JOURNAL OF VIROLOGY, Dec. 2005, p. 14956–14961 Vol. 79, No. 23
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.23.14956–14961.2005


# NOTES

## Inhibition of E2 Binding to Brd4 Enhances Viral Genome Loss and Phenotypic Reversion of Bovine Papillomavirus-Transformed Cells

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Received 12 June 2005/Accepted 1 September 2005

**The bovine papillomavirus E2 protein tethers the viral genomes to mitotic chromosomes in dividing cells through binding to the C-terminal domain (CTD) of Brd4. Expression of the Brd4-CTD competes the binding of E2 to endogenous Brd4 in cells. Here we extend our previous study that identified Brd4 as the E2 mitotic chromosome receptor to show that Brd4-CTD expression released the viral DNA from mitotic chromosomes in BPV-1 transformed cells. Furthermore, stable expression of Brd4-CTD enhanced the frequency of morphological reversion of BPV-1 transformed C127 cells resulting in the complete elimination of the viral DNA in the resulting flat revertants.**

The papillomaviruses are a group of small DNA viruses that cause benign lesions in higher vertebrates, including humans. The "high-risk" human papillomaviruses (HPVs) are associated with a number of human cancers including cervical cancer (21). The papillomaviruses have a specific tropism for squamous epithelial cells and infect cells within the basal epithelial layer to establish an infection. Late gene expression, lytic DNA amplification, and virus production are restricted to the more terminally differentiated cells of the epithelium (9).

During the life cycle of the papillomaviruses, the viral DNA is maintained as an extrachromosomal plasmid at a low-copy level in infected cells (9). Mouse cells transformed by BPV-1 maintain the viral DNA in a stable extrachromosomal plasmid state and have served as an excellent model for studying viral DNA replication and genome maintenance (8, 12, 14). The maintenance of the transformed phenotype requires the continued presence of viral genomes; cells cured of the viral genomes revert to a flat, nontransformed phenotype (17).

To ensure that the viral genomes are not lost upon breakdown and reassembly of the nuclear membrane during cellular mitosis, papillomaviruses, like Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus, use strategies to maintain their genomes in the nuclear space through the noncovalent association of their genomes to cellular mitotic chromosomes via a virally encoded DNA-binding protein (2, 10, 11, 16).

Papillomavirus genome maintenance has been best studied for BPV-1 (3, 11, 13, 15, 16). The persistence of the viral genomes is mediated through the multiple E2-binding sites of BPV-1 genome (15). E2 binds these specific sites through its DNA-binding domain and tethers BPV-1 DNA to mitotic chromatin in dividing cells through its transactivation domain (3, 13, 16). E2 mutations abrogating the mitotic chromosome attachment lead to the dramatic loss of viral genomes from BPV-1 transformed cells (13). Mutations in the transactivation domain have also been shown to disrupt the tethering of viral genomes to mitotic chromosomes (1, 4, 20).

Our previous work identified the bromodomain-containing protein 4 (Brd4) as the chromosome associated protein through which E2 and the viral DNA bind mitotic chromosomes (19). Brd4 is a member of the BET family proteins and associates with mitotic chromosomes during mitosis (6, 7). E2 binds Brd4 through the C-terminal domain (CTD) of Brd4. The Brd4-CTD can be stably expressed in cells where it inhibits the binding of E2 to endogenous Brd4 on mitotic chromosomes, prevents the tethering of BPV-1 DNA to Brd4 and blocks BPV-1 transformation of mouse C127 cells (19). Additional evidence has confirmed the role of Brd4 as the tether for E2 and viral genomes on mitotic chromosomes (4, 5).

In the present study, we have further examined the functional significance of E2-Brd4 interaction in BPV-1 genome maintenance in BPV-1 transformed H2 cells and tested the potential of the Brd4-CTD to cure BPV-1 transformed cells of viral DNA.

