Regulation of Human Papillomavirus Type 16 E7 Activity through Direct Protein Interaction with the E2 Transcriptional Activator

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In order to ensure a productive life cycle, human papillomaviruses (HPVs) require fine regulation of their gene products. Uncontrolled activity of the viral oncoproteins E6 and E7 results in the immortalization of the infected epithelial cells and thus prevents the production of mature virions. Ectopically expressed E2 has been shown to suppress transcription of the HPV E6 and E7 region in cell lines where the viral DNA is integrated into the host genome, resulting in growth inhibition. However, it has been demonstrated that growth control of these cell lines can also occur independently of HPV E2 transcriptional activity in high-risk HPV types. In addition, E2 is unable to suppress transcription of the same region in cell lines derived from cervical tumors that harbor only episomal copies of the viral DNA. Here we show that HPV type 16 (HPV-16) E2 is capable of inhibiting HPV-16 E7 cooperation with an activated ras oncogene in the transformation of primary rodent cells. Furthermore, we demonstrate a direct interaction between the E2 and E7 proteins which requires the hinge region of E2 and the zinc-binding domain of E7. These viral proteins interact in vivo, and E2 has a marked effect upon both the stability of E7 and its cellular location, where it is responsible for recruiting E7 onto mitotic chromosomes at the later stages of mitosis. These results demonstrate a direct role for E2 in regulating the function of E7 and suggest an important role for E2 in directing E7 localization during mitosis.

Human papillomaviruses (HPVs) are a large family of small, double-stranded DNA viruses. They infect cutaneous and mucosal epithelial tissue at different anatomical locations, resulting in a variety of clinical symptoms ranging from benign warts to invasive genital cancers (13, 29). Infection with the high-risk types, most commonly HPV type 16 (HPV-16) and HPV-18, has been associated with the development of more than 99% of cervical cancer cases. The tumorigenicity of HPV is dependent on the activity of two virally encoded oncoproteins, E6 and E7. These bind at a high affinity and disrupt the function of p53 (46) and the retinoblastoma tumor suppressor protein pRB (6, 20), respectively. While E6 plays a role in inhibiting apoptosis and interfering with cell adhesion and polarity (30), E7 acts by driving S-phase progression, regulating gene expression, and interfering with the activities of cyclins and cyclin-dependent kinases (23). In addition to inactivating the function of pRB, other cellular targets of E7 include the TATA box-binding protein (TBP) (31), TBP-associated factors (35), members of the AP-1 transcription factor family (2), and histone deacetylases (7). E7 is a small phosphoprotein that shares some sequence homology with the adenovirus E1a protein and the simian virus 40 large T antigen (8). E7 is 98 amino acids in length and contains a zinc-binding domain in the C-terminal region whose structural integrity is necessary for the activity of E7 (2, 26). While both E6 and E7 cooperate to induce immortalization of keratinocytes, expression of E7 alone is sufficient to induce DNA synthesis in differentiated keratinocytes (9) and invasive cervical cancer in transgenic mice (41).

Clinical studies have revealed a long latency period between primary infection with HPV and the development of invasive tumors (61). HPV-driven tumors are invariably characterized by elevated levels of E6 and E7 expression and the frequent integration of the viral episo...
not episomal, DNA in cell lines derived from cervical tumors. We were therefore interested in investigating possible roles for E2 in controlling the activity of E7 in a manner independent of its transcriptional function. By using baby rat kidney (BRK) transformation assays, we show that E2 inhibits E7-induced transformation and that this inhibition is not due to repression of E7 expression by the E2 protein. In addition, we show that E2 and E7 interact directly and that this binding maps to a region in the C-terminal half of E7 which overlaps the site of interaction of several important E7 binding partners. In addition, E2 stabilizes E7 and recruits it to mitotic chromosomes at telophase.

**MATERIALS AND METHODS**

**Cells and transfection.** U2OS (human osteosarcoma), BRK, and CaSkI (human cervical carcinoma, HPV-16-positive) cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum. U2OS and BRK cells were transfected by calcium phosphate precipitation as described previously (34).

