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Initiation of DNA Synthesis by Human Papillomavirus E7 Oncoproteins Is Resistant to p21-Mediated Inhibition of Cyclin E-cdk2 Activity

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The E6 and E7 proteins from the high-risk human papillomaviruses (HPVs) bind and inactivate the tumor suppressor proteins p53 and Rb, respectively. In HPV-positive cells, expression of E6 proteins from high-risk types results in increased turnover of p53, which leads to an abrogation of p21-mediated G_1/S arrest in response to DNA-damaging agents. In contrast, keratinocytes which express E7 alone have increased levels of p53 but, interestingly, also fail to undergo a G_1/S arrest. We investigated the mechanism by which E7 bypasses this p21 arrest by using both keratinocytes which stably express E7 as well as U20S cells which stably or transiently express E7. We observed that E7 does not affect the induction of p21 synthesis by p53. While glutathione *S*-transferase (GST)-E7 bound a low level of in vitro-translated p21, we were unable to detect E7 and p21 in the same complex by GST-E7 binding assays or immunoprecipitations from cell extracts. Furthermore, E7 did not prevent p21-mediated inhibition of cyclin E kinase activity. In keratinocytes expressing E7, increased levels of p53, p21, and cyclin E, as well as increased cyclin E kinase activity, were observed. To determine if this increase in cyclin E activity was necessary for E7's ability to overcome p21-mediated G_1/S arrest, we examined U20S cells in which cyclin E levels are not increased in response to E7 expression. U20S cells which stably express E7 were found to initiate DNA synthesis in the presence of DNA-damaging agents despite the inhibition of cyclin E activity by p21. In transient assays, cotransfection of E7 or E2F-1 along with p21 into U20S cells rescued G_1 arrest and resulted in S-phase entry, as measured by the ability to incorporate bromodeoxyuridine. These data indicate that E7 is able to overcome G_1/S arrest without directly affecting p21 function and likely acts through deregulation of E2F activity.

Human papillomaviruses (HPVs) are small DNA viruses which induce hyperproliferative lesions in epithelial tissues. High-risk genital HPV strains (HPV type 16 [HPV-16], HPV-18, HPV-31, and HPV-54) are associated with the development of anogenital cancer, particularly malignant carcinoma of the cervix, while low-risk types (HPV-6 and HPV-11) induce benign genital warts (29, 34, 38, 45, 68). In the high-risk HPV types, the E6 and E7 genes encode transforming proteins which act through their associations with the tumor suppressor proteins p53 and Rb, respectively (18, 44, 59, 63). In a productive infection in vivo, E6 and E7 most likely act to control cell cycle progression and so it is not surprising that, in some viral types, these genes can contribute to cellular transformation (8, 23, 29, 34).

In uninfected epithelia, keratinocytes undergo terminal differentiation as they stratify and migrate toward the surface. Once cells leave the basal layer and begin to differentiate, they exit the cell cycle, and their nuclei are degraded in the stratum granulosum layer (29). In an HPV infection, the expression of differentiation markers is similar to that of uninfected epithelia but suprabasal cells retain their nuclei and remain replication competent (3). Using amphotropic retroviruses, Halbert et al. (23) and Cheng et al. (8) demonstrated that expression of E7 alone blocks exit from the cell cycle and prevents the differentiation-dependent degradation of nuclei in suprabasal layers. It is likely that disruption of Rb function contribute to this property of E7, but it is not clear if it is sufficient (11, 18, 44, 59). The E7 proteins from low-risk viruses also bind Rb but with substantially reduced affinity, and it is not known whether this level of binding is sufficient to maintain cells in a replication-competent state (8, 26, 45). In an uninfected cell, hypophosphorylated Rb represses transcription from promoters containing E2F sites during early G_1 by binding the E2F family of transcription factors (5, 28, 62). E2F sites are found in the promoters of a large number of genes required for DNA synthesis during S phase, and phosphorylation of Rb results in the disruption of the Rb-E2F complex, leading to the stimulation of S-phase-dependent transcription (35, 61). The binding of E7 to Rb renders E2F transcription constitutive and independent of the phase of the cell cycle (6).

In addition to Rb, E7 also binds the Rb-related proteins p107 and p130 (9, 17). All three pocket proteins, Rb, p107, and p130, negatively regulate E2F transcription, and recent data suggest that each may associate with different groups of E2F family members. Some reports suggest that Rb binds only E2F-1, E2F-2, and E2F-3, while p107 and p130 associate with E2F-4 and E2F-5 (22, 53, 58, 64). Other groups report a functional role for an Rb–E2F-4 complex (30, 43). Unlike Rb, p107 and p130 bind cyclin E-cdk2 and cyclin A-cdk2 complexes in a cell cycle-regulated manner coincident with their appearance just prior to and during S phase, respectively (20, 36, 37). The E7 proteins associate with cyclin-cdk proteins through the binding of p107 as well as p130, and these complexes exhibit histone H1 kinase activity (1, 9, 40). While it is likely that the association of E7 with non-Rb pocket proteins and cyclin-cdk complexes contributes to S-phase progression, the targets of these complexes are only poorly understood.

Further insight into how E7 modulates the cell cycle has been provided by studies using DNA-damaging agents such as

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actinomycin D and gamma irradiation. Following exposure to these agents, the levels of p53 are increased, resulting in stimulation of expression of the cyclin regulatory protein p21 through p53 binding sites located in the p21 promoter (15, 19, 25). This increase in p21 protein levels results in a G_1 arrest which is dependent on its association with cyclin-cdk complexes (7, 13, 46). At low levels, p21 associates with active cyclin D-cdk4, cyclin E-cdk2, and cyclin A-cdk2 complexes, while at high stoichiometric concentrations, it inhibits cyclin kinase activities, resulting in G_1/S arrest (66). Cells which express the E6 oncoprotein have low levels of p53 due to an increase in the rate of turnover and are resistant to DNA damage-induced G_1/S arrest (21, 32, 54). Surprisingly, expression of E7 alone also renders cells refractory to G_1 arrest in response to DNA damage despite the presence of functional p53 proteins, suggesting that other mechanisms are involved (12, 27, 56). E7 could abrogate the G_1/S checkpoint either by directly inhibiting p21 action or by targeting activities downstream of cyclin-cdk complexes. We have investigated E7's role in this process to provide insight into how it modulates cell cycle progression.

