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Abrogation of a mitotic checkpoint by E2 proteins from oncogenic human papillomaviruses correlates with increased turnover of the p53 tumor suppressor protein

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Human papillomavirus (HPV) E2 and E1 proteins are required for the replication of viral genomes *in vivo*. We have examined the effects of increasing the level of E2 on viral and cellular replication using recombinant adenoviruses. Infection of cells which maintain HPV 31 DNA episomally with E2 recombinant adenoviruses resulted in a 5-fold increase in genome copy number as well as an S phase arrest allowing for the continued replication of cellular DNA. Similar effects on cell cycle progression were seen following infection of normal human foreskin keratinocytes, the natural host cell. The DNA content of these cells increased beyond 4N indicating that multiple rounds of replication had occurred without an intervening mitotic event. In addition, increased cyclin A and E associated kinase activity was observed, while no change was detected in cyclin B associated kinase activity or in the activation state of cdc2 kinase. Interestingly, the levels of the p53 tumor suppresser protein were dramatically reduced through a post-transcriptional mechanism following infection. These data suggest a role for E2 in regulating viral and cellular replication by abrogation of a mitotic checkpoint, which is, at least in part, controlled by p53.
Keywords: cell cycle/DNA replication/HPV/p53/S phase

Introduction

Progression through the eukaryotic cell cycle is regulated at distinct checkpoints to insure the ultimate fidelity of DNA replication. These checkpoints insure that transit into the next part of the cell cycle occurs following successful completion of the previous phase. Transition through these checkpoints is controlled by cellular proteins which detect incomplete replication or DNA damage. The cellular proteins that regulate these processes consist of complexes of a regulatory cyclin subunit and a catalytic subunit with kinase activity referred to as a cyclin-dependent kinase (CDK) (Grana and Reddy, 1995; Morgan, 1995). These cyclin–CDK complexes act to phosphorylate other proteins, such as the retinoblastoma protein, which regulate the G_1, S and G_2/M phases of the cell cycle. In the fission yeast, *Schizosaccharomyces pombe*, a single CDK ($p34^{cdc2}$) regulates cell cycle progression in combination with different phase-specific cyclin proteins (Enoch and Nurse, 1991; Hayles *et al.*, 1994; King *et al.*, 1994; Nurse, 1994). However, in mammalian cells, a series of cyclin proteins are synthesized sequentially during the cell cycle and form active complexes with multiple CDKs (Heichman and Roberts, 1994; Grana and Reddy, 1995; Morgan, 1995; Su *et al.*, 1995). The D-type cyclins associate with several CDK proteins in mid-G_1, while cyclins E and A associate with cdk2 in late G_1 and S phase, respectively. The transition from S phase into mitosis and the induction of spindle formation is controlled by complexes of cyclins A and B with the *cdc2* gene product (Enoch and Nurse, 1991; King *et al.*, 1994; Nurse, 1994). Following completion of each phase of the cell cycle, the active complexes are specifically degraded or rendered inactive in order to prevent any interference with progression.

Cell cycle progression is also regulated through negative feedback on the cyclin–CDK activity by inhibitors of the CDKs which include p21, p27, p16, p18, p19, p20 and p57 (Grana and Reddy, 1995; Morgan, 1995). The transcriptional activator, p53, senses damage to DNA and, in response, activates the expression of the p21 gene product (El-Deiry *et al.*, 1993). This increase in p21 protein level inhibits cyclin E–cdk2 and cyclin A–cdk2 activities, resulting in cell cycle arrest. Another important checkpoint exists at the end of S phase, insuring that DNA replication has been completed faithfully prior to progression into mitosis (Enoch and Nurse, 1991; Heichman and Roberts, 1994; Nurse, 1994; Su *et al.*, 1995). The process of DNA replication is highly coordinated and dictates that each replication origin is activated once per cell cycle even though initiation occurs at different times during S phase. The cellular factors that regulate this process remain largely undefined, and examples of abrogation of this checkpoint in higher eukaryotes are rare. Several gene products have been identified recently in fission yeast which, when deleted (or overexpressed), have been shown to abrogate the normal checkpoint control mechanism linking DNA replication to mitosis. These events include destruction or inhibition of cdc2 kinase; overexpression of the rum1 protein, which is a direct inhibitor of the cyclin B–cdc2 kinase activity; deletion of the *cdc13* gene product which encodes the cyclin B protein; and deletion/overexpression of the *cdc18* gene product which has been shown to be directly involved in coupling S phase to cell division in fission yeast (Kelly *et al.*, 1993; Hayles *et al.*, 1994; Heichman and Roberts, 1994; Nurse, 1994; Su *et al.*, 1995; Jallepalli and Kelly, 1996; Muzi-Falconi *et al.*, 1996; Schneider *et al.*, 1996).

Information on the regulators of this mitotic checkpoint comes from cell fusion studies (Rao and Johnson, 1970).

When G_1-arrested nuclei are transplanted into cells in S phase, a new round of DNA synthesis is initiated, implicating a *trans*-acting factor in the regulation of entry into S phase. However, G_2 nuclei transplanted into S phase cells do not induce re-replication (or endoreduplication) nor do they not prevent the continued replication of S phase nuclei. This indicates that nuclear localized negative regulatory signals exist to prevent re-replication. Using an *in vitro* replication system, Blow and Laskey (1988) demonstrated the ability to induce a second round of DNA replication without an intervening mitosis by permeabilizing *Xenopus* nuclei with detergents. These studies suggested the existence of 'licensing factors' which allow for replication and prohibit replication in their absence. Candidate genes, the minichromosome maintenance (MCM) proteins, have been identified in yeast, but none has been identified yet in mammalian cells (Romanowski and Madine, 1996). Genetic studies in yeast have identified cyclins, namely Clb5 and Clb6, as being important for faithful completion of S phase, and mutant alleles of cdc2 and cyclin B have been isolated which lead to re-replication (Schwob and Nasmyth, 1993; Hayles *et al.*, 1994). Abrogation of replication control has also been demonstrated in *Drosophila melanogaster* (Sauer *et al.*, 1995). This occurs as part of the normal process of terminal differentiation and results in polytene chromosomes or polyploid cells. Therefore, several mechanisms exist which allow for the abrogation of the normal cell cycle checkpoint at the end of S phase.

In contrast to studies in yeast and *Drosophila*, little is known about the mammalian factors regulating the mitotic checkpoint and controlling the fidelity of DNA replication. A single conditional mutant Chinese hamster ovary cell line has been isolated which can accumulate up to 32N DNA at the restrictive temperature (Handeli and Weintraub, 1992). The gene product responsible, however, has not been isolated, and the mechanism remains undefined. Based on studies of fibroblasts derived from p53 knockout mice, a role has recently been suggested for this tumor suppressor protein in regulating the mitotic checkpoint (Cross *et al.*, 1995). Upon passage in culture, these p53-deficient cells rapidly become aneuploid. In addition, treatment of these cells with the mitotic spindle inhibitor, nocodazole, resulted in an S phase arrest with continued chromosomal replication.

Viral systems have often provided useful insights into the biochemistry of eukaryotic DNA replication as well as in cell cycle control. Viral proteins target cell cycle progression to facilitate their own replicative cycle. Prominent examples are the targeting of Rb family members by the simian virus 40 (SV40) large T antigen, adenovirus E1A and the E7 protein of high risk human papillomaviruses (HPVs), which results in the constitutive activation of the E2F family of transcription factors (Nevins, 1992). HPVs, in particular, provide a unique example of viral targeting of cell cycle progression. HPVs infect the basal cells of stratified epithelia and establish their genomes as low copy viral episomes (Laimins, 1993; Howley, 1996). In these infected cells, viral episomes replicate in synchrony with cellular chromosomes. As infected cells migrate from the basal layer and undergo differentiation, lytic viral replication and late gene expression are induced (Bedell *et al.*, 1991; Meyers *et al.*, 1992; Laimins, 1993; Frattini *et al.*, 1996; Howley, 1996). In normal epithelia, differentiating suprabasal cells exit the cell cycle after leaving the basal layer. In contrast, in HPV-infected epithelia which are actively producing virus, viral proteins arrest suprabasal cells at the G_1–S boundary until they reach terminal differentiation, at which point cells are induced to re-enter S phase (Laimins, 1993; Howley, 1996). The viral replication proteins, E1 and E2, are both necessary and sufficient to activate viral replication in transient transfection assays, while the E7 oncoprotein, in the absence of other viral proteins, has been shown to activate cellular replication in a stratified epithelium (Ustav and Stenlund, 1991; Blanton *et al.*, 1992; Chiang *et al.*, 1992; DelVecchio *et al.*, 1992; Frattini and Laimins, 1994a; Cheng *et al.*, 1995). The E1 protein functions as the viral initiator protein, and the E2 protein, while having the structure of a classical transcriptional activator, has been shown to be involved in replication through heteromeric complex formation with E1 (Androphy *et al.*, 1987; Dostatni *et al.*, 1988; Mohr *et al.*, 1990; Blitz and Laimins, 1991; Lambert, 1991; Yang *et al.*, 1991; Hegde *et al.*, 1992; Seo *et al.*, 1993; Thorner *et al.*, 1993; Frattini and Laimins, 1994b; Li and Botchan, 1994).

Previous studies from our laboratory used recombinant amphotrophic retroviruses expressing the E2 gene product of the oncogenic papillomavirus, HPV 31, to infect monolayer cultures of a human keratinocyte cell line (CIN 612-9E) which stably maintains HPV 31 episomes at ~50 copies per cell. Following infection by the E2-expressing retrovirus, these cells exhibited a 5- to 9-fold increase in viral copy number which was not seen following infection with retroviruses expressing other HPV gene products (Frattini and Laimins, 1994b). The nuclei of these E2-expressing cells were significantly enlarged, and the cells appeared growth arrested. Small cells quickly grew out of the large cell colonies and, since a period of drug selection was required, these smaller cells constituted the population which was analyzed for viral copy number. The changes in cell morphology observed at early time points suggested that increased levels of E2 might have an immediate effect on cell cycle progression. This is consistent with reports that high level E2 expression is toxic to cells, and cell lines which stably overexpress E2 have been difficult to isolate (M.G.Frattini and L.A.Laimins, unpublished observation).

In this study, we investigated the effects of the E2 protein on cell cycle progression using an adenovirus-mediated expression system. High titer recombinant adenoviruses expressing the E2 gene product were constructed and, following infection of either immortalized or normal human keratinocytes, we observed that infected cells arrested in S phase and continued to replicate cellular DNA. This loss of replication control correlated with an increased turnover of the p53 tumor suppressor gene product, consistent with a role for this protein in control of the mitotic checkpoint in mammalian cells.

