Activation of NF-κB by Human Papillomavirus 16 E1 Limits E1-Dependent Viral Replication through Degradation of E1

Tomomi Nakahara, Katsuyuki Tanaka, Shin-ichi Ohno,* Nagayasu Egawa,* Takashi Yugawa, Tohru Kiyono
Division of Carcinogenesis and Cancer Prevention, National Cancer Center Research Institute, Tokyo, Japan

ABSTRACT
NF-κB is a family of transcription factors that regulate gene expression involved in many processes, such as the inflammatory response and cancer progression. Little is known about associations of NF-κB with the human papillomavirus (HPV) life cycle. We have developed a tissue culture system to conditionally induce E1-dependent replication of the human papillomavirus 16 (HPV16) genome in human cervical keratinocytes and found that expression of HPV16 E1, a viral helicase, results in reduction of IκBox and subsequent activation of NF-κB in a manner dependent on helicase activity. Exogenous expression of a degradation-resistant mutant of IκBox, which inhibits the activation of NF-κB, enhanced E1-dependent replication of the viral genome. Wortmannin, a broad inhibitor of phosphoinositide 3-kinases (PI3Ks), and, to a lesser extent, VE-822, an ATR kinase inhibitor, but not KU55933, an ATM kinase inhibitor, suppressed the activation of NF-κB and augmented E1-dependent replication of the HPV16 genome. Interestingly, the enhancement of E1-dependent replication of the viral genome was associated with increased stability of E1 in the presence of wortmannin as well as the IκBox mutant. Collectively, we propose that expression of E1 induces NF-κB activation at least in part through the ATR-dependent DNA damage response and that NF-κB in turn limits E1-dependent replication of HPV16 through degradation of E1, so that E1 and NF-κB may constitute a negative feedback loop.

IMPORTANCE
A major risk factor in human papillomavirus (HPV)-associated cancers is persistent infection with high-risk HPVs. To eradicate viruses from infected tissue, it is important to understand molecular mechanisms underlying the establishment and maintenance of persistent infection. In this study, we obtained evidence that human papillomavirus 16 (HPV16) E1, a viral DNA helicase essential for amplification of the viral genomes, induces NF-κB activation and that this limits E1-dependent genome replication of HPV16. These results suggest that NF-κB mediates a negative feedback loop to regulate HPV replication and that this feedback loop could be associated with control of the viral copy numbers. We could thus show for the first time that NF-κB activity is involved in the establishment and maintenance of persistent HPV infection.

Human papillomaviruses (HPVs) are a large family of nonenveloped, small DNA viruses with an approximately 8-kbp double-stranded circular genome that infect stratified squamous epithelium in various anatomical sites such as skin, the anogenital tract, and the oral cavity. Virus infection can cause hyperproliferative lesions, ranging from verrucae to cancer. To date, at least 180 types of HPV have been cloned from clinical lesions and classified as either cutaneous or mucosal types based on their predilection for different sites of infection. A subset of mucosal HPV's, high-risk HPVs (HR-HPVs), such as HPV16, are strongly associated with anogenital cancers, including nearly 100% of cervical cancers and some proportion of head and neck cancers. Altogether, it is estimated that HR-HPV infections are responsible for more than 5% of all cancers worldwide. A major risk factor for HR-HPV-associated carcinogenesis is persistent infection with HR-HPVs. Therefore, it is important to elucidate molecular mechanisms underlying the establishment and maintenance of persistent infection with HR-HPVs in order to allow eradication of the viruses from infected tissue (1).

The life cycle of HPVs is characterized by tight associations with differentiation processes in the stratified epithelium. The production of progeny virions is exclusively restricted to upper differentiated layers of stratified epithelium. The viruses enter nuclei of basal, mitotic cells and establish their genomes as nuclear episomes at 50 to 100 copies per cell following transient initial amplification. In basal cells, viral early genes are expressed at minimal levels and the copy number is kept constant by limiting replication of the viral genome to once in S phase on average, termed maintenance replication. As the infected cells differentiate, levels of the viral early genes are increased and the expression of late viral genes, including major and minor capsid proteins, L1 and L2, respectively, is induced. The viral genomes are drastically amplified to many thousands by continued replication at a high level, termed productive amplification, and eventually encapsidated into progeny virions (2). Two viral early proteins, E1 and E2, are necessary for HPV genome replication along with the cel-
lular replication machinery. A basic mechanism of E1-dependent replication of papillomavirus genomes is well elucidated in which E1 binds to the replication origin of the viral genome in cooperation with E2, forming a hexameric DNA helicase and unwinding the viral DNA by the power of ATP hydrolysis. E1 also recruits host cell replication factors such as DNA polymerase α, topoisomerase I, and replication protein A (RPA). However, it is poorly understood how this E1-dependent replication is regulated during the three phases of viral genome replication—initial amplification, maintenance replication, and productive amplification—in the viral life cycle (3, 4).

Recently, several studies have indicated that DNA damage response (DDR) pathways are involved in HPV genome replication. Key regulators of DDR are phosphoinositide 3-kinase-like kinases (PI3Ks), ataxia telangietasia mutated (ATM), ATM and Rad3 related (ATR), and DNA-dependent protein kinase (DNA-PKcs). Upon sensing DNA damage, these PIKKs initiate signaling inducing the cell cycle checkpoints and DNA repair machinery or apoptosis (5–7). Moody and Laimins reported a series of studies demonstrating that activation of ATM is required for productive amplification but not for stable maintenance of HPV31 in CIN612 cells isolated from a cervical intraepithelial neoplasia grade 1 (CIN) biopsy specimen. HPV31 E7, an oncogene of HR-HPV, was shown to activate ATM and its downstream mediator checkpoint kinase 2 (CHK2) (8–10). Sakakibara et al. and Fradet-Turcotte et al. reported that E1 proteins of various HPVs, including HPV16 and HPV31 and Bos taurus papillomavirus 1 (BPV1), also cause activation of ATM (11, 12). In addition, it is reported that transient replication of HPV18 genomes in U2OS cells induces activation of ATR-mediated DDRs which depend on the expression of E1 and E2 (13).

Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) comprises a family of transcription factors that activate or repress expression of a large number of genes in a myriad of physiological and pathological processes contributing to immune and inflammatory responses, cellular stress responses, differentiation, cell proliferation, and apoptosis. Currently, five members of the family are known in mammals, RELA/p65, p50/p105, p52/p100, RELB, and c-REL, which form homo- or heterodimers. The activity of NF-κB is negatively regulated primarily through its cytoplasmic retention by binding to inhibitors of NF-κB (IκBs), including IκBα. Upon stimulation, phosphorylation-dependent degradation of IκBs by a ubiquitin proteasome pathway liberates NF-κBs into the nucleus (14, 15). Such activation of NF-κB is a fundamental immediate early step of immune activation, inducing expression of interferons (IFNs) as well as inflammatory cytokines. Many viruses have been shown to target NF-κB pathways to evade innate immune responses. On the other hand, accumulated studies have revealed that activation of NF-κB signaling is induced and incorporated in the viral life cycle and is important for pathogenicity of oncogenic viruses such as Epstein-Barr virus (EBV), Kaposi’s sarcoma-associated herpesvirus (KSHV), and human adult T cell leukemia virus 1 (HTLV-1) (16).

Increased expression of NF-κB has been described for HPV-associated lesions such as cervical cancers as well as their precursor lesions and laryngeal papillomas and cancers (17–22). Several studies have shown that E6 and E7 proteins of HPVs modulate NF-κB pathways (23–30). However, the results of these studies have been contradictory as to whether E6 and/or E7 activate or suppresses. NF-κB p65 (RELA) has been shown to bind to the long control region (LCR) of HPV16 and suppress the activity of its promoter, p97 (31). Recently, it was also reported that E2 proteins of various HPVs enhance tumor necrosis factor alpha (TNF-α)-induced activation of NF-κB in HaCaT cells (32). NF-κB plays an important role in maintaining homeostasis of epithelium and cellular differentiation of keratinocytes (33, 34). Altogether, the available data suggest involvement of NF-κB in the pathogenesis and the life cycle of HPVs. In the present study, we investigated the relationship between NF-κB activity and replication of HPV16 genomes. As the outcomes and pathways of NF-κB signaling are known to vary depending on the cell type, we developed a tissue culture system to efficiently induce E1-dependent replication of the HPV16 genome in human cervical keratinocytes (HCKs), a natural host of HPV replication. We found that NF-κB was activated upon E1 expression, which in turn limits E1-dependent replication of HPV16 genome in mitotic, undifferentiated HCKs. The activation of NF-κB was induced through DDR upon E1 expression. Implications of the NF-κB-mediated suppression of E1-dependent replication for the three phases of viral genome replication in the viral life cycle will be discussed.