MATERIALS AND METHODS

Cells and exposure to DNA-damaging agents. Normal human keratinocytes (NHKs) (Clonetics, San Diego, Calif.) were maintained in KGM (Clonetics) prior to retroviral infection. Retrovirus-infected human keratinocytes (HKs) or control NHKs were maintained either in KGM or in serum-containing medium with fibroblast feeders (41). U20S (wild-type Rb and wild-type p53), C33A (mutant Rb and mutant p53), and HaCat (mutant p53) cells were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum (Gibco BRL, Grand Island, N.Y.). DNA damage was induced in NHK and HK lines with 0.5 nM actinomycin D treatment for 22 to 24 h, and cells were then harvested for Western blotting or histone H1 assays (12). U20S cells were treated with 5.0 nM actinomycin D for 22 to 24 h. Cells were then harvested for the preparation of whole-cell extracts or treated with 50 μM bromodeoxyuridine (BrdU) for an additional 18 h.

Western blot analysis. The following antibodies were used at 0.5 μg/ml: anti-cyclin E antibody (HE12; Pharmingen, San Diego, Calif.), anti-p53 antibody (Ab-2; Oncogene Science, Uniondale, N.Y.), anti-p21 antibody (15091A; Pharmingen), anti-proliferating cell nuclear antigen (PCNA) antibody (PC10; Pharmingen), and anti-cdk2 antibody (sc-163; Santa Cruz Biotechnology, Santa Cruz, Calif.). Whole-cell extracts were prepared by placing cells in lysis buffer consisting of 250 mM NaCl, 50 mM HEPES (pH 7.0), 5 mM EDTA, 1 mM dithiothreitol (DTT), 100 mM sodium fluoride, 200 μM sodium orthovanadate, 10 μM zinc chloride, 0.5% Nonidet P-40, 5 mM benzamidine hydrochloride, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, and 10 mM phenylmethylsulfonyl fluoride. Cells were resuspended in lysis buffer and incubated on ice for 10 min before insoluble proteins were pelleted in a tabletop microcentrifuge at 4°C. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, Calif.). Fifty micrograms of whole-cell extract per lane was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting was performed with the above antibodies and the proteins were visualized by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions. Band intensity was quantitated with a densitometer (model GS-670) with Molecular Analyst software (both from Bio-Rad). The nonlinear effects of ECL at high exposures were not corrected for in determination of the levels of induction, so the actual fold induction was likely underestimated. However, all comparisons of the levels of expression were made from one exposure of protein samples run on the same gel.

Retroviruses. PA317 cells producing 16E6E7 and 16E7 amphotropic retroviruses, conferring neomycin resistance, were a gift of D. Galloway (24). The 18E7 retrovirus was constructed by cloning an 18E7 *Bam*HI/*Bgl*II fragment into the *Bam*HI site of pMX1112 (gift of Nissam Hay) which confers hygromycin resistance. Ecotropic virus was harvested from transient transfections of the psi-2 packaging line. Amphotropic virus was produced after infection of PA317 cells with ecotropic virus and selection with hygromycin (400 μg/ml) (2). A 1.5-ml sample of supernatant containing amphotropic retrovirus was used to infect cells in 5 ml of total medium containing 9 μg of Polybrene (Sigma, St. Louis, Mo.) per ml. Infected HK cells were selected with 250 μg of G418 (Gibco BRL) per ml or 30 μg of hygromycin (Calbiochem, Cambridge, Mass.) per ml, and colonies were pooled and analyzed as mass cultures. U20S, C33A, and HaCat cells were selected with 100 μg of hygromycin per ml, and E7 expression was confirmed by Western blot analysis.

Histone H1 kinase assays. Whole-cell extracts were prepared in lysis buffer as described above. Equal amounts of protein were diluted to a volume of 1 ml in lysis buffer and incubated with 1 μg of anti-cyclin E antibody (sc-248; Santa Cruz), 1 μg of anti-p107 antibody (SD-9; Santa Cruz), or 1 μg of anti-cdk2 antibody (sc-163; Santa Cruz). After a 1-h incubation at 4°C, immune complexes were collected with protein G Sepharose (Pharmacia, Piscataway, N.J.). For E7 kinase assays, an affinity-purified antibody directed to the carboxyl terminus of 18E7 was used (40). In vitro kinase assays were performed by the method of Koff et al. (33) with histone H1 as a substrate. Briefly, immunoprecipitates from 0.3 to 1.0 mg of whole-cell extract were collected with protein A or G Sepharose beads and washed four or five times with cold lysis buffer and then washed four times with cold kinase buffer (50 mM Tris [pH 7.0], 10 mM $MgCl_2$). Beads were then incubated for 20 min at 37°C in 50-μl portions of reaction mixture prepared in kinase buffer (30 μM ATP, 1 mM DTT, 0.1 mg of bovine serum albumin per ml, 1 μg of histone H1, 10 μCi of [γ-^{32}P]ATP). Fifty microliters of 6× SDS-PAGE sample buffer containing DTT was added, and complexes were boiled before separation by SDS-PAGE. Radioactivity incorporated was quantitated with a phosphorimager (Fuji, Tokyo, Japan).

In vitro binding assay. Proteins labeled with [^{35}S]methionine (Amersham) were synthesized by using TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, Wis.) according to the manufacturer's instructions with the following plasmids: pSG5-Rb (gift of D. McCance), pWaf-1-T7 (gift of B. Vogelstein [19]), pBS-cyclin E (40), and pBS-p27 (gift of T. Hunter) (57). Glutathione *S*-transferase (GST) and GST-E7 proteins were prepared as described previously (40). The indicated amount of each in vitro-translated product was diluted in 0.4 ml of lysis buffer and precleared with 6 μg of GST for 1 h at 4°C followed by 50 μl of glutathione-agarose for 30 min at 4°C (Sigma). The precleared supernatant was then mixed with 3 μg of GST-E7 or GST and incubated for 1 h at 4°C. Glutathione-agarose beads (50 μl) were added and incubated an additional 30 min before collection by centrifugation. The proteins complexed to the glutathione-agarose were then washed five times with 1 ml of cold lysis buffer, released by boiling in 6× SDS-PAGE sample buffer containing DTT, and separated by SDS-PAGE (40). Gels were fixed, placed in Amplify (Amersham), and dried. One-fourth of the input amount was also separated by SDS-PAGE and examined with a densitometer to check that equivalent amounts were used.