**E2 and Brd4 bind directly.** We previously showed that Brd4 interacts with E2 in cells to form a molecular bridge linking the papillomavirus genomes to host mitotic chromosomes (19). However, we did not establish whether the E2 and Brd4 interaction was directly or mediated by an intermediate factor. We therefore tested whether their binding is direct. Purified recombinant Brd4-CTD was incubated with purified glutathione *S*-transferase (GST) fusion proteins containing HPV-16E2, BPV-1 E2TA (full-length E2) or BPV-1 E2TR (truncated E2 lacking the transactivation domain) (Fig. 1A) immobilized on glutathione beads. E2TR that does not bind endogenous Brd4 served as a negative control (19). Brd4-CTD bound efficiently to both GST-16E2 and GST-E2TA but not to GST-E2TR (Fig. 1B), demonstrating that the binding between E2 and Brd4 is direct.

**Brd4-CTD dissociates BPV-1 viral genomes from the host**

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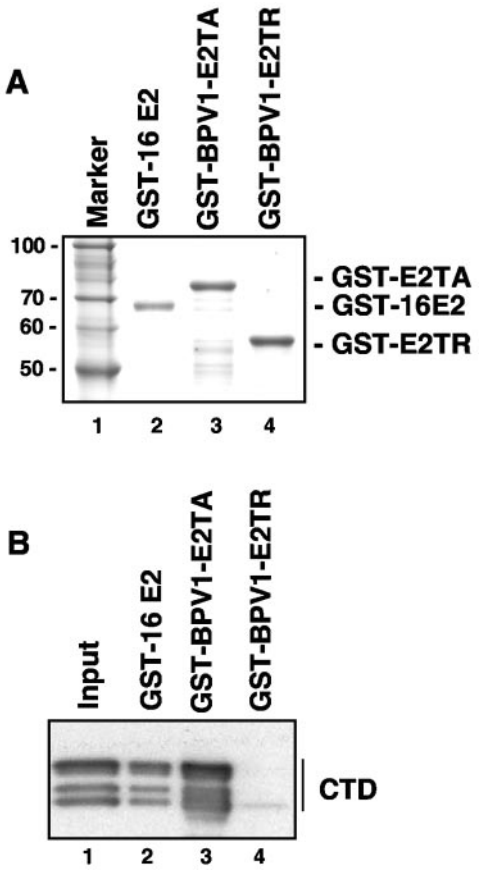


FIG. 1. Direct interaction between E2 and the Brd4-CTD. (A) Purification of GST-E2 proteins. GST fusion proteins were produced in *E. coli*. The fusion proteins, purified and immobilized on glutathione resin, were eluted by sodium dodecyl sulfate (SDS) sample buffer, resolved on SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by Coomassie blue staining as input control. (B) In vitro binding of recombinant Brd4-CTD to E2 protein. The Brd4-CTD fragment was produced in *E.coli* from the pET32a plasmid encoding the His tagged Brd4-CTD. After purification over a Ni-nitrilotriacetic acid column, the tag was cleaved with enterokinase. Then, 10 μg of Brd4-CTD was mixed with 5 μl of immobilized GST-E2, and the binding was performed as described previously (19). Eluates from GST-E2 beads were resolved by SDS-PAGE, along with 60% of the Brd4-CTD input, and detected by Western blotting using a Brd4 antibody C-MCAP (7). Note that the smaller fragments shown in panel B represent the proteolytic cleavage products of Brd4-CTD. They are still recognized by the antibody against the C-terminal 14 amino acids of Brd4, suggesting that the cleavages occurred near the N terminus of the Brd4-CTD. Consistent with our previous result showing that the E2-binding domain can be further mapped to the last 138 amino acids (amino acids 1224 to 1362) of Brd4, these C-terminal fragments of Brd4-CTD all bound to E2.