MATERIALS AND METHODS

Cells and tissue culture. Human cervical keratinocytes immortalized with telomerase reverse transcriptase (TERT) (HCK1T) were described previously (35). HCK1T-HPV16 cells were generated by cotransfecting 1.25 μg of pAd/HPV16/neO with 1.25 μg of pxCANCre to HCK1T using Lipofectamine 2000 (Life Technologies, Grand Island, NY) according to the manufacturer’s protocol. Cells were selected with 150 μg/mL of G418 for 2 days starting from 2 days posttransfection and then maintained until G418-resistant colonies became visible. These colonies were isolated, and the presence of HPV16 genomes as episomes was confirmed by Southern blotting. Oligonucleotide HCK1T that contained HPV16 genomes as episomes was named HCK1T-HPV16 and used in this study. HCK1T tetON E1, tetON E1 + E2, and tetON E2 were generated by retrovirus gene transfer and subsequent drug selection as indicated below. HCK1T-HPV16 tetON E1 + E2, tetON E1K483A + E2, tetON E1, and tetON E2 were generated by lentivirus infection. HCK1T-HPV16 tetON E1 + E2 clones A5 and B2 introduced with expression of IκBα wild type, the IκBα mutant, and the multiconing site (MCS) control were generated by retrovirus infection and subsequent drug selection. HCK1T-HPV16 and its derivatives were maintained in keratinocyte-serum-free medium (K-SFM) at 37°C in a 5% CO2 incubator. HCK1T and its derivatives not containing HPV16 genome were maintained in EpiLife medium (Life Technologies, Grand Island, NY). CIN612-9E cells were maintained on feeder cells in E medium as described previously (36). Inhibitors and doxycycline were dissolved in dimethylsulfoxide (DMSO) and stored as high-concentration stocks at −20°C until used at the indicated concentrations. The concentrations of the stock solutions were as follows: doxycycline (catalog no. Z1311N; TaKaRa Bio Inc., Shiga, Japan) at 1 mg/mL and KU55933 (catalog no. 118500; Merck Millipore, Billerica, MA, USA), wortmannin (catalog no. 230-02341; Wako Pure Chemical Industries, Ltd., Osaka, Japan), SB203580 (catalog no. 20-2173; Funakoshi Co., Ltd., Tokyo, Japan), VE-822 (catalog no. S7102; Selleck Chemicals, Houston, TX, USA), and NU7062 (catalog no. 2828; Tocris Bioscience, Minneapolis, MN, USA) at 10 mM. Human TNF-α (catalog no. 203-15263; Wako Pure Chemical Industries, Ltd.) was dissolved and stored in phosphate-buffered saline (PBS) at 10 μg/mL.

Plasmids. pAd/HPV16/neO containing the full length of the HPV16 genome flanked with two loxP sites was constructed by replacing an XbaI-NotI fragment encoding enhanced green fluorescent protein (EGFP) of the original vector, pAd/HPV16/Cre (37), with an XbaI-NotI fragment containing a neomycin resistance gene of pMSCV-neo (TaKaRa Bio Inc., Shiga, Japan). pxCANCre was obtained from the Riken DNA bank (RDB10748; Riken Bioresource center, Tsukuba, Japan). Lentivirus vec-
tors, CSII-CMV-tetON-ADV and CSII-TRE-Tight-HA16E1 were described previously (38). The codon-optimized HPV16 E2 cDNA (GenScript, Piscataway, NJ) was used to construct CSII-TRE-Tight-HA16E2, in which the N-terminal hemagglutinin (HA)-tagged HPV16 E2 was inserted under a tetracycline-responsive promoter. CSII-TRE-Tight-HA16E1K483A was generated by site-directed mutagenesis (QuickChange site-directed mutagenesis kit; Agilent Technologies, Santa Clara, CA). CSII-TRE-Tight-HA16E1-PB and CSII-TRE-Tight-3xFLAG16E2-PB were generated by inserting an expression cassette of the bacterial (PB) resistance gene under the mouse phosphoglycerate kinase 1 (PGK) promoter downstream of HA16E1 or 3xFLAG16E2. pQCXIZeo-tetON was generated by inserting rtTA (tetON) from pRevTet-ON (catalog no. 631007; TaKaRa Bio Inc., Shiga, Japan) under the cytomegalovirus (CMV) promoter of pQCXIZeo, in which a puromycin resistance gene was inserted previously (38). The codon-optimized HPV16 E2 cDNA (Ser32/36Ala) derived from pCMV-IkB (Life Technologies, Grand Island, NY) was inserted into pQCXIZeo using Gateway recombination kit (catalog no. A2361; Promega, Madison, WI, USA). For Southern blotting, 10 μg of total genomic DNA was digested with either BamHI or XhoI and digested genome from pUC-HPV16 plasmid DNA by BamHI digestion were used as standards to measure the amount of HPV16 DNA. All real-time PCRs were run in triplicate, and at least three independent experiments were performed. The copy number of HPV16 per cell was calculated based on the assumption that total human genomic DNA is 6.6 pg/diploid cell. The experiments were repeated at least three times. P values were determined by Student’s t test. Two sets of primer pairs were used for qPCR to confirm that the viral copy numbers estimated by each pair were consistent. The pairs of primers used were as follows: E6 pairs (HPV16-97F, 5′-GAACATGGATGTTGACAGCACC-3′, and HPV16- 174R, 5′-TGTTAGGTTGCTGACGTTG-3′) and L2 pairs (HPV16-4481F, 5′-ACAGATACACTGTCTGAGAACC-3′, and HPV16-4656R, 5′-GGAAGTTTGGATCTGAGATGA-3′).

RNA extraction and RT-qPCR. Total RNA was isolated using an RNeasy Plus mini kit (catalog no. 74136; Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions. Micrograms of total RNA was subjected to a 10-μl reverse transcription (RT) reaction mixture using a PrimeScript RT reagent kit (catalog no. RR037A; TaKaRa Bio Inc., Shiga, Japan), and 1 μl of the RT products was used for real-time PCR to measure mRNAs of the interest. In the case of β-actin mRNA, 10-times-diluted RT products were used due to the abundance of the mRNA. The PCR mixtures were prepared using Kapa SYBR Fast qPCR kits, and the PCR run was achieved with StepOnePlus. The relative mRNA levels of the interest were calculated by a threshold cycle (ΔΔCt) method using β-actin mRNA as an internal control. All PCRs were run in triplicate. All experiments were repeated at least three times. P values were determined by Student’s t test. The following primers were used: β-actin, forward, 5′-ACCAACCTGGAAGACATGGAACAA-3′, and reverse, 5′-TACGAC AGCCCTGATACGACATCTA-3′; TNFα, forward, 5′-CAAGTGAACCC GGTAGAC-3′, and reverse, 5′-GGTGTTGGAGGAGCAGCAT-3′; interleukin-6 (IL-6), forward, 5′-AAATTCGTGATCATCTCTCCAGCC-3′, and reverse, 5′-AGTGCTCCCTGGGTCCTCTAC-3′; E2, forward, 5′-AGCCTCTTGTCTGACCTCC-3′, and reverse, 5′-AATTCTGTGTGCGCACTGTG-3′; E4, forward, 5′-GTCGATCT CGTCAAGCAGAAGTAC-3′, and reverse, 5′-GATGGAACGAGCTCA CTAGG-3′. These primers were designed based on the published data (40).

Western blotting. Cells were collected as described above, lysed in lysis buffer (50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 1% NP-40, 20% glycerol, 0.1% SDS, 1% deoxycholate) containing a protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and phosphatase inhibitors (500 μM sodium orthovanadate, 100 mM sodium fluoride, 10 mM sodium pyrophosphate), and then subjected to brief sonication in an ice-cold water bath. A protein concentration of cell lysates was measured by using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) after centrifugation at 20,000 × g for 15 min. When necessary, an insoluble fraction was lyzed in an SDS sample buffer (50 mM Tris-HCl, 2% SDS, and 10% glycerol). The equivalent protein amounts of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot analysis was conducted as described previously (38). The primary antibodies used in this study were as follows: mouse monoclonal antibodies (Mabs) HA (16B12; Covance, Princeton, NJ), FLAG (M2; Sigma-Aldrich, St. Louis, MO, USA), vinculin (hVIN-1; Sigma-Aldrich), pATM(S1981) Phototope kit (New England BioLabs, Ipswich, MA, USA). The linearized plasmid containing the full length of HPV16 was used as a template. Hybridization and chemiluminescent detection were performed as described previously. The LAS3000 charge-coupled device (CCD) imaging system (Fujifilm Co., Ltd., Japan) was employed for detection. Quantification of images was conducted using software programs NIH Image J and Multi Gauge (Fujimco Co., Ltd., Japan). Real-time PCR to quantify the viral genome copy number was performed as described previously. In brief, 10 to 100 ng of total genomic DNA was mixed with a master mix of Kapa SYBR Fast qPCR kits (Kapa Biosystems, Woburn, MA) and 300 nM (each primer and then subjected to a real-time PCR using StepOnePlus (Life Technologies, Grand Island, NY). Serial dilutions of linearized HPV16 genome from pUC-HPV16 plasmid DNA by BamHI digestion were used as standards to measure the amount of HPV16 DNA. All real-time PCRs were run in triplicate, and at least three independent experiments were performed.
His-Ub, an 0.8-μg amount of CSII-CMV-HA16E1 vaccine (Wetzlar, Germany). The cells were examined using a Leica FW4000 microscope (Leica Microsystems, Wetzlar, Germany).

