

## Modulation of the Cell Division Cycle by Human Papillomavirus Type 18 E4

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Received 11 March 2002/Accepted 20 July 2002

**The life cycle of human papillomaviruses (HPVs) is tightly coupled to the differentiation program of their host epithelial cells. HPV E4 gene expression is first observed in the parabasal layers of squamous epithelia, suggesting that the E4 gene product contributes to the mechanism of differentiation-dependent virus replication, although its biological function remains unclear. We analyzed the effect of HPV type 18 E4 on cell proliferation and found that E4 expression induced cell cycle arrest at the G<sub>2</sub>/M boundary. The functional region of E4 necessary for the growth arrest activity was located in the central portion of the molecule, and this activity was independent of the E4-mediated collapse of cytokeratin intermediate filament structures.**

Human papillomaviruses (HPVs) infect epithelial cells, causing hyperproliferative lesions such as warts and condylomas. Over 70 HPV types have been identified (12). According to their tissue tropism, they are categorized into two major groups, the cutaneous and mucosal HPVs. The mucosal HPVs are further grouped into high-risk and low-risk types. Lesions caused by high-risk HPVs have a propensity to progress to malignant tumors, most prominently cervical carcinomas. In contrast, lesions caused by low-risk HPVs have a much lower risk for malignant progression (51).

The HPV E6 and E7 oncoproteins are known to play key roles in HPV-associated cancer formation (21, 22, 24, 33, 34). This fact is supported by the finding that most HPV-positive cancer cells maintain the expression of E6 and E7 (4, 52). E6 forms a complex with p53 and, in combination with the cellular ubiquitin ligase E6AP, induces the degradation of p53 through the proteasome (25, 48, 49, 60). E7 binds to and functionally inactivates pRB (20, 35). Both p53 and pRB play critical roles as regulators of the cell cycle and apoptosis. By inhibiting these and other regulatory mechanisms, HPV E6 and E7 allow for the accumulation of genetic mutations and the survival of mutated cells (8, 42, 61). E6 and E7 expression also contributes to the immortalization of infected cells; E6 can enhance telomerase activity through an unknown mechanism (29, 57), whereas E7 inhibits a p16<sup>ink4A</sup>-dependent pathway that limits cellular proliferation in epithelial cells (28). It is generally believed that these functions of the viral oncoproteins contribute in important ways to HPV-induced cancer formation. However, it is likely that additional viral genes also contribute to the establishment of hyperproliferative potentially precancerous lesions caused by high-risk HPVs. In addition, the relatively

low incidence of malignant progression of high-risk HPV-positive lesions indicates that additional mutations of cellular genes may also be necessary for malignant progression.

Studies of the viral life cycle are important for understanding the process of HPV-induced cancer formation. HPV infects the basal layer cells of cutaneous or mucosal membranes. The HPV genome is maintained in an episomal state and at a low copy number in basal cells. The basal cells divide, and the descendant cells move to the upper layers of the epithelium and undergo a program of terminal differentiation. The HPV genome is amplified to a high copy number in differentiated cells, late genes are expressed, and virus particle formation is observed. This tight association between viral replication and the host cell differentiation program is a prominent feature of the HPV life cycle. Several reports have described the regulatory mechanisms of viral replication and how these are connected to the cellular differentiation program. Certain transcription factors, including YY1, skn-1, and CDP, have been implicated in differentiation-dependent HPV gene expression (1–3, 30, 38, 63).

The expression profile of the HPV E4 gene is clearly linked to cellular differentiation status (6, 9, 10, 13, 14, 36, 39). The E4 open reading frame is located in the region of early viral genes, such as E1 and E2, even though other studies have suggested that its expression pattern is more characteristic of a late gene. E4 expression is first detected in the parabasal layers, where vegetative HPV DNA replication is initiated. The E4 protein is encoded by a spliced E1<sup>Δ</sup>E4 mRNA and is expressed as a fusion protein with the N terminus of the E1 protein. It has been reported that in HPV type 1 (HPV1)-infected warts, the E4 protein accumulates at high levels and constitutes approximately 20% of the total proteins (6, 13). Since the expression of E4 is tightly linked to host cell differentiation, it likely plays an important role in the viral life cycle (13, 26). Several biological activities of E4 have been reported; the best defined is its association with cytokeratins and the concomitant destabilization of cytokeratin networks (17, 41, 44, 47, 54). It has been proposed that this activity of E4 may contribute to the efficient transmission of HPV, as progeny virus is shed within terminally

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differentiated epithelial squamae (17). These studies, however, do not rule out the possibility that E4 may have additional biological activities that also contribute to the viral life cycle.

In this report, high-risk HPV-derived E4 proteins were expressed in cultured cells, and their effect on cell growth was analyzed. E4 expression suppressed cell growth and arrested cell cycle progression at the G<sub>2</sub>/M boundary. No apoptosis was observed, however. Moreover, this activity of E4 was independent of its ability to disrupt cyokeratin networks. Our findings suggest that E4 may also contribute to the regulation of the viral life cycle by modulating the host cell division cycle.