Results

Construction of recombinant adenoviruses

In order to study the immediate effects of increasing the levels of the papillomavirus replication protein, E2, on

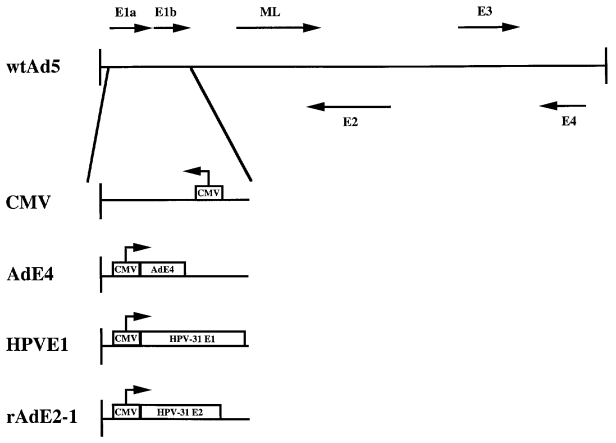


Fig. 1. Diagram of the recombinant adenoviruses used in these studies. Recombinant adenoviruses expressing the HPV 31 E1 and E2 proteins and a control virus expressing the adenovirus E4 ORF 6/7 protein were generated as described in Materials and methods. These recombinant viruses were constructed by replacing the E1A coding sequences of the adenovirus type 5 mutant, dl309, with the indicated CMV expression cassette. The control CMV recombinant virus replaces both the adenovirus E1A and E1B coding sequences with an empty CMV expression cassette as indicated. Adenovirus coding sequences are shown and ML represents the major late promoter.

cellular proliferation and HPV copy number, we constructed a high titer recombinant adenovirus expressing the HPV 31 E2 gene product (Figure 1). The HPV 31 E2 gene was cloned under the control of a cytomegalovirus (CMV) promoter into a plasmid which contained 452 bp of the 5′ end of the adenovirus genome. In this construct, the adenovirus E1A gene was replaced by the CMV promoter and the E2 gene. A fragment containing the 5′ end of the adenovirus genome and the E2 expression cassette was then ligated to purified restricted recombinant adenovirus genomes which lacked both the 452 bp 5′ end and the E1A coding sequences. The ligation products were then transfected into 293 cells, which express the E1A gene products. The resultant viruses were plaque purified, and a single recombinant E2-expressing adenovirus (rAdE2-1) was used in the majority of the following studies, although identical effects were also seen with the initial heterogeneous viral stock. A similar methodology was used to construct a recombinant adenovirus expressing the HPV 31 E1 gene product.

rAdE2-1 expresses active E2 protein

To demonstrate that the rAdE2-1 virus was expressing active E2 protein, we used a functional assay for E2 DNA binding. A squamous cell carcinoma cell line, SCC-13, was infected with a high multiplicity of rAdE2-1. Nuclear extracts were then prepared at various time points following infection and subjected to an electromobility shift assay (EMSA) using a ^{32}P-labeled DNA probe which contained two E2 binding sites. As shown in Figure 2, E2 DNA binding was first detected at 12 h post-infection and increased steadily throughout the length of the time course. This pattern of E2 protein activity correlated with the level of E2 gene expression as determined by Western blot analyses at similar times post-infection (data not shown).

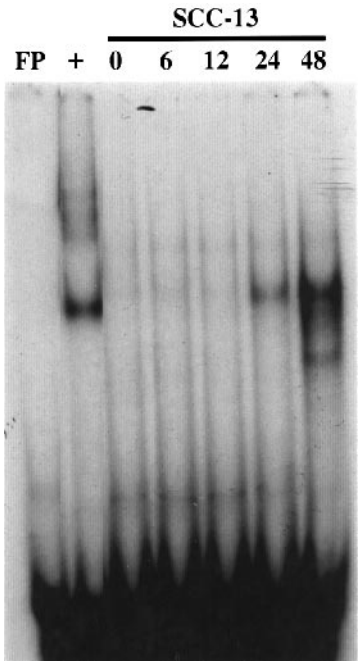


Fig. 2. The recombinant adenovirus, rAdE2-1, expresses active E2 protein. SCC-13 cells were infected, and nuclear extracts prepared at the indicated time points (in hours) post-infection as described in Materials and methods. For the EMSA analysis, 7.5 μg of extract was mixed with a ^{32}P-labeled DNA probe containing two E2 binding sites. FP represents free probe, and the plus sign (+) represents 70 ng of a purified GST–E2 fusion protein containing the carboxy-terminal DNA binding domain, a positive control for E2 DNA binding. Protein–DNA complexes were visualized by autoradiography.

Infection of CIN 612-9E cells with rAdE2-1 results in an increase in viral copy number and an accumulation of cells with greater than 4N DNA content

We next examined if, as we previously observed using recombinant retroviruses (Frattini and Laimins, 1994b), increasing the level of E2 protein in cells which stably maintain HPV genomes as episomes (CIN 612-9E) would result in an increase in viral genomic copy number. Monolayer cultures of CIN 612-9E cells were infected with a control AdE4 open reading frame (ORF) 6/7-expressing recombinant adenovirus (AdE4) or recombinant HPV 31 E1- or E2-expressing adenoviruses, and viral copy number was determined by Southern analyses (Figure 3). While infection with AdE4- or E1-expressing viruses had minimal effect on HPV 31 copy number, infection by rAdE2-1 alone increased the copy number ~5-fold, and infection by both E1- and E2-expressing viruses increased copy number ~20-fold, as quantitated by phosphoimager analysis. Cells infected with rAdE2-1, but not the other recombinant viruses, exhibited enlarged nuclei similar to what we observed previously following infection with E2-expressing recombinant retroviruses (data not shown). To examine if E2 had an effect on cell cycle progression, rAdE2-1-infected cells were fixed with ethanol at 48 h post-infection, stained with propidium iodide and examined by fluorescence-activated cell sorter (FACS) analyses. As seen in Figure 4, the majority of cells in mock- or AdE4 control-infected cells were in G_0/G_1. In contrast, the majority of cells infected with the E2 recombinant adenovirus were consistent with cells in S or G_2/M phases of

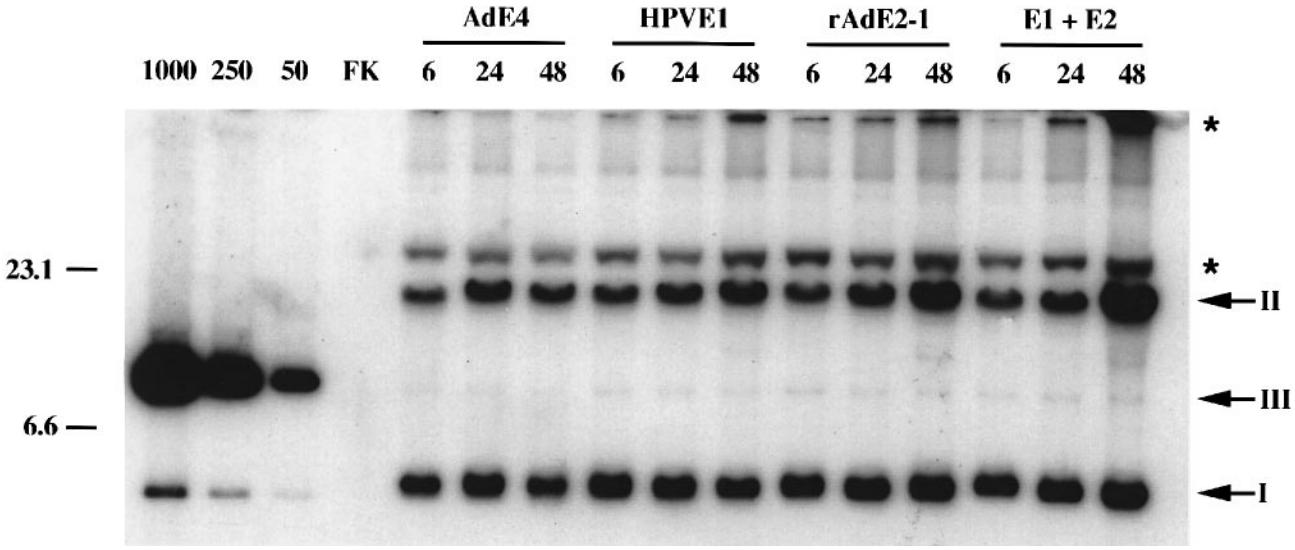


Fig. 3. Infection of CIN 612-9E cells with recombinant adenoviruses expressing the E2 protein results in an increase in viral DNA copy number. Recombinant adenoviruses expressing the AdE4, E1 or E2 proteins were used to infect CIN612-9E cells as described in Materials and methods. Total genomic DNA was isolated at 6, 24 and 48 h post-infection, and 10 μg of total cellular DNA was examined by Southern analyses using a ^{32}P-labeled HPV 31-specific DNA probe from the non-coding region. Hybridizing species were visualized by autoradiography. Lanes 1, 2 and 3 represent 1000, 250 and 50 copies per cell of linearized HPV 31 DNA, respectively. Lane 4 is 10 μg of normal human foreskin keratinocyte DNA. Molecular weight standards are indicated in kb. I, II and III indicate monomeric supercoiled, nicked and linearized viral genomes, respectively. The asterisk (*) represents multimeric concatenated genomic species. The fold increase in viral genome copy number was quantitated using a phosphoimager and normalized to the values obtained for the copy number controls.

the cell cycle. In addition, a significant number of these rAdE2-1-infected cells had DNA contents >4N (Figure 4).

rAdE2-1-infected NHK cells contain greater than 4N DNA content and display an aberrant cell/nuclear size

Since the cells used in the previous experiments were from an immortalized cell line (CIN 612-9E) which express HPV gene products, it was not clear whether the effects on cell cycle progression we observed were the result of overexpression of E2 alone or a synergistic effect of E2 with other papillomaviral proteins. Studies were initiated, therefore, using normal human foreskin keratinocytes (NHK), the natural host cell for HPV infection. As seen in Figure 5A, a similar switch in the proportion of cells in G_0/G_1 to those in S and G_2/M was observed following infection of NHK cells with rAdE2-1. No such change was seen in cells infected with the control CMV adenovirus, and similar results were observed with the recombinant AdE4 virus (data not shown). The appearance of cells with >4N DNA content was most pronounced at 66 h post-infection, at which time we also observed the appearance of cells with <2N DNA content, indicative of apoptotic cells in which the DNA was being degraded (Darzynkiewicz *et al.*, 1992). Figure 5B shows a representative phase contrast micrograph of control CMV- and rAdE2-1-infected NHK cells at 22 and 66 h post-infection. The photograph demonstrates that as early as 22 h post-infection large cells with enlarged nuclei can be detected specifically in the rAdE2-1-infected cells. At 66 h post-infection, the control CMV-infected cells approached confluency with no change in cell/nuclear size. In contrast, the average size of the rAdE2-1-infected population continued to increase with the appearance of smaller refractile cells. The presence of small cells in the rAdE2-1 infections