**mitotic chromosomes.** Brd4-CTD can abolish the Brd4/mitotic chromosome association of E2 as well as the tethering of BPV-1 DNA to Brd4 (19). We therefore tested whether Brd4-CTD could dissociate the viral DNA from host mitotic chromosomes in H2 cells, a clonal line of C127 cells harboring exclusively extrachromosomal BPV-1 DNA. H2-CTD and H2-V cell lines were established previously by transduction of retroviruses expressing either the Xpress-tagged Brd4-CTD or empty vector (19). Immunofluorescent staining using anti-Xpress antibody verified that Brd4-CTD was expressed in ~90% of H2-CTD cells. As expected, BPV-1 DNA was detected as punctuate dots associated with host mitotic chromosomes in H2-V cells (Fig. 2) (13, 16). However, the fluorescence in situ hybridization (FISH) signal was not detected in H2-CTD cells. In each of the 15 H2-V mitotic spreads examined, metaphase chromosomes were positive for BPV-1 DNA. In contrast, for H2-CTD cells, all 15 metaphase chromosome spreads analyzed were negative for BPV-1 DNA. This result demonstrated that, in blocking the E2-Brd4 interaction, the Brd4-CTD efficiently disrupts the association of BPV-1 DNA with mitotic chromosomes, further confirming that the viral genome-host chromosome interaction is mediated by E2/Brd4 binding. The cells analyzed were at passage 3 after retrovirus transduction. As described below, the H2-CTD cells at passage 3 and at later passages still contain BPV-1 DNA. Therefore, the lack of any detectable DNA associated with the mitotic chromosomes in these cells reflects the fact that the viral genomes, while still present in the cell, are no longer tightly associated with mitotic chromosomes. The dissociated genomes are presumably washed away by the hypotonic washes and the Carnoy's fixative used in the FISH procedure.

**Brd4-CTD induces morphological reversion in H2 cells.** The FISH data predicted that Brd4-CTD expression might lead to the curing of the extrachromosomal DNA from H2 cells since they were no longer tightly associated with host mitotic chromosomes. To address this question, both H2-CTD and H2-V cells were continuously split at 1:10 ratio. In early-passage cells, there were no obvious morphological differences between the control and CTD-treated cells. At passage 4, however, we observed some flat cells resembling nontransformed parental C127 cells in the H2-CTD culture but not in H2-V cells. This flat phenotype became more evident with continued passage (Fig. 3A). By passage 12, the majority of the Brd4-CTD-expressing cells showed a nontransformed morphology with only occasional transformed cells intermingled among the flat cells (data not shown), whereas the H2-V cells retained the transformed morphology throughout the analysis (Fig. 3A). Therefore, Brd4-CTD expression led to a progressive reversion from the transformed phenotype to a flat cell morphology resembling the parental C127 cells. Furthermore, stable expression of the Brd4-CTD had no effect on the morphology or growth characteristics of non-transformed C127 cells, HeLa cells or C33A cells (data not shown).

**CTD lowers the BPV-1 plasmid number in H2 cells.** To test whether the phenotypic reversion observed in H2-CTD cells after multiple cell passages results from the loss of viral DNA in these cells, we compared the levels of BPV-1 DNA in H2-CTD and H2-V cells at each passage. Real-time PCR analysis for BPV-1 genome demonstrated that the BPV-1 DNA copy numbers remained nearly unchanged for the first three passages in both cell lines (data not shown). At passages 3 to 6, the H2-V cells maintained approximately 35 ± 14 copies of viral DNA/cell compared to the 40 copies/cell for H2 cells. Despite some experimental variability, there was no significant trend of DNA reduction detected in H2-V cells. In contrast, analysis of the same passages from H2-CTD cells showed a dramatic decrease of BPV-1 DNA level at passages 4 and higher. By passage 6, the relative abundance of viral DNA in H2-CTD cells was reduced to approximately 25% of the level in H2-V cells (or approximately 8 copies/cell). This decrease in BPV-1