#### MATERIALS AND METHODS

**Cell cultures and transfection.** HeLa, CV1, and C33A cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum. DNA transfections were performed by using a standard calcium phosphate precipitation method (37). Cells ( $2 \times 10^5$ ) were seeded in a 6-cm dish 1 day prior to transfection. Plasmid and carrier DNAs (total, 10  $\mu$ g) were incubated with 500  $\mu$ l of HEPES-buffered saline transfection buffer (140 mM NaCl, 0.75 mM Na<sub>2</sub>HPO<sub>4</sub>, 25 mM HEPES, 110 mM CaCl<sub>2</sub> [pH 6.90]) for 30 min at room temperature and then added to a culture dish. At 20 h after transfection, cells were washed once with phosphate-buffered saline (PBS), and fresh growth medium was added.

**Plasmids.** The E1<sup>Δ</sup>E4 cDNA expression plasmid was created by joining the corresponding E1 and E4 sequences by PCR. The information for the HPV16 E1<sup>Δ</sup>E4 (16E4) and HPV18 E1<sup>Δ</sup>E4 (18E4) mRNA structures was obtained from the HPV database (18). The E1<sup>Δ</sup>E4 DNA was cloned into plasmid pCMV-FLAG1 (Stratagene Inc., San Diego, Calif.) to fuse a FLAG epitope tag to the N terminus. The FLAG-E1<sup>Δ</sup>E4 sequence was subsequently transferred to a pCMV4 expression plasmid (37). A series of truncation mutations were introduced by using a PCR-mediated mutagenesis strategy (11); see Fig. 4A for the individual E1<sup>Δ</sup>E4 mutants. A commercially available green fluorescent protein (GFP) expression plasmid, pGreenLantern-1 (Invitrogen Corp., Carlsbad, Calif.), was used to mark transfected cells. For transfection experiments, herring sperm DNA (Roche Diagnostics GmbH, Mannheim, Germany) was used as carrier DNA. The human immunodeficiency virus type 1 (HIV-1) Vpr gene was amplified from an HIV-1 LAI DNA clone (40) by PCR, and a FLAG-Vpr expression plasmid was constructed as described for the FLAG-E1<sup>Δ</sup>E4 expression plasmid.

**Immunofluorescence microscopy and immunoblotting.** The transfected cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. FLAG-tagged E4 proteins were detected with an anti-FLAG polyclonal antibody (Sigma, St. Louis, Mo.) and Alexa Fluor 488 goat anti-rabbit immunoglobulin G (Molecular Probes, Eugene, Oreg.). Cytokeratins were detected with an anticytokeratin 8/18 monoclonal antibody (MAb) (Progen Biotechnik GmbH, Heidelberg, Germany) and Alexa Fluor 546 goat anti-mouse immunoglobulin G (Molecular Probes). Nuclei were visualized by staining with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Nacalai Tesque, Inc., Kyoto, Japan).

Whole-cell extracts were prepared from transfected cells with radioimmunoprecipitation (RIPA) buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 0.1% sodium dodecyl sulfate [SDS], 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM dithiothreitol, 16  $\mu$ g of benzamidine HCl/ml, 10  $\mu$ g of phenanthroline/ml, 10  $\mu$ g of aprotinin/ml, 10  $\mu$ g of leupeptin/ml, 10  $\mu$ g of pepstatin A/ml, 1 mM phenylmethylsulfonyl fluoride). A 500- $\mu$ l portion of RIPA buffer was added directly to a culture dish and incubated for 20 min at 4°C with rocking. The cell lysate was transferred to a microcentrifuge tube and centrifuged at 12,000  $\times$  g for 10 min at 4°C. The supernatant was used as the detergent-soluble fraction. The pellet was resuspended in 500  $\mu$ l of SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (50 mM Tris-HCl [pH 6.8], 2% SDS, 100 mM dithiothreitol, 10% glycerol, 0.1% bromophenol blue) and used as the non-detergent-soluble fraction. Both fractions were analyzed by SDS-15% PAGE and transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Ltd., Little Chalfont, England). An anti-FLAG MAb M5 (Sigma) was used for the detection of FLAG-tagged proteins. For visualization, a chemiluminescence detection reagent (Lumi-Light Western blotting substrate; Roche Diagnostics GmbH) was used.