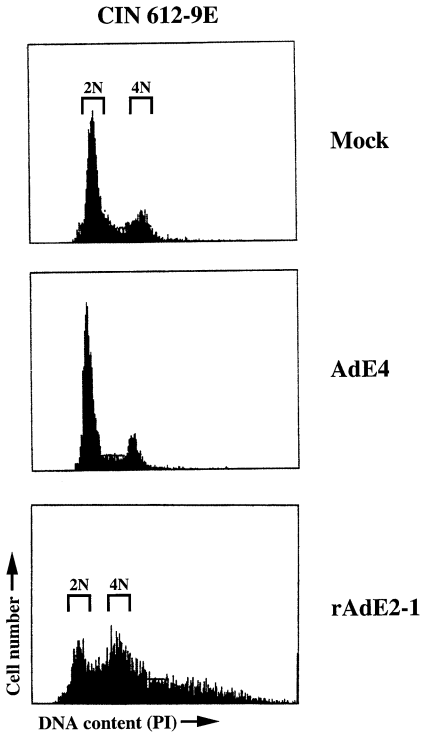
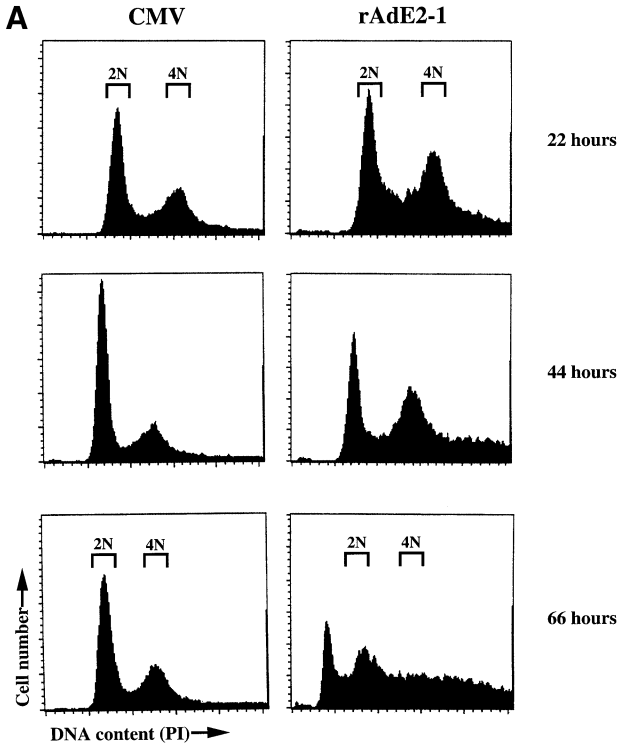


Fig. 4. Infection of CIN 612-9E cells with recombinant adenoviruses expressing the E2 protein results in an accumulation of cells with >4N DNA content. Recombinant adenoviruses expressing the AdE4 or E2 proteins were used to infect CIN612-9E cells and, at 48 h post-infection, cells were fixed, stained with propidium iodide and subjected to FACS analyses as described in Materials and methods. Mock represents uninfected cells; 2N and 4N indicate cells in G_0/G_1 and G_2/M phases of the cell cycle, respectively.



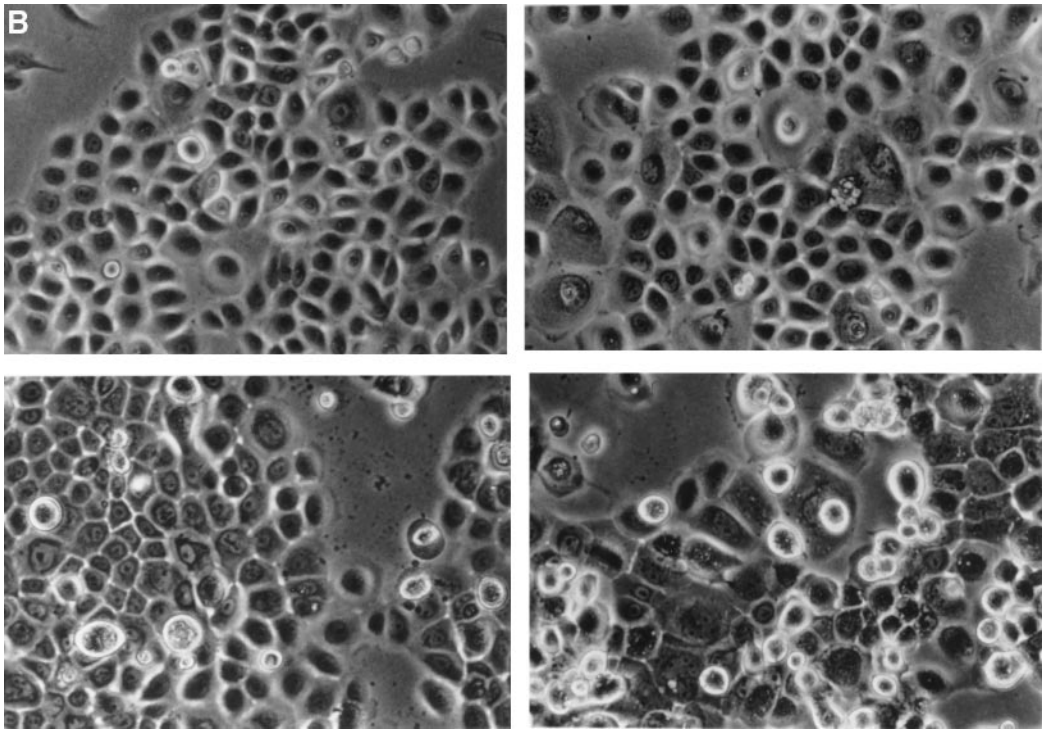


Fig. 5. Infection of NHK cells with recombinant adenoviruses expressing the E2 protein results in an accumulation of cells with >4N DNA content and aberrant cell/nuclear size. (**A**) CMV and rAdE2-1 recombinant adenoviruses were used to infect NHK cells and, at the indicated time points post-infection, cells were fixed, stained and subjected to FACS analyses as described in Materials and methods. 2N and 4N indicate cells in G_0/G_1 and G_2/M phases of the cell cycle, respectively. The peak of cells with <2N DNA content at 66 h post-infection with the rAdE2-1 virus represents an apoptotic cell population. (**B**) Phase contrast microscopy of CMV (upper left quadrant, lower left quadrant) and rAdE2-1 (upper right quadrant, lower right quadrant) infected NHK cells at 22 (upper left and right quadrants) and 66 h (lower left and right quadrants) post-infection.

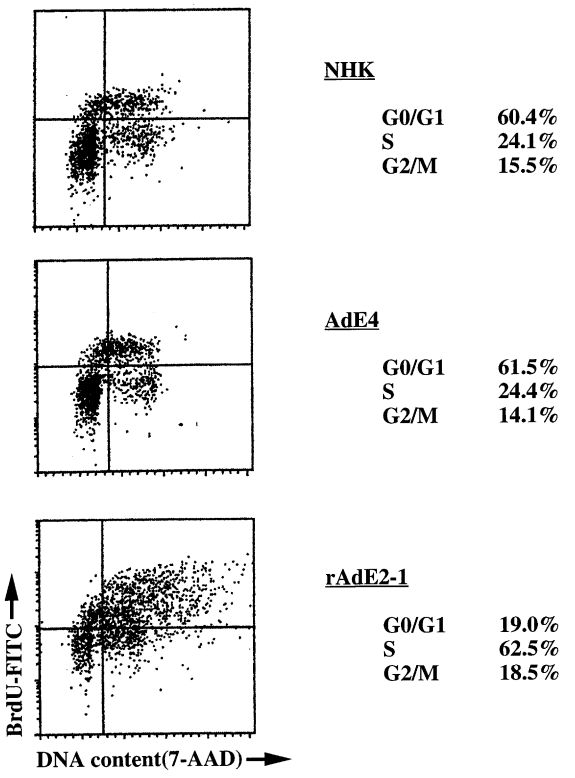


Fig. 6. Two-dimensional FACS analyses demonstrate that >60% of the cells infected with rAdE2-1 are actively synthesizing DNA. Recombinant adenoviruses expressing the AdE4 or E2 proteins were used to infect NHK cells. At 40 h post-infection, cells were pulsed for 1 h with BrdU and subsequently fixed, stained and subjected to FACS analyses as described in Materials and methods. The NHK panel represents uninfected cells. The upper left and right quadrants correspond to cells actively synthesizing DNA, as determined by BrdU incorporation, while cells in the lower left and right quadrants represent cells in G_0/G_1 and G_2/M, respectively.

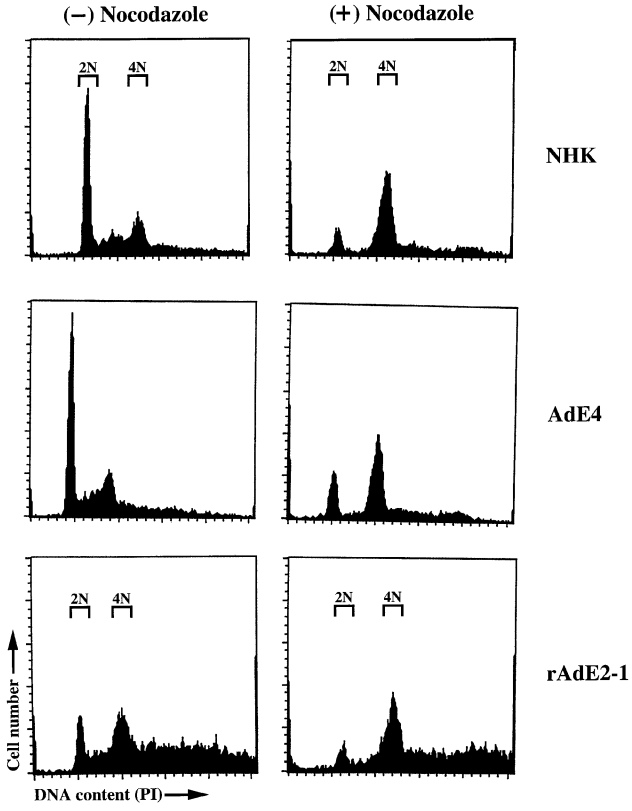


Fig. 7. rAdE2-1-infected NHK cells are arrested in S phase. Recombinant adenoviruses expressing the AdE4 or E2 proteins were used to infect NHK cells. At 24 h post-infection, cells were treated with (+) or without (–) the mitotic spindle inhibitor, nocodazole, for 20 h. At 44 h post-infection, cells were fixed, stained and subjected to FACS analyses as described in Materials and methods. NHK represents uninfected cells; 2N and 4N indicate cells in G_0/G_1 and G_2/M phases of the cell cycle, respectively.

most likely represents the apoptotic population detected in the FACS analysis in Figure 5A.