Acute expression assay. Transfections and subsequent β-galactosidase (βgal) and BrdU staining were performed by the method of Johnson et al. (31) as modified by DeGregori et al. (10). CMV-E2F-1 was a gift of W. Krek, pCep-WAF-1 was a gift of B. Vogelstein, and pSG5-18E7 was described previously (39). Two micrograms of CMV-βgal was transfected along with 2 μg of each plasmid, except for 120 ng of CMV-E2F-1. The concentration of total DNA was increased to 20 μg with pUC-18, and the DNA was transfected into U20S cells by calcium phosphate precipitation (51). Transfected cells were split onto coverslips and incubated for 24 to 48 h before addition of 50 μM BrdU (Boehringer Mannheim, Indianapolis, Ind.) and then incubated for an additional 18 h. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature, made permeable by treatment with methanol-acetone (1:1) for 30 s at room temperature, and stained first with an anti-βgal antibody (5 Prime-3 Prime, Boulder, Colo.) (diluted 1:250) and then with a second antibody, a Texas red-conjugated anti-rabbit immunoglobulin (Ig) antibody (Amersham) (diluted 1:50). Cells were fixed again in paraformaldehyde, treated with 2 N HCl for 30 min, and stained first with an anti-BrdU antibody (Boehringer Mannheim) (diluted 1:20) and then with a second antibody, a fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig antibody (Amersham) (diluted 1:10).

BrdU immunofluorescence. Stable U20S-1112 and U20S-18E7 cells were grown on coverslips or 10-cm-diameter dishes and treated with 5 nM actinomycin D for 22 to 24 h. BrdU was then added to a final concentration of 50 μM, and the cells were incubated for an additional 18 h. Cells on coverslips were stained for BrdU as described for the acute expression assays above.

RESULTS

E7-expressing keratinocytes contain elevated levels of p53, p21, cyclin E, PCNA, and cdk2. To examine the mechanism by which the oncogenic E7 proteins modulate cell cycle progression, we first isolated cell lines following infection of NHKs with E7-expressing amphotropic retroviruses. Recombinant retroviruses expressing the HPV-16 E6 and E7 genes (HK-E6E7) or the HPV-16 or HPV-18 E7 open reading frames alone (HK-E7) were used to infect monolayer cultures of NHK cells. Following drug selection, pooled resistant colonies were examined. In agreement with a previous report (23), infection with these retroviruses extended the life span of normal foreskin keratinocytes. We also observed that the frequency of establishment of HK-E7 lines in serum-free medium was much lower than for HK-E6E7 lines. These HK-E7 cells were difficult to maintain in culture and grew more slowly than HK-E6E7 or NHK cells. When HK-E7 cells were isolated in medium containing serum, lines were more easily established,

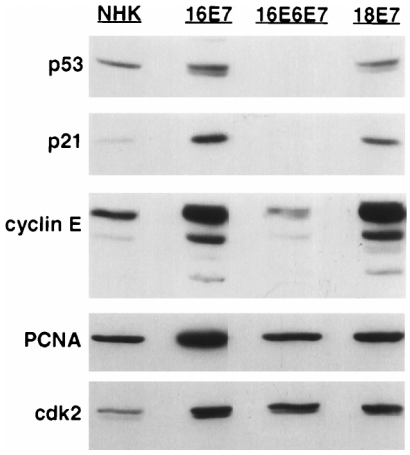

FIG. 1. Expression of cell cycle proteins in NHK, HK-E7, and HK-E6E7 cells. Equivalent amounts of whole-cell extracts of NHK, HK-E7, and HK-E6E7 cells were separated by SDS-PAGE and examined by Western blot analysis with anti-p53, anti-p21, anti-cyclin E, anti-PCNA, and anti-cdk2 antibodies as indicated. Proteins were visualized by chemiluminescence.

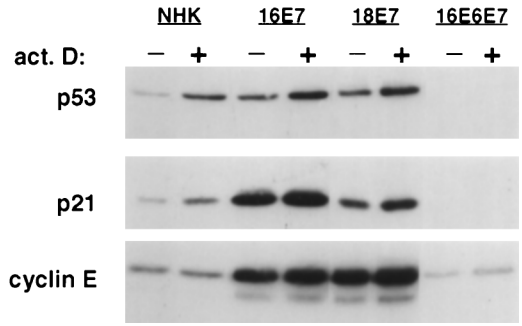

FIG. 2. E7 does not affect p21 increase in response to actinomycin D treatment. Equivalent amounts of whole-cell extracts of NHK, HK-E7, and HK-E6E7 cells, not treated (−) or treated (+) with actinomycin D (act. D), were separated by SDS-PAGE and examined by Western blot analysis with anti-p53, anti-p21, and anti-cyclin E antibodies. Proteins were visualized by chemiluminescence and quantitated by densitometry. Data for quantitated Western blots are as follows: for four representative Western blots using the E7 lines, the mean increase in p21 was 47%, with a range of 26 to 84%, and the mean increase in p53 was 68%, with a range of 53 to 77%. For two representative Western blots using the NHK lines, the increases in p21 were 29 and 47% and the increases in p53 were 41 and 74%.

grew more rapidly, and less cell death was observed than in HK-E7 lines in serum-free medium.

We next examined these lines for the expression of proteins which are known to play roles in regulation of the G_1/S checkpoint. Keratinocytes infected with HPV-16 or HPV-18 E7 retroviruses were found to contain increased levels of p53 protein than in NHK cells, while E6E7-infected cells expressed reduced levels (Fig. 1). Consistent with the levels of p53, HK-E7 cells also contained elevated levels of p21 while HK-E6E7 cells had reduced levels. Interestingly, cyclin E levels in HK-E7 cells were two- to threefold higher than in NHKs. The levels of PCNA and cdk2 were also increased in HK-E7 cells, but to a lesser degree than for cyclin E. In contrast, the levels of cyclin E and PCNA expressed in HK-E6E7 cells were similar to those seen in NHK cells, although HK-E6E7 cells expressed elevated levels of cdk2. The increase in expression of p53, p21, and cyclin E in HK-E7 cells was observed regardless of the culture medium and in all five pooled lines examined. We conclude that keratinocytes expressing E7 oncoproteins in the absence of E6 contain high basal levels of cell cycle regulators, such as cyclin E, p53, p21, and PCNA. In contrast, p53 and p21 levels in keratinocytes expressing both E7 and E6 were reduced, while cyclin E and PCNA levels were similar to those seen in uninfected cells.