Indirect immunofluorescence analysis. The cells were first rinsed with PBS twice and fixed in 4% paraformaldehyde (PEA) for 15 min at room temperature. The fixed cells were incubated with 0.01% Triton-X in PBS for 10 min after being rinsed twice with PBS and then incubated with the blocking buffer (2% bovine serum albumin [BSA] in PBS) for 30 min at room temperature. Incubation with the primary antibody was done for 30 min at room temperature, followed by further incubation. At 18 h posttransfection, the cells were subjected to Western blotting (Fig. 1F). Expression levels of HA16E1 and -E2 were also analyzed by Western blotting (Fig. 1F). Episomal copies of HPV16 genomes increased by approximately 100-fold in HCKIT-HPV16 cells designed to express both E1 and E2 (tetON E1 + E2) upon incubation with DOX (Fig. 1A, lanes 4, 5, 8, and 9, and B), whereas no increase was detectable in those designed to express E1K483A and E2 (tetON E1K483A + E2) with the DOX treatment (Fig. 1A, lanes 6, 7, 10, and 11, and B) even though the level of E1K483A appeared higher than that of the wild-type E1 (Fig. 1F, lanes 2 and 4). The copy number of HPV16 genomes increased slightly (by approximately 3-fold) in cells designed to express E1 alone (tetON E1) upon DOX treatment, while no increase was detected in those designed to express E2 alone (tetON E2). The basal copy numbers of HPV16 genomes were comparable among those cells in the absence of DOX. These results clearly indicated that exogenous E2 expression enhanced E1-dependent replication of the HPV16 genome, although we failed to detect HA-tagged E2 by Western blotting. The slight increase in the copy number of HPV16 genomes in the tetON E1 cells could depend on E2 derived from episomal HPV16 genomes. The expression level of E1 from episomal genomes appeared to be too low to support detectable replication even when excess amounts of E2 were provided exogenously. In the tetON E1 + E2 cells, the copy number of the viral genomes increased in a time-dependent manner (Fig. 1C); the increase was detected as early as at 4 h and continued up to 24 h after DOX addition, whereas no further increase was detected thereafter. The maximal level of E1 as well as the copy number of HPV16 genomes was observed at the highest concentration of DOX tested (1 μg/ml) (data not shown). Therefore, E1-dependent replication of the HPV16 genome was evaluated at 24 h after addition of 1 μg per ml of DOX in subsequent analyses unless otherwise indicated. As the estimated copy number of the viral genomes by qPCR was well correlated with that obtained by

RESULTS

Conditional expression of E1 and E2 induces amplification of the HPV16 genome as episomes in HCKs. In order to study E1-dependent replication of the HPV16 genome in human cervical keratinocytes, we first established immortal human cervical keratinocytes (HCKs) stably maintaining HPV16 genomes as episomes as described in Materials and Methods. We confirmed that HCKIT-HPV16 cells were able to maintain HPV16 genomes as episomes at a constant copy number (approximately 30 copies/cell) for at least 3 months and several freeze and thaw cycles (Fig. 1A, lanes 4, 6, 8, and 10). They were coinfected with lentivirus vectors expressing the reverse tetracycline-regulated transactivator (rtTA; tetON) together with those expressing HA-tagged HPV16 E1 (HA16E1), a helicase-defective mutant of E1 (HA16E1K484A), or HA-tagged HPV16 E2 under the control of a tetracycline-responsive promoter or combinations of HA16E2 and HA16E1 or HA16E1K484A. The same numbers of those cells were seeded the day before and then incubated with either vehicle or 1 μg per ml of doxycycline (DOX) for 24 h. The total genomic DNA was isolated, and the copy number of HPV16 genomes was measured by Southern blotting and quantitative real-time PCR (qPCR) (Fig. 1A and B). Expression levels of HA16E1 and -E2 were also analyzed by Western blotting (Fig. 1F). Episomal copies of HPV16 genomes increased by approximately 100-fold in HCKIT-HPV16 cells designed to express both E1 and E2 (tetON E1 + E2) upon incubation with DOX (Fig. 1A, lanes 4, 5, 8, 9, and B), whereas no increase was detectable in those designed to express E1K483A and E2 (tetON E1K483A + E2) with the DOX treatment (Fig. 1A, lanes 6, 7, 10, and 11, and B) even though the level of E1K483A appeared higher than that of the wild-type E1 (Fig. 1F, lanes 2 and 4). The copy number of HPV16 genomes increased slightly (by approximately 3-fold) in cells designed to express E1 alone (tetON E1) upon DOX treatment, while no increase was detected in those designed to express E2 alone (tetON E2). The basal copy numbers of HPV16 genomes were comparable among those cells in the absence of DOX. These results clearly indicated that exogenous E2 expression enhanced E1-dependent replication of the HPV16 genome, although we failed to detect HA-tagged E2 by Western blotting. The slight increase in the copy number of HPV16 genomes in the tetON E1 cells could depend on E2 derived from episomal HPV16 genomes. The expression level of E1 from episomal genomes appeared to be too low to support detectable replication even when excess amounts of E2 were provided exogenously. In the tetON E1 + E2 cells, the copy number of the viral genomes increased in a time-dependent manner (Fig. 1C); the increase was detected as early as at 4 h and continued up to 24 h after DOX addition, whereas no further increase was detected thereafter. The maximal level of E1 as well as the copy number of HPV16 genomes was observed at the highest concentration of DOX tested (1 μg/ml) (data not shown). Therefore, E1-dependent replication of the HPV16 genome was evaluated at 24 h after addition of 1 μg per ml of DOX in subsequent analyses unless otherwise indicated. As the estimated copy number of the viral genomes by qPCR was well correlated with that obtained by
FIG 1 Conditional induction of E1-dependent replication in immortalized human cervical keratinocytes containing HPV16 genome (HCK1T-HPV16). (A) Representative image of Southern blotting for HPV16 genomes in 10 µg of total genomic DNA isolated from cells with the indicated gene expression at 24 h after DOX (1-µg/ml) addition. The serially diluted, linearized full lengths of HPV16 genomes were included as copy number standards (lanes 1 to 3). The DNAs with BamHI digestion, which linearizes the HPV16 genome, and XhoI digestion, which does not cut the HPV16 genome, are shown in lanes 4 to 7 and lanes 8 to 11, respectively. Arrowheads indicate HPV16 DNAs which correspond to Form I, a supercoiled DNA, and Form II, a relaxed circular DNA. (B and C) qPCR results for the copy number of HPV16 genomes with the indicated gene expression at 24 h (B) and at the indicated time points following DOX incubation (C). (D) Indirect immunofluorescence analysis (IFA) for HA-E1 and E2. As a negative control, HCK1T-HPV16 with tetON E1 incubated with a vehicle control is shown. (E) The number of cells with the indicated gene expression that stayed attached to tissue culture plates is shown. (F) Western blot analysis for the levels of E1 and the markers of apoptosis, PARP-1 and caspase 3, in HCK1T-HPV16 cells with the indicated gene expression following incubation with DOX (lanes 2, 4, 6, and 8) or vehicle as a control (lanes 1, 3, 5, and 7). Arrowheads indicate cleaved forms of PARP-1 and caspase 3. α-Tubulin was detected as a loading control. The averages of results from at least three independent experiments are shown. P values were evaluated by Student’s t test.
Southern blotting, the copy number of HPV16 genomes was measured by qPCR in the subsequent experiments.

The intracellular localization of HA-tagged E1 and E2 was examined by immunofluorescence analysis (IFA) using an anti-HA tag antibody at 24 h after addition of vehicle or DOX (Fig. 1D). In the presence of DOX, HA-positive signals were observed in roughly 40% of cells, indicating heterogeneity in expression levels of E1 and E2 in the population. In a fraction of the tetON E1+E2 cells, positive signals with anti-HA antibody were found accumulated at nuclear foci (Fig. 1D, E1+E2 DOX+). In contrast, no obvious accumulation of HA-positive signals at nuclear foci was observed in the tetON E1K483A+E2 cells. Instead, strong HA-positive signals were diffusely distributed throughout nucleus and weaker signals were also detected in the cytoplasm. In the tetON E1 cells, a diffuse pattern of HA-positive signals was detected in nuclei and weaker signals were also detected in cytoplasm in a majority of the positive cells. On the other hand, homogeneous HA-positive signals were found mostly confined to nucleus and the majority of the positive cells. On the other hand, homogeneous HA-positive signals were found mostly confined to nucleus and no obvious accumulation at foci was detected in the tetON E2 cells and the tetON E2 DOX+ cells. To investigate whether the expression suppresses cell proliferation (11, 12). Consistent with their results, we noted that the tetON E1+E2 cells and the tetON E1 cells but not the tetON E1K483A+E2 cells and the tetON E2 cells appeared to be not proliferating very well, and some of those cells were detached from the bottom of tissue culture plates by 24 h when incubated with DOX. We counted the cells which stayed attached to the culture plates and found that the number of the tetON E1+E2 cells and the tetON E1 cells grown in the presence of DOX for 24 h was significantly less than the number of those grown in the presence of a vehicle control (Fig. 1D; data not shown for the tetON E1 cells). On the other hand, no such difference in the cell numbers was observed in the tetON E1K483A+E2 or the tetON E2 cells (Fig. 1E; data not shown for E2 alone). To investigate whether the apparent inhibition of cell proliferation was caused by induction of apoptosis, markers of apoptosis such as caspase 3 and poly(ADP-ribose) polymerase 1 (PARP-1) were analyzed by Western blotting. The cells detached from a tissue culture plate as well as the cells remaining attached were collected, and equivalent protein amounts of cell lysates were subjected to SDS-PAGE and subsequent Western blot analysis (Fig. 1F). The cleaved forms of PARP-1 and caspase 3 were detected in the cells expressing E1 and E2 or E1 alone but not in the cells expressing E1K483A and E2 or E2 alone, suggesting that E1 expression induces apoptosis depending on its helicase activity. HPV16 E1 induces activation of NF-κB in human cervical keratinocytes containing episomal HPV16 genomes. To explore associations of NF-κB with viral genome replication, we first studied the status of NF-κB p65 (RELA) and IkBα in HCK1T-HPV16 cells after induction of E1, E1K483A, and/or E2 expression. We found that the steady-state level of IkBα was reduced upon induction of E1 and E2 expression, while RELA was localized to the cytoplasm in a majority of the cells incubated with a vehicle control (Fig. 2B). These results indicated that NF-κB is activated upon induction of E1 and E2 expression. Then, transcriptional activation of several NF-κB target genes was examined (Fig. 2C). Total RNA was isolated following incubation of DOX or vehicle and subjected to reverse transcription (RT)-qPCR. All the NF-κB target genes examined were upregulated upon induction of E1 and E2 expression. The mRNA levels of TNF-α, interleukin-6 (IL-6), and IL-8 were increased by 2.5±0.11, 4.9±1.17, and 14.2±3.46-fold, respectively, whereas no significant increase of these mRNA levels was detected upon induction of E1K483A and E2 expression. Taken together, these results indicate that the expression of E1 and E2 induces activation of NF-κB most likely through promoting degradation of IkBα. Viral early transcripts containing spliced E1′E4 open reading frame (ORF) were slightly reduced in the tetON E1+E2 cells (0.88±0.12) even though the viral copy number increased (Fig. 1A). It is possible that exogenous E2 expression suppressed the viral early promoter p97. However, in the E1K483A+E2 cells, similar reduction of the viral early transcripts was observed (0.73±0.12) when neither NF-κB activation nor viral genome replication was induced. Therefore, no increase of viral early transcripts despite the increased viral genome copies was likely associated with suppression of p97 by NF-κB activation in the tetON E1+E2 cells (31). HPV16 E1 induces activation of NF-κB in human cervical keratinocytes in the absence of an episomal HPV genome. We next examined whether E1 can reduce the IkBα level in the absence of an episomal HPV16 genome. Parental HCK1T cells were used to establish new cell lines expressing HA-tagged E1 and/or 3×FLAG-tagged E2 of HPV16 upon DOX administration. The steady-state level of IkBα was reduced in the parental HCK1T cells upon expression of E1 and E2 as well as E1 alone but not E2 alone (Fig. 3A, lanes 4 and 6 compared to lanes 3 and 5, respectively), indicating that replication of the viral genomes is not necessary for NF-κB activation, E1 expression per se rather causing the activation. Reduction of the IkBα level appeared more prominent in the cells expressing only E1 than in cells expressing E1 and E2, most likely due to higher expression of E1 (Fig. 3A, lanes 4 and 6). By switching the tag from HA to 3×FLAG, we were able to detect E2 by Western blotting. Consistent with reduction of the IkBα level, NF-κB target genes were upregulated upon expression of E1 and E2 as well as E1 alone in the absence of an HPV16 genome (Fig. 3B). We noted that endogenous mRNA levels of NF-κB target genes were also higher in CIN612-9E cells harboring episomal HPV31 genomes, compared to HCK1T, suggesting constitutive activation of NF-κB in the presence of episomal HPV genomes, and this was...
not specific to HCK1T-HPV16 (Fig. 3C). Similarly to HCK1T-HPV16, cell proliferation of HCK1T was also inhibited and cleaved forms of caspase 3 and PARP-1 were detected upon expression of E1 alone or E1 and E2, but not E2 alone (data not shown). These results are consistent with previous findings that E1-mediated growth suppression does not require E2 or viral genome replication (12,13).

