the wild-type E7 virus (Fig. 1). Therefore, it was used mainly as an infection control. We did not detect any significant replication of these viruses in primary human fibroblasts during a 36-h infection period. The replication of viral DNA was measured by assaying for an increase in E7 DNA relative to total cellular DNA. No significant increase in E7 DNA relative to total DNA was observed (Fig. 2). Also, we assayed for expression of the E4 gene, which is critical for adenovirus DNA replication. We did not see any difference in E4 gene expression in cells infected with the wild-type E7 virus and the mutant E7 virus (not shown).

The E7 virus was used to infect primary human fibroblasts. Accumulation of the E7 protein in serum-deprived fibroblasts stimulated the cells to enter S phase without requiring any growth factor (Table 2). The E7-induced entry into S phase coincided with an accumulation of free E2F (Fig. 3), which is believed to be the activator form of the E2F factors. The activator form of E2F has been shown to stimulate expression of several genes involved in DNA replication (9). It is possible that E7, by generating the activator form of E2F factors, stimulates entry into S phase. E7 was shown to stimulate expression of cyclin A and cyclin E genes (58). In our experiments, serum-deprived human fibroblasts contained the cyclins, and the accumulation of E7 did not show any significant increase in their levels (not shown). This could be a result of high background signals. Also, we did not observe any significant increase in the cdk activity (Fig. 4). In addition, we did not detect any increase in the phosphorylation of Rb, p107, E2F-4, and DP1 (not shown). Therefore, these results suggested the possibility that E7 stimulated S-phase entry by increasing the levels of free E2F and that an increase in cdk activity or Rb phosphorylation is not essential. This would be apparently different from the observations made in cells constitutively expressing E7. It was shown that keratinocytes constitutively expressing E7 are not arrested by DNA damage and that the Rb protein remains in the hyperphosphorylated forms (11, 23). Our results, on the other hand, are consistent with the observations made with E2F-1 overexpression. It was shown that cells overexpressing E2F-1 bypass G_1 arrest induced by γ-irradiation without an accompanying increase of G_1 cdk activities (9). It is, however, possible that E7 causes hyperphosphorylation of Rb with a much delayed kinetics which is beyond the time frame of the experiments presented here. A delayed phosphorylation of Rb was also observed in the case of E2F-1-induced S-phase entry (9). Nevertheless, in keeping with an observation made by Arroyo et al. (1), we detected an interaction of E7 with a cyclin-containing complex of E2F (Fig. 3). Therefore, an involvement of cyclin-cdk2 cannot be ruled out.

To further investigate the role of the cdks in the E7-mediated entry into S phase, we coinfected cells with recombinant adenovirus that encodes the cdk inhibitor p21. Infection of fibroblasts with the p21 virus resulted in an inhibition of the serum-stimulated entry into S phase, which coincided with an inhibition of the cdks. Interestingly, coinfection with the E7 virus abrogated the p21 inhibition, and the cells entered S phase in the presence of a high level of p21. p21 inhibits cell cycle progression by inhibiting cdks (12, 18). E7 expression did not reverse the p21 inhibition of cdk activity (Fig. 5). This observation confirms that an increase in cdk activity is not essential for the E7-mediated S-phase entry. However, we cannot completely rule out an involvement of these kinases because there was always a residual of level of these kinases in our experiments. Nevertheless, the results are consistent with the notion that E7 bypasses the need for cdks by acting on targets downstream of the cdks, which is also in agreement with the fact that E7 interacts with the Rb family proteins, which are downstream targets of the cdks. We suggest that an E7-mediated disruption of the repressor complexes involving the E2F factors plays a crucial role in stimulating entry into S phase.

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REFERENCES

1. **Arroyo, M., S. Bagchi, and P. Raychaudhuri.** 1993. Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex. Mol. Cell. Biol. **13:**6537–6546.
2. **Babiss, L. E., and H. S. Ginsberg.** 1984. Adenovirus type 5 early region 1b gene product is required for efficient shutoff of host protein synthesis. J. Virol. **50:**202–212.