E7 does not alter p21 synthesis or its inhibition of cyclin E-cdk2 kinase activity. To investigate E7's ability to modulate progression through the G_1/S checkpoint, we examined the effect of actinomycin D treatment on cell cycle progression. Actinomycin D treatment has been previously shown to induce a p53-dependent arrest in keratinocytes (12). When HK-E7 and HK-E6E7 cell lines grown in either the presence or absence of serum were treated with actinomycin D, they continued to cycle as determined by fluorescence-activated cell sorter analysis. In contrast, NHK cells were found to accumulate in G_1 with a decrease in S-phase populations (data not shown). This is in agreement with previous reports demonstrating that cells expressing E7 alone are at least partially refractory to p53-dependent arrest at the G_1/S checkpoint (12, 27, 56). One way that E7 could overcome G_1/S checkpoint control is by interfering directly with the function of p53's effector, p21. E7 could prevent the activation of p21 expression or prevent the inhibition of cyclin-cdk complexes by p21. To investigate these possibilities, the levels of p53 and p21 proteins in HK cells were examined before and after exposure to DNA-damaging agents. As shown in Fig. 2, the levels of p53 and p21 in NHKs increased upon treatment with low concentrations of actinomycin D, while neither was increased in HK-E6E7 cells. In cells infected with either HPV-16 or HPV-18 E7-expressing retroviruses, p53 and p21 also increased by an amount similar to that seen in NHKs (Fig. 2). It therefore appears that the presence of E7 does not alter the activation of p21 expression by p53, despite the high basal expression of this gene.

Another mechanism by which E7 could block p21 function is through direct binding. We therefore investigated whether E7 could form complexes with p21 proteins isolated from cell extracts or synthesized in vitro. In a previous study (40), immunoprecipitation of E7 from retrovirus-infected cells as well as incubation of NHK cell extracts with GST-E7 demonstrated that E7 was associated with cyclin E through p107 binding. Using a similar assay, we incubated purified GST-E7 with U20S cell extracts, isolated E7-associated complexes, and screened for the presence of p21 by Western blot analysis. No p21 binding was detected in GST-E7 complexes despite the presence of significant levels of cyclin E (Fig. 3A). We have also been unable to coimmunoprecipitate a complex of E7 and p21 from HK-E7 cell extracts (data not shown). In additional experiments, in vitro-translated Rb, p21, $p27^{KIP1}$, and cyclin E were incubated with GST and GST-E7 proteins. Complexes were then isolated and analyzed by SDS-PAGE. In these assays, Rb association with E7 was easily detected, while no association was observed with p21, p27, and cyclin E (Fig. 3B and C). The lack of cyclin E association is due to a requirement for p107 protein in the complex (40). A very low level of p21 binding was detected in this assay upon prolonged exposure (Fig. 3C), while no binding was detected in control reactions. Since the level of binding was very low, it is unlikely that this represents a significant association.

Next, it was important to determine if the increased amount of p21 present in HK-E7 cells after actinomycin D treatment was still functional by measuring cyclin E-associated kinase activity. For these experiments, cyclin E, in association with cdk2, was immunoprecipitated from E7-expressing cells and examined for kinase activity with histone H1 as a substrate. As shown in a representative experiment in Fig. 4, following treatment of NHK cells with actinomycin D, cyclin E-associated

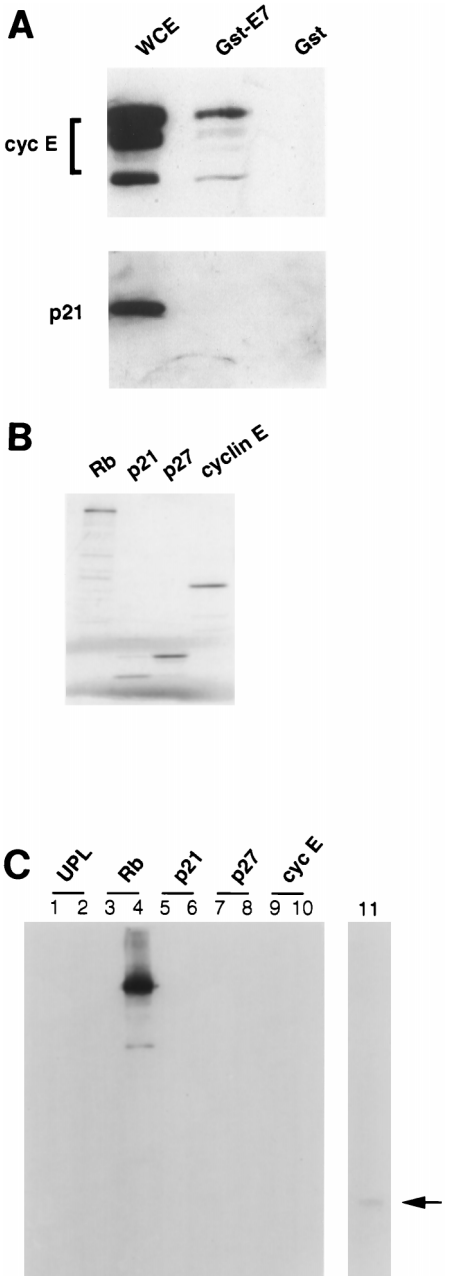

FIG. 3. E7 does not bind p21 in vitro. (A) GST or GST-E7 protein was mixed with 1.2 μg of U20S whole-cell extracts (WCE), and the resulting protein complexes were precipitated with glutathione-agarose. Associated proteins were separated by SDS-PAGE, and Western blot analysis was performed with anti-cyclin E or anti-p21 antibodies. Proteins were visualized by chemiluminescence. The p21 Western blot was exposed 6 times longer than the cyclin E Western blot. (B) In vitro-translated product inputs. One-fourth of the amount of each in vitro-translated product used in the GST-E7 mixing experiment was separated by SDS-PAGE. (C) Each in vitro-translated product or unprogrammed lysate (UPL), as indicated, was mixed with GST (lanes 1, 3, 5, 7, and 9) or GST-E7 (lanes 2, 4, 6, 8, and 10) protein, collected with glutathione-agarose, and separated by SDS-PAGE. Lane 11 is a longer exposure of lane 6.

kinase activity was reduced. In three different experiments, cyclin E kinase activity of actinomycin D-treated NHKs was 43% ± 14% of untreated NHKs. In HK-E6E7 cells, a high level of cyclin E-associated kinase activity was observed, despite the fact that the total amount of cyclin E protein was

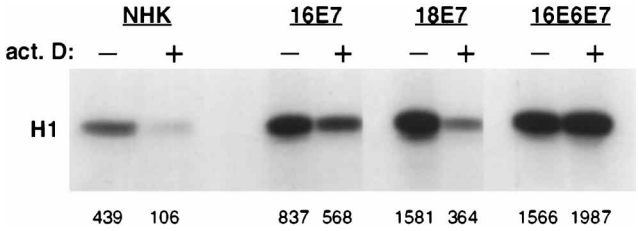

FIG. 4. Cyclin E-associated kinase activity in HK-E7 cells is reduced following exposure to actinomycin D. Cells were exposed (+) or not exposed (−) to actinomycin D (act. D). Equivalent amounts of whole-cell extracts (1.5 mg) were immunoprecipitated with anti-cyclin E antibodies, and in vitro kinase assays were performed as described in Materials and Methods with histone H1 as a substrate. The incorporated radioactivity as measured by a phosphorimager is shown below each lane.