**Plasmids.** Glutathione S-transferase (GST) fusion plasmids were all cloned in pGEX-2T, including HPV-16 E2 (39), E7 (33), E1 (53), mutant forms of E2 (32), 11E2 (25), and TBP (31). The truncations below were cloned into pGEX-2T by PCR with the following primers: for the truncation of E2 with residues spanning positions 269 to 365, forward primer 5′ GACACCGGATCCCTGCGCAACC and reverse primer 5′ CTTTGGATCCATGATACGTATA; for the N-terminal half of 16E7, forward primer 5′ AGCTGAGGGATCCCCAGCT GTAATC and reverse primer 5′ CTTTGAGTCCTGACCATCTAT; and for the C-terminal half of 16E7, forward primer 5′ CCAGGATCCCCAGCA GACCCGGAC and reverse primer 5′ CTTCTTGGATCCATGATACGTGCAA. His-tagged E7 was cloned as described in reference 40. For in vitro translations, 16E7 was cloned in plasmid pSP64 (33), 16E2 was cloned in pcDNA (5), the N- and C-terminal halves of 16E2 were cloned in pSP64 (39), mutant forms of 16E7 were cloned in pSP64 (33), and 11E7 was cloned in pcDNA from p40M plasmids (54) for the EcoRI and HindIII restriction sites. For in vivo expression, the following plasmids were used: pGFP-N1 (Clontech), 16E2 in pCMV (5), p4M:16E7 (54), adenovirus E1a (47), and Ras (pEJH6) (52). The Δ3 and Δ4 mutant forms of E7 were cloned from plasmid pSP64 into pcDNA at the EcoRI and BamHI restriction sites.

**Antibodies.** Anti-hemagglutinin (HA) monoclonal antibody 12C5 (Roche) was used to detect HA-tagged proteins in Western blot and immunofluorescence assays. Mouse monoclonal antibody against E2 and anti-E2 polyclonal antibody have been described previously (27, 32). In addition, the following commercial antibodies were used: anti-β-gal (anti-β-galactosidase; Promega), polyclonal rabbit anti-α-actin (Sigma), mouse anti-16E7 (ED17; Santa-Cruz), and polyclonal rabbit anti-HA (Santa-Cruz).

**BRK transformation assay.** Primary BRK cells from 9-day-old Wistar rats were transfected with 6 μg of either p4M:HPV-16E7 or an adenovirus E1a expression plasmid encoding HA-tagged E7 and E2. At 24 h later, E1A extraction was performed and the soluble fraction was incubated with anti-HA beads (Sigma) for 3 to 4 h on a rotating wheel at 4°C. Precipitated proteins were analyzed by Western blotting with an anti-E2 polyclonal antibody.

**Half-life experiments.** CaSkI cells were transfected with 3 μg of a plasmid expressing E2 by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At 24 h after transfection, cells were treated for different times (0, 15, 30, 60, and 120 min) with cycloheximide (50 μg/ml in dimethyl sulfoxide [DMSO]) to block protein synthesis. Total cellular extracts were then analyzed by Western blot assay, and the intensities of the bands on the X-ray film were measured with Adobe Photoshop. The standard deviation was calculated from three independent assays.

**RT-PCR.** Total RNA was isolated from CaSkI cells (transfected as above) and U2OS cells transfected as in the transformation assay 24 h after transfection with TRI Reagent (Sigma) according to the manufacturer’s instructions. A total of 1 μg of RNA was subjected to reverse transcription (RT) with the RETROscript system (Ambion). A control without reverse transcriptase was also added for assaying contamination with DNA. PCR was performed with 20 cycles and an annealing temperature of 55°C for E2, green fluorescent protein (GFP), and actin and 58°C for E7. PCR primers for actin, E7 (59), and GFP (25) have been described previously. 16E2 primers were as follows: forward, 5′ ATGGGACTCTTTGCCAA; reverse, 5′ TCATATAGACATATCCGAGCAG. Immunofluorescence and microscopy. Cells were stained and fixed for immunofluorescence as described previously (25). Briefly, cells were fixed with 3.7% paraformaldehyde in PBS and permeabilized with 0.1% Triton X-100 in PBS. Primary antibodies were incubated for 1.5 h at 37°C, followed by extensive washing in PBS and incubation for 30 min at 37°C with secondary anti-rabbit or anti-mouse antibody conjugated with fluorescein or rhodamine (Molecular Probes). For visualization of chromosomes, cells were stained with Hoechst stain (bisbenzimide; Sigma no. 33258). Samples were then washed several times with water and mounted with Vectashield mounting medium (Vector Laboratories Inc.) on glass slides.