The majority of rAdE2-1-infected NHK cells are in S phase and re-replicate cellular DNA

In order to confirm that the cells with >4N DNA content were synthesizing DNA; mock-, AdE4- and rAdE2-1-infected NHK cells were labeled for 1 h at 40 h post-infection with bromodeoxyuridine (BrdU), fixed and stained. A two-dimensional FACS analysis of these cells (Figure 6) demonstrated that a majority of the mock- and AdE4-infected cells were in G_0/G_1 (60.4 and 61.5%, respectively) while the majority of the rAdE2-1-infected cells were in S phase (62.5%). In addition, many of the rAdE2-1-infected cells in S phase had DNA contents >4N. We therefore conclude that infection of normal human keratinocytes with recombinant adenoviruses expressing the E2 protein induces a specific accumulation in S phase resulting in re-replication of cellular DNA.

rAdE2-1-infected NHK cells are arrested in S phase

This accumulation in S phase could either be the result of cells arrested in S phase or, alternatively, a distinct population of rapidly cycling cells. To distinguish between these possibilities, control and rAdE2-1-infected NHK cells were treated with nocodazole, a mitotic spindle inhibitor, at 24 h post-infection. At 44 h post-infection, the cells were fixed, stained with propidium iodide and examined by FACS analyses. As shown in Figure 7, mock- or AdE4-infected NHK cells treated with nocodazole were arrested in G_2 and exhibited the expected cell cycle distribution. In contrast, the distribution of rAdE2-1-infected cells, including those cells with >4N DNA content, remained unchanged following treatment with nocodazole. It appears that the cells infected by rAdE2-1 were not cycling rapidly, but were arrested in S phase and continued to replicate cellular DNA. These cells failed to progress into mitosis and were, therefore, unaffected by treatment with nocodazole.

rAdE2-1-infected NHK cells contain increased cyclin A- and E-associated histone H1 kinase activity, but the activation state of cdc2 kinase remains unchanged

The mechanism by which HPV E2 proteins induced an S phase arrest most likely involved the altered activity or expression of cellular proteins which regulate passage through the cell cycle. We therefore investigated the levels and activities of cellular proteins which regulate transit through the G_1 and S phases. The level of cyclin A, E and B kinase activity was examined by associated histone H1 kinase activity of immunoprecipitated protein complexes. At 40 h post-infection, NHK cell extracts were

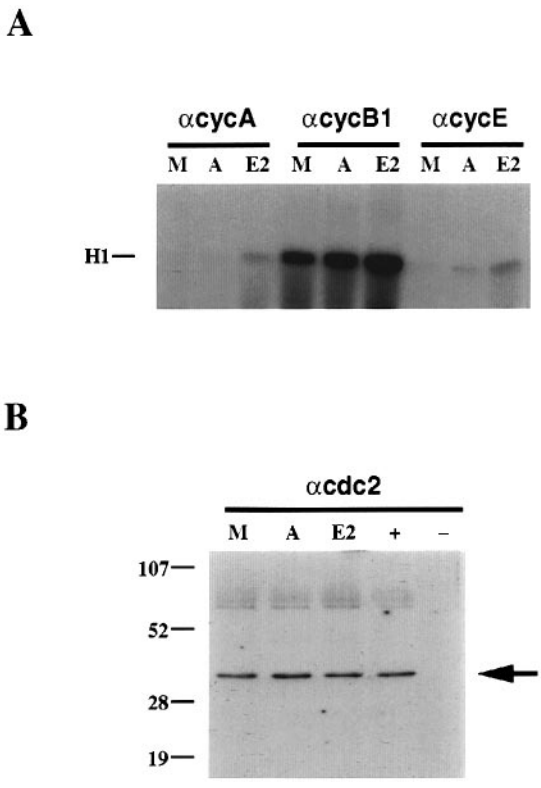


Fig. 8. Infection of NHK cells with rAdE2-1 results in increased cyclin A- and E-associated histone H1 kinase activity, but no change in the activation state of cdc2 kinase. Recombinant adenoviruses expressing the AdE4 (A) or E2 (E2) proteins were used to infect NHK cells. (M) represents uninfected cells. At 40 h post-infection, total cell extracts were prepared as described in Materials and methods. (**A**) Immunoprecipitation and associated histone H1 kinase activity assay. Equal amounts of protein extract were immunoprecipitated with the indicated antibody and subsequently incubated with [γ-^{32}P]ATP and histone H1. Following SDS–PAGE, phosphorylated histone H1 was visualized by autoradiography. (**B**) Cdc2 immunoprecipitation and anti-phosphotyrosine Western blot analyses. Equal amounts of protein extract were immunoprecipitated with a cdc2 antibody and, following SDS–PAGE, the amount of cdc2 containing phosphorylated tyrosine residues was visualized by Western analyses using an anti-phosphotyrosine antibody. The arrow indicates phosphorylated cdc2. The plus sign (+) represents a sample of purified cdc2–cyclin B as a positive control, while the minus sign (–) indicates a negative control immunoprecipitation done in the absence of extract. Molecular weight markers are indicated in kDa.

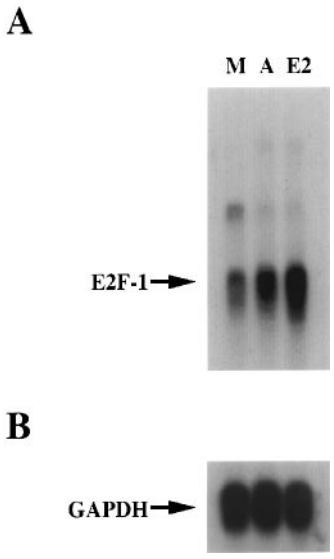


Fig. 9. Infection of NHK cells with rAdE2-1 results in increased E2F-1 gene expression. Recombinant adenoviruses expressing the AdE4 (A) or E2 (E2) proteins were used to infect NHK cells. (M) represents uninfected cells. (**A**) At 40 h post-infection, mRNA was isolated, and equal amounts were subjected to Northern analyses using a ^{32}P-labeled E2F-1-specific DNA probe as described in Materials and methods. Hybridizing species were visualized by autoradiography. The fold increase in mRNA transcripts was quantitated using a phosphoimager and normalized to the values obtained for the uninfected control. (**B**) The Northern blot in (A) was rehybridized with the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) to confirm equal loading of mRNA.

prepared, and cyclin proteins immunoprecipitated with the corresponding antibodies. The immunoprecipitated complexes were then incubated with [γ-^{32}P]ATP and histone H1, and the levels of phosphorylation examined following SDS–PAGE. As shown in Figure 8A, increased levels of kinase activity associated with cyclins A and E were observed specifically in rAdE2-1-infected cells, while the level of kinase activity associated with cyclin B remained relatively unchanged. The presence of equivalent amounts of cyclin B kinase activity in control and rAdE2-1-infected cells is in agreement with approximately equal distribution of cells in G_2/M as shown in Figure 6. The increase in cyclin A and E kinase activity in rAdE2-1-infected cells is consistent with the majority of these infected cells being arrested in S phase and not progressing into mitosis.

Because studies in the fission yeast, *Schizosaccharomyces pombe*, have demonstrated that alteration of the activation state of cdc2 kinase is involved in progression from S phase into mitosis (Hayles *et al.*, 1994), we next examined if infection with rAdE2-1 had a similar effect on cdc2 kinase activity. Since cdc2 kinase activity is dependent on dephosphorylation of certain tyrosine residues, we immunoprecipitated cdc2 proteins from cell extracts of mock-, AdE4- or rAdE2-1-infected cells and examined the activation state of cdc2 kinase by Western analyses with an anti-phosphotyrosine antibody. Figure 8B demonstrates no change in the phosphorylation state of cdc2 in rAdE2-1-infected cells, indicating that alterations in the activation state of cdc2 kinase are not responsible for the observed S phase arrest.

rAdE2-1 infection results in increased E2F-1 gene expression

Since previous reports have shown that an increase in the total amount of the transcription factor E2F-1 in quiescent fibroblasts can result in the induction of S phase (Johnson *et al.*, 1993; Kowalik *et al.*, 1995), we also investigated if there was an alteration in the amount of E2F-1 in rAdE2-1-infected NHK cells. Mock-, AdE4- and rAdE2-1-infected cells were harvested at 40 h post-infection, and Northern analyses of mRNA transcripts revealed an ~3-fold increase (as quantitated by phosphoimager analysis) in E2F-1 mRNA in rAdE2-1-infected cells (Figure 9A). EMSA analyses of E2F complexes in mock-, AdE4- and rAdE2-1-infected cells at 48 h post-infection demonstrated a predominance of the S phase-specific E2F–cyclin A–cdk2–p107 protein complex in E2-expressing cells, which was not seen in the control extracts (data not shown). Taken together, these data indicate that the majority of rAdE2-1-infected cells were in S phase.

rAdE2-1 infection results in a specific reduction in the levels of the p53 tumor suppressor protein and the cyclin-dependent kinase inhibitor, p21, but has no effect on p53 gene expression

Another protein which recently has been suggested to play a role in regulating transit into the M phase of the cell cycle is the p53 tumor suppressor protein, which has a well characterized role in monitoring the G_1 to S phase transition. When p53 protein levels were examined by immunoprecipitation (at 48 h post-infection) using mock-, AdE4- and rAdE2-1-infected [^{35}S]cysteine-labeled cell extracts, a dramatic reduction in p53 protein level was observed specifically in rAdE2-1-infected cells (Figure 10A, lanes 1–3). Similar results were also seen in Western analyses of mock-, AdE4- and rAdE2-1-infected cell extracts (Figure 10B). The reduction in p53 protein level was not the result of specific repression of p53 transcription by the E2 protein, as Northern analyses of p53 mRNA levels indicated no difference in levels of p53 transcripts in mock-, AdE4- and rAdE2-1-infected cells (Figure 10C). The decreased amount of p53 protein in rAdE2-1-infected cells was specific, as immunoprecipitation of cyclin B protein revealed equal amounts in all cells (Figure 10A, lanes 4–6). The presence of equivalent amounts of cyclin B protein is in agreement with equal amounts of cyclin B kinase activity observed (Figure 8A). Immunoprecipitation analyses of [^{35}S]cysteine-labeled cell extracts also revealed equivalent amounts of cyclin A and E protein in all cells; however, a specific reduction was observed in the level of the CDK inhibitor, p21, in rAdE2-1-infected cells (data not shown). This specific reduction in p21 protein levels was confirmed in Western analyses of mock-, AdE4- and rAdE2-1-infected cell extracts (Figure 11).

The reduction in p53 protein level in rAdE2-1-infected cells is due to a decrease in protein half-life

We next investigated if the reduction in p53 protein level was due to post-transcriptional effects such as a reduced translation rate or a decreased half-life of the protein. At 40 h post-infection, mock-, CMV- and rAdE2-1-infected cells were pulsed with [^{35}S]cysteine for 1 h and then chased with an excess of cold cysteine for 1, 3 and 6 h. As seen in Figure 12, the normal half-life of p53 in normal human keratinocytes is ~3 h (as quantitated by phosphoimager analysis), which is consistent with previous reports (Lechner *et al.*, 1992). Following infection with control CMV recombinant adenoviruses, the half-life of the p53 protein is $>$2 h, which is similar to results obtained with the other control recombinant virus, AdE4 (data not shown). In contrast, in rAdE2-1-infected cells, the p53 half-life was reduced to $<$30 min. In this study, the majority of rAdE2-1-infected cells were arrested in S phase while the majority of mock-infected cells were in G_0/G_1, as indicated by the two-dimensional FACS analysis shown in Figure 6. It was important, therefore, to determine whether the p53 half-life varied as a function of the cell cycle and whether the reduction in half-life was merely the result of arrest in the S phase of the cell cycle. The half-life of p53 in normal human keratinocytes in S phase was determined by synchronizing cells in late G_1 with hydroxyurea and examining the half-life of p53 at 6 h following release when the majority of cells were in