similar to that seen in NHK cells. This is likely the result of reduced levels of p21 in HK-E6E7 cells (Fig. 1 and 2). Consistent with the reduced levels of p53 and p21, cyclin E activity in HK-E6E7 cells was not affected or only slightly inhibited upon exposure to actinomycin D (Fig. 4). HK-E7 cells also exhibited a high basal level of cyclin E-associated kinase activity which, unlike HK-E6E7 cells, was reduced following actinomycin D treatment. These experiments were repeated using HK-E7 lines from five different infections and consistently demonstrated that cyclin E activity in HK-E7 cells was inhibited by actinomycin D treatment. The cyclin E kinase activity of actinomycin D-treated HK-E7 cells from six experiments was 46% ± 10.2% of untreated HK-E7 cells. While its activity was still inhibited by p21, the residual level of cyclin E activity was high and comparable to that seen in untreated NHK cells (Fig. 4). We conclude that the increased p21 protein in HK-E7 cells remained functional, although the residual levels of cyclin E-cdk2 activity in treated cells were high.

As a result of binding to the pocket protein p107, E7 has been shown to associate with cyclin-cdk proteins, and these complexes exhibit kinase activity (1, 9, 40). We next investigated if the kinase activity associated with E7, as well as p107, was reduced following actinomycin D treatment. In Fig. 5, the in vitro kinase activity associated with cyclin E, p107, and 18E7 is shown. A high level of background kinase activity was precipitated from NHK cells with the E7 antibody. The kinase activity associated with p107, as well as the activity which is specifically associated with E7 in HK-E7 cells, when corrected for background, was relatively low compared to the total cyclin E kinase activity. While cyclin E activity was inhibited in both NHKs and HK-E7 cells following actinomycin D treatment, p107 and E7 kinase activities were not appreciably affected.

Increased cyclin E levels in response to E7 expression is limited to HK-E7 cells. Since cyclin E activity in HK-E7 cells remained high after actinomycin D treatment, this could account for the absence of G_1 arrest. Alternatively, it was possible that this increased expression of cyclin E was not essential for the abrogation of G_1 arrest by E7. It was also interesting that keratinocytes expressing E6 together with E7 did not show any increased expression of cyclin E (Fig. 1 and 2).

To determine if expression of E7 alone always resulted in increased cyclin E expression, the same 18E7-expressing retrovirus was used to infect two HPV-negative keratinocyte cell lines, C33A and HaCat, as well as an osteosarcoma line, U20S. While C33A and HaCat contain mutant p53, U20S cells contain functional p53 protein. Following infection, drug-resistant colonies were pooled and screened by Western blot analysis for the levels of cyclin E. Expression of E7 in these cell lines did not result in increased expression of cyclin E (Fig. 6). The

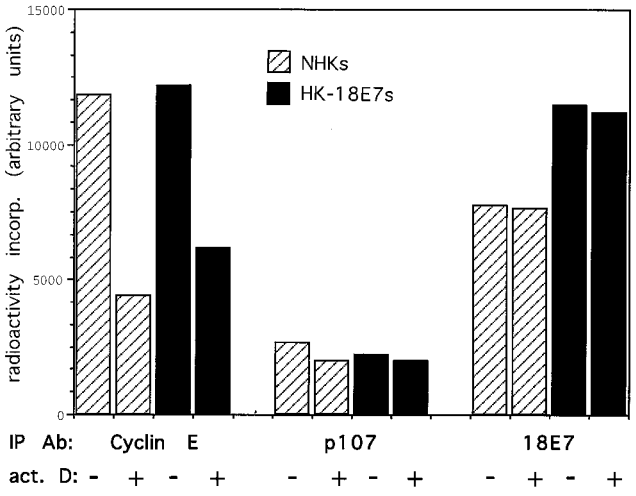

FIG. 5. Kinase activities associated with p107 and E7 are relatively unaffected compared to cyclin E-associated kinase activity. Cells were exposed (+) or not exposed (−) to actinomycin D (act. D). Equivalent amounts of whole-cell extracts were immunoprecipitated with anti-cyclin E, anti-p107, or anti-E7 antibodies, and in vitro kinase assays were performed as described in Materials and Methods with histone H1 as a substrate. The radioactivity incorporated (incorp.) as measured by a phosphorimager is represented on the *y* axis, and the antibody used for immunoprecipitation (IP Ab) is listed below each group of bars.

levels of E7 expression in HK-E7, C33A-E7, HaCat-E7, and U20S-E7 cell lines were similar as determined by Western blot analysis (data not shown). We conclude that the increased level of cyclin E is not seen in all cells expressing E7.

E7 is able to overcome p21 arrest in cells which do not contain increased steady-state levels of cyclin E. Since U20S cells retain functional p53 proteins and undergo a p21-mediated G_1/S arrest following treatment with actinomycin D, we used these cells to examine if increased cyclin E levels were required for E7's abrogation of p21-mediated G_1/S arrest (67). Stable U20S lines were created using the 18E7-expressing retrovirus (U20S-E7) or a control retrovirus (U20S-1112). U20S-E7 cells did not exhibit higher levels of p53 or p21 compared with those in U20S-1112 cells (Fig. 7, lanes 1 and 3). Importantly, U20S-E7 cells also did not contain increased levels of cyclin E compared to those of control virus-infected U20S-1112 cells (Fig. 6B). To determine if U20S-E7 lines are able to initiate DNA synthesis despite the presence of elevated p21, we again utilized actinomycin D as a DNA-damaging agent. Following treatment with actinomycin D, the levels of p53 and p21 were increased in both U20S-1112 and U20S-E7 lines (Fig. 7). BrdU was added to both untreated and treated cells to identify cells entering S phase. The cells were incubated for another 18 h before detection of BrdU-positive nuclei by immunofluorescence staining. Representative photographs and the mean percentages of BrdU-positive U20S-1112 and U20S-E7 cells after actinomycin D treatment are shown in Fig. 8. In the presence of DNA-damaging agents, only 7% of the control U20S-1112 cells entered S phase, while 30% of U20S-E7 cells were able to initiate DNA synthesis (Fig. 8B and C). Since more than 90% of untreated cells were labeled with BrdU over the 18-h incubation, E7 expression did not completely rescue the actinomycin D-induced arrest (Fig. 8A). U20S-E6E7 cells were also not completely refractory to DNA damage arrest in this system, with 49% of cells incorporating BrdU after actinomycin D treatment (data not shown).