Inhibition of NF-κB activation enhances E1-dependent replication of HPV16 genomes. As induction of NF-κB activation by E1 has never been described, we were interested in whether this activation of NF-κB affects E1-dependent replication of the viral genome. To this end, we examined effects of IkBα overexpression, which prevents activation of NF-κB, on E1-dependent replication. As shown in Fig. 1, the tetON E1+E2 cells proved to be a heterogeneous cell population in which roughly 40% of the cells express HA-tagged proteins upon DOX treatment. We therefore tried to obtain a clonal cell population expressing E1 and E2 more homogenously upon induction. A total of 50 clones were isolated by limiting dilution and examined for the copy number of HPV16 genomes and the levels of E1 and E2 expression in the presence or absence of DOX. There were variations in the basal copy number of HPV16 genomes and the induction levels of E1 among clones; some clones contained only a few copies of HPV16 genomes while others contained 1,000 copies (data not shown). In most clones, the induction levels of E1 were correlated with the increase in HPV16 copy numbers and levels of E1 and IκBα were inversely correlated (Fig. 4A), as expected from the results of the pooled population (Fig. 2A). In clones A5 and B2, we were able to detect a band at a size corresponding to HA16E2 (Fig. 4A, HA long exposure), which could not be detected in the parental pooled population upon DOX treatment (Fig. 1F). We chose clones A5 and B2 for further studies as these showed the sharpest increase of the viral genomes after DOX addition. The basal copy numbers of HPV16 genomes in clones A5 and B2 were the averages of the clones. Wild-type IκBα (IκBα WT); an IκBα S32A/S36A mutant (IκBα MT), which is resistant to phosphorylation and subsequent degradation; or an empty vector (MCS) as a control were transduced to clones A5 and B2 by retroviral gene transfer. Accumulation of exogenous IκBαWT as well as IκBαMT regardless of E1 and E2 expression was confirmed, while reduction of endogenous IκBα level upon induction of E1 and E2 expression was evident in the control cells (Fig. 4B, shown for clone B2). TNF-α treatment, which activates NF-κB via degradation of IκBα to parental HCK1T-HPV16, was also included as a positive control (Fig. 4B, lanes 1 and 2). The NF-κB target genes, TNF-α, IL-6, and IL-8, were extremely upregulated following incubation with DOX in
**FIG 3** Activation of NF-κB upon induction of E1 and/or E2 expression in HCK1T in the absence of an episomal HPV16 genome. (A) The level of IκBα and expression of E1 and E2 were analyzed in parental HCK1T cells that do not contain an HPV16 genome by Western blotting. HCK1T-tetON cells were included as a negative control. Cell lysates were prepared at 24 h following incubation with DOX (lanes 2, 4, 6, and 8) or a vehicle (lanes 1, 3, 5, and 7). Vinculin was detected as a loading control. (B) qRT-PCR analysis for mRNA levels of the NF-κB target genes in HCK1T-tetON E1+E2 or E1 alone. The mRNAs of the indicated genes were normalized to β-actin mRNA. Fold increases of the indicated mRNAs in DOX-treated samples over those in vehicle-treated samples are shown. The averages of at least three independent experiments are shown. (C) RT-PCR analysis for mRNA levels of the NF-κB target genes in HCK1T, HCK1T-HPV16, and CIN612-9E cells. As a negative control, an RNA isolated from HCK1T not subjected to RT reaction was used as a template for PCRs (lane 1).

**Possible Negative Feedback Loop between E1 and NF-κB**

Activation of NF-κB seen in the presence of HPV16 genomes (Fig. 3C). We then analyzed replication of the HPV16 genome and cell proliferation upon E1 and E2 expression in the presence of IκBα overexpression (Fig. 4D and E). Interestingly, the copy number of HPV16 genomes following DOX incubation in the cells expressing IκBαWT as well as IκBαMT was significantly higher than that in the control cells, suggesting that the activation of NF-κB suppresses E1-dependent replication of the HPV16 genome (Fig. 4D). The basal copy number of HPV16 genomes in the cells expressing IκBαWT as well as IκBαMT was slightly higher than that in the control cells. Although the difference was not statistically significant, it is possible that the basal activity of NF-κB also influences the control of HPV16 copy numbers in the absence of exogenous E1 as discussed below. We noted that the protein level of HA-E1 in the cells expressing IκBαMT appeared to be higher than that in MCS control cells. The elevated level of HA-E1 might have contributed to augmented replication of the HPV16 genome in cells expressing IκBαMT. In contrast, the inhibitory action of E1 on cell proliferation was not affected by overexpression of IκBαWT or IκBαMT (Fig. 4E), suggesting that activation of NF-κB is not involved in E1-induced growth suppression or apoptosis. The levels of viral transcripts containing spliced E1'-E4 ORF were approximately 1.5- and 1.7-fold higher in the cells expressing IκBαWT and IκBαMT, respectively, than in the control cells in the absence of DOX, implying that the basal activity of NF-κB might suppress p97 to some extent. The viral transcripts were increased by 2-fold in the control cells and by 5.6-fold in the cells with IκBαWT or IκBαMT upon DOX treatment. We still considered these results to show that activation of NF-κB suppresses p97 and that overexpression of IκBαWT or IκBαMT substantially alleviates the NF-κB-mediated suppression (Fig. 3D). Overall, these data suggest that activation of NF-κB limits E1-dependent replication of the HPV16 genome while it is not involved in suppressive effects of E1 on cell proliferation. We repeated the same experiments using clone A5 and obtained essentially the same results as with clone B2 (data not shown).