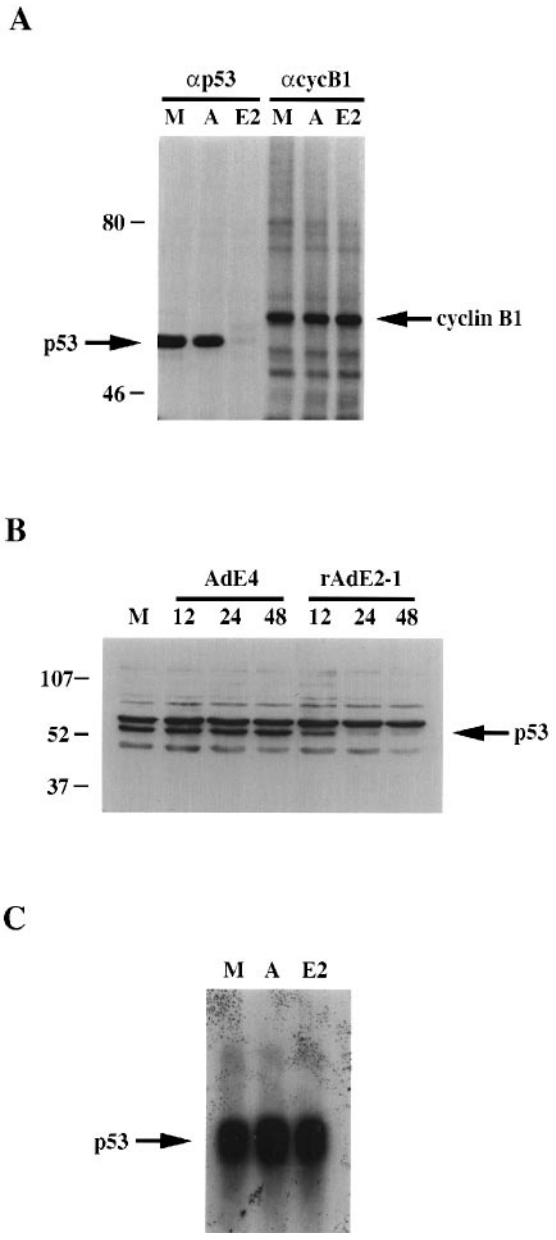


Fig. 10. NHK cells infected with rAdE2-1 display a reduction in the level of p53 protein, but contain equivalent amounts of p53 transcripts. Recombinant adenoviruses expressing the AdE4 (A) or E2 (E2) proteins were used to infect NHK cells. (M) represents uninfected cells. (**A**) Immunoprecipitation analysis of p53 and cyclin B1 proteins. At 44 h post-infection, cells were metabolically labeled with [^{35}S]cysteine, and equal amounts of acid-precipitable counts were subjected to immunoprecipitation with the indicated antibody. Following SDS–PAGE, labeled proteins were visualized by fluorography. Molecular weight markers are indicated in kDa. (**B**) Western blot analyses of p53 protein levels in AdE4- and rAdE2-1-infected NHK cells at 12, 24 and 48 h post-infection. (M) represents uninfected cells. At the indicated time points post-infection, nuclear extracts were prepared, and 20 μg was used for Western blot analyses as described in Materials and methods. Molecular weight markers are indicated in kDa, and the position of the p53 protein is indicated. (**C**) Northern analysis of p53 transcripts. The Northern blot shown in Figure 9 was rehybridized with a ^{32}P-labeled p53-specific DNA probe as described in Materials and methods. Hybridizing species were visualized by autoradiography. The fold increase in mRNA transcripts was quantitated using a phosphoimager and normalized to the values obtained for the uninfected control.

S phase, as indicated by FACS analyses of duplicate cultures (data not shown). The half-life of p53 in normal NHK cells in S phase was found to be ~3 h, which is consistent with that seen in randomly cycling cells (Figure 12). We conclude that the reduction in p53 levels was the direct result of infection by E2-expressing recombinant adenoviruses.

Discussion

In our studies, infection of normal human keratinocytes with recombinant adenoviruses expressing the HPV 31 E2 ORF was found to induce an S phase arrest that allowed for the re-replication of cellular DNA. Normally, the function of the S–G_2/M checkpoint is to insure that each replicon has completed replication prior to entry into mitosis, guaranteeing completion of S phase and preventing re-replication (Heichman and Roberts, 1994; Nurse, 1994; Su *et al.*, 1995). An examination of the cell cycle distribution of rAdE2-1-infected cells revealed that at 40 h post-infection the majority of cells were arrested in S phase, and BrdU labeling demonstrated that these cells continued to synthesize DNA. In these studies, we have not determined whether each replicon continues to be replicated with equal efficiency or only a subset of origins re-initiate. In either case, the normal regulatory mechanism that insures faithful completion of S phase has been overridden by infection with E2-expressing recombinant adenoviruses. The recombinant viruses themselves are replication deficient, as indicated by Southern analyses (data not shown) and, therefore, do not contribute to the amount of cellular DNA replication that was observed. While we have observed similar effects on template number and cellular morphology using E2-expressing recombinant retroviruses (Frattini and Laimins, 1994b), we cannot exclude completely the possibility that in our experiments, E2, because of its function as a transcriptional activator, is acting in synergy with an aberrantly expressed adenovirus protein. Whether E2 is acting directly or in combination with adenovirus gene products, it is clear that the E2 protein is essential for this process. These studies demonstrate that viruses can specifically target the mitotic checkpoint and alter cell cycle progression.

Our initial motivation for construction of these recombinant adenoviruses was to examine the effects of a rapid increase in the levels of the viral replication factors, E1 and E2, on HPV copy number. Transient assays have demonstrated that both proteins are necessary and sufficient for replication of an origin-containing construct (Ustav and Stenlund, 1991; Chiang *et al.*, 1992; DelVecchio *et al.*, 1992; Frattini and Laimins, 1994a). *In vivo*, papillomaviruses target stratified epithelial cells, linking their vegetative life cycle to the differentiation process. Initially, HPV infects the basal cell population, but amplification of viral genomes occurs upon terminal differentiation, concurrent with the induction of late gene expression from a promoter located within the E7 ORF (Bedell *et al.*, 1991; Meyers *et al.*, 1992; Laimins, 1993; Hummel *et al.*, 1995; Frattini *et al.*, 1996; Howley, 1996). This promoter has the coding potential to increase expression of both E1 and E2 proteins. Our studies demonstrated that infection of immortal keratinocyte cell lines which stably maintain HPV episomes (CIN 612-9E)

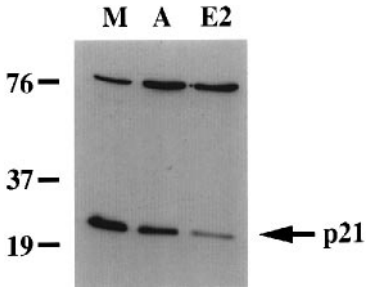


Fig. 11. NHK cells infected with rAdE2-1 display a reduction in the level of the cyclin-dependent kinase inhibitor, p21. Western blot analyses of p21 protein levels in AdE4 (A) and rAdE2-1 (E2) infected NHK cells at 48 h post-infection. (M) represents uninfected cells. At 48 h post-infection, cell extracts were prepared, and 40 μg was used for Western blot analyses as described in Materials and methods. Molecular weight markers are indicated in kDa, and the position of the p21 protein is indicated.

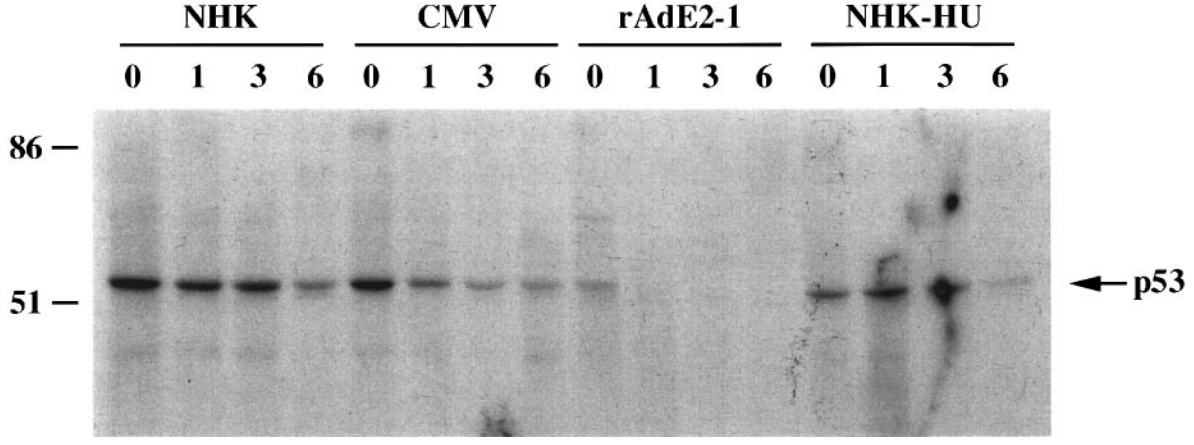


Fig. 12. Pulse–chase analysis of p53 protein in rAdE2-1-infected NHK cells demonstrates a decrease in the half-life of the protein. Control CMV or rAdE2-1 recombinant adenoviruses were used to infect NHK cells as described in Materials and methods. At 40 h post-infection, cells were pulsed with [^{35}S]cysteine for 1 h and then chased with an excess of cold cysteine for 0, 1, 3 or 6 h. Equal amounts of acid-precipitable counts were subjected to immunoprecipitation with a p53-specific antibody. Following SDS–PAGE, labeled protein was visualized by fluorography. NHK represents uninfected cells. NHK-HU represents NHK cells which were synchronized in G_1 with hydroxyurea and used to determine the half-life of the p53 protein 6 h after release, when the majority of cells were in S phase. Molecular weight markers are indicated in kDa. The protein half-life was determined using a phosphoimager, and values were normalized to the 0 h control for each set.

with recombinant E1- and E2-expressing adenoviruses resulted in an increase in viral DNA copy number to levels similar to those seen during differentiation-dependent genome amplification. Similar increases in viral copy number were observed following infection of CIN612-9E cells with recombinant retroviruses expressing the E1 and E2 proteins (Frattini and Laimins, 1994b), while no change in template number was seen following infection with recombinant retroviruses expressing the other viral early region proteins (E5, E6, E7, or E6 and E7; M.G.Frattini and L.A.Laimins, unpublished observation). This suggests that increased activity or expression of the E1 and E2 proteins may play a determining role in the initiation of late viral functions.

During viral infection *in vivo*, amplification of viral DNA and virion assembly occur in terminally differentiating cells which are often characterized by large pycnotic aberrantly shaped nuclei. Since these cells are terminally differentiated, maintenance of normal cell cycle control would not be essential. It is possible that E2 induces a general S phase arrest in these differentiated cells to allow for continued replication of viral and, therefore, cellular DNA. Further studies are in progress to demonstrate an increase in E2 protein or activity upon differentiation in a productively infected epithelium.