**Flow cytometry.** Cells were cotransfected with E4 or Vpr expression plasmid (2  $\mu$ g) and GFP expression plasmid pGreenLantern-1 (0.5  $\mu$ g), fixed with 1% paraformaldehyde-ethanol for 10 min at various times after transfection, and treated with 0.5 mg of RNase A/ml-0.1% Triton X-100 in PBS for 30 min at

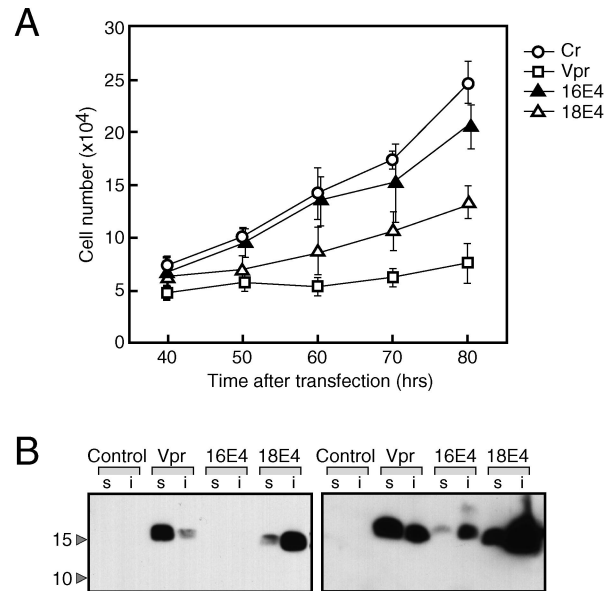


FIG. 1. Growth-inhibitory effect of HPV E4. (A) HeLa cells ( $2 \times 10^5$ ) were transfected with 2  $\mu$ g of expression plasmid for 16E4, 18E4, or HIV-1 Vpr. A GFP expression plasmid, pGreenLantern-1 (Cr), was cotransfected to distinguish the transfected cells. GFP-positive cells were counted at the indicated times after transfection. Transfection efficiency was over 80%. The results shown are derived from an experiment performed in triplicate; error bars indicate standard deviations. (B) At 48 h after transfection, cell extracts were prepared with RIPA buffer. Both detergent-soluble (s) and non-detergent-soluble (i) fractions were analyzed by SDS-PAGE, and E4 and Vpr were detected by immunoblot analysis with an anti-FLAG antibody. The two panels represent short (left) and long (right) exposures of the same film. The positions of molecular weight markers (in thousands) are indicated on the left.

37°C. Cells were stained with 0.1 mg of propidium iodide (PI)/ml-PBS and analyzed by flow cytometry (FACScan; Becton Dickinson and Company, Franklin Lakes, N.J.). To selectively analyze transfected cells, only GFP-positive cells were counted for PI staining.

The detection of apoptotic cells was performed with an Annexin-V-FLUOS staining kit (Roche Diagnostics GmbH).

**Growth suppression assays.** HeLa cells ( $2 \times 10^5$ ) transfected with 2  $\mu$ g of E4 or Vpr expression plasmid, 0.5  $\mu$ g of pGreenLantern-1, and 7.5  $\mu$ g of carrier DNA were seeded at a density of  $0.5 \times 10^5$  cells/6-cm dish at 24 h after transfection. Cell numbers per dish were determined at various times after transfection. Only live cells, determined by trypan blue exclusion, were counted.

#### RESULTS

**18E4 expression induces cellular growth suppression.** The HPV E4 gene product is expressed as an E1<sup>Δ</sup>E4 fusion protein containing the 5 amino-terminal amino acids of E1 at its N terminus (18). To investigate the biological activity of high-risk HPV E1<sup>Δ</sup>E4 proteins, we constructed FLAG-tagged E1<sup>Δ</sup>E4 expression plasmids for HPV16 and HPV18. HeLa cells were transfected with these E1<sup>Δ</sup>E4 expression plasmids, and the growth of the transfected cells was analyzed (Fig. 1A). In each experiment, transfection efficiency was monitored by cotransfection of a GFP expression plasmid, and over 80% of the cells were confirmed to be GFP positive. Cell growth was diminished in 18E4-expressing cells compared to control transfected cells. Similar results were obtained for the expression of an