**E1 induces NF-κB activation by eliciting DDR pathways**

NF-κB can be activated by a variety of upstream signals, including DNA damage response (DDR). Upon generation of double-stranded breaks (DSBs), ATM has been shown to activate NF-κB through phosphorylation-dependent degradation of IκBα (43). Because the wild type but not the helicase-defective mutant of HPV16 E1 has been shown to activate ATM, we initially hypothesized that E1 induces NF-κB activation by eliciting DDR, most likely via activation of ATM. To test this hypothesis, activation of DDR was analyzed in heterogeneous cell populations of tetON E1+E2, tetON E1K483A+E2, tetON E1, or tetON E2 or several clones of the tetON E1+E2 cells by Western blotting. Checkpoint kinases 1 and 2 (CHK1 and CHK2, respectively) are known phosphorylation targets of ATR and ATM that mediate signal transduction downstream of ATR and ATM, respectively. Antibodies specific to phosphorylated ATM, pATM(S1981), and CHK2, pCHK2(T68), or CHK1, pCHK1(S345), were used as markers for ATM or ATR activation, respectively. NBS1 was also analyzed as it has been shown to be phosphorylated by ATM (5, 44). Upon DOX incubation, phosphorylation of ATM and CHK1 was increased in the cell populations of tetON E1+E2 and tetON E1, although basal levels of the phosphorylated CHK1 appeared also higher in these cells (Fig. 5A, left panel). A slower-migrating pattern of NBS1 and CHK2 upon DOX incubation was detected, indicating
FIG 4 Effects of overexpression of IκBα on E1-dependent replication of HPV16 and cell proliferation of subclones of HCK1T-HPV16 cells with tetON E1+E2. (A) Shown are results of Western blot analysis (left) for E1 and E2 levels and the steady-state levels of IκBα in representative clones and the results of qPCR for HPV16 genomes (right) following incubation with DOX or vehicle for 24 h. Images with short and long exposures with the anti-HA antibody were included to indicate expression of HA-E2. α-Tubulin is shown as a loading control for the top three panels, and vinculin is shown for the HA panel comparing clones to the parental population. (B) Western blot analysis to verify overexpression of IκBα in clone B2 transduced with retroviruses carrying an empty vector designated MCS (lanes 3 and 4) or the vector expressing IκBα S32/36A mutant (IκBα MT) (lanes 5 and 6) or wild-type IκBα (IκBα WT) (lanes 7 and 8). Parental HCK1T-HPV16 treated with TNF-α for 15 min was included as a positive control for degradation of IκBα (lanes 1 and 2). Vinculin was detected as a loading control. (C) RT-qPCR analysis of NF-κB target genes and viral early transcripts containing spliced E1^E4 ORF. Shown are levels of the indicated mRNAs relative to MCS control without DOX treatment. Two asterisks indicate a P value of <0.01 for the comparison of the mRNAs in MCS with those in IκBα WT- or IκBα MT-expressing cells in the absence of DOX. Differences for all of the mRNA levels between MCS and IκBα WT- or IκBα MT-expressing cells with DOX treatment were statistically significant (P < 0.01). The highlighting of statistical significance was omitted for DOX-treated samples to avoid making graphs too busy. (D) The copy number of HPV16 genomes was measured by qPCR with indicated gene expression. An asterisk indicates a P value of <0.05. (E) The number of cells staying attached to tissue culture plates is shown. An asterisk indicates a P value of <0.05. The averages of at least three independent experiments are shown.
FIG 5 Effects of inhibitors of DDR on activation of NF-κB, E1-dependent replication of HPV16 genomes, and cell proliferation. (A) Western blot analysis for activation of ATM- and ATR-mediated DDR pathways. The left panel (lanes 1 to 8) shows the results of original cell populations of HCK1T-HPV16 with the indicated gene control in each panel. White arrowheads indicate a slower migration pattern of NBS1 and CHK2. (B) Time course experiment for DDR activation and the level of IκBα in the presence of DMSO as a control, 10 μM wortmannin, or 2.5 μM KU55933. (C) Transcriptional activation of NF-κB target genes and the viral early transcripts was examined by RT-qPCR in the presence or absence of the inhibitors incubated with or without DOX for 24 h. An asterisk indicates a P value of <0.05 for the comparison of the mRNAs between DMSO-treated and wortmannin-treated cells in the absence of DOX. The mRNAs of NF-κB target genes but not the viral early transcripts in wortmannin- or KU55933-treated cells compared to those in DMSO-treated cells in the presence of DOX were significantly reduced (P < 0.05). The highlighting of P values less than 0.05 was omitted for DOX-treated samples. (D) The copy number of HPV16 genomes was measured by qPCR at 24 h after DOX addition in the presence of the indicated inhibitors. (E) The number of cells staying attached to tissue culture plates in the presence or absence of inhibitors was counted. An asterisk indicates a P value of <0.05. (F) Relative mRNA levels of HA-E1 were analyzed by RT-qPCR in the presence or absence of wortmannin following incubation with DOX or vehicle for 24 h. (G) Western blot analysis for solubility of E1 in the presence of wortmannin or vehicle. S and I indicate soluble (lanes 1, 3, and 5) and insoluble (lanes 2, 4, and 6) fractions, respectively. The averages or representative images of at least three independent experiments are shown.
that this was attributable to ATM-mediated phosphorylation of these proteins. In clones of the tetON E1 + E2 cells, the phosphorylation of ATM, CHK1, and CHK2 was also increased following incubation with DOX and a slower-migrating pattern of NBS1 and CHK2 was detected. On the other hand, none of those inductions for DDR activation could be detected in the cell populations of tetON E1K483A + E2 and tetON E2, as expected. These results indicated that ATM and ATR pathways are both activated upon induction of E1 and that the activation of DDR depends on its helicase activity. We then examined reduction of the IkBa level in the presence of DDR inhibitors such as wortmannin and KU55933 upon E1 and E2 expression. Wortmannin is a broad inhibitor of PI3Ks shown to inhibit ATM, ATR, and DNA-PKcs, while KU55933 is a specific inhibitor of ATM. Clone B2 was pre-incubated with indicated inhibitors at 2 h prior to addition of DOX, and the cell lysates were collected at the indicated time points in the presence or absence of the inhibitors. Activation of DDR and reduction of the IkBa level were analyzed by Western blotting (Fig. 5B). A high level of E1 expression was detected at 8 h following DOX addition. Concomitantly, a slower-migrating pattern of NBS1 and CHK2 and the phosphorylated form of CHK1 at S345 and CHK2 at T68 were detected, and they continued to accumulate up to 24 h in DMSO-treated control cells. Reduction of the IkBa level was also evident as early as 8 h following DOX addition and continued thereafter (Fig. 5B, lanes 1 to 4). In the presence of wortmannin, a slower-migrating pattern of NBS1 was less prominent though present, and appearance of the phosphorylated CHK1 and CHK2 was delayed; they were first detected at 16 h following incubation with DOX compared to 8 h in DMSO-treated controls. The time-dependent accumulation of the phosphorylated CHK1 and CHK2 was reduced although not completely abrogated in the presence of wortmannin, indicating that ATM- and ATR-mediated signal transduction was suppressed, albeit not completely. Interestingly, the IkBa level following DOX addition was maintained in the presence of wortmannin, suggesting that activation of DDR signals results in reduction of the IkBa level (Fig. 5B, lanes 5 to 8). In the presence of KU55933, a slower-migrating pattern of NBS1 was almost completely absent while the phosphorylation of CHK1 was increased at a level and with kinetics similar to that seen in DMSO-treated cells. The phosphorylated form and slower-migrating pattern of CHK2 were reduced though not completely abrogated in the presence of KU55933, indicating that ATM- but not ATR-mediated signals were suppressed as expected (Fig. 5B, lanes 9 to 12). The inhibitory effects of KU55933 on reduction of the IkBa level following induction of E1 and E2 expression were much weaker than those of wortmannin. In the presence of KU55933, reduction of the IkBa level was not observed at 8 h but became evident at 24 h following DOX incubation, suggesting that ATM signals partially contribute to reduction of the IkBa level upon E1 and E2 expression. Furthermore, activation of the NF-kB target genes was strongly suppressed in the presence of wortmannin upon induction of E1 and E2 expression, corresponding with the level of IkBa (Fig. 5C, left panel). Similar to the overexpression of IkBa, the basal levels of the NF-kB target genes in the absence of DOX were significantly lower in the presence of wortmannin than that in a control. The activation of NF-kB target genes was also suppressed in the presence of KU55933. The difference in those mRNA levels between the presence and absence of KU55933 was statistically significant. However, the suppression by KU55933 treatment was not as potent as that by wortmannin treatment, consistent with the extent of the IkBa reduction (Fig. 5C, right panel). The levels of the NF-kB target genes in the absence of DOX were comparable in the presence and absence of KU55933. The levels of viral early transcripts containing E1-E4 spliced ORF were comparable between DOX-treated and untreated samples regardless of the presence of the inhibitors. These results suggested that the activation of DDR upon E1 expression results in activation of NF-kB, most likely by promoting IkBa degradation. We also examined the effects of wortmannin and KU55933 on E1-dependent replication of the HPV16 genome (Fig. 5D). The viral copy number following DOX incubation was significantly higher in the presence of wortmannin than that in a DMSO control. On the other hand, the copy numbers were only slightly higher in the presence of KU55933 following DOX incubation, without statistical significance. The copy numbers of HPV16 genomes without DOX incubation were comparable among the cells treated with inhibitors and a DMSO control. These results suggested that activation of DDR results in suppression of E1-dependent replication of the viral genome and that this suppression is primarily mediated by DDR pathways other than the ATM-mediated pathways. Furthermore, when the effects of wortmannin and KU55933 were investigated, reduction of cell numbers after incubation with DOX was observed regardless of the presence of inhibitors (Fig. 5E). Given the fact that wortmannin as well as KU55933 treatment itself modestly inhibited cell proliferation in the absence of DOX, it was not clear whether activation of DDR is involved in inhibitory action of E1 on cell proliferation. Similar results were obtained upon treatment of parental cell populations of tetON E1 + E2 and some of the other clones with those inhibitors (data not shown). Importantly, the effects of wortmannin and KU55933 on E1-dependent replication of the viral genome were well correlated with those on reduction of the IkBa level and proved colinear with overexpression of IkBa (Fig. 4). Collectively, these results suggest that activation of DDR signals limits E1-dependent replication of the HPV16 genome through activation of NF-kB. The subtle effects of KU55933 on E1-dependent replication and suppression of cell growth were consistent with previous reports (12, 13).

We also noted that the protein level of E1 was highest at 8 h following DOX incubation and then gradually decreased, despite the continued presence of DOX (Fig. 5B, lanes 1 to 4). In contrast, such a decrease in the E1 protein level was completely blocked and accumulation during the observation period was apparent up to 24 h with wortmannin treatment (Fig. 5B, lanes 5 to 8). The decrease in the E1 level was modestly alleviated by KU55933 treatment (Fig. 5B, lanes 9 to 12). The results were reproducible in several independent experiments. These observations led us to hypothesize that the DDR-dependent activation of NF-kB disturbs the protein stability of E1. To eliminate the possibility that wortmannin somehow enhanced transcription of E1 from a tetracycline-inducible promoter, the mRNA level of exogenous HA-E1 was analyzed in the presence or absence of wortmannin (Fig. 5F). The mRNA levels of HA-E1 at 24 h following DOX induction were comparable between the cells treated with wortmannin and those treated with DMSO, indicating that the higher level of E1 protein in the presence of the inhibitor was not due to enhanced transcription. We also considered the possibility that lysis of E1 is partial under our lysis conditions and that treatment with wortmannin may alter the solubility of E1. To address whether our lysis method is...
responsible for the seemingly increased level of E1 in the presence of wortmannin, we lysed an insoluble fraction in an SDS sample buffer after extraction of a soluble fraction and the levels of E1 were analyzed in those fractions from equal numbers of cells. In addition, the cells were directly lysed in an SDS sample buffer for comparison (Fig. 5G). Minichromosome maintenance protein 2 (MCM2), histone H3, and vinculin were also detected to indicate solubility of nuclear, chromatin, and cytoplasmic proteins, respectively, under these conditions. Under our lysis conditions, E1 was detected mostly in the soluble fraction (Fig. 5G, lanes 1, 3, and 5), and very little E1 remained in the insoluble fraction (Fig. 5G, lanes 2, 4, and 6). MCM2 and vinculin were detected mostly in the soluble fraction, while histone H3 was detected in both. All proteins were well extracted after direct lysis in an SDS sample buffer as expected (Fig. 5G, lanes 7 to 9). The level of E1 was higher in the presence of wortmannin than in the vehicle-treated control regardless of the lysis methods tested, demonstrating that the increased level of E1 was not due to increased solubility (Fig. 5G, lanes 3, 5, 8, and 9).