3. **Blake, M., and J. C. Azizkhan.** 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. **9:**4994–5002.
4. **Chang, M. W., E. Barr, M. M. Lu, K. Burton, and J. M. Leiden.** 1995. Adenovirus-mediated over-expression of the cyclin/cyclin-dependent kinase inhibitor, p21 inhibits vascular smooth muscle cell proliferation and neointima formation in the rat carotid artery model of balloon angioplasty. J. Clin. Invest. **96:**2260–2268.
5. **Chellapan, S., V. B. Kraus, B. Kroger, K. Munger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA **89:**4549–4553.
6. **Cheng, S., D. Schmidt-Grimminger, T. Murant, T. Broker, and L. Chow.** 1995. Differentiation-dependent up-regulation of the human papillomavirus E7 gene reactivates cellular DNA replication in suprabasal differentiated keratinocytes. Genes Dev. **9:**2335–2349.
7. **Crook, T., J. A. Tidy, and K. H. Vousden.** 1991. Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. Cell **67:**547–556.
8. **Davies, R., R. Hicks, T. Crook, J. Morris, and K. Vousden.** 1993. Human

papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. J. Virol. **67:**2521–2528.
9. **DeGregori, J., T. Kowalik, and J. R. Nevins.** 1995. Cellular targets for activation by the E2F-1 transcription factor include DNA synthesis- and G_1/S-regulatory genes. Mol. Cell. Biol. **15:**4215–4224.
10. **DeGregori, J., G. Leone, K. Ohtani, A. Miron, and J. R. Nevins.** 1995. E2F-1 accumulation bypasses a G1 arrest resulting from the inhibition of G1 cyclin-dependent kinase activity. Genes Dev. **9:**2873–2887.
11. **Demers, G., S. Foster, C. Halbert, and D. Galloway.** 1994. Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7 protein. Proc. Natl. Acad. Sci. USA **91:**4382–4386.
12. **Deng, C., P. Zhang, J. Harper, S. Elledge, and P. Leder.** 1995. Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell **82:**675–684.
13. **Dynlacht, B. D., O. Flores, J. A. Lees, and E. Harlow.** 1994. Differential regulation of E2F trans-activation by cyclin/cdk2 complexes. Genes Dev. **8:** 1772–1786.
14. **Dyson, N., P. Guida, K. Munger, and E. Harlow.** 1992. Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. J. Virol. **66:**6893–6902.
15. **Dyson, N., P. M. Howley, K. Munger, and E. Harlow.** 1989. The human papillomavirus 16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science **243:**934–937.
16. **Ginsberg, D., G. Vairo, T. Chittenden, A. Xiao, G. Xu, K. Wydner, J. DeCaprio, J. Lawrence, and D. Livingston.** 1994. E2F-4, a new member of the E2F transcription factor family, interacts with p107. Genes Dev. **8:**2665–2679.
17. **Halbert, C., G. Demmers, and D. Galloway.** 1991. The E7 gene of human papillomavirus type 16 is sufficient for transformation of human epithelial cells. J. Virol. **65:**473–478.
18. **Harper, J., G. Adami, N. Wei, K. Keyomarsi, and S. Elledge.** 1993. The p21 cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell **75:**805–816.
19. **Hawley-Nelson, P., K. H. Vousden, N. L. Hubbert, D. R. Lowy, and J. T. Schiller.** 1989. HPV16 E6 and E7 cooperate to immortalize human foreskin keratinocytes. EMBO J. **8:**3905–3910.
20. **Hearing, P., and T. Shenk.** 1983. The adenovirus type 5 E1A transcriptional control region contains a duplicated enhancer element. Cell **33:**695–703.
21. **Hecks, D. V., C. L. Yee, P. M. Howley, and K. Munger.** 1992. Efficiency of binding to the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses. Proc. Natl. Acad. Sci. USA **89:**4442–4446.
22. **Helin, K., and E. Harlow.** 1993. The retinoblastoma protein as a transcriptional repressor. Trends Cell Biol. **3:**43–46.