Examples of abrogation of the mitotic checkpoint are rare in mammalian cells but have been documented in *Drosophila* and yeast. Recently, an example of loss of this checkpoint control in mammalian cells has been reported in fibroblasts derived from p53 knockout mice which had been treated with the mitotic spindle inhibitor, nocodazole (Cross *et al.*, 1995). These cells arrested in S phase and continued to replicate cellular DNA in a manner similar to what we have observed in cells infected with E2-expressing adenoviruses. Most of the current data involving this cell cycle checkpoint, however, has come from the fission yeast, *S.pombe*, where several gene products have been identified which, when deleted (or overexpressed), have been shown to abrogate the normal checkpoint control mechanism resulting in re-replication of chromosomal DNA. These gene products include cdc2 kinase, rum1, cdc13 and cdc18. Recently, Jallepalli and Kelly (1996) demonstrated that overexpression of the cdc2 kinase inhibitor, rum1 [or its budding yeast equivalent, sic1 (Schneider *et al.*, 1996)] in fission yeast resulted in accumulation of the *cdc18* gene product through a post-transcriptional mechanism and, therefore, re-replication. These experiments suggested that the initiation of DNA replication in fission yeast might be mediated through cdc2 kinase inhibition, resulting in a specific increase in cdc18 protein. Cdc18, like its budding yeast homolog cdc6, is normally a short-lived protein which is most likely involved in protein–protein and protein–DNA interactions at the origin of replication (Kelly *et al.*, 1993; Liang *et al.*, 1995; Jallepalli and Kelly, 1996; Muzi-Falconi *et al.*, 1996). Since our studies demonstrated no increase or decrease in the activation state of mammalian cdc2 kinase, it is intriguing to speculate that E2 might be functioning as a transcriptional activator of the mammalian cdc18 homolog (mcdc18) gene expression, allowing for mcdc18 accumulation and re-replication. Alternatively, it is possible that E2 could be functioning as a transcriptional repressor of the gene(s) responsible for mcdc18 degradation or through protein–protein interactions by interfering with the degradation process.

The most surprising observation from our studies was the reduced level of p53 protein detected in rAdE2-1-infected cells. Although this reduction in p53 protein level appears to be due to increased protein turnover, we cannot rule out an additional effect on p53 translation in E2-expressing cells. The ability to accelerate the degradation of p53 previously has been demonstrated for the E6 proteins of the oncogenic HPV types (Wernesss *et al.*, 1989; Scheffner *et al.*, 1990; Crook *et al.*, 1991; Lechner *et al.*, 1992). The E6 proteins from these high risk viruses bind E6Ap, a ubiquitin ligase, and this complex associates with p53 resulting in an increase in protein degradation (Scheffner *et al.*, 1990). Whether the observed effect of E2 overexpression is also mediated by E6Ap is currently under study. Since E2 is a transcriptional activator, it is also possible that the increase in p53 turnover is an indirect effect resulting from the activation of expression of enzymes in the p53 degradation pathway. In cells infected with the high risk HPV types, the E6 protein increases turnover of the p53 protein; therefore, a role for E2 in this process would appear redundant. However, in other non-oncogenic HPV types, E6 proteins bind p53 poorly, and no effect is seen on p53 protein levels. The E2 proteins are highly conserved among HPV types, and each type-specific protein can substitute for the other in transient replication assays utilizing various HPV origin fragments (Chiang *et al.*, 1992). Therefore, unlike the ability of E6 proteins from only the high risk HPV types to accelerate the turnover of p53, the ability of the E2 protein to induce a reduction in p53 levels may be conserved among human papillomavirus types. Since E6 is expressed from a viral promoter which is repressed by the E2 protein (Thierry and Yaniv, 1987; Bernard *et al.*, 1989; Romanczuk *et al.*, 1990; Dong *et al.*, 1994; M.G.Frattini and L.A.Laimins, unpublished observation), it is possible that this apparent redundancy could be required in oncogenic viral infections where a terminally differentiated cell undergoing genome amplification and, therefore, viral propagation, would be dependent on increasing E2 protein level or activity.

While p53 appears to play an important role in regulating the mitotic checkpoint, our studies as well as those of Cross *et al.* (1995) suggest that loss of p53 alone is not sufficient for S phase arrest. Even though fibroblasts from p53 knockout mice quickly become aneuploid when grown in culture (implicating the involvement of p53 at this checkpoint), it is only in the presence of the mitotic spindle inhibitor, nocodazole, that these cells become arrested in S phase. In *Drosophila*, the process of endoreduplication allows for re-replication of certain chromosomal origins and occurs during various stages of embryonic development. This arrest is mediated by the continued expression of cyclin E, a gene which recently has been shown to be under the control of the transcription factor E2F-1 (Duronio and O'Farrell, 1995; Duronio *et al.*, 1995; Ohtani *et al.*, 1995). In *Drosophila* embryos, cells undergoing endoreduplication maintain elevated levels of cyclin E kinase activity which are sufficient to induce S phase arrest (Duronio *et al.*, 1995). A role for cyclin proteins is also suggested from examination of yeast strains containing conditional mutations in several cyclin genes which exhibit unregulated replication at the non-

permissive temperature (Schwob and Nasmyth, 1993). In rAdE2-1-infected cells, elevated levels of cyclin A and E kinase activity were detected while the level of cyclin B kinase activity was not changed when compared with control infected cells. The increase in cyclin A and E kinase activity, like the increase in E2F-1 mRNA, could be a reflection of the majority of E2-expressing cells being arrested in S phase. However, there was also a specific reduction in the level of the CDK inhibitor, p21, in rAdE2-1-infected cells. This reduction in the total amount of the p21 CDK inhibitor, which is probably due to the decrease in the amount of the p53 protein in rAdE2-1-infected cells, could account for the increase in cyclin A and E kinase activity. Interestingly, Waldman *et al.* (1996) have demonstrated recently that, following exposure to radiation and various chemotherapeutic agents, colorectal carcinoma cells, in which the p21 genes were deleted by homologous recombination, underwent multiple rounds of S phase without an intervening mitotic event. After several successive S phases, cell death occurred through apoptosis, suggesting that p21 (and, therefore, p53) could be responsible for control of the mitotic checkpoint (Waldman *et al.*, 1996). In our studies, rAdE2-1-infected cells undergo a similar fate and provide a unique example of abrogation of a cellular growth control mechanism by viral proteins from a DNA tumor virus. Although viral infection culminates in the death of the host cell, interference with the mitotic checkpoint provides the virus with a selective advantage, allowing for viral amplification and, therefore, propagation.

Previous studies from several investigators have examined the effects of overexpression of the bovine papillomavirus type 1 (BPV-1) E2 protein in cells which express the HPV 18 E6 and E7 oncoproteins or immortalized tumor cell lines (Hwang *et al.*, 1993, 1996; Dowhanick *et al.*, 1995). In the HPV-positive cells, increased BPV-1 E2 protein resulted in decreased E6/E7 expression, through E2-mediated transcriptional repression. This decrease in E6 expression led to an immediate increase in p53 levels, G_1 arrest and, in some studies, reduced colony-forming ability. Our studies concentrated on HPV 31 E2 proteins, and we have not observed similar effects on HPV 31 copy number, or the resultant S phase arrest, using the BPV-1 E2 SV40 expression-based viruses to infect CIN 612-9E cells (M.G.Frattini and L.A.Laimins, unpublished observation). It is possible that species-specific differences, as observed with the E6 and E7 proteins, also exist with the E2 proteins. In agreement with this hypothesis are the previously published reports which demonstrate that the BPV-1 E2 protein does not substitute efficiently for the HPV E2 protein in transient replication assays (Chiang *et al.*, 1992). Alternatively, the level of E2 expression from our recombinant adenoviruses may be significantly higher than that observed with the BPV-1 E2 studies. The differences, therefore, could be the result of quantitative effects. Based on the data currently available, however, we favor a species-specific difference.

These experiments have identified an important mitotic checkpoint which is abrogated by the expression of a viral protein. This checkpoint normally controls genomic stability by coupling the completion of the DNA synthesis phase of the cell cycle to the initiation of cell division. Modulation of this control point of the eukaryotic cell cycle is likely to play an important role not only in the process of viral pathogenesis but also in the development and progression of human malignancies. Insights into the mechanisms through which this checkpoint is controlled may ultimately provide the basis for novel anticancer therapies.

Materials and methods

Cell lines

Both SCC-13 cells, which were derived from a squamous cell carcinoma, and CIN 612-9E cells, which were derived from a HPV-31b-positive CIN I grade cervical lesion, were grown in E medium with mitomycin C-treated fibroblast feeders as described (Bedell *et al.*, 1991; Meyers *et al.*, 1992; Frattini *et al.*, 1996). Secondary NHK cells (Clonetics) were grown in a serum-free medium, KGM (Keratinocyte Growth Media, Clonetics). 293 cells, a human lung epithelial cell line which has been transformed with the adenovirus E1A gene, were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 50 μg/ml gentamycin.

Construction of E1 and E2 recombinant adenoviruses

Replication-defective recombinant adenoviruses were constructed in the context of the mutant adenovirus type 5 genome, 309 (dl309) (Jones and Shenk, 1979; Swaminathan and Thimmapaya, 1996). The 309 mutant has a unique *Xba*I restriction site in the 5′ end of the viral genome which, when restricted, results in the removal of the E1A coding sequences. These gene products have been shown to be essential for viral transcription and replication functions. In order to construct recombinant genomes, it was first necessary to clone the E1 and E2 gene products (Goldsborough *et al.*, 1989; Frattini and Laimins, 1994a), containing 5′ and 3′ *Bam*HI restriction sites, into the *Bgl*II restriction site of the transfer vector, pCMV#2 (Swaminathan and Thimmapaya, 1996). The plasmid pCMV#2 uses the CMV promoter to direct high level expression of cloned genes in eukaryotic cells. This plasmid also carries the first 452 bp of the 5′ end of the adenovirus type 5 genome directly upstream of the promoter sequences. The E1 and E2 recombinant pCMV#2 clones were then restricted with *Nhe*I, made blunt with the Klenow fragment of *Escherichia coli* DNA polymerase I, dephosphorylated using calf intestinal alkaline phosphatase (CIAP, Boehringer Mannheim) and restricted with *Xba*I. The resulting DNA fragment containing the 452 bp 5′ end of the adenovirus genome, the CMV promoter sequences, the E1 or E2 coding sequence and the SV40 polyadenylation sequence was purified from the remaining plasmid sequences by agarose gel electrophoresis. This fragment was then ligated to the adenovirus 309 genome which had been restricted with *Xba*I, dephosphorylated with CIAP and purified from the E1A coding sequences by agarose gel electrophoresis. The resulting replication-defective recombinant adenovirus genomes replaced the adenovirus E1A coding sequences with the CMV promoter and HPV E1 or E2 genes. The DNA from these ligations was precipitated with ethanol and used to transfect 293 cells, which constitutively express the E1A gene products allowing for replication and, therefore, propagation of these recombinant viruses.

In order to obtain sufficient adenovirus genomic DNA to make the recombinant viruses, adenoviral DNA was purified from 50 plates of 293 cells which were infected with a similar recombinant adenovirus expressing the adenovirus E4 ORF 6/7 gene product from the CMV promoter (Swaminathan and Thimmapaya, 1996). A cell lysate was made by resuspending the infected cells in 100 mM Tris–HCl, pH 8.0 and freeze–thawing the suspension three times. The adenoviral DNA was then purified from the cell lysate by gradient centrifugation. This included two CsCl isopycnic ultracentrifugation steps, protease digestion of the viral proteins, phenol/chloroform extraction of the viral DNA and precipitation of the DNA with ethanol. The viral DNA was then dissolved in 1× TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA), and portions restricted with *Xba*I and gel purified as outlined above.