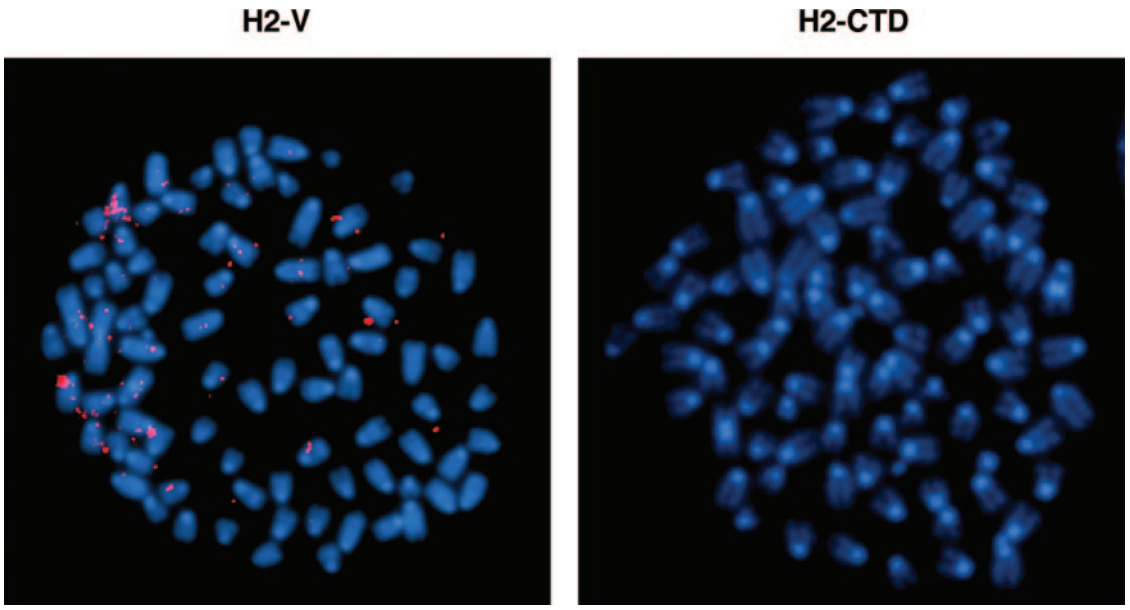


FIG. 2. Expression of the Brd4-CTD abrogates association of BPV-1 genomes with host mitotic chromosomes. Passage 3 H2-CTD and H2-V cells were cultured in chamber slides and arrested at metaphase by a 2-h incubation with 1 μg of Colcemid/ml. Cells were lysed with hypotonic solution (0.56% KCl) and fixed to glass slides by using Carnoy's fixative (75% methanol and 25% acetic acid) before hybridization with a BPV-1 probe labeled in red with fluorescence labeling reagents from Vysis. Cells were also counterstained with Vysis DAPI (4′,6′-diamidino-2-phenylindole) II antifade and examined using an Olympus AX70 microscope with red-green-DAPI filters and Genus software from Applied Imaging.

DNA in H2-CTD cells after passage 3 was confirmed by the Southern blot analysis (data not shown). The timing of the significant genome loss in H2-CTD cells at passage 4 coincided well with the initial appearance of flat revertants in cell culture, suggesting that the flat H2-CTD cells were due to the loss of viral DNA in these cells.

This influence of Brd4-CTD on the transformed cell morphology and viral genome level was observed several passages (≥4) after the CTD was transduced into the cells. This result is consistent with our previous chromatin immunoprecipitation result showing that Brd4-CTD expression in H2 cells did not cause a significant dilution and/or loss of the viral DNA in a single passage but did dissociate the viral DNA from Brd4 (19). With continued passage, some of the Brd4-CTD-expressing cells may eventually lose the viral genomes and revert to a nontransformed phenotype. We have recently found that the E2 transcriptional activation function is also dependent upon Brd4 and that the Brd4-CTD inhibits this function (M.-R. Schweiger, J. You, and P. M. Howley, submitted for publication). Whereas it is possible that the inhibition of E2 transcriptional activation by Brd4-CTD could contribute to the morphological reversion of H2 cells, previous studies have shown that BPV-1 genomes mutated for the E2 transactivation function are still transformation competent (18). Thus, the nearly complete dissociation of viral DNA from host mitotic chromosomes at the early stage of Brd4-CTD expression as shown by FISH analysis argues strongly that disrupted tethering is responsible for the loss of viral genomes and the resulting morphological reversion in the cells. The ability of Brd4-CTD to completely dissociate viral plasmid from mitotic chromosomes makes it likely that Brd4 might be the sole receptor for BPV-1 E2 and viral DNA during mitosis.