23. **Hickman, E., S. Picksley, and K. Vousden.** 1994. Cells expressing HPV16 E7 continue cell cycle progression following DNA damage induced by p53 activation. Oncogene **9:**2177–2181.
24. **Hu, Q., N. Dyson, and E. Harlow.** 1990. The regions of the retinoblastoma protein needed for binding to adenovirus E1A or SV40 large T antigen are common sites for mutations. EMBO J. **9:**1147–1155.
25. **Ikeda, M., L. Jakoi, and J. R. Nevins.** 1996. A unique role for the Rb protein in controlling E2F accumulation during cell growth and differentiation. Proc. Natl. Acad. Sci. USA **93:**3215–3220.
26. **Jewers, R. J., P. Hilderbrandt, J. W. Ludlow, B. Kell, and D. J. McCance.** 1992. Regions of human papillomavirus type 16 E7 oncoprotein required for immortalization of human keratinocytes. J. Virol. **66:**1329–1335.
27. **Johnson, D., J. Schwarz, W. Cress, and J. R. Nevins.** 1993. Expression of transcription factor E2F-1 induces quiescent cells to enter S phase. Nature **365:**349–352.
28. **Laimins, L.** 1993. The biology of human papillomaviruses: from warts to cancer. Infect. Agents Dis. **2:**74–86.
29. **Lam, E. W. P., J. D. H. Morris, R. Davies, T. Crook, R. J. Watson, and K. H. Vousden.** 1994. HPV16 E7 oncoprotein deregulates B-myb expression: correlation with targeting of p107/E2F complexes. EMBO J. **13:**871–878.
30. **Lechner, M., D. Mack, A. Finicle, T. Crook, K. Vousden, and L. Laimins.** 1992. Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. EMBO J. **11:**3045–3052.
31. **Lees, J. A., M. Saito, M. Vidal, M. Valentine, T. Look, E. Harlow, N. Dyson, and K. Helin.** 1993. The retinoblastoma protein binds to a family of E2F transcription factors. Mol. Cell. Biol. **13:**7813–7825.
32. **McIntyre, M., M. Frattini, S. Grossman, and L. Laimins.** 1993. Human papillomavirus type 18 E7 protein requires intact Cys-X-X-Cys motifs for zinc binding, dimerization, and transformation but not for Rb binding. J. Virol. **67:**3142–3150.
33. **McIntyre, M., M. Ruesch, and L. Laimins.** 1996. Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. Virology **215:**73–82.
33a. **Meyerson, M., and E. Harlow.** 1994. Identification of G_1 kinase activity for cdk6, a novel cyclin D partner. Mol. Cell. Biol. **14:**2077–2086.
34. **Moberg, K., M. A. Starz, and J. A. Lees.** 1996. E2F-4 switches from p130 to p107 and pRb in response to cell cycle entry. Mol. Cell. Biol. **16:**1436–1449.
35. **Moran, E.** 1993. DNA tumor virus transforming proteins and the cell cycle. Curr. Opin. Genet. Dev. **3:**63–70.
36. **Morozov, A., W. C. Phelps, and P. Raychaudhuri.** 1994. Activation of the c-fos gene by the HPV16 oncoproteins depends upon the cAMP-response element at −60. J. Biol. Chem. **269:**18434–18440.
37. **Morozov, A., J. Subjeck, and P. Raychaudhuri.** 1995. HPV16 E7 oncoprotein induces expression of a 110 kD heat shock protein. FEBS Lett. **371:**214–218.
38. **Munger, K., W. C. Phelps, V. Bubb, P. M. Howley, and R. J. Schlegel.** 1989. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. J. Virol. **63:**4417–4421.
39. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science **258:**424–429.
40. **Phelps, W. C., S. Bagchi, J. A. Barnes, P. Raychaudhuri, V. Kraus, K. Munger, P. M. Howley, and J. R. Nevins.** 1991. Analysis of *trans* activation by human papillomavirus type 16 E7 and adenovirus 12S E1A suggests a common mechanism. J. Virol. **65:**6922–6930.