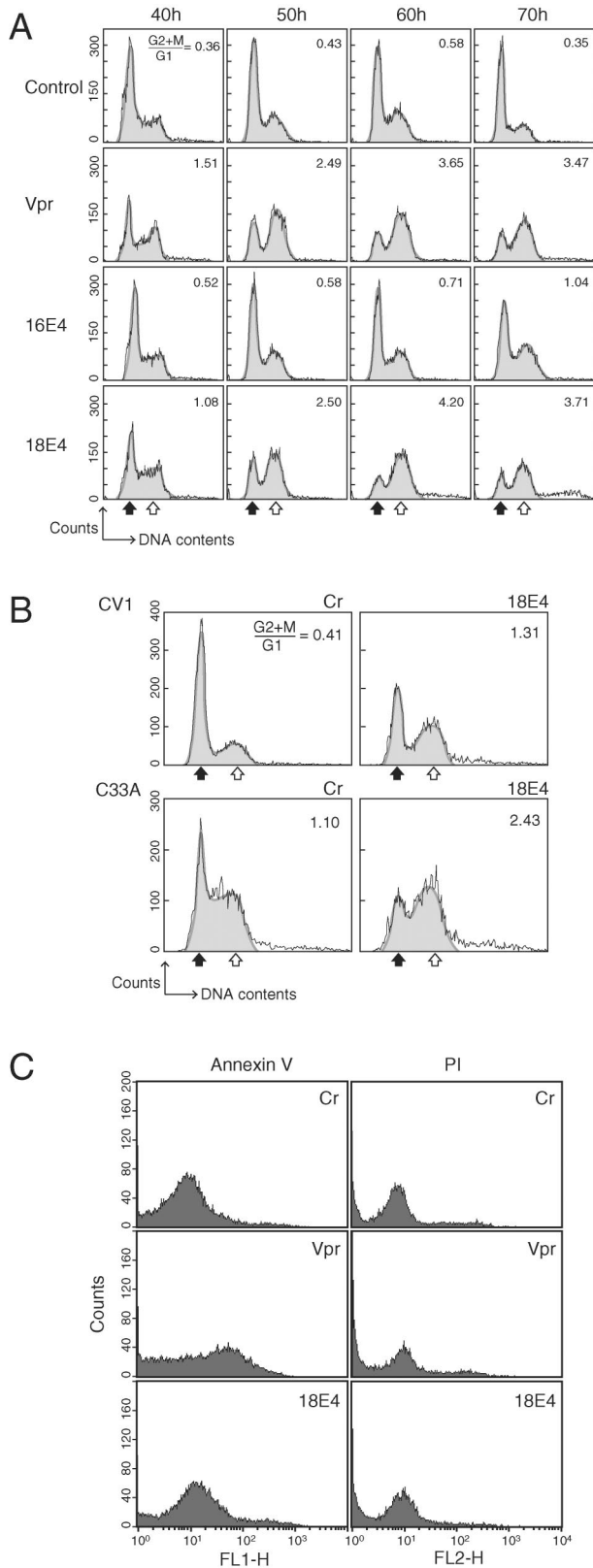


FIG. 2. Cell cycle analysis of E4- and Vpr-expressing cells. (A) HeLa cells were transfected with 2  $\mu$ g of Vpr or the indicated E4 expression plasmid in combination with pGreenLantern-1. Cells were collected at the indicated times after transfection, and the DNA contents were analyzed by flow cytometry (FACSscan). Nuclei were stained

untagged E1<sup>Δ</sup>E4 protein (data not shown). 16E4 also showed weak but significant activity for suppressing cell growth. As a positive control for growth suppression, we expressed a FLAG-tagged HIV-1 Vpr regulatory protein which was previously reported to interfere with cellular proliferation (31). The HIV-1 Vpr protein was able to induce growth suppression to a similar extent as 18E4 (Fig. 1A).

Next, we analyzed the levels of expression of the 16E4, 18E4, and HIV-1 Vpr proteins by immunoblot analysis with FLAG epitope-specific antibodies. 18E4 and HIV-1 Vpr were expressed at similar levels, whereas the steady-state levels of 16E4 appeared much lower (Fig. 1B), a finding which might account for its weak growth-inhibitory activity (Fig. 1A). As expected, the Vpr protein was localized to the nucleus (data not shown) and was recovered in the detergent-soluble fraction (Fig. 1B). In contrast, 18E4 and 16E4 were mostly cytoplasmic (data not shown) and were detected in the non-detergent-soluble fraction (Fig. 1B).

**18E4 expression induces G<sub>2</sub> cell cycle arrest.** We next analyzed whether 16E4 and 18E4 caused growth arrest at a specific phase of the cell cycle. It has been reported that HIV-1 Vpr induces growth arrest at the G<sub>2</sub> phase of the cell cycle (23, 27). HeLa cells transfected with the Vpr expression plasmid exhibited an increased proportion of cells in G<sub>2</sub>/M at 40 h after transfection, consistent with G<sub>2</sub> arrest (Fig. 2A). This effect was even more dramatic at later times. Like Vpr-expressing cells, HeLa cells transfected with 18E4 also exhibited a dramatic increase in the G<sub>2</sub>/M population (Fig. 2A). As expected, HeLa cells transfected with 16E4 showed a less dramatic increase in the G<sub>2</sub>/M population, particularly at early times after transfection, but this increase became more noticeable at later times (Fig. 2A). The difference between 16E4 and 18E4 is most likely a consequence of the lower steady-state levels of 16E4 in the transfected HeLa cells (Fig. 1B). These results suggest that 18E4 is expressed at higher levels and therefore that its biological activities are more noticeable. Hence, we focused mostly on 18E4 for the rest of our studies. The results suggest that, like the expression of HIV-1 Vpr protein, the expression of high-risk HPV E1<sup>Δ</sup>E4 proteins can induce growth arrest in cells at the G<sub>2</sub>/M boundary of the cell division cycle.