Slides were analyzed with either a Leica DMLB fluorescence microscope equipped with a Leica photo camera (A101M871016) or a Zeiss LSM 510 confocal microscope with two lasers giving excitation lines at 480 and 510 nm. The data were collected with a 100 × objective oil immersion lens.

**RESULTS**

**HPV-16 E2 inhibits E7-induced transformation.** Previous studies have shown that overexpression of the E2 protein resulted in growth arrest in cell lines containing integrated HPV DNA due to suppression of E6 and E7 gene transcription (14, 22). We were interested in whether E2 had any direct effects upon the transformation activity of E7 in the absence of an E2-responsive promoter. To do this, we used primary BRK cells transfected with HPV-16 E7 and EJ-ras, with or without HPV-16 E2 (31). For comparison, we also included in parallel adenovirus E1a and EJ-ras transfections. After 2 weeks of selection, the cells were fixed and stained and the colonies counted. The results obtained are shown in Fig. 1a, where it can be seen that HPV-16 E2 is a potent inhibitor of E7 transforming activity. This effect of E2 appears to be specific since, in contrast, E2 has no effect on the transforming activity of adenovirus E1a (Fig. 1b). To verify that the expression of E7 from the p4M plasmid was not inhibited by the E2 expression

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plasmid, we analyzed the level of E7 gene expression 24 h after transfection into BRK cells by RT-PCR analysis. Figure 1c shows that E2 does not inhibit E7 expression in this assay system, suggesting that E2 suppression of E7-induced transformation is at the posttranscriptional level.

**E2 and E7 bind directly.** The above result indicates a specific effect of E2 on E7 function that is independent of E2’s transcriptional activities with respect to the HPV promoter. Since previous studies have shown that E2 interacts with E6 (25), we were interested in investigating whether E2 could also associate with E7 and thereby modulate E7 activities. To do this, we performed GST pulldown assays with bacterially expressed, GST-tagged proteins immobilized on resin. All binding assays were performed for 1 h on ice, and the reaction mixture was extensively washed with a detergent-containing buffer. As shown in Fig. 2a, HPV-16 E2 bound strongly to the positive control of GST-16E1, as well as to GST-16E7. No interaction was detected with GST alone. To investigate whether E7 could also pull down E2 expressed in vivo, GST-16E7 was incubated with an extract from U2OS cells transiently transfected with a 16E2 expression plasmid and increasing amounts of E2, along with a GFP-expressing plasmid as a control for transfection efficiency. One microgram of total cell RNA was reverse transcribed, and the expression of E2, E7, and GFP was analyzed by PCR with specific primers. -RT, controls without RT.

FIG. 1. The transformation activity of E7 is inhibited in the presence of E2. Primary BRK cells from 9-day-old Wistar rats were transfected with 6 μg of plasmids expressing HPV-16 E7 (a) or adenovirus E1a (b) together with Ras as a cooperating oncogene and pcDNA carrying a selectable marker. Cells were maintained in medium containing 200 μg/ml G418 for 2 weeks and then fixed and stained, and the colonies were counted (as shown at the top). The chart shows the mean of three independent experiments, and error lines indicate standard deviations. (c) BRK cells treated as described for panel a were transiently transfected with 6 μg of plasmid pJ40 expressing E7 and increasing amounts of E2, along with a GFP-expressing plasmid as a control for transfection efficiency. One microgram of total cell RNA was reverse transcribed, and the expression of E2, E7, and GFP was analyzed by PCR with specific primers. -RT, controls without RT.
sively washed and the amount of E7 retained was then detected by Western blotting with an anti-E7 monoclonal antibody. The results, shown in Fig. 2c, demonstrate that E7 binds E2 directly and that this interaction is comparable to the interaction between E7 and TBP. No interaction was seen between E7 and GST alone.