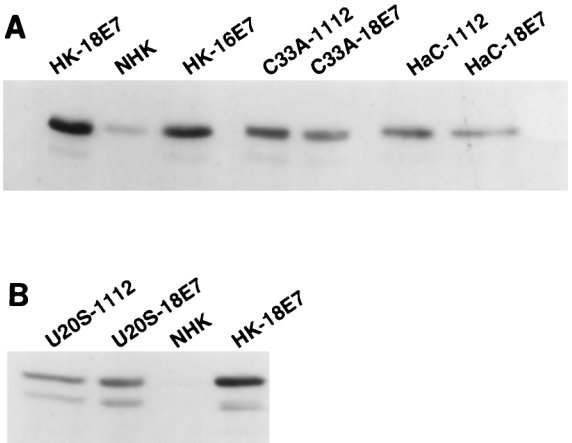

FIG. 6. Cyclin E levels are not increased in all cell lines in response to E7 expression. C33A, HaCat, and U20S cells were infected with a control retrovirus (1112) or an 18E7-expressing retrovirus (18E7). After selection, whole-cell extracts were separated by SDS-PAGE and examined by Western blot analysis with anti-cyclin E antibodies and chemiluminescence. (A) Cyclin E Western blot analysis of C33A and HaCat infections. Analyses of whole-cell extracts from NHK, HK-18E7, and HK-16E7 cells are also shown. (B) Cyclin E Western blot analysis of U20S infections. Whole-cell extracts from NHK and HK-18E7 cells are also shown.

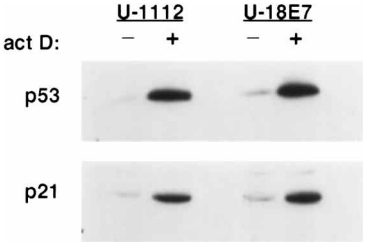

FIG. 7. U20S cells do not contain elevated levels of p53 or p21 upon stable expression of E7. Upon actinomycin D treatment, p53 and p21 are increased in both lines. Cells from U20S-1112 and U20S-18E7 lines were exposed (+) or not exposed (−) to actinomycin D (act D), and then whole-cell extracts were prepared, separated by SDS-PAGE, and examined by Western blot analysis. Western blots using anti-p53 and anti-p21 antibodies are shown.

We next determined if p21 was functional in these stable cell lines by examining the kinase activity associated with cyclin E in U20S-E7 and control U20S-1112 cells treated with actinomycin D. As shown in Fig. 8D, cyclin E activity in both U20S-1112 and U20S-E7 cells was inhibited to similar levels after actinomycin D treatment. This indicates that p21 after treatment is functional in both these lines. Since cyclin A-cdk2 complexes are also inhibited by p21, we also tested kinase activity associated with cdk2. The total cdk2 activity in U20S-E7 cells was dramatically inhibited following actinomycin D treatment, indicating that other cdk2 complexes are not compensating for the inhibited cyclin E activity (Fig. 8E). From these experiments, we conclude that U20S-E7 cells are able to initiate DNA synthesis, as measured by the ability to incorporate BrdU, despite elevated levels of a functional p21 protein.

Expression of E7 and overexpression of E2F-1 have similar abilities to overcome p21 arrest. To look at the immediate effects of E7 on p21-mediated G_1/S arrest, we next used transient-transfection assays. The use of transient-transfection assays minimizes the effects of any secondary changes which may arise during the selection of stable cell lines. Based on an assay developed by Johnson et al. (31), a CMV-βgal expression plasmid was transfected into U20S cells along with expression vectors for p21, E7, or both. At 48 h posttransfection, BrdU

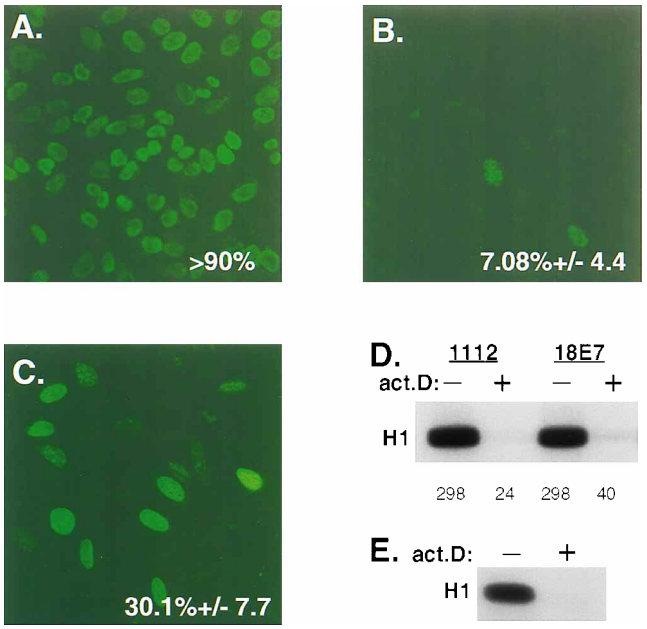

FIG. 8. Stable U20S cells expressing E7 are able to initiate DNA synthesis despite inhibition of cyclin E kinase activity. Stable U20S-E7 lines and control U20S-1112 lines were treated with actinomycin D for 24 h, incubated for 18 h with BrdU, and then stained for BrdU incorporation by using a mouse anti-BrdU antibody and an FITC-conjugated anti-mouse Ig secondary antibody. In all untreated cell lines, more than 90% of cells incorporated BrdU during the labeling time; a representative photo is shown in panel A. In panels B and C, the number of BrdU-positive cells is expressed as a mean percentage of cells in a field ± the standard deviation after at least 18 independent fields from two independent experiments were counted. Actinomycin D-treated U20S-1112 (B) and U20S-E7 (C) cells are shown. (D) Cyclin E kinase activity is similar in control U20S cells (1112) and E7-expressing U20S cells (18E7) before (−) and after (+) actinomycin D (act. D) treatment. Cyclin E immunoprecipitation and in vitro kinase assays were performed as described in Materials and Methods. Radioactivity units as measured by a phosphorimager are listed below the lanes. (E) Kinase activity associated with cdk2 is inhibited in U20S-E7 cells following actinomycin D treatment. cdk2 immunoprecipitation and in vitro kinase assays were performed as described in Materials and Methods.

was added and incubated for 18 h to identify S-phase cells. The cells were then analyzed by immunofluorescence to identify cells which had taken up transfected DNA (βgal-positive cells) as well as those which had entered S phase (BrdU-positive cells). Figure 9A shows the results of a representative assay identifying two transfected cells, one BrdU positive and one BrdU negative.