**Colony morphology analysis of Brd4-CTD-induced reversion.** Although we detected a significant decrease of BPV-1 DNA levels in passage 4 to 6 of H2-CTD cells, the DNA content leveled off after passage 6. We speculated that the continued culture of H2-CTD cells would provide a selective advantage for transformed cells harboring BPV-1 DNA. To establish a direct link between Brd4-CTD-mediated viral genome loss and morphological reversion, we examined H2-CTD cells at a single-cell level by analyzing colony morphology. H2-CTD and H2-V cells from each passage were plated as single cells at 20 to 30 cells per plate to evaluate the morphology of colonies derived. After staining with methylene blue, transformed colonies that were not contact inhibited and grew to high cell density stained dark blue. In contrast, flat revertants, which form a contact inhibited monolayer, stained light blue (Fig. 3B). Interestingly, a third population of colonies containing both light and dark blue staining, hence termed "mix clones," was also observed (Fig. 3B). In contrast to the homogenous populations of cells in the transformed and flat colonies, the mixed colonies had both cell types. We examined ~100 colonies for each cell line at each passage for 10 passages and quantitated each colony type. A representative analysis is shown for passage 6 in Fig. 3C. For H2-CTD cells, the majority of the colonies were either flat (32%) or mixed (62%) and only 6% of the colonies were fully transformed, whereas for H2-V cells, 50% of the colonies retained a fully transformed morphology.

Notably, a large percentage of the colonies derived from the H2-CTD cell line showed mixed colony morphology. In some cases, only the cells in the middle of the colony retained the transformed morphology, whereas in others transformed cells form a pie-shape patch emanating from the center of the colonies. The sectoring was not a function of cell plating density because H2-V cells plated at the same density gave rise to a much lower number of such colonies. Rather, these sectoring patterns were reminiscent of the plasmid sectoring phenotype of a BPV-1 mutant in which the plasmid segregation is compromised by an E2 mutation (13). This sectoring pattern sug-