Having found that HPV-16E2 and HPV-16E7 could interact, we wanted to assess whether this was conserved between low- and high-risk HPV types. To do this, we assayed binding between the respective E2 and E7 proteins derived from HPV-11 and HPV-16. As can be seen in Fig. 2d, HPV-16 E7 binds HPV-16 E2 at a significantly higher level than that seen between HPV-11 E2 and E7, suggesting that the E2-E7 interaction is stronger for the high-risk HPV type.

We then proceeded to map the sites of interaction between HPV-16 E2 and E7. The N- and C-terminal halves of E2 (as indicated in Fig. 3a) were first in vitro translated and incubated with GST-16E7 bound to resin. After extensive washing, the bound proteins were analyzed by SDS-PAGE and autoradiography. Figure 3b shows that E7 binds preferentially to the C-terminal half of E2. To further map the site of interaction, a series of truncations along the C-terminal region of E2 fused to GST were purified and tested for the ability to bind in vitro-translated E7. As can be seen in Fig. 3c, E7 only interacts with those E2 proteins which retain an intact hinge region, thereby mapping the E7 site of interaction on E2 to a region spanning amino acid residues 202 to 249, although a role for additional amino acid residues extending to position 306 cannot be excluded.

We then performed a similar mutational analysis to map the site of interaction of E2 on the E7 protein. As can be seen in Fig. 3d, in vitro-translated E2 binds preferentially to the GST-tagged C-terminal half of E7. With a series of deletion mutant forms of E7 (31) translated in vitro, it can be seen that the E7 mutant lacking residues 79 to 83 (∆H9004) is defective in the ability to bind GST-tagged E2 (Fig. 3e). In contrast, the other three mutant forms with changes within the zinc-binding domain of E7 still retain the ability to bind E2.

**HPV-16 E2 and E7 interact in vivo.** Having shown that E2 and E7 can interact in vitro, we then proceeded to investigate whether we could detect the interaction in vivo. U2OS cells were cotransfected with HA-tagged 16E7 and untagged 16E2, and after 24 h cell extracts were incubated with anti-HA antibody cross-linked to agarose beads (Sigma). After extensive washing, the precipitated proteins were analyzed by SDS-PAGE and Western blotting with a polyclonal antibody against 16E2 (32). As shown in Fig. 4, E2 coimmunoprecipitates with E7 but is not precipitated by the anti-HA antibody if E7 is absent. These results demonstrate that E2 and E7 can form a complex in vivo.
HPV-16 E2 increases the stability of HPV-16 E7. Upon the coexpression of E2 and E7 in U2OS cells, we consistently observed that E7 levels were increased in the presence of E2, compared with the expression of E7 alone. As shown in Fig. 5a (lanes 2, 3, and 7), E7 is weakly detectable when expressed alone in either the soluble or the insoluble fraction of the cellular extract (see Materials and Methods), compared with the expression of β-gal, which was used as a marker of transfection efficiency. However, in the presence of E2 the levels of E7 increase markedly (Fig. 5a, lanes 4, 5, 8, and 9). To inves-
and monoclonal anti-HA antibodies. The expression of different combinations of E2 and E7 probed with polyclonal anti-E2 of soluble and insoluble fractions of the cellular extracts expressing E7 were transfected with or without E2. Shown are Western blot assays probed with 16E2 in U2OS cells. (a) Different amounts of HA-tagged proteins were detected by Western blotting (WB) with anti-E2 or anti-HA antibody. Fifteen percent of each cell extract used for the precipitation was included as inputs. The arrow indicates the immunoglobulin G light chain.