The HeLa cell line is a cervical cancer cell line that contains integrated copies of HPV18, and the HPV18 E6 and E7 oncoproteins are expressed in these cells (5, 50). High-risk HPV E6 and E7 proteins are known to subvert G<sub>1</sub>/S as well as G<sub>2</sub>/M checkpoint control, raising the possibility that these viral oncoproteins may somehow mask additional effects of E4 expression (19, 32, 53, 58, 59, 62). To clarify this issue, we analyzed the effects of E4 expression in two other, HPV-negative epithelium-derived cell lines: C33A, an HPV-negative human cer-

with PI. Only the population of transfected GFP-positive cells was counted. Closed and open arrows indicate peaks corresponding to G<sub>1</sub> and G<sub>2</sub>/M phases, respectively. The ratio of G<sub>2</sub>/M to G<sub>1</sub> is shown in each panel. (B) CV1 and C33A cells were transfected with an 18E4 expression plasmid, and the cell cycle profiles were analyzed as described for panel A. Cr, control. (C) Induction of apoptosis by E4 or Vpr expression, as determined by Annexin V staining at 50 h after transfection. The samples were costained with PI for the detection of necrotic cells. Apoptotic cells are positive for Annexin V staining (FL1-H) and negative for PI staining (FL2-H).



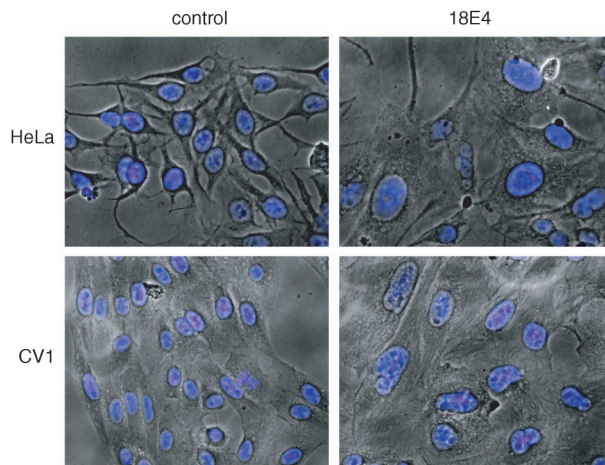


FIG. 3. Morphology of E4-expressing cells. The cells were transfected with 4  $\mu$ g of an 18E4 expression plasmid, fixed with 50% acetone-methanol at 70 h after transfection, and stained with methylene blue. Nuclei were visualized by DAPI staining. Control cells were transfected with the empty expression plasmid.

vical carcinoma cell line, and CV1, a monkey kidney-derived cell line. 18E4 induced G<sub>2</sub> arrest in both cell lines, indicating that the G<sub>2</sub>/M growth arrest effect observed was independent of E6 and E7 functions (Fig. 2B).

In addition to inducing G<sub>2</sub> arrest, Vpr is also known to elicit an apoptotic response (55, 56). Vpr expression increased the Annexin V-positive cell population in HeLa cells. The samples were costained with PI to distinguish apoptotic cells from necrotic cells. Vpr-induced Annexin V-positive cells were negative for PI staining, indicating that Vpr induced apoptotic cell death (Fig. 2C). In contrast to the expression of Vpr, the expression of 18E4 did not increase the incidence of apoptosis in HeLa cells. It has been reported that the mechanisms by which Vpr induces G<sub>2</sub> arrest and apoptosis are linked (55). Since 18E4 induced an increase in the G<sub>2</sub>/M population almost as efficiently as HIV-1 Vpr (Fig. 1) but exhibited no proapoptotic activity, these results suggest that the mechanism of G<sub>2</sub> cell cycle arrest induction by E1<sup>Δ</sup>E4 is different from that of Vpr.

Under normal conditions, cell cycle arrest at the G<sub>2</sub>/M boundary is induced in response to DNA damage and unfavorable environmental conditions. If high-risk HPV E1<sup>Δ</sup>E4 expression induces similar G<sub>2</sub> arrest, then cells will be maintained at the G<sub>2</sub> phase without DNA synthesis or nuclear division. Microscopic observations of 18E4-transfected HeLa and CV1 cells at 70 h after transfection revealed that the cells and the nuclei were frequently enlarged. Especially with HeLa cells, cells containing several gigantic nuclei and micronuclei were observed (Fig. 3). These results suggest that 18E4-expressing cells may continue to synthesize DNA and undergo aberrant nuclear division. This notion is supported by the FACScan results shown in Fig. 2A, where a cell population with a greater than 4N DNA content was observed upon expression of 18E4 in HeLa cells.