**ATR, but not DNA-PKcs and p38MAPK, is involved in E1-dependent activation of NF-κB.** It is reported that p38 mitogen-activated protein kinase (p38MAPK) activated upon UV exposure induces NF-κB activation through cassease kinase II (CKII)- and p38MAPK-dependent phosphorylation of IkBα at its C-terminal proline, glutamic acid, serine, and threonine-rich (PEST) domain (45, 46). UV-induced activation of p38MAPK is thought to be independent of ATM. Because our results suggested that E1 induces reduction of the IkBα level by eliciting DDR and that this reduction is not primarily mediated by kinase activity of ATM, we questioned whether p38MAPK might be involved. Activation of p38MAPK was investigated using an antibody specific to phosphorlated p38MAPK at T180 and Y182, an indicator of p38MAPK activation. Phosphorylation of p38MAPK was increased following incubation with DOX in the pooled cell populations of tetON E1 + E2 and tetON E1 (Fig. 6A) and in clones of tetON E1 + E2 cells (Fig. 6B, lanes 1 and 2), while it remained unaffected in the cell populations of tetON E1K483A + E2 and tetON E2. These results suggested that E1 induces activation of p38MAPK in a manner dependent on its helicase activity, akin to the activation of DDR. To investigate if the E1-induced activation of p38MAPK is mediated by DDR, the effects of wortmannin and NU7026 on p38MAPK were examined in clone B2 (Fig. 6B). In the presence of NU7026, the phosphorylation of p38MAPK was increased to a level comparable to that in the presence of the DMSO control upon incubation with DOX, indicating that the E1-induced activation of p38MAPK is independent of ATM. Phosphorylation of p38MAPK was increased during 2 h of preincubation with wortmannin. Although further accumulation of the phosphorylated form was not observed after incubation with DOX, it was not clear whether the E1-induced activation of DDR other than ATM is involved in the activation of p38MAPK. We also examined activation of DNA-PKcs upon E1 and E2 expression, as phosphorylation of DNA-PKcs at serine 2056 (S2056) has been shown to occur in response to DNA damage (47). Phosphorylation of DNA-PKcs was indeed increased upon E1 and E2 expression, and this was abrogated in the presence of wortmannin (Fig. 6B). The treatment with NU7026 showed little to no effect on the phosphorylation of DNA-PKcs, suggesting that the activation of DNA-PKcs upon E1 and E2 expression is independent of ATM. To explore which one of these molecules mediates the E1-dependent activation of NF-κB, the levels of E1 and IkBα at 24 h after addition of DOX were investigated in the presence of VE-822, NU7026, and SB203580, specific inhibitors of ATR, DNA-PKcs, and p38MAPK, respectively (Fig. 6C). In comparison with a vehicle-treated control, increased levels of E1 were detected in the presence of wortmannin and VE-822, among those inhibitors, implying that ATR activity is involved in regulation of the protein stability of E1 (Fig. 6C, bottom panel for quantification). On the other hand, apparent inhibition of IkBα reduction was seen only in the presence of wortmannin. To further analyze the effects of VE-822 on the levels of IkBα and E1, a time course experiment was carried out with HCKIT-HPV16 tetON E1 + E2 clone B2 as described in Fig. 5. In the presence of VE-822, the phosphorylation of CHK1 was almost completely abrogated, verifying that VE-822 indeed inhibits ATR activity. Importantly, progressive decrease of E1 as well as IkBα levels following DOX addition was prevented, at least until 16 h, and then became evident at 24 h after DOX addition in the presence of VE-822, suggesting that ATR is involved in the E1-dependent activation of NF-κB and subsequent destabilization of E1. We then examined E1-dependent replication of the HPV16 genome and found that it was enhanced in the presence of VE-822, compared to the control. The basal copy number of HPV16 genomes was also slightly increased in the absence of DOX (Fig. 6E). On the other hand, inhibition of cell proliferation was still seen in the presence of VE-822 upon expression of E1 and E2 (Fig. 6F). Because the treatment of VE-822 itself modestly suppressed cell proliferation in the absence of DOX, it was not clear whether activation of ATR mediates inhibition of cell proliferation by E1 expression. We found no difference in the levels of E1 and IkBα examined by time course experiments and in E1-dependent replication of the viral genomes in the presence of Nu7026 or SB203580 compared to the control (data not shown). The effect of VE-822 in inhibiting reduction of the IkBα level was more potent than that of NU55933 but not wortmannin, suggesting that ATR and ATM play major and minor roles, respectively, in the E1-dependent activation of NF-κB. Since the expression level of E2 could also affect viral genome replication, we analyzed the stability of E2 in the presence of wortmannin and VE-822 (Fig. 6G). To this end, we generated a heterogeneous cell population of HCKIT-HPV16 with tetON-inducible expression of HA-E1 and 3×FLAG-tagged E2 by lentivirus gene transfer, as HA-tagged E2 was very difficult to detect in our hands. HCKIT-HPV16 tetON HA-E1 and FLAG-E2 induced replication of the HPV16 genome as well as original tetON E1 + E2 cells did with DOX incubation (data not shown). A time course experiment revealed that the expression level of FLAG-E2 was overall constant for at least 24 h and not affected by the presence of wortmannin or VE-822, indicating that DDR- and/or NF-κB-induced destabilization is specific to E1. All results could be replicated with clone A5 (data not shown).

**Activation of NF-κB mediates DDR-induced destabilization of E1 and promotes polyubiquitination of E1.** To address whether the activation of NF-κB indeed mediates DDR-induced destabilization of E1, a time course experiment was performed with HCKIT-HPV16 tetON E1 + E2 clone B2 transduced with IkBoWT, IkBoMT, or MCS. Following DOX incubation, phosphorylation of CHK1 was increased and a slower-migrating pattern of NBS1 appeared, with kinetics and levels comparable among the cells transduced with MCS, IkBoWT, and IkBoMT.
DOX incubation, cell lysates of HCK1T-HPV16 tetON E1 cells were isolated in the presence or absence of epoxomicin at the time at which destabilization of E1 could be detected. As expected, progressive decrease of the E1 level was evident in the absence of DOX, further reduction of IκBα and E1 expression was abrogated in the presence of epoxomicin. These results could be replicated with clone A5 (data not shown). While expression of IκBα was increased in the absence of DOX, the accumulation of E1 protein was not as evident as in wortmannin-treated cells. In the cells expressing IκBα WT, though accumulation of E1 protein was not as evident as in the presence of inhibitors as described in the Fig. 5 legend. The averages or representative images of at least three independent experiments are shown.

(A) Activation of p38MAPK was examined in HCK1T-HPV16 populations with the indicated gene expression. (B) Effects of wortmannin and KU55933 on activation of p38MAPK and DNA-PKcs in clone B2. Cell lysates were prepared at 0 and 24 h after DOX addition following preincubation with the inhibitors for 2 h. Vinculin was detected as a loading control. (C) Western blot analysis for the levels of IκBα and E1 at 24 h after incubation with DOX in the presence of indicated inhibitors. A bottom graph shows intensities of E1 with inhibitors relative to that with a vehicle control. The intensities of E1 were normalized to that of vinculin. Concentrations of inhibitors used were 10 μM wortmannin, 0.5 nM VE-822 (ATR inhibitor), 20 μM Nu7026 (DNA-PKcs inhibitor), 2.5 μM KU55933, and 10 μM SB203580 (p38MAPK inhibitor). (D) Western blot analysis for a time course experiment in the presence of wortmannin (lanes 5 to 8), VE-822 (lanes 9 to 12), or a vehicle control (lanes 1 to 4). (E) The copy number of HPV16 genomes was measured by qPCR. (F) The number of cells staying attached was examined after incubation with DOX or vehicle in the presence of DMSO or VE-822 for 24 h. An asterisk indicates a \( P \) value of \(<0.05\). (G) Western blot analysis for the stability of E2 in the presence of indicated inhibitors as described in the Fig. 5 legend. The averages or representative images of at least three independent experiments are shown.
control of the CMV promoter, CSII-CMV-HA16E1, and an expression vector of 6×His-tagged ubiquitin (pCMV-6×His-Ub) were cotransfected into 293FT cells with an expression vector of IκBαMT or an empty vector, and cell lysates were collected at 48 h thereafter. The HA-E1 was immunoprecipitated using magnetic beads conjugated with anti-HA antibodies and subsequently analyzed by Western blotting. The effect of wortmannin was investigated by incubation from 20 h posttransfection until harvesting. The GFP expression vector was also included in all transfections to monitor the efficiency of the transfections. We found that E1 expression did not conspicuously reduce the IκBα level in 293FT cells (Fig. 7D, lanes 7 and 8). To rule out the possibility that coexpression of His-tagged ubiquitin hinders the E1-induced reduction of IκBα. Regardless of coexpression of His-tagged ubiquitin, reduction of the IκBα level was barely detected and effects of wortmannin on levels of E1 and IκBα were inconspicuous (Fig. 7D, lanes 1, 2, and 3 and lanes 7, 8, and 12). To potentiate NF-κB activation in 293FT cells expressing E1, TNF-α was added at 20 h posttransfection. As expected, this TNF-α treatment reduced the IκBα level and, to a lesser extent, that of E1 compared to the vehicle-treated control (Fig. 7D, compare lane 2 with lane 4 and lane 8 with lane 10). In cells cotransfected with an IκBαMT expression vector, the level of E1 appeared lower, probably due to the lower efficiency of the transfection as indicated by GFP expression (Fig. 7D, lanes 5, 6, 11, and 12). Nonetheless, protein ladders were detected in the immunoprecipitates at a size above 100 kDa, bigger than the E1 (around 70 kDa), indicating polyubiquitination of E1, and this was abolished with the expression of IκBαMT (Fig. 7D, lanes 17 and 18). Consistent with the results of Western blotting, effects of wortmannin on polyubiquitination of E1 were barely detected. These results suggest that activation of the NF-κB pathway promotes ubiquitin-dependent proteasomal degradation of E1, though the precise mechanisms remain unclear.