41. **Phelps, W. C., K. Munger, C. L. Yee, J. A. Barnes, and P. M. Howley.** 1992. Structure-function analysis of the human papillomavirus type 16 E7 oncoprotein. J. Virol. **66:**2418–2427.
42. **Pondaven, P., L. Meijer, and D. Beach.** 1990. Activation of M-phase-specific histone H1 kinase by modification of the phosphorylation of its p34cdc2 and cyclin components. Genes Dev. **4:**9–17.
43. **Sardet, C., M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, and R. Weinberg.** 1995. E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle. Proc. Natl. Acad. Sci. USA **92:** 2403–2407.
44. **Sellers, W. R., J. W. Rodgers, and W. G. Kaelin, Jr.** 1995. A potent transrepression domain in the retinoblastoma protein induces a cell cycle arrest when bound to E2F sites. Proc. Natl. Acad. Sci. USA **92:**11544–11548.
45. **Sheffner, M., B. A. Wereness, J. M. Huibregtse, A. J. Levine, and P. M. Howley.** 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell **63:**1129–1136.
46. **Shiyanov, P., S. Bagchi, G. Adami, J. Kokontis, N. Hay, M. Arroyo, A. Morozov, and P. Raychaudhuri.** 1996. p21 disrupts the interaction between cdk2 and the E2F-p130 complex. Mol. Cell. Biol. **16:**737–744.
47. **Slansky, J. E., Y. Li, W. G. Kaelin, Jr., and P. J. Farnham.** 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. Mol. Cell. Biol. **13:**1610–1618.
48. **Tommasino, M., J. P. Adamczewski, F. Carlotti, C. F. Barth, R. Manetti, M. Contorni, F. Cavalieri, T. Hunt, and L. Crawford.** 1993. HPV16 E7 protein associates with the protein kinase p33 cdk2 and cyclin A. Oncogene **8:**195–202.
49. **Vairo, G., D. Livingston, and D. Ginsberg.** 1995. Functional interaction between E2F-4 and p130: evidence for distinct mechanisms underlying growth suppression by different retinoblastoma protein family members. Genes Dev. **9:**869–881.
50. **Vindelov, L. L., I. J. Christensen, and N. I. Nissen.** 1983. A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. Cytometry **3:**323–327.
51. **Watanabe, S., T. Kanda, and K. Yoshiike.** 1989. Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. J. Virol. **63:**965–969.
52. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature **358:**259–261.
53. **Weintraub, S. J., K. N. B. Chow, R. X. Luo, S. H. Zhang, S. He, and D. Dean.** 1995. Mechanism of active transcriptional repression by the retinoblastoma protein. Nature **375:**812–815.
54. **White, A. E., E. M. Livanos, and T. D. Tlsty.** 1994. Differential disruption of genomic integrity and cell cycle regulation in normal human fibroblasts by the HPV oncoproteins. Genes Dev. **8:**666–677.
55. **Wu, E. W., K. E. Clemens, D. V. Heck, and K. Munger.** 1993. The human papillomavirus E7 oncoprotein and the cellular transcription factor E2F bind to separate sites on the retinoblastoma suppressor protein. J. Virol. **67:**2402–2407.
56. **Yee, A. S., P. Raychaudhuri, L. Jakoi, and P. Raychaudhuri.** 1989. The adenovirus-inducible factor E2F stimulates transcription after specific DNA binding. Mol. Cell. Biol. **9:**578–585.
57. **Yin, Y., M. A. Tainsky, F. Z. Bischoff, L. C. Strong, and G. M. Wahl.** 1992. Wild type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell **70:**937–948.
58. **Zerfass, K., A. Schulze, D. Spitkovsky, V. Friedman, B. Henglein, and P. Jansen-Durr.** 1995. Sequential activation of cyclin E and cyclin A gene expression by human papillomavirus type 16 E7 through sequences necessary for transformation. J. Virol. **69:**6389–6399.