**The central region of the 18E4 protein is necessary for G<sub>2</sub>/M cell cycle arrest.** Next, we mapped the domain(s) in the 18E4 protein that is required for the G<sub>2</sub> arrest effect. Thus far, most studies on E4 proteins have been performed with HPV1 and

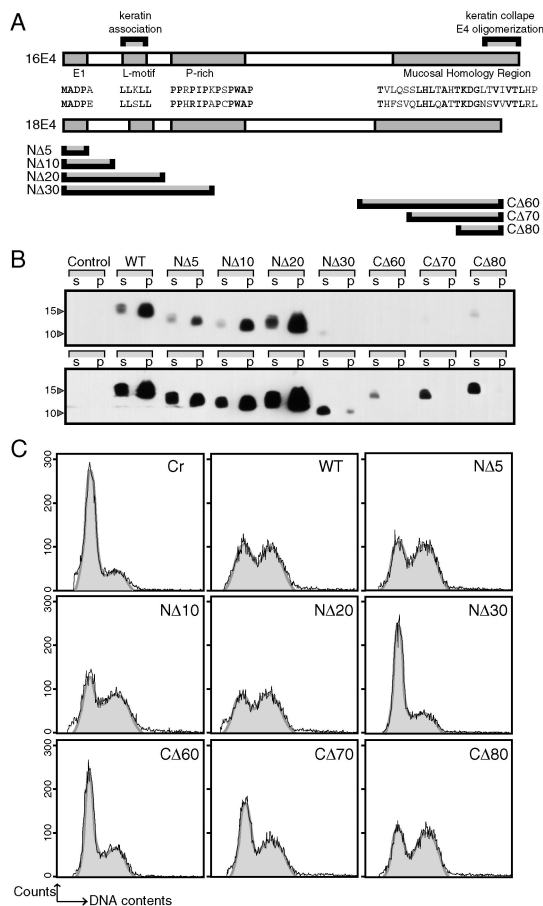


FIG. 4. Identification of the region within the 18E4 protein that is necessary for G<sub>2</sub>/M arrest. (A) Schematic representation of the mutant 18E4 proteins tested. The truncated regions of the FLAG-tagged 18E4 protein are represented by black-gray bars. The motifs and functional regions reported for 16E4 are indicated (45). The corresponding regions in 18E4 are represented by gray boxes, and the amino acid sequence alignment of the conserved regions is presented. (B) Steady-state levels of mutant 18E4 proteins expressed in transfected cells. Both detergent-soluble (s) and non-detergent-soluble (i) fractions were analyzed by SDS-PAGE, followed by immunoblot analysis with an anti-FLAG antibody. The two panels represent short (upper) and long (lower) exposures of the same film. The positions of molecular weight markers (in thousands) are indicated on the left. (C) Cell cycle profiles of HeLa cells expressing the indicated 18E4 mutants. Cells were transfected with 4  $\mu$ g of an E4 expression plasmid and pGreen-Lantern-1. FACScan analysis was performed as described in the legend to Fig. 2A. Cr, control; WT, wild type.

HPV16 (18). Mapping studies with 16E4 have indicated that the N-terminal region is required for an association with cyto-keratin, whereas the C-terminal region is involved in the collapse of the cyto-keratin intermediate filament (IF) network as well as E4 oligomerization (16, 43, 45). The functional motifs of 18E4 were extrapolated by sequence comparison with 16E4 and are shown in Fig. 4A. Given the importance of the N- and C-terminal domains of 16E4, we constructed mutant 18E4 expression plasmids with serial deletions at the N and C termini.

E4 is a relatively small protein, consisting of approximately 100 amino acids, and deletions introduced into such a small protein may severely affect protein stability. Therefore, steady-

state levels of the mutant 18E4 proteins were analyzed prior to functional analyses. HeLa cells were transfected with the mutant FLAG-18E4 plasmids, and steady-state levels of the E4 proteins in both detergent-soluble and non-detergent-soluble fractions were determined by immunoblot analysis with an anti-FLAG MAb (Fig. 4B). Only amino-terminal mutant N $\Delta$ 20 was expressed at levels similar to those of wild-type 18E4 protein. Steady-state levels of amino-terminal mutants N $\Delta$ 5 and N $\Delta$ 10 were reduced but, like the N $\Delta$ 20 mutant, these mutants showed distributions in detergent-soluble and non-detergent-soluble fractions similar to those of wild-type 18E4 protein. Steady-state levels of the N $\Delta$ 30 mutant and all the C-terminal deletion mutants were dramatically reduced, and these proteins were mainly detected in the detergent-soluble fraction, suggesting that the intracellular localization of these mutant proteins is altered compared to that of wild-type 18E4 protein.

To analyze which region(s) of E4 may be essential for the induction of G<sub>2</sub> arrest, we expressed the mutant proteins in HeLa cells and analyzed cell cycle distribution by FACScan analysis at 50 h posttransfection. Except for the expression of the N $\Delta$ 30 and C $\Delta$ 60 mutants, the expression of each of the other mutant 18E4 proteins induced a marked increase in the G<sub>2</sub>/M population similar to that seen with wild-type 18E4 protein (Fig. 4C). The effect of C $\Delta$ 70 was relatively weak, but this result is difficult to interpret due to the low level of expression of this mutant. When we transfected larger amounts of expression plasmids, we observed a more dramatic increase in the G<sub>2</sub>/M population with C $\Delta$ 60 but not with N $\Delta$ 30 (data not shown). However, from these results it is not possible to discriminate whether N $\Delta$ 30 lacks the essential domain to induce G<sub>2</sub> arrest or whether the level of expression of the mutant protein was too low to exhibit its G<sub>2</sub> arrest effect. Nevertheless, these results indicate that the domain of 18E4 essential to induce G<sub>2</sub>/M arrest is minimally contained within amino acids 21 to 59 of 18E4.