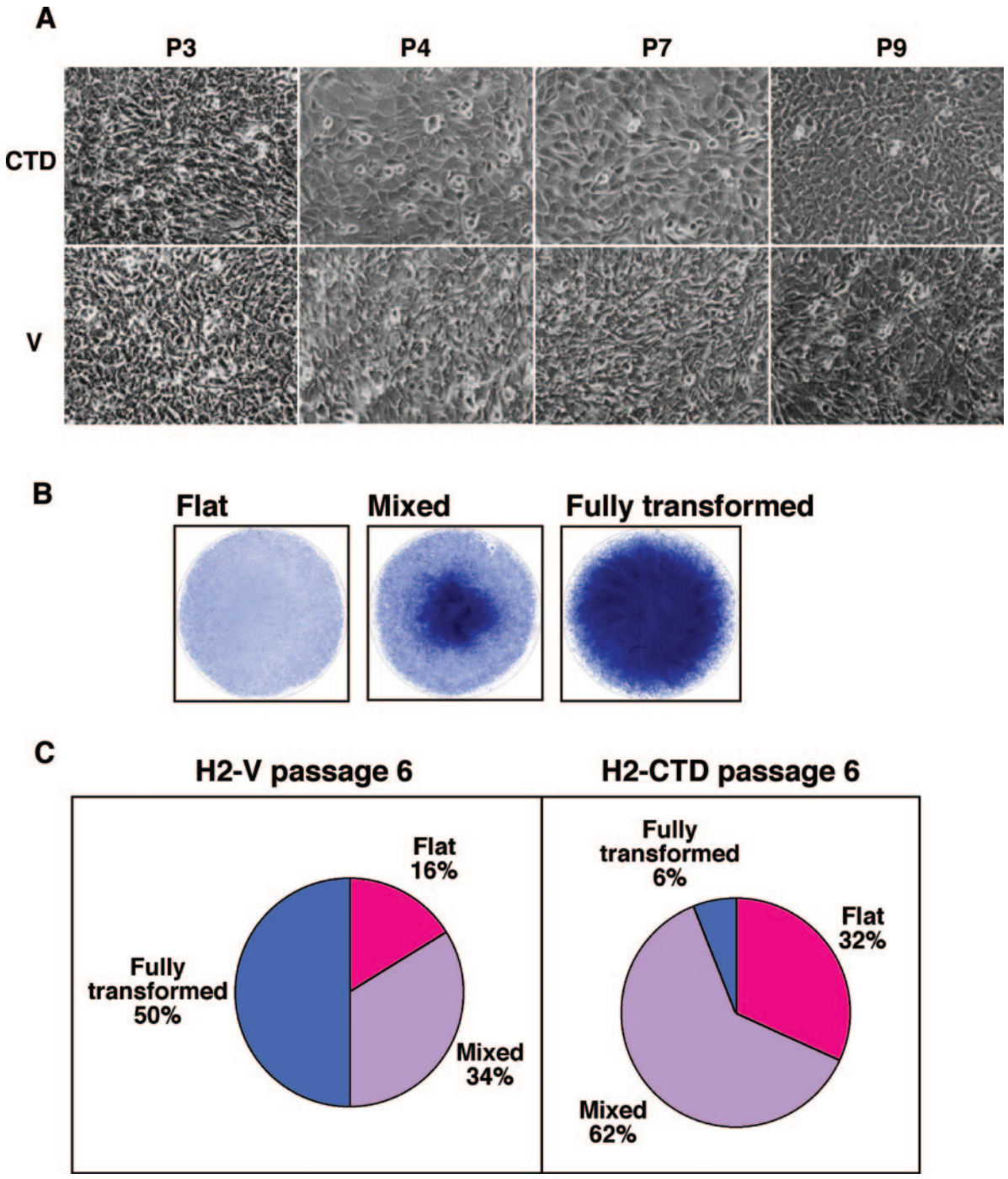


FIG. 3. (A) Morphological analysis of Brd4-CTD-expressing H2 cells. Both H2-V and H2-CTD cells were split at 1:10 and grown for the indicated passages. The H2-V cells retained a transformed morphology characterized by spindle-shaped cells that grow to high saturation density. In contrast, many of the H2-CTD cells reverted to the flat morphology typical of nontransformed C127 cells by passage 4. The cell morphology was examined by using a Nikon DIAPHOT 200 microscope equipped with a Nikon N6000 camera. Magnification, ×100. (B and C) Morphological analysis of individual colonies isolated from H2-V and H2-CTD cells. Cells seeded at low density were cultured for 20 days to allow colony formation. The plates were stained with methylene blue as described previously (19). The colonies were scanned using a UMAX Power Look 1120 scanner. Quantitation of the three different types of clones from cells at passage 6 is shown in panel C.

gested a plasmid maintenance defect in H2-CTD cells where the BPV-1 plasmids are no longer tightly associated with host chromosomes. We reasoned that the sectored colonies arose by an asymmetrical distribution of BPV-1 molecules to daughter cells.

At passage 11, single-cell clones were also isolated by cloning into 96-well plates. Among 30 single clones isolated from H2-V cell line, 4 showed a completely flat morphology and the others were either mixed or fully transformed. In contrast, 12 of 18 single clones isolated from H2-CTD culture showed the revertant flat morphology. Immunofluorescence staining of Brd4-CTD in H2-CTD cells showed that, whereas 80% of the cells still expressed the Brd4-CTD at passage 4, only 5% of the cells at passage 6 and <1% of the cells at passage 10 were