Transfection of the 293 cells was done using the Lipofectamine reagent (GIBCO). A total of 580 000 cells were seeded into a 60 mm tissue culture dish and allowed to grow until reaching 70–80% confluence (usually overnight). Then 3 μg of DNA was mixed with 0.25 ml of OPTI-MEM I reduced serum medium (GIBCO), combined with an equal volume of the same medium containing 26 μl of Lipofectamine reagent, and incubated at room temperature for 30 min in a polystyrene tube. After this incubation, 0.5 ml of serum-free DMEM was added to the

lipid–DNA complexes, and the resulting solution was added immediately to a plate of cells containing 0.5 ml of serum-free DMEM. The cells were incubated in the presence of the lipid–DNA complexes for 5 h at 37°C. The transfection mixture was then diluted with 1.5 ml of DMEM containing 20% FBS and incubated overnight before changing the medium. The cells were monitored daily for the appearance of a cytopathic effect (CPE). At 7 days post-transfection, the cells were harvested, buffered by the addition of 30 mM Tris–HCl, pH 7.4, and subjected to three rounds of freeze–thawing. After removing the cell debris by centrifugation, the supernatant was used to infect a 60 mm dish of 293 cells by incubating an 80% confluent plate with 1 ml of the supernatant from the original transfection. After 2 h at 37°C, the inoculum was aspirated and replaced with 5 ml of fresh medium. CPE was evident after 6 days of infection on the E1 and E2 recombinant virus plates. The cells were harvested as above, and a 10 cm plate of 293 cells was infected with 2 ml of the second round supernatant. When ~80% of the infected cells were floating (day 3 post-infection), high titer recombinant adenoviruses were harvested as usual, aliquoted in 1 ml fractions and stored at –80°C. The AdE4 (Swaminathan and Thimmapaya, 1996) and CMV (Kowalik *et al.*, 1995) recombinant adenoviruses were propagated in a similar fashion.

CIN 612-9E or NHK cells, on 60 or 100 mm plates, which were ~60% confluent, were infected with the AdE4, CMV, HPV E1 or rAdE2-1 recombinant adenoviruses with a multiplicity of infection (m.o.i.) of 50–100 as described above.

Electromobility shift assays (EMSA)

Nuclear extracts were prepared from infected cells at the indicated times post-infection essentially as described (Schreiber *et al.*, 1989). For the E2 protein EMSA analyses, 7.5 μg of extract was incubated with 2 μg of poly (dI:dC) and 1 ng of a ^{32}P-labeled 35 bp oligonucleotide containing two E2 binding sites (Androphy *et al.*, 1987; Hegde *et al.*, 1992) in binding buffer [20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamidine, 10% (v/v) glycerol] for 20 min at 25°C. Protein–DNA complexes were separated by 5% native polyacrylamide gel electrophoresis (PAGE), dried and visualized by autoradiography. E2F EMSA analyses were performed as described (Kowalik *et al.*, 1995; Ohtani *et al.*, 1995) with ^{32}P-labeled wild-type and mutant E2F-1-specific oligonucleotides (Santa Cruz Biotechnology, Inc.).

Antibodies and Western blot analyses

Antibodies directed against cyclins A, B1 and E as well as those against cdc2, p21 and phosphotyrosine were obtained from Santa Cruz Biotechnology, Inc. The p53 antibody, Ab-2, was obtained from Oncogene Science Inc. The HPV 31 E2 antibody was as described (Frattini and Laimins, 1994a), and purified cdc2–cyclin B was obtained from New England Biolabs. Following separation of protein complexes by SDS–PAGE, proteins were transferred to a PVDF membrane (Millipore), and Western blot analyses were performed using chemiluminescent detection methods as described by the manufacturer (ECL, Amersham).

Southern and Northern blot analyses

At the indicated times post-infection, infected cells were harvested and genomic DNA isolated as described (Frattini *et al.*, 1996). Southern blot analyses were performed using 10 μg of total cellular DNA. Following restriction with *Bam*HI, which does not restrict the HPV 31 genome, DNA samples were separated on 0.8% agarose gels, transferred to a nylon membrane (GeneScreen Plus, DuPont) and probed with a ^{32}P-labeled HPV 31-specific probe. Hybridizing species were visualized by autoradiography. For Northern analyses, RNA was isolated from monolayer cultures using the Trizol reagent (GIBCO), and polyadenylated RNA (mRNA) was purified with the PolyATract system as described by the manufacturer (Promega). Equal amounts of mRNA, as determined by UV absorption, were separated on 1% agarose–formaldehyde gels, transferred to a nylon membrane (GeneScreen Plus, DuPont) and hybridized with the indicated ^{32}P-labeled DNA probe. Hybridizing transcripts were visualized by autoradiography. Northern blots were stripped and reprobed with the glyceraldehyde-3-phosphate dehydrogenase gene to confirm equal loading of mRNA. The fold increase in viral genomic copy number or mRNA transcripts was quantitated using a phosphoimager (Fuji, Japan) and normalized to the values obtained for the copy number controls or the uninfected control sample, respectively.

FACS analysis

At the indicated time points, cells were harvested, washed with phosphate-buffered saline (PBS) and fixed with –20°C 70% ethanol. Where indicated, cells were incubated in 0.1 M Na_2HPO_4 and 0.05 M citric acid buffer, pH 7.8 for 30 min at 25°C to extract cleaved DNA fragments (Darzynkiewicz *et al.*, 1992). Cells were then washed with PBS and resuspended in 3.8 mM sodium citrate, 125 μg/ml RNase A, and either 50 μg/ml propidium iodide (PI, Sigma) or 25 μg/ml 7-amino-actinomycin D (7-AAD, Calbiochem) as indicated.

For proliferation analysis, cells were pulsed with 10 μM BrdU for 30 min prior to harvest. Cells were then washed with PBS, fixed with –20°C 70% ethanol and stained intracellularly for BrdU incorporation with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU antibody (Boehringer-Mannheim) for 30 min at 25°C. For cell cycle arrest studies, infected cells were incubated with hydroxyurea (Sigma) or nocodazole (Sigma) as described (Lees *et al.*, 1992).

Fixed and stained cells were analyzed on a Becton-Dickinson FACScan (Mountain View, CA) with LYSIS II software. Samples were gated by forward and 90° scatter for intact, single cells for each treatment. Voltages for FL-1 (BrdU-FITC) and FL-3 (7-AAD or PI) and quadrants were set based on negatively stained samples.

Immunoprecipitation and pulse–chase analysis

Immunoprecipitation analyses of uninfected and infected cells were performed essentially as described (McIntyre *et al.*, 1993; Frattini and Laimins, 1994a). At the indicated times post-infection, cells were starved in cysteine- and methionine-free KGM (Clonetics) for 1 h and then metabolically labeled for 3–4 h with 0.5 mCi of [^{35}S]cysteine (Amersham). Unlabeled cells were harvested at the indicated time points. Cells were lysed in ELB lysis buffer [50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 100 mM NaF, 200 μM Na_3-*o*-VO_4, 1 mM PMSF, 5 mM benzamidine, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 1.0% (v/v) NP-40] and cleared with a control antibody. Labeled samples were normalized by equal amounts of trichloroacetic acid-precipitable counts, and unlabeled samples were normalized by total protein concentration. Following immunoprecipitation with protein A–Sepharose (Pharmacia), immune complexes were washed, resuspended in 4× Laemmli sample buffer, and electrophoresed on SDS–PAGE gels. Gels containing labeled proteins subsequently were fixed, soaked in Amplify (Amersham), dried and proteins visualized by fluorography.

For pulse–chase analysis, infected cells were starved and pulse labeled for 1 h as indicated above. Cells were either harvested immediately or washed with PBS and incubated in chase medium containing excess unlabeled cysteine for 1, 3 or 6 h. Proteins were immunoprecipitated, electrophoresed and visualized as described above. The protein half-life was determined using a phosphoimager (Fuji, Japan), and values were normalized to the 0 h control for each set.

Kinase assays

Kinase assays were performed as described (McIntyre *et al.*, 1996). At the indicated times post-infection, cells were harvested, lysed and immunoprecipitated as outlined above. Immune complexes were washed twice with ELB buffer and then twice with kinase buffer (50 mM HEPES, pH 7.0, 15 mM $MgCl_2$, 1 mM DTT) containing 0.1 mg/ml bovine serum albumin. Kinase reactions were then performed in 50 μl of kinase buffer with 10 μCi of [γ-^{32}P]ATP and 25 μM unlabeled ATP at 30°C for 30 min. Reactions were stopped by the addition of an equal volume of 4× Laemmli sample buffer and separated on an SDS–PAGE gel. Labeled proteins were visualized by autoradiography.

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References

Androphy,E., Lowy,D.R. and Schiller,J.T. (1987) Bovine papillomavirus E2 *trans*-activating gene product binds to specific sites in papillomavirus DNA. *Nature*, **325**, 70–73.

Bedell,M.A., Hudson,J.B., Golub,T.R., Turyk,M.E., Hosken,M., Wilbanks,G.D. and Laimins,L.A. (1991) Amplification of human