**Cytokeratin association is dispensable for E4-mediated G<sub>2</sub> arrest.** In contrast to wild-type 18E4 and the N $\Delta$ 5, N $\Delta$ 10, and N $\Delta$ 20 mutants of 18E4, which were detected in the non-detergent-soluble fraction, the C $\Delta$ 70 and C $\Delta$ 80 mutants were found mostly in the detergent-soluble fraction (Fig. 4C). Since each of these mutants was similarly active in inducing G<sub>2</sub>/M arrest, it appears unlikely that the ability of E4 to form non-detergent-soluble complexes is linked to its growth-inhibitory function. To analyze this notion in more detail, we performed an immunofluorescence analysis of cytokeratin 8/18 and E4 in HeLa cells transfected with a control vector or a vector expressing wild-type 18E4 or the N $\Delta$ 20 or C $\Delta$ 70 mutant (Fig. 5). Cytoplasmic aggregates presumably corresponding to the non-detergent-soluble E4 material were detected in cells expressing wild-type 18E4. Cytokeratin 8/18 staining of normal cells revealed a filamentous network throughout the cytoplasm. In 18E4-expressing cells, the staining pattern for cytokeratin 8/18 was markedly different, suggesting that the IF network might have collapsed, presumably due to 18E4 expression. In support of this notion, cytokeratin 8/18 and 18E4 colocalized in cytoplasmic aggregates, as was previously reported for 16E4 (17). Although the N $\Delta$ 20 mutant formed aggregates similar to those seen with wild-type 18E4, there was less dramatic colocalization with cytokeratin 8/18-positive structures. The C $\Delta$ 70 mu-

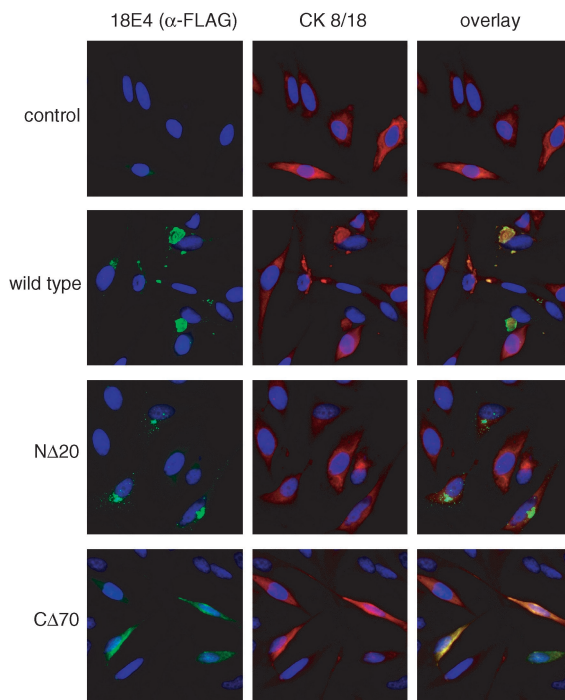


FIG. 5. Immunofluorescence analysis of 18E4 proteins and cytokeratin 8/18 in transfected cells. Cells transfected with wild-type, N $\Delta$ 20, and C $\Delta$ 70 18E4 expression plasmids (5  $\mu$ g) were fixed at 48 h after transfection. E4 proteins (left panels) and cytokeratin (CK) (middle panels) were detected by using anti-FLAG and anticytokeratin 8/18 antibodies, respectively. Nuclei (right panels) were visualized by DAPI staining.

tant displayed a diffuse cytoplasmic and perinuclear staining pattern, similar to that of cytokeratin 8/18 in normal cells. The cytokeratin IF network was maintained intact in C $\Delta$ 70-expressing cells. These results are consistent with studies on 16E4 that mapped the domains for multimerization to the C terminus and the ability to interact with cytokeratins to the N terminus (45). These results indicate that neither the ability of E4 to form oligomeric aggregates nor its association with cytokeratins is required for the ability of E4 to arrest cells in G<sub>2</sub>/M. The failure of the C $\Delta$ 70 mutant to induce the formation of E1<sup>+</sup>E4 aggregates and/or collapse of the cytokeratin network may be attributable to the low levels of expression of this mutant. Regardless, since this mutant still efficiently induced G<sub>2</sub>/M arrest, the formation of E1<sup>+</sup>E4 aggregates and/or collapse of the cytokeratin network are not necessary prerequisites for the E1<sup>+</sup>E4-mediated induction of G<sub>2</sub>/M growth arrest.