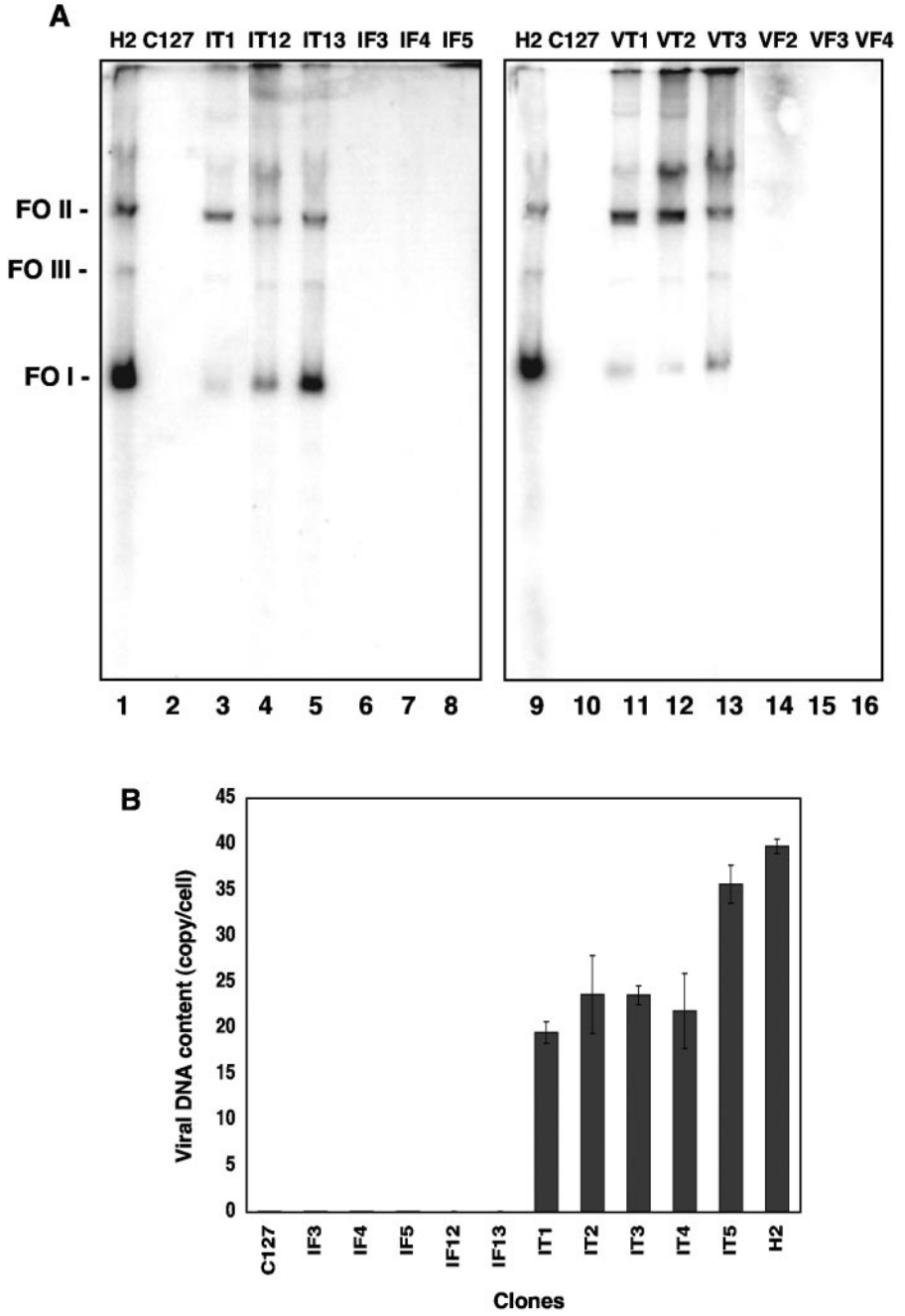


FIG. 4. (A) Analysis of BPV-1 DNA in transformed (IT and VT) and flat revertant (IF and VF) subclones. Total cellular DNA was extracted and assayed for the presence of BPV-1 genomes by Southern hybridization as described previously (12). A total of 20 μg of DNA was cleaved with SalI, and the resulting fragments were separated on a 0.8% agarose gel, denatured, and transferred to a Hybond N+ membrane. Cloned BPV-1 DNA was labeled using a Prime-It random primer labeling kit (Stratagene) and hybridized to DNA immobilized on the membrane. After washing, the filters were exposed to Kodak MS film. FO I and FO II, supercoiled and nicked circular extrachromosomal DNA, respectively; FO III, linear viral DNA. (B) Real-time PCR quantitation of the viral genomes in both transformed and reverted clones isolated from H2-CTD cell line. Cellular DNA was extracted from the cells using Hirt's lysis buffer (0.6% SDS and 10 mM EDTA [pH 8.0]) at 4°C for 15 min, followed by extraction in 1 M NaCl at 4°C overnight. After centrifugation at 14,000 × *g* for 30 min at 4°C, the supernatant was analyzed using a LightCycler machine (Roche) according to the manufacturer's instructions. The PCR primers used in the analysis were designed to amplify a 432-bp region spanning nucleotides 2601 to 3032 of BPV-1 DNA.