FIG. 4. E2 and E7 interact in vivo. U2OS cell extracts expressing 16E2 alone or with HA-16E7 were incubated with anti-HA antibody linked to agarose beads, and the immunoprecipitated (IP) proteins were detected by Western blotting (WB) with anti-E2 or anti-HA antibody. Fifteen percent of each cell extract used for the precipitation was included as inputs. The arrow indicates the immunoglobulin G light chain.

tigate whether this increase in the levels of E7 in the presence of E2 was due to the interaction between the two proteins, we repeated the assay but included two E7 mutant forms. The results obtained are shown in Fig. 5b, where it can be seen that the mutant form of E7 (Δ4) that is defective for E2 binding is unaffected by the presence of E2 while the Δ3 mutant form of E7, which retains E2-binding activity, is stabilized by E2 in a manner similar to that seen with wild-type E7.

We were then interested in investigating the effects of E2 on E7 protein expressed endogenously in CaSKi cells. Therefore, CaSKi cells were transfected with E2 and the levels of E7 protein in a total cell extract were analyzed by Western blotting with antibodies against E7 and actin as a loading control. As demonstrated in Fig. 6a, the levels of E7 protein are markedly increased in the presence of E2. This is independent of E7’s mRNA levels, as tested by RT-PCR on cells transfected in a parallel experiment (Fig. 6b). To test whether the increase in the levels of E7 was due to its increased stability, CaSKi cells were transfected with E2 and 24 h later cycloheximide was added to block protein synthesis. Cell extracts were then made at different time points and E7 expression analyzed by Western blotting. As can be seen in Fig. 6c, the levels of E7 by itself are significantly decreased between 15 and 30 min into the chase, compared with the levels of actin, which was used as a loading control for equal levels of cell extract. In contrast, in the presence of E2, the initial level of E7 is approximately twofold higher and this remains constant for much longer and can still be detected up to 2 h into the chase (Fig. 6d). Similar results were also obtained by transient transfection in U2OS cells, which again was independent of any effects on E7 gene expression (data not shown). Taken together, these studies demonstrate that E2 increases the stability of the E7 protein.

E7 colocalizes with E2 on mitotic chromosomes. Having shown that E2 binds and stabilizes the E7 protein (Fig. 5 and 6) but at the same time inhibits its transforming activity (Fig. 1), we were next interested in investigating the potential effects of E7 upon E2 function. To do this, we analyzed the effects of E7 upon E2 transcriptional activity and upon the ability of E2 to bind DNA. In both cases, no significant effects were observed (data not shown).

A recently described novel function of the E2 protein involves binding to mitotic chromosomes, which is thought to be important for the segregation of viral genomes into daughter cells. This activity of E2 has mainly been studied with the E2 protein derived from bovine papillomavirus type 1 (BPV-1), which was shown to require binding to the cellular protein Brd-4 in mitotic cells (60). Prior to investigating the potential effects of E7 upon E2 localization during mitosis, we first sought to examine whether HPV-16 E2 could bind to mitotic chromosomes similarly to what has been reported for BPV E2. To do this, E2 was overexpressed in U2OS cells and 48 h later the cells were fixed and E2 was detected with a polyclonal antibody against HPV-16 E2. The cells were also stained with Hoechst stain to visualize cellular chromosomes. The cells were not synchronized by drug treatment, and visualization of the chromosomal patterns was used to determine the stage of mitosis (48) in which the cells were fixed. As can be seen in Fig. 7a, HPV-16 E2 shows diffused nuclear staining in interphase cells and during the initial stages of mitosis it is excluded from condensed chromosomes (Fig. 7b). As the cell enters telophase, E2 localizes to mitotic chromosomes (Fig. 7b, bottom).