## DISCUSSION

The life cycle of papillomaviruses is tightly associated with the differentiation program of epithelial cells via mechanisms that remain to be fully investigated. The ability of HPVs to induce hyperproliferation of epithelial cells is an important aspect of the oncogenic activities of high-risk HPVs. To fully understand the molecular mechanisms that contribute to this activity of HPVs, it is important to investigate the cross talk between HPVs and their epithelial host cells on a molecular

level. The expression of the E4 gene product is coupled to the keratinocyte differentiation program, and it has been proposed that E4 contributes to the viral life cycle by interacting with cytokeratins and inducing collapse of the IF network (17, 43). This process presumably facilitates the egress of progeny HPVs when they are shed within the terminally differentiated keratinocyte squamulae. In this report, we have analyzed the effect of E4 expression on cell growth. These studies revealed that 18E4 can induce G<sub>2</sub>/M growth arrest. Since this effect was observed in HPV18-positive and -negative cells, the E6 and E7 oncoproteins do not interfere with this activity of E4. The growth arrest activity of 18E4 was independent of its ability to induce collapse of the cytokeratin IF network.

Both 16E4 and 18E4 induced G<sub>2</sub>/M arrest in HeLa cells, suggesting that growth inhibition may be an activity shared by other HPV E4 proteins. Compared to that of 18E4, the activity of 16E4 appeared weaker, but this finding may be a consequence of the lower levels of 16E4 expression in HeLa cells. Additional experiments will be necessary to define the molecular targets of 18E4 that mediate this G<sub>2</sub>/M arrest.

The 18E4 protein was as efficient as HIV-1 Vpr in suppressing the growth of HeLa cells. However, in contrast to that of Vpr, 18E4-mediated growth suppression was not accompanied by apoptosis. Since the p53 pathway in HeLa cells is functionally compromised by the expression of HPV18 E6, Vpr-induced apoptosis is likely independent of p53. Although the mechanism is largely unknown, it has been suggested that the abilities of Vpr to induce G<sub>2</sub>/M arrest and apoptosis may be functionally related (55). Since no apoptosis was observed in 18E4-expressing cells, it may be concluded that HIV-1 Vpr-induced G<sub>2</sub>/M arrest and 18E4-induced G<sub>2</sub>/M arrest are mediated by different mechanisms. Alternatively, it is possible that 18E4 contains additional antiapoptotic activities that prevent G<sub>2</sub>/M-arrested cells from being eliminated by apoptosis (46).

18E4-expressing HeLa cells showed marked nuclear enlargement and multinucleation, and FACS analysis revealed the appearance of a hyperploid cell population. This finding suggests that a small percentage of 18E4-expressing HeLa cells, after arresting at the G<sub>2</sub>/M boundary for various amounts of time, will undergo additional rounds of DNA synthesis and nuclear division in the absence of cellular division. It is interesting that high-risk HPV E6 and E7 can also induce aberrant DNA synthesis in G<sub>2</sub>/M-arrested epithelial cells (58). Moreover, functional disruption of p53 or p21<sup>cip1/WAF1</sup> by high-risk HPV E6 also causes DNA rereplication in G<sub>2</sub>/M-arrested cells (7). Hence, it is possible that the observed multinucleation and the appearance of hyperploid cells are a manifestation of compromised G<sub>2</sub>/M checkpoint control caused by HPV18 E6 and/or E7 proteins that are synthesized from the integrated HPV18 genomes in HeLa cells. HPV E4 gene expression in an HPV-associated lesion is first detected in the parabasal layers, and it is expected that the other viral genes, including those for E6 and E7, are coexpressed in these cells. Hence, the cooperative interaction between E4-expressing cells and E6- and E7-expressing cells that likely causes the endoreduplication and multinucleation observed in 18E4-expressing HeLa cells may be physiologically significant.

Our studies showed that the N- and C-terminal domains of 18E4 fulfill functions similar to those in 16E4. The N-terminal domain contributes to the ability of E4 to interact with cyto-

keratins, whereas the C-terminal domain is necessary for E4 to form cytoplasmic aggregates and to cause the collapse of the cytokeratin IF network structure (43, 45). The C-terminal domain of E4 is also the site of interaction for a recently identified putative RNA helicase, E4-DBP (15). Our analysis showed that the C- and N-terminal domains were largely dispensable for the growth-suppressing activity of E4. Our mapping experiments revealed that a sequence of approximately 40 amino acid residues located in the central region of E4 may constitute a novel domain necessary for E4-mediated G<sub>2</sub>/M growth arrest. Additional mutagenesis experiments with this domain will be necessary to map the structural determinants of this novel activity of E4 in greater detail and to perform a targeted search for cellular factors that can interact with this region of E4.

#### ACKNOWLEDGMENTS

We thank Atsue Ueda for technical assistance and manuscript preparation. We are grateful to Karl Münger (Harvard Medical School) for critical review of the manuscript.

This research was supported in part by grants to H.S. from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

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