positive for Brd4-CTD expression. Therefore, these data suggested that the frequency of revertants continued to increase even after the loss of Brd4-CTD expression, perhaps reflecting inefficient partitioning of the viral genomes once the copy number was reduced. The reduction of Brd4-CTD expression might be a factor that limited the capacity of Brd4-CTD to "cure" viral genomes in all of the cells treated. As the Brd4-CTD expression is lost with passage, it is expected that E2

would regain its ability to bind Brd4 and mitotic chromosomes. Nonetheless, this morphological analysis of the individual colonies and clones demonstrated that the Brd4-CTD expression significantly enhanced the reversion of transformed H2 cells to a flat nontransformed phenotype.

**Phenotypic reversion resulted from the loss of viral genomes.** We next tested whether the flat cell reversion seen in H2-CTD cells was due to the loss of viral genomes. Independent colonies from passage 11 were expanded to determine whether the cells still harbored BPV-1 DNA. The clones derived from the H2 expressing the Brd4-CTD "inhibitor" were labeled either as "IT" for transformed or as "IF" for flat revertant. Similarly, the clones derived from H2-V cells carrying the retrovirus "vector" were labeled as "VT" and "VF" depending upon their morphology. Immunofluorescent staining of Brd4-CTD in the isolated "IF" or "IT" clones showed that none of these cells retained Brd4-CTD expression in agreement with our analysis that Brd4-CTD expression was lost during cell passaging. The fact that the reverted morphology persisted even after the Brd4-CTD expression was lost indicated that the reversion was due to the permanent loss of the viral genome rather than an effect of the Brd4-CTD on either cellular or viral gene expression. We next analyzed the viral DNA levels in the isolated clones. Total cellular DNA was digested with SalI (recognizes no sites in BPV-1 DNA) before Southern hybridization using a BPV-1 probe as described in reference 12). As shown in Fig. 4A, no BPV-1 DNA was detected in C127 cells or any of the flat revertant clones. In the transformed cell lines and in H2 cells, viral DNA was detected in its circular extrachromosomal forms (12). Some of the viral DNA was also converted to full-length linear DNA due to mild shearing of the DNA. This result was confirmed by Southern blot analysis with the single-cut enzyme BamHI (data not shown). Both data suggested that, like H2 cells, the transformed cells harbored the viral DNA in an extrachromosomal state. We also quantitated the viral genome level in H2-CTD clones by real-time PCR. Neither parental C127 cells nor flat revertants contained detectable BPV-1 DNA under conditions sensitive enough to detect 0.1 viral genome per cell (Fig. 4B). The "IT" clones showed some reduction (up to 50%) in the BPV-1 level compared to the parental H2 cells (Fig. 4B). This lower level of DNA could also be seen in Fig. 4A (compare lanes 3 to 5 with lane 1) and suggested that the initial Brd4-CTD expression in H2-CTD cells may have contributed to a reduction of the viral DNA levels. This analysis thus established a direct correlation between viral DNA loss and phenotypic reversion of BPV-1 transformed cells.

Previously, we showed that by blocking E2/Brd4 interaction, Brd4-CTD can inhibit BPV-1 transformation of C127 cells (19). In this study, we show that the Brd4-CTD reduces the BPV-1 genome levels in transformed cells, underscoring the role of E2/Brd4 association in the papillomavirus plasmid maintenance. The ability of the Brd4-CTD to cure infected cells of the PV genomes suggests that targeting E2/Brd4 binding might represent a new strategy for the development of papillomavirus antivirals. BPV-1 transformation provides an excellent model for analyzing plasmid maintenance and for investigating antiviral compounds.

We thank Carl Baker (National Cancer Institute, Bethesda, MD) for the H2 cell line, Suzanne Masotta of the DF/HCC Cytogenetics Core for the FISH analysis, and Jennie Croyle and Carrie Wu for excellent technical support. We are grateful to Jennifer Altreuter and Joseph DeMasi for critical readings of the manuscript.

This work was supported by grants from the National Cancer Institute to P.M.H. J.Y. has been supported by a fellowship from Charles A. King Trust/Bank of America (cotrustees). M.-R.S. was supported by the Studienstiftung des Deutschen Volkes from Germany.

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