papillomavirus genomes *in vitro* is dependent on epithelial differentiation. *J. Virol.*, **65**, 2254–2260.
Bernard,B.A., Bailly,C., Lenoir,M.-C., Darmon,M., Thierry,F. and Yaniv,M. (1989) The human papillomavirus type 18 (HPV18) E2 gene product is a repressor of the HPV18 regulatory region in human keratinocytes. *J. Virol.*, **63**, 4317–4324.
Blanton,R., Colterra,M.D., Gown,A., Halbert,C. and McDougall,J.K. (1992) Expression of the HPV 16 E7 gene generates proliferation in stratified squamous cell cultures which is dependent upon p53 levels. *Cell Growth Differ.*, **3**, 791–802.
Blitz,I.L. and Laimins,L.A. (1991) The 68 kilodalton E1 protein of BPV-1 is a DNA binding phosphoprotein which associates with the E2 transcriptional activator *in vitro*. *J. Virol.*, **65**, 649–656.
Blow,J.J. and Laskey,R.A. (1988) A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature*, **332**, 546–548.
Cheng,S., Schimdt-Grimminger,S.-D., Murant,T., Broker,T.R. and Chow,L.T. (1995) Differentiation-dependent up-regulation of HPV E7 reactivates cellular DNA replication in suprabasal differentiated cells. *Genes Dev.*, **9**, 2335–2349.
Chiang,C.M., Ustav,M., Stenlund,A., Ho,T., Broker,T.R. and Chow,L.T. (1992) Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. *Proc. Natl Acad. Sci. USA*, **89**, 5799–5803.
Crook,T., Tidy,J.A. and Vousden,K.H. (1991) Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and transactivation. *Cell*, **67**, 547–556.
Cross,S.M., Sanchez,C.A., Morgan,C.A., Schimke,M.K., Ramel,S., Idzerda,R.L., Raskind,W.H. and Reid,B.J. (1995) A p53-dependent mouse spindle checkpoint. *Science*, **267**, 1353–1356.
Darzynkiewicz,Z., Bruno,S., Del Bino,G., Gorczyca,W., Hotz,M.A., Lassota,P. and Traganos,F. (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry*, **13**, 795–808.
DelVecchio,A.M., Romanczuk,H., Howley,P.M. and Baker,C.C. (1992) Transient replication of human papillomavirus DNAs. *J. Virol.*, **66**, 5949–5958.
Dong,G., Broker,T.R. and Chow,L.T. (1994) Human papillomavirus type 11 E2 proteins repress the homologous E6 promoter by interfering with the binding of host transcription factors to adjacent elements. *J. Virol.*, **68**, 1115–1127.
Dostatni,N., Thierry,F. and Yaniv,M. (1988) A dimer of BPV-1 E2 containing a protease resistant core interacts with its DNA target. *EMBO J.*, **7**, 3807–3816.
Dowhanick,J.J., McBride,A.A. and Howley,P.M. (1995) Suppression of cellular proliferation by the papillomavirus E2 protein. *J. Virol.*, **69**, 7791–7799.
Duronio,R.J. and O'Farrell,P.H. (1995) Developmental control of the G1 to S transition in *Drosophila*: cyclin E is a limiting downstream target of E2F. *Genes Dev.*, **9**, 1456–1468.
Duronio,R.J., O'Farrell,P.H., Xie,J.-E., Brook,A. and Dyson,N. (1995) The transcription factor E2F is required for S phase during *Drosophila* embryogenesis. *Genes Dev.*, **9**, 1445–1455.
El-Deiry,W.S. *et al*. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.
Enoch,T. and Nurse,P. (1991) Coupling M phase and S phase: controls maintaining the dependence of mitosis on chromosome replication. *Cell*, **65**, 921–923.
Frattini,M.G. and Laimins,L.A. (1994a) The role of the E1 and E2 proteins in the replication of human papillomavirus type 31b. *Virology*, **204**, 799–804.
Frattini,M.G. and Laimins,L.A. (1994b) Binding of the human papillomavirus E1 origin-recognition protein is regulated through complex formation with the E2 enhancer-binding protein. *Proc. Natl Acad. Sci. USA*, **91**, 12398–12402.
Frattini,M.G., Lim,H.B. and Laimins,L.A. (1996) *In vitro* synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. *Proc. Natl Acad. Sci. USA*, **93**, 3062–3067.
Goldsborough,M.D., DiSilvestre,D., Temple,G.F. and Lorincz,A.T. (1989) Nucleotide sequence of human papillomavirus type 31: a cervical neoplasia-associated virus. *Virology*, **171**, 306–311.
Grana,X. and Reddy,E.P. (1995) Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene*, **11**, 211–219.
Handeli,S. and Weintraub,H. (1992) The ts41 mutation in Chinese hamster cells leads to successive S phases in the absence of intervening G2, M, and G1. *Cell*, **71**, 599–611.
Hayles,J., Fisher,D., Woollard,A. and Nurse,P. (1994) Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2–mitotic B cyclin complex. *Cell*, **78**, 813–822.
Hegde,R.S., Grossman,S., Laimins,L.A. and Sigler,P. (1992) Crystal structure at 1.7 Å of the BPV-1 E2 DNA-binding domain bound to its target DNA. *Nature*, **359**, 505–512.
Heichman,K.A. and Roberts,J.M. (1994) Rules to replicate by. *Cell*, **79**, 557–562.
Howley,P.M. (1996) Papillomavirinae: the viruses and their replication. In Fields,B.N., Knipe,D.M. and Howley,P.M. (eds), *Fundamental Virology*. Raven Press, New York, pp. 947–978.
Hummel,M., Lim,H.B. and Laimins,L.A. (1995) Human papillomavirus type 31b late gene expression is regulated through protein kinase C-mediated changes in RNA processing. *J. Virol.*, **69**, 3381–3388.
Hwang,E.-S., Riese,D.J.,II, Settleman,J., Nilson,L.A., Honig,J., Flynn,S. and DiMaio,D. (1993) Inhibition of cervical carcinoma cell line proliferation by the introduction of a bovine papillomavirus regulatory gene. *J. Virol.*, **67**, 3720–3729.
Hwang,E.-S., Naeger,L.K. and DiMaio,D. (1996) Activation of the endogenous p53 growth inhibitory pathway in HeLa cervical carcinoma cells by expression of the bovine papillomavirus E2 gene. *Oncogene*, **12**, 795–803.
Jallepalli,P.V. and Kelly,T.J. (1996) Rum1 and Cdc18 link inhibition of cyclin-dependent kinase to the initiation of DNA replication in *Schizosaccharomyces pombe*. *Genes Dev.*, **10**, 541–552.
Johnson,D.G., Schwarz,J.K., Cress,W.D. and Nevins,J.R. (1993) Expression of transcription factor E2F1 induces quiescent cells to enter S phase. *Nature*, **365**, 349–352.
Jones,N. and Shenk,T. (1979) Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell*, **17**, 683–689.
Kelly,T.J., Martin,G.S., Forsburg,S.L., Stephen,R.J., Russo,A. and Nurse,P. (1993) The fission yeast cdc18+ gene product couples S phase to START and mitosis. *Cell*, **74**, 371–382.
King,R.W., Jackson,P.K. and Kirschner,M.W. (1994) Mitosis in transition. *Cell*, **79**, 563–571.
Kowalik,T.F., DeGregori,J., Schwarz,J.K. and Nevins,J.R. (1995) E2F1 overexpression in quiescent fibroblasts leads to induction of cellular DNA synthesis and apoptosis. *J. Virol.*, **69**, 2491–2500.
Laimins,L.A. (1993) The biology of human papillomaviruses: from warts to cancer. *Infect. Agents Dis.*, **2**, 74–86.
Lambert,P. (1991) Papillomavirus DNA replication. *J. Virol.*, **65**, 3417–3420.
Lechner,M., Mack,D., Finicle,A., Crook,T., Vousden,K.H. and Laimins,L.A. (1992) HPV E6 proteins bind p53 *in vivo* and abrogate p53-mediated repression of transcription. *EMBO J.*, **11**, 3045–3052.
Lees,E., Faha,B., Dulic,V., Reed,S. and Harlow,E. (1992) Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. *Genes Dev.*, **6**, 1874–1885.
Li,R. and Botchan,M.R. (1994) Acidic transcription factors alleviate nucleosome-mediated repression of BPV-1 DNA replication. *Proc. Natl Acad. Sci. USA*, **91**, 7051–7055.
Liang,C., Weinreich,M. and Stillman,B. (1995) ORC and cdc6p interact and determine the frequency of initiation of DNA replication in the genome. *Cell*, **81**, 667–676.
McIntyre,M.C., Frattini,M.G., Grossman,S.R. and Laimins,L.A. (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.
McIntyre,M.C., Ruesch,M.N. and Laimins,L.A. (1996) Human papillomavirus E7 oncoproteins bind a single form of cyclin E in a complex with cdk2 and p107. *Virology*, **215**, 73–82.
Meyers,C., Frattini,M.G., Hudson,J.B. and Laimins,L.A. (1992) Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science*, **257**, 971–973.
Mohr,I.J., Clark,R., Sun,S., Androphy,E., MacPherson,P. and Botchan,M. (1990) Targeting the E1 replication factor to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science*, **250**, 1694–1699.
Morgan,D.O. (1995) Principles of CDK regulation. *Nature*, **374**, 131–134.
Muzi-Falconi,M., Brown,G.W. and Kelly,T.J. (1996) cdc18+ regulates initiation of DNA replication in *Schizosaccharomyces pombe*. *Proc. Natl Acad. Sci. USA*, **93**, 1566–1570.
Nevins,J.R. (1992) E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science*, **258**, 424–429.

Nurse,P. (1994) Ordering S phase and M phase in the cell cycle. *Cell*, **79**, 547–550.
Ohtani,K., DeGregori,J. and Nevins,J.R. (1995) Regulation of the cyclin E gene by transcription factor E2F1. *Proc. Natl Acad. Sci. USA*, **92**, 12146–12150.
Rao,P.N. and Johnson,R.T. (1970) Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature*, **225**, 159–164.
Romanczuk,H., Thierry,F. and Howley,P.M. (1990) Mutational analysis of *cis* elements involved in E2 modulation of human papillomavirus type 16 P97 and type 18 P105 promoters. *J. Virol.*, **64**, 2849–2859.
Romanowski,P. and Madine,M. (1996) Mechanisms restricting DNA replication to once per cell cycle: MCMs, pre-replicative complexes, and kinases. *Trends Cell Biol.*, **6**, 184–189.
Sauer,K., Knoblich,J.A., Richardson,H. and Lehner,C.F. (1995) Distinct modes of cyclin E/cdc2c kinase regulation and S phase control in mitotic and endoreduplication cycles of *Drosophila* embryogenesis. *Genes Dev.*, **9**, 1327–1339.
Scheffner,M., Werness,B.A., Hubregiste,J.M., Levine,A.J. and Howley, P.M. (1990 The E6 oncoprotein encoded by HPV 16 and 18 promotes the degradation of p53. *Cell*, **63**, 1129–1136.
Schneider,B.L., Yang,Q.-H. and Futcher,A.B. (1996) Linkage of replication to Start by the cdk inhibitor sic1. *Science*, **272**, 560–562.
Schreiber,E., Matthias,P., Muller,M.M. and Schaffner,W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.*, **17**, 6419.
Schwob,E. and Nasmyth,K. (1993) CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. *Genes Dev.*, **7**, 1160–1175.
Seo,Y.-S., Muller,F., Lusky,M., Gibbs,E., Kim,H.-Y., Phillips,B. and Hurwitz,J. (1993) BPV-1 encoded E2 proteins enhance binding of E1 to the BPV replication origin. *Proc. Natl Acad. Sci. USA*, **90**, 2865–2869.
Su,T.T., Follette,P.J. and O'Farrell,P.H. (1995) Qualifying for the license to replicate. *Cell*, **81**, 825–828.
Swaminathan,S. and Thimmapaya,B. (1996) Transactivation of adenovirus E2-early promoter by E1A and E4 6/7 in the context of viral chromosome. *J. Mol. Biol.*, **258**, 736–746.
Thierry,F. and Yaniv,M. (1987) The BPV-1 E2 *trans*-acting protein can be either an activator or a repressor of the HPV 18 regulatory region. *EMBO J.*, **6**, 3391–3397.
Thorner,L.K., Lim,D.A. and Botchan,M. (1993) DNA-binding domain of BPV-1 E1 helicase: structural and functional aspects. *J. Virol.*, **67**, 6000–6014.
Ustav,M. and Stenlund,A. (1991) Transient replication of BPV-1 requires two polypeptides encoded by the E1 and E2 open reading frames. *EMBO J.*, **10**, 449–457.
Waldman,T., Lengauer,C., Kinzler,K.W. and Vogelstein,B. (1996) Uncoupling of S phase and mitosis induced by anticancer agents in cells lacking p21. *Nature*, **381**, 713–716.
Wernesss,B., Levine,A.J. and Howley,P.M. (1989) Association of HPV 16 and 18 E6 proteins with p53. *Science*, **248**, 76–79.
Yang,L., Li,R., Mohr,I.J., Clark,R. and Botchan,M.R. (1991) Activation of BPV-1 replication *in vitro* by the transcription factor E2. *Nature*, **353**, 628–633.

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