E1 and E2 expression results in accumulation of cells in S and G2 phases. Several studies have shown that expression of E1 and E2 results in accumulation of cells in S and G2 phases (11–13). To investigate a temporal relationship between the levels of E1 and the cell cycle progression in our system, the cell cycle profile was analyzed at the indicated time points following DOX addition in clone B2 (Fig. 8). Accumulation of cells in S and G2/M phases was already evident at 8 h and increased by 16 h following induction of E1 and E2 expression. The percentage of the cells in S phase was consistently higher (32% in S and 23% in G2 at 8 h) than that in G2.

**FIG 7** Activation of NF-κB mediates destabilization of E1 and promotes polyubiquitination. (A) Time course experiments for clone B2 transduced with MCS (lanes 1 to 4), IκBαWT (lanes 9 to 12), or IκBαMT (lanes 5 to 8) following incubation with DOX. A white arrowhead indicates a slower-migrating pattern of posttransfection. As expected, this TNF-activation in 293FT cells expressing E1, TNF-IκB indicates cotransfection with an expression vector of IκBαMT.
phase until 16 h after DOX addition (46% in S and 26% in G₂ phase at 16 h), suggesting that expression of E1 and E2 delayed S-phase progression, consistent with previous reports (12, 13). A proportion of the cells progressed to G₂ phase by 24 h. The percentage of the cells in G₂ phase (40%) exceeded that of the cells in S phase (33%) at 24 h and remained consistently higher up to 40 h with incubation of DOX. Cells containing less than 2 N DNA (sub-G₁) appeared at 24 h, coinciding with the detection of cleaved forms of PARP-1 and caspase 3 (Fig. 1F). These results were replicated with clone A5 (data not shown). Taken together with the observation that the level of E1 was highest between 8 and 16 h and progressively reduced by 24 h (Fig. 4, 5, and 6), these results suggest that E1 and E2 expression initially induces cell cycle retardation in S phase, which is attenuated as the level of E1 decreases.

NF-κB activity regulates copy number of the HPV genome during maintenance replication. As mentioned above, endogenous levels of the NF-κB target genes were consistently higher in human keratinocytes stably maintaining episomal HPV genomes than in HCK1T cells (Fig. 3C and D). We also noted a slight increase of the HPV16 genome copy number in HCK1T-HPV16 tetON E1+E2 cells to which IkBαMT was introduced compared to MCS in the absence of DOX (Fig. 4D). Since our results suggested that NF-κB activation limits E1-dependent replication, we postulated that NF-κB activity could be involved in maintenance of the HPV genome in proliferating keratinocytes. Therefore, IkBαMT was introduced into parental HCK1T-HPV16 by retrovirus gene transfer as described for Fig. 4, and the copy number of HPV16 genomes was measured at 5 days after drug selection was completed (Fig. 9A, black bars). As expected, the copy number was increased by 30% with statistical significance in the cells expressing IkBαMT compared to the MCS control. Conversely, the copy number of HPV16 genomes was decreased by approximately 50% with incubation of TNF-α for 5 days. The copy number of the HPV31 genome in CIN612-9E cells was also decreased by TNF-α in a dose-dependent manner (Fig. 9B, black bars). While expression of IkBαMT did not affect cell proliferation, TNF-α treatment modestly suppressed cell proliferation in both cell lines (Fig. 9A and B, white bars). These results imply that constitutive activation of NF-κB in the presence of episomal HPV is involved in regulation of maintenance replication of the viral genome.

DISCUSSION
Papillomaviruses undergo three phases of genome replication, the initial amplification, maintenance replication, and productive amplification (38). While the underlying mechanisms for the differentiation-dependent productive amplification are becoming gradually clear, virtually nothing is known about the molecular mechanisms for transition from the initial amplification to maintenance replication within the basal compartment. Our results...
showed that NF-κB is activated when E1-dependent replication of the HPV16 genome is induced in undifferentiated, basal cell-like human cervical keratinocytes and that inhibition of this NF-κB activation enhances E1-dependent replication of the HPV16 genome. Thus, our results suggest that E1 and NF-κB may form a negative feedback loop to regulate E1-dependent replication of the HPV16 genome (Fig. 9C). The stability of E1 was augmented when activation of NF-κB was abrogated in the presence of DDR inhibitors or IκBαMT, and activation of NF-κB by TNF-α facilitated polyubiquitination of E1, suggesting that DDR-NF-κB activation may promote degradation of E1. Furthermore, constitutive activation of NF-κB was evident in human keratinocytes stably maintaining episomal HPV genomes, and inhibition or potentiation of the NF-κB activity resulted in increase or decrease, respectively, of the viral copy numbers (Fig. 9A and B). Based on these results, we propose a possible model featuring involvement of an E1-NF-κB feedback loop in the establishment and maintenance of persistent HPV infection. This feedback loop could be further ensured by transcriptional inhibition of the viral early promoter, which drives the expression of early genes, including E1, by active NF-κB. To our knowledge, this is the first evidence implicating NF-κB activity in regulation of papillomavirus genome replication.

**Activation of NF-κB by E1 through DDR.** Our results showed that NF-κB is activated as a consequence of E1-induced DDR, primarily via the ATR-mediated pathway (Fig. 6). These results were rather surprising to us since the best-studied pathway of NF-κB activation upon DNA damage is ATM-mediated induction of IκBα degradation. ATM has been shown to activate the IκB kinase (IKK) complex by directly phosphorylating its regulatory subunit, the NF-κB essential modulator (NEMO), and this activation of IKK leads to phosphorylation-dependent degradation of IκBα (49). Interestingly, it was recently shown that degradation of NEMO is enhanced in human keratinocytes containing episomal HPV16 genomes, compared to primary human keratinocytes (50). Therefore, activation of NF-κB by the ATM-mediated pathway through NEMO could be inherently limited in HCK1T-HPV16 cells. We failed to detect phosphorylation of the IKK complex upon E1 and E2 expression and found that an inhibitor of IKKs, IKK inhibitor VII, had no effect on E1-dependent replication (T. Nakahara and T. Kiyono, unpublished data). These results are consistent with a modest effect of KU55933 on reduction of the IκBα level, as the ATM-mediated activation of NF-κB was shown to be dependent on IKKs. The absence of IKK phosphorylation also implies that ATR-mediated reduction of IκBα might be independent of IKKs. Similar to our results, it was reported that
degradation of IkBα was induced by doxorubicin, a DNA-damaging agent, in mouse embryonic fibroblasts (MEFs) devoid of IKKs and that this was independent of phosphorylation at S32 and S36 as well as the PEST domain of IkBα. LY294002, a PI3K inhibitor, partially blocked the degradation of IkBα (51). Whereas the level of IkBα was maintained throughout a 24-h observation period in the presence of wortmannin, it was decreased after 16 h following DOX addition even though phosphorylation of CHK1 was more efficiently suppressed throughout in the presence of VE-822 (Fig. 6D). The greater effect of wortmannin in inhibiting E1-induced reduction of IkBα could be due to its pleiotropic effects, suppressing both ATM and ATR pathways. Alternatively, it is possible that other PI3Ks are involved. Since no data are available directly implicating ATR in NF-κB activation in the literature thus far, further studies are necessary to elucidate the mechanism(s) for ATM-mediated activation of NF-κB. We also found that E1 induces activation of p38MAPK in a manner dependent on its helicase activity but independent of DDR (Fig. 6). Although it is beyond the scope of our current study, the significance of p38MAPK activation in the HPV life cycle warrants future investigation.