Transfection of the βgal reporter plasmid along with a simian virus 40 expression vector control typically resulted in 60 to 90% of βgal-positive cells also staining positive for BrdU. The number of BrdU-positive cells in the βgal control transfection was set at 100, and the other transfections within each experiment were expressed as a percentage of the control. The average values from three independent experiments are shown in Fig. 9B. Transfection of p21 was found to result in a dramatic decrease in the number of cells entering S phase to 21%. Cotransfection of an 18E7 expression plasmid consistently resulted in a rescue of p21 arrest, with an average of 57% of transfected cells found in S phase (Fig. 9B). We confirmed by Western blot analysis that transfection of p21 and E7 plasmids resulted in expression of these proteins and that cotransfection of E7 or E2F-1 did not inhibit expression of p21 (data not shown).

In previous studies, DeGregori et al. (10) and Dimri et al. (14) demonstrated that E2F-1 expression was able to overcome p21 arrest with similar assay systems. E2F-1 is a downstream target of cyclin-cdk complexes such as cyclin E-cdk2 (49, 55). Since it has been shown that E7 deregulates E2F activity by binding Rb, as well as p107 and p130, we next tested the effect of transfecting an E2F-1 expression plasmid on p21 arrest in U20S cells. High-level expression of E2F-1 was also able to overcome p21 arrest, with 68% of the cells in S phase (Fig. 9B). Since the percentages of cells induced to enter S phase were similar in both E7- and E2F-1-expressing plasmid-transfected cells, we conclude that expression of HPV-18 E7 and overexpression of E2F-1 have similar abilities to overcome a G_1 arrest induced by transfection of p21 in U20S cells.

DISCUSSION

Investigation of the mechanism by which E7 expression overcomes p21-mediated arrest provides insight into how E7 functions to deregulate G_1/S control and promote S-phase progression. This is particularly important in viral infections of differentiating epithelia where E7 has been implicated in activation of suprabasal DNA replication which allows for virion production (8, 23). Using treatment with actinomycin D to induce DNA damage, we observed that E7-expressing cells retained the ability to induce p53 and p21 expression. Moreover, the elevated levels of p21 were able to inhibit cyclin E kinase activity in both keratinocytes and U20S cells expressing E7, indicating that E7 was not directly interfering with p21 function.

While the results of our experiments demonstrated that GST-E7 could associate with in vitro-translated p21, the levels of binding were so low that we do not believe it constituted a significant physiological association. In addition, we did not detect p21 from whole-cell extracts in association with GST-E7, nor have we been able to coimmunoprecipitate p21 and E7 from keratinocytes immortalized by E7 (HK-E7). Recent reports indicate that binding of p107 and p21 by cyclin E-cdk2 is mutually exclusive, since p107 and p21 associate with cyclin-cdk complexes through homologous sequences (67). Since we and others have previously shown that E7 associates with cyclin-cdk2 complexes through p107, it is unlikely that p21 binds these complexes (1, 9, 40). E7 also exhibits associated kinase activity, and like p107-associated kinase activity, it was found not to be dramatically reduced following induction of p21. Since the E7-associated kinase activity is low in comparison to total cyclin E kinase activity, it is not clear if it plays a significant role in E7 function or alters the targets of p107-bound cyclin E-cdk2 complexes. Likewise, the role of p107 cyclin-associated complexes in cell cycle progression is unclear, and the cellular targets of the kinase activity are not known.

Using transient-transfection assays in U20S cells, the ability of E7 to overcome p21-mediated arrest was observed to be similar in effect to that seen with E2F-1 overexpression. This is consistent with previous studies which have shown that overexpression of E2F-1 alone can overcome p21-induced cell cycle arrest (10, 14). Dimri et al. (14) also demonstrated that E2F activity is repressed as a result of inhibition of cyclin-cdk activity, which suggests that it may be the ultimate downstream effector of p21 arrest. The results of our experiments suggest that E7, like E2F-1 overexpression, acts downstream of cyclin-cdk complexes to overcome p21 arrest. The similarity between the effects of E7 and the overexpression of E2F-1 is consistent with E7's ability to deregulate E2F activity through the binding of Rb and possibly other pocket proteins. This is also in agreement with the results of a study by Slebos et al. (56) which demonstrated that mouse embryonic fibroblasts with germline

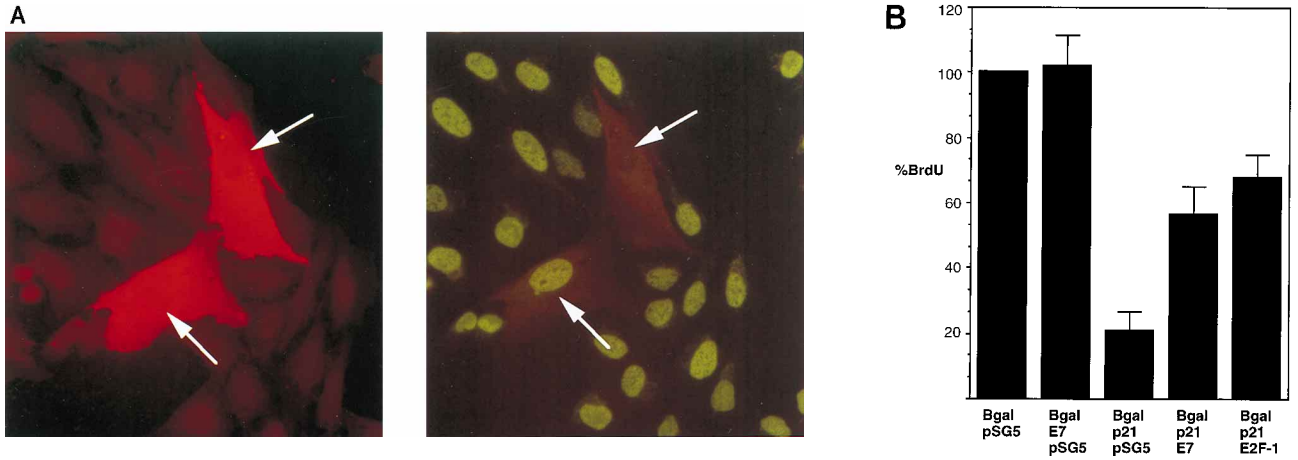

FIG. 9. HPV-18 E7, like E2F-1, expression can overcome p21-mediated G_1 arrest. (A) Immunofluorescence of transfected U20S cells. U20S cells were transfected with a βgal expression plasmid and incubated for 24 to 48 h before the addition of BrdU for an 18-h labeling period. Cells were stained by indirect immunofluorescence as described in Materials and Methods. The left panel shows the rabbit anti-βgal staining with a Texas red-conjugated anti-rabbit Ig secondary antibody. The right panel shows the mouse anti-BrdU staining of the same field with a FITC-conjugated second anti-mouse Ig secondary antibody. The βgal-positive cells are indicated by arrows; the upper right-hand cell is negative for BrdU staining, and the lower left-hand cell is positive for BrdU staining. (B) E7 or E2F-1 expression results in the initiation of DNA synthesis in the presence of p21. A total of 200 to 250 βgal (βgal)-positive cells were counted and recorded as BrdU positive or negative. The percent BrdU-positive cells in the control βgal transfection was set at 100%. The error bars represent the standard deviations of at least three experiments.