Since both E6 and E7 are known to induce chromosomal segregation defects (19), we were then interested to see whether they exert these effects by binding directly to the
chromosomal arms. Neither E7 nor E6, when transfected alone, was detected on mitotic chromosomes in any of the stages of mitosis; instead, they showed diffuse staining and chromosomal exclusion (Fig. 7c and d). However, when E7 was coexpressed with E2 it could clearly be detected on mitotic chromosomes together with E2 at telophase (Fig. 8a, bottom). The colocalization of E2 and E7 was also confirmed by scanning confocal microscopy (Fig. 8a, upper right part). As an additional control, E2 was also detected with a previously described monoclonal antibody (27) together with a rabbit anti-HA antibody to detect E7 (Fig. 8a, lower right part), and a similar pattern of expression was observed. To further verify the specificity of the antibodies used in the fluorescence experiments, telophase cells expressing either E2 or HA-tagged E7 alone were also stained with both rabbit anti-E2 and mouse anti-HA antibodies. Figure 8b shows that in the absence of the antibody-specific protein, very low background staining was obtained, indicating no cross-reaction between the different antibodies and proteins. To verify that E2-induced relocalization of E7 onto chromosomes was dependent upon the association between E2 and E7, we repeated the assay with the Δ4 mutant form of E7 which cannot bind to E2. As can be seen in Fig. 8c, E2 shows clear costaining with the chromosomes while the E7 Δ4 mutant form does not. Finally, the specificity of the relocalization was further confirmed by the inclusion of HA-tagged 16E6, which was previously shown to interact with E2 (25) and where no E2-induced alteration of the pattern of E6 expression was seen during mitosis (Fig. 8d). These results demonstrate a specific relocalization of the E7 protein onto mitotic chromosomes as a direct result of its interaction with E2 during telophase.

DISCUSSION

Unlike lytic viruses, HPVs can replicate their DNA and release infectious virions without resulting in cellular death or transformation. The virus initially infects the basal layer of the epithelium, and production of mature virions is only observed in the upper granular layer (29). With respect to the papillomavirus life cycle, cellular immortalization and viral genome integration are both disadvantageous for the virus; the replicative capacity of the virus is lost with the loss of the E2
protein, and immortalized cells are unable to differentiate into the stratum corneum, where mature virions are formed and shed. Therefore, to ensure an efficient and reproductive life cycle, HPVs finely modulate the activities of cellular proteins and their own viral gene products through various interactions.

Several studies have shown the regulation of expression of viral oncoproteins E6 and E7 by the E2 protein (14, 24). In this study, we have identified a direct interplay between the HPV-16 E2 and E7 proteins. Initially, we observed E2 inhibition E7's growth-promoting function in the absence of any E2-mediated transcriptional modulation (Fig. 1). This result provides evidence of a transcription-independent ability of E2 to counteract the activity of the viral oncoprotein. This is in agreement with previously published data showing that both HPV-16 and -18 E2 proteins can induce apoptosis in HPV-positive cell lines in the absence of their DNA-binding domains (11, 58). In addition, it has been previously shown that mutations in the E2-binding sites proximal to the p97 promoter do not fully alleviate E2-mediated repression of HPV-16-induced immortalization of primary human keratinocytes (43).