Inhibition of E1-dependent replication of the viral genome by NF-κB. The time course experiments in the present study revealed that the expression level of E1 progressively decreased and this was abrogated in the presence of wortmannin, VE-822, and IkBoMT expression, suggesting that activation of NF-κB causes destabilization of E1 (Fig. 5, 6, and 7). Since the results with a proteasome inhibitor, epoxomicin, did not provide a direct answer to whether NF-κB-dependent-reduction of E1 is mediated by a ubiquitin-proteasome pathway, we carried out a ubiquitin conjugation assay in 293FT cells and found polyubiquitination of E1 to be greatly enhanced by TNF-α treatment (Fig. 7D). Thus, it is likely that NF-κB promotes ubiquitin-dependent proteasomal degradation of E1. On the other hand, E1 expression scarcely induced reduction of IkBα in 293FT cells. We observed increased phosphorylation of ATM and CHK1 in 293FT cells expressing E1 (data not shown). The lack of conspicuous effect of E1 expression on the IkBα level in 293FT cells might be due to the presence of adenovirus E1A, since it has been shown to suppress IkBα degradation (52), or to a cell type difference downstream of DDR activation. It was previously shown that E1 of BPV1 is targeted by anaphase-promoting complex (APC) for degradation (33, 34). Although the question of whether E1 proteins of HPVs, including HPV16, are also targeted by APC has not been resolved, it is possible that the reduction of HPV16 E1 seen in this study was mediated by APC as HPV16 E1 contains three putative D boxes (RxxL) and one KEN box, known recognition motifs for APC. APC is a multicomponent ubiquitin ligase well known to regulate the cell cycle. Several recent studies have indicated that APC is activated upon DNA damage in an ATM-dependent as well as an ATM-independent manner (55–57). Although it is not clear whether NF-κB was involved in the DDR-induced APC activation in these studies, it is reasonable to speculate that E1-dependent activation of DDR induces activation of APC, possibly through NF-κB. The fact that wortmannin treatment completely blocked the reduction of E1 while overexpression of IkBoMT blocked reduction of E1 somewhat less efficiently suggests that NF-κB-dependent as well as -independent mechanisms for E1 degradation may exist downstream of DDR (Fig. 5, 6, and 7). In the BPV1 study, polyubiquitination of BPV1 E1 was enhanced in the presence of BPV1 E2 and a plasmid containing the replication origin of BPV1 in vivo. We observed that a majority of the cells accumulated in S phase by 16 h and a proportion of these cells progressed to G1 phase by 24 h following DOX treatment, indicating that the degradation of E1 in our experiments most likely occurs during S phase in the presence of E2 and the viral genome (Fig. 8). These results are consistent with the finding that polyubiquitination of BPV1 E1 is facilitated in the S-phase extract of 293 cells in vitro. Therefore, it seems likely that APC-mediated degradation of BPV1 E1 in vivo also occurs in S phase and APC is activated by E1-induced DDR. However, our results do not rule out the possibility that other E3 ubiquitin ligases are involved in DDR- and/or NF-κB-dependent degradation of E1. Whereas several studies have highlighted a significance of ATM activation for productive amplification, contribution of ATR activation to the HPV life cycle has not been indicated. Our results also suggest that ATR may negatively regulate viral genome replication through NF-κB activation. Previously, King et al. reported that activation of DDR by etoposide had no effect on E1-dependent replication in a transient replication assay using 293T cells (58). Their results are in agreement with our finding of a minor effect of ATM since etoposide primarily induces DSBs by inhibiting topoisomerase II (59). However, they showed elevated phosphorylation of CHK1 by etoposide treatment, suggesting that ATR was also activated. The limited effect of ATR activation on E1-dependent replication in their study could be because 293T cells were used. The effect of E1 expression on IkBα reduction in 293FT cells was likewise minor in our hands. On the other hand, Edwards et al. recently showed that knockdown of ATR by small interfering RNA (siRNA) and treatment with a CHK1 inhibitor for 2 days resulted in reduction of the viral genome in W12 cells, isolated from a CIN1 biopsy specimen containing HPV16, in contrast to our results (60). Since we incubated VE-822 at most for 26 h, the longer duration and/or differences in approaches to inhibition of the ATR-CHK1 pathway might have affected the results through an NF-κB-independent pathway.

It was shown recently that DNA damage induces simultaneous activation of NF-κB and IRF7 and subsequent production of interferon alpha (IFN-α) and IFN-α in HeLa cells (61). Several studies have demonstrated that chronic IFN-β treatment reduces episomal copies of the papillomavirus genome in W12 cells for HPV16, in CIN612 cells for HPV31, and in mouse C127 cells containing BPV1 (62–65). Conversely, it was found that IFN-inducible genes were downregulated in cells containing an episomal HPV genome and that loss of viral episomes is associated with endogenous activation of type 1 IFN-induced antiviral genes (30, 66). The gene product of IFN-induced protein with tetratricopeptide repeats 1 (IFIT1, also called ISG56) was demonstrated to inhibit E1-dependent replication of viral DNA by direct binding to HPV18 E1 in transient replication assays (67, 68). Our results do not exclude the possibility that E1-dependent activation of NF-κB results in upregulation of type I IFNs and that these subsequently inhibit the activity of E1 with regard to viral genome replication. This might explain our finding that expression of IkBoMT enhanced E1-dependent replication of the viral genome very well while it attenuated the degradation of E1 less efficiently than did wortmannin treatment.

A possible function of the NF-κB-mediated feedback loop in the viral life cycle. In our previous study (38), we demonstrated that E1 of HPV16 is required for initial and productive amplification while it is dispensable for maintenance replication. Interest-
ingly, Hoffmann et al. (69) reported that HPV16 genomes in W12 cells are maintained by replicating in a once-per-S-phase fashion while HPV31 genomes in CIN612-9E cells are maintained by a random-choice mechanism and that introduction of E1 expression from an exogenous promoter in W12 cells switched HPV16 genome replication to a random-choice mechanism. The authors concluded that there are two modes of genome replication, at least for HPV16 during the maintenance phase, and that choice of modes is associated with the expression level of E1. In agreement with previous studies (11, 12), our results also showed that excess expression of E1 could be detrimental to cell proliferation in basal cell-like cervical keratinocytes (Fig. 1). Thus, regulation of the E1 protein level appeared to be an essential element in the viral life cycle for establishing viral persistence and determining the mode of viral genome replication. Results of our present study suggest that NF-κB activity is involved in regulation of the E1 level and that NF-κB activation is induced by E1 itself, constituting a negative feedback loop in proliferating keratinocytes. We propose that E1-dependent initial amplification following virus infection induces NF-κB activation and that this in turn terminates the initial amplification by inducing degradation of E1, mediating a transition to maintenance replication. Although the expression level of exogenous E1 in our experiments could be much higher than that during initial amplification, a lower level of E1 might be sufficient to induce NF-κB activation. Karim et al. reported that an increase in NF-κB activity was detected within 24 h after infection with HPV16 virions isolated from raft cultures (50). Although their interpretation was that NF-κB is activated as part of an innate immune response, their results did not exclude the possibility that E1-dependent initial amplification contributes to activation of NF-κB. Indeed, it is plausible that innate immune responses to virus infection potentiate E1-induced activation of NF-κB during the initial amplification. In cervical keratinocytes stably maintaining episomal HPV genomes, constitutive activation of NF-κB was detected and manipulation of NF-κB activity resulted in increase or decrease of the viral copy numbers (Fig. 3 and 9). Although we cannot provide direct evidence that the endogenous E1 level was changed by such manipulation, given that an antibody sufficiently sensitive to detect endogenous E1 has yet to be developed, the results imply that endogenous E1, if present, may induce constitutive activation of NF-κB in the presence of episomal HPV genomes and that the NF-κB activity regulates viral copy numbers through limiting or completely abolishing E1. Thus, we speculate that NF-κB activity may play a key role in determining the mode of maintenance replication. Since several groups have shown that E1s of various papillomaviruses induce activation of DDR (11–13), we believe that a negative feedback loop between E1 and NF-κB might constitute a common mechanism to regulate E1 levels in the basal compartment of the infected tissue. Interestingly, it has been reported that NF-κB is upregulated in basal cells of a fraction of CIN1 compared with normal tissues (22). The reported percent NF-κB positivity in low-grade cervical lesions has varied from study to study; some authors have indicated that upregulation of NF-κB is detected only in cervical cancer while others indicated that it is already present in the basal compartment in 30% of CIN1 lesions (17, 19). The varied patterns of NF-κB activation in the basal compartment of CIN1 may reflect the presence or absence of E1 during the stable maintenance phase. It is also possible that expression of other viral proteins such as E6 contributes to constitutive activation of NF-κB in the presence of HPV genomes, as E6 of HPV16 has been shown to induce NF-κB by at least two mechanisms (28, 70). Recently, Gunasekaran and Laimins (71) reported that a cellular microRNA, miR-145, contains a seed sequence matching E1 and E2 ORFs of HPV31 and that its overexpression resulted in decrease of viral copy numbers in CIN612-9E under undifferentiating as well as differentiating culture conditions. The level of miR-145 was found to be high in a monolayer culture but suppressed upon differentiation, suggested that miR-145 suppresses E1 and/or E2 levels in basal cells and that the suppression is removed upon differentiation. Interestingly, they found two RELA binding sites in the promoter of miR-145. Although experimental data showing that NF-κB upregulates miR-145 have not yet been provided, effects of TNF-α on viral genomes could also have been mediated by up-regulation of miR-145 in the case of CIN612-9E. Together with our results, these studies clearly underscore an importance of NF-κB activity for viral persistence of HPVs. Currently, we are not sure whether NF-κB activation and/or its inhibition of E1-dependent replication occurs during productive amplification. As NF-κB activity and components of NF-κB pathways are intricately associated with differentiation processes in keratinocytes, the outcomes as well as the pathways of NF-κB activation could be different in differentiating cells. Interestingly, Satsuka et al. reported that TNF-α treatment slightly reduces the copy number of HPV18 replications in monolayer cultures of keratinocytes whereas it enhanced productive amplification in raft cultures (72). On the other hand, it is also conceivable that NF-κB activation is prevented by increased levels of E6 and E7, as these may suppress NF-κB activity by various mechanisms (24, 30).

NF-κB is a major transcription factor that upregulates inflammatory cytokines, including TNF-α, IL-6, and IL-8, which in turn activate NF-κB itself. A number of studies have provided compelling evidence that chronic inflammation increases risk of cancer development, including that of cervical neoplasms. The ability of NF-κB to suppress viral genome replication may facilitate loss of episomal HPV and emergence of viral genome integration, potentiating cancer progression when ectopically activated in basal cells. Clearly, uncovering NF-κB functions in the viral life cycle is important to elucidate molecular mechanisms underlying viral persistence and pathogenesis of HPV infection.

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