disruptions in both Rb alleles are partially refractory to p53-dependent G_1 arrest.

We observed that NHKs expressing E7 exhibited increased levels of cyclin E. However, neither expression of E7 together with E6 in NHKs nor expression of E7 alone in C33A, HaCat, or U20S cells resulted in an increase in cyclin E. Interestingly, only the cells which contain increased levels of p53 and p21 in response to E7 expression, the HK-E7 cells, exhibited increased cyclin E protein. It is possible that increased cyclin E activity may be selected for in these stable cell lines to overcome the effects of elevated p21 levels and allow for their efficient proliferation in culture. Since cyclin E has additional targets besides E2F-1 which are required for a normal S phase, HK-E7 cells would likely still require cyclin E activity for multiple divisions (16). It is also possible that loss of Rb function, through E7 expression, results in the derepression of the cyclin E promoter through its E2F sites, although the lack of a similar increase in cyclin E in HK-E6E7 cells or U20S-E7 cells, which both contain functional Rb, would argue against this mechanism (47).

Regardless of the mechanism responsible for the increase in steady-state levels of cyclin E in HK-E7 cells, our data using the transient-transfection assay and stable U20S-E7 cells indicate that elevated levels are not required for E7's ability to overcome a G_1/S checkpoint block and initiate DNA synthesis. Since the cyclin E present in the U20S-E7 cells is inhibited by the increased p21 to a degree similar to that found in control cells, E7 is not interfering with the function of p21. To assay for DNA synthesis in our experiments, we used BrdU incorporation which measures only S-phase entry, not passage through or completion of S phase. Despite the fact that 30% of stable U20S-E7 cells were able to incorporate BrdU after actinomycin D treatment, analysis of DNA content by propidium iodide staining indicated that there was still an increase of U20S-E7 cells with diploid (2N) DNA content which was similar to control cells (52a). Therefore, while E7 expression results in the initiation of S phase, these cells may not proceed normally through S phase and accumulate greater than 2N DNA contents. It is likely that, while E7-expressing cells initiate S phase in the presence of p21-inhibited cyclin E, this S phase is not complete. This would be consistent with an E7-induced S phase which is mediated by E2F-1 deregulation. Other studies have demonstrated that, while E2F-1 overexpression is able to induce S phase in serum-starved or p21-arrested cells, this S phase is abortive and results in p53-mediated apoptosis (10, 31, 52, 65). Perhaps not surprisingly, high-risk HPVs encode a protein, E6, which abrogates p53 functions by promoting its degradation (54).

In the differentiating suprabasal cells of an HPV-infected epithelium, E7 expression may result in the initiation of an S phase like that induced by E2F-1 expression alone. This would allow cells to remain competent to replicate viral genomes but would not result in a complete S phase like that seen in uninfected cycling cells. Recent experiments with *Drosophila melanogaster* demonstrate that cyclin E has E2F independent targets which are required for S phase (16). Our experiments do not imply that E7 can act to replace the role of cyclin E in S phase. While we have shown that E7 does not affect p21's actions at cyclin-cdk complexes, it is likely that all lines which stably express E7 retain a requirement for cyclin E activity to proceed through S phase and reinitiate subsequent divisions in culture. Previous studies have demonstrated that E7 cells which contain increased p53 still contain hyperphosphorylated Rb, indicating that these cells which continue through S phase do contain some kinase activity (12, 27, 56). Duronio et al. (16) have proposed that since cyclin E and E2F-1 positively regulate each other's activity, the activation of either one alone would be sufficient to promote S-phase entry but that both activities are still required for a normal S phase.

The ability of E7 to overcome p21-mediated arrest may also provide insight into how E7 functions to retain DNA replication competence in differentiating cells during an HPV infection. Upon epithelial differentiation, p21 transcripts have been shown to be increased through a p53-independent pathway (42, 48). E7 expression, possibly through deregulating E2F-1, would permit differentiating cells to remain in S phase despite an increase in p21. This would allow for amplification of viral DNA in suprabasal cells in the presence of significant levels of p21. It is also possible, however, that additional targets besides

pocket proteins are required for overcoming G_1 arrest in a differentiating epithelium.

A recent study by Demers et al. (11) examined mutant E7 proteins for their ability to overcome p21-mediated G_1 arrest, transforming growth factor β-induced G_1 arrest, and suprabasal quiescence in differentiated epithelium. Each E7 construct was found to exhibit similar activities in all three assays, suggesting a common mechanism of action. E7 proteins with mutations in the pocket protein binding domain of E7 were impaired in their ability to overcome G_1 arrest in all systems, in agreement with the hypothesis that abrogation of this checkpoint requires pocket protein binding and subsequent deregulation of E2F activity. Interestingly, two E7 proteins with mutations in the amino terminus of E7 which retained the ability to bind Rb were also defective in overcoming G_1 arrest, and it is therefore possible that other functions of E7 besides pocket protein binding are required for the abrogation of G_1 arrest. Alternatively, these relatively drastic mutations in E7's amino terminus, which include a substitution with proline or a five-amino-acid deletion, may affect the efficiency with which these proteins disrupt Rb-E2F interactions. In support of this idea, some groups have reported that similar mutant E7 proteins exhibit impaired ability to transactivate an E2F-responsive promoter, although conflicting data exist on this point (4, 50, 60). Therefore, the impaired ability of these mutant E7 proteins to overcome p21 arrest may still be consistent with the hypothesis that E7 is acting through deregulation of E2F-1. Further studies are still required, however, to address if there are additional targets of E7 which are important for retention of replication competence, as well as if there are additional functions of E7 which are required in the vegetative life cycle of HPVs.

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