By providing evidence of a direct interaction between E2 and E7, both in vivo and in vitro, we propose a novel mechanism by which E2 interferes with the oncogenicity of E7. The transforming activity of E7 depends on its interference with the activity of cell cycle regulatory proteins. Although binding to and disrupting the function of pRB are considered to be the major activities of E7, many studies have shown that this binding is dispensable in some E7-induced phenotypes and is insufficient for E7-induced transformation (3, 18, 50). In vitro binding assays showed that E2 binds directly to the C-terminal protein.
half of E7 at a region where several cellular targets of E7 bind, such as TBP (31), the Mi2-NURD histone deacetylase complex (7), and the AP1 transcription factor (2). This suggests that the binding of E2 to E7 might compete with the binding of E7 to some of its cellular targets and thus inhibits its activity. Another mechanism by which E2 could repress the activity of E7 could be by reducing the cellular levels of the E7 protein. However, our data do not support this hypothesis. The cellular levels of E7, both endogenous and overexpressed, were markedly stabilized by the addition of E2. Although E2 can act as both a transcriptional activator and a repressor in a concentration-dependent manner (51), we excluded the role of E2's transcriptional activity in increasing the levels of E7 for the following reasons. First, E7's levels were stabilized by E2 even in the absence of an E2-binding site on the E7 promoter when both proteins were overexpressed in U2OS cells. Second, in the CaSkI cell line, which contains the LCR, the half-life of E7 was increased, independently of the HPV mRNA levels at the specific concentrations used in this study. Finally, the levels of the Δ4 deletion mutant form of E7, which is defective in binding E2, were not changed upon the introduction of E2, thus providing further evidence that the stability of E7 is increased due to direct protein-protein interaction between E2 and E7. Since E7 has been shown to be ubiquitinated and cleaved by the SCF-ubiquitin ligase complex (37), it will be interesting to investigate whether E2 perturbs this activity.
In this study, we observed both a repression of E7’s transforming activity and an increase in its stability in the presence of E2; we were then interested in seeing whether E7 might interfere with the functions of E2. While we saw no effects on E2-mediated transcriptional activation or DNA-binding activity, we found intriguing colocalization of E7 with E2 on mitotic chromosomes. The first suggestion that a papillomavirus-encoded protein can associate with mitotic chromosomes came from a study of BPV E2 (49). BPV-1 E2 was shown to bind to the bromodomain-containing protein Brd4 (60), which attaches to acetylated chromatin during interphase and mitosis (12). Although BPV and HPV E2 proteins display identical DNA sequence specificities and similar functions, they vary in some of their characteristics (5, 28). Here, we have found that E2 from HPV-16 also possesses a chromosome-binding phenotype but, unlike BPV-1 E2, binds chromosomes only at late stages of mitosis. Therefore, we anticipate that 16E2 may use an alternative method to that used by BPV-1 E2 to localize to and bind mitotic chromosomes. Previously, HPV E2 was shown to be excluded from metaphase chromosomes (57). Here we confirm the exclusion of 16E2 from mitotic chromosomes in metaphase; however, in contrast to that study (57), we did not observe a pattern of HPV-16 E2 expression that indicates its localization to mitotic spindles or centrosomes. This difference might be due to the use of different cell lines, different E2 expression constructs, and different detection and fixation procedures. Further studies are required to clarify these issues.

Most interestingly, we observed that 16E2 relocates E7, but not E6, to mitotic chromosomes at telophase. This suggests that the relocalization of E7 by E2 is a highly specific event. This was further borne out by the use of an E7 mutant form that fails to bind E2, which likewise also failed to colocalize with E2 on mitotic chromosomes. We cannot exclude the possibility that this E7 mutant form has other properties which preclude E2 stabilization and recruitment to chromosomes, and future studies will aim to clarify this by using E2 mutant forms that are defective for binding to E7. The biological function of this specific localization is also still unidentified, and it remains to be determined whether E2 alters E7’s patterns of expression in the context of a normal viral life cycle. Studies are in progress to investigate this in raft culture models of HPV infection. However, these studies are particularly intriguing when one considers the effects of E6 and E7 upon mitosis, where both have been shown to separately induce mitotic abnormalities when stably expressed in cultured cell lines or cells derived from transgenic mice (17, 38, 45). Further dissection of the role of each protein showed that when each protein is transiently expressed, only E7 results in immediate chromosomal abnormalities (15, 16). This suggests that E7 has a direct effect in inducing centrosomal abnormalities, while the effects induced by E6 might be an indirect consequence of the abrogation of p53 function. Provided with the direct role of E7 in interfering with the mitotic machinery, the tethering of E7 to mitotic chromosomes may be required to directly inhibit cellular checkpoint proteins that might be activated in response to the detection of E2 on mitotic chromosomes, thus avoiding cell cycle arrest in mitosis. Another possible function would be to segregate E7 itself and thus ensure that appropriate amounts of the protein will be present in newly divided daughter cells. E7 might also be required to stabilize the binding of E2 to the viral episomes or mitotic DNA during cell division, and current studies are aimed toward assessing the effects of E7 upon E2 during exit from mitosis.

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