Variations in the association of papillomavirus E2 proteins with mitotic chromosomes

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The E2 protein segregates episomal bovine papillomavirus (BPV) genomes to daughter cells by tethering them to mitotic chromosomes, thus ensuring equal distribution and retention of viral DNA. To date, only the BPV1 E2 protein has been shown to bind to mitotic chromosomes. We assessed the localization of 13 different animal and human E2 proteins from seven papillomavirus genera, and we show that most of them are stably bound to chromosomes throughout mitosis. Furthermore, in contrast to the random association of BPV1 E2 with mitotic chromosomes, several of these proteins appear to bind to more specific regions of mitotic chromosomes. Using human papillomavirus (HPV) type 8 E2, we show that this region is adjacent to centromeres/kinetochore. Therefore, E2 proteins from both HPV and animal papillomavirus bind to mitotic chromosomes, and there are variations in the specificity of this binding. Only the α-papillomavirus E2 proteins do not stably associate with mitotic chromatin throughout mitosis. These proteins closely associate with prophase chromosomes and bind to chromosomes in telophase but not in metaphase. However, extraction of mitotic cells before fixation results in α-E2 proteins binding to the pericentromeric region of metaphase chromosomes, as observed for HPV8 E2. We postulate that this is the authentic target of these E2 proteins but that additional factors or a specialized cellular environment is required to stabilize this association. Thus, E2-mediated tethering of viral genomes to mitotic chromosomes is a common strategy of papillomaviruses, but different viruses have evolved different variations of this theme.

human papillomavirus | mitosis | replication | segregation

There are 17 distinct genera of Papillomaviridae (1); each virus infects a specific region of cutaneous or mucosal epithelium in a particular host. For example, human papillomavirus (HPV) types 1, 2, 3, and 4 cause plantar and palmar warts whereas HPV5 and HPV8 are found in cutaneous lesions of epidermodysplasia verruciformis. Mucosal HPV types such as HPV6, HPV11, HPV16, and HPV31 infect genital and sometimes oral epithelia. These infections are usually persistent, and so papillomaviruses need a mechanism to efficiently retain the extrachromosomal genomes within dividing cells. The viral E1 and E2 proteins initiate viral DNA replication by binding to the replication origin. Multiple E2 binding sites are required in addition for the genome to be stably maintained in dividing cells (2). The bovine papillomavirus (BPV) type 1 E2 protein tethers the genomes by means of the E2 binding sites to cellular mitotic chromosomes (3–5) to ensure that they are retained in the nucleus and distributed to daughter cells. This mechanism is a common one in persistent episomal viruses; Epstein–Barr virus and human herpesvirus 8 also encode a protein that tethers the viral genomes to mitotic chromosomes to ensure their faithful segregation.

The E2 protein is also a transcriptional regulator, consisting of a transactivation domain and a dimerization/DNA-binding domain, linked by a nonconserved flexible hinge region. The functional domains have 35% similarity among papillomaviruses. The transactivation domain of BPV1 E2 is necessary for interaction with mitotic chromosomes (6) whereas the DNA binding domain is required to tether the viral genomes. Shorter E2 repressor proteins, which contain only the DNA binding/dimerization domain, antagonize the functions of the full-length protein and are unable to associate with mitotic chromosomes. The cellular bromodomain protein Brd4 is important for the association of the BPV1 E2 protein and viral genomes with mitotic chromosomes (7–9). Brd4 binds acetylated histones and is associated with chromatin throughout mitosis (10, 11). Brd4 colocalizes with E2 in distinct speckles on mitotic chromosomes (7), and disruption of this interaction dissociates E2 from chromosomes (8). Thus, the E2–Brd4 association is crucial for BPV1 episomal genome segregation. However, the association of Brd4 with other E2 proteins is complex and requires further investigation.

Among the papillomaviruses, genome segregation and chromosomal attachment have been best studied for BPV1. Since the BPV1 E2 protein and genome chromosomal attachment was first discovered, there have been no published reports of other E2 proteins attached to mitotic chromosomes, although there is one report that the HPV11 protein associates with the mitotic spindle (12). This failure to observe chromosomal attachment of other E2 proteins is not due to lack of effort. Rather, it is mainly due to difficulties expressing these proteins in a functional state in mitotic cells. The E2 proteins are often difficult to detect, are prone to misfolding and aggregation, and can induce cellular growth arrest and apoptosis (13, 14). To gain insight into the mechanism by which other papillomavirus genomes are stably maintained, we examined the interaction of multiple papillomavirus E2 proteins with mitotic chromatin.

We find that many E2 proteins associate with mitotic chromosomes like BPV1. Furthermore, certain E2 proteins, such as HPV8, interact with specific regions on mitotic chromosomes adjacent to kinetochores. And, finally, E2 proteins from α-papillomaviruses are not stably associated with chromosomes throughout mitosis. However, prefixation treatments of cells expressing these proteins result in a chromosomal binding pattern similar to that of HPV8 E2. Thus, tethering genomes to mitotic chromosomes by the E2 protein is a common strategy that might involve different mechanisms.

Results

Cloning and Expression of the Papillomavirus E2 Proteins. Thirteen papillomavirus E2 proteins were tested for their ability to associate with mitotic chromosomes. Seven human viruses (HPV1a, HPV4, HPV8, HPV11, HPV16, HPV31, and HPV57) and six animal viruses [cottontail rabbit papillomavirus (CRPV), rabbit oral papillomavirus (ROPV), canine oral papillomavirus (COPV), European elk papillomavirus (EEPV), deer papillo-
mavirus (DPV), and BPV1] were chosen. These viruses are widely representative of the Papillomaviridae family, as shown in the phylogenetic tree based on the E2 amino acid sequences in Fig. 1.

The E2 proteins were expressed from the pMEP4 vector with a FLAG epitope tag at the N termini. Earlier experiments had shown that HPV11, HPV16, and HPV31 E2 proteins with C-terminal epitope tags were not stably expressed in mitotic cells (data not shown). Other experiments demonstrated that an N-terminal vesicular stomatitis virus glycoprotein tag resulted in nonnuclear localization of HPV11 and HPV16 E2 proteins. Only proteins with an N-terminal FLAG tag showed the characteristic nuclear E2 pattern (data not shown). Therefore, each papillomavirus E2 protein was expressed with an N-terminal FLAG tag.

pMEP4 is an episomally maintained vector that expresses genes from an inducible metallothionein promoter. It contains a hygromycin resistance gene that allows transfected cells to be selected and pooled into a stable cell line. By varying the induction conditions for the metallothionein promoter, we have shown that correctly localized and functional levels of BPV1 E2 protein were expressed from this vector (15). The E2 chromosome-binding assay requires moderate expression levels of E2 in a large percentage of cells. E2 levels that are too low are difficult to detect in mitotic cells, and levels that are too high result in aggregated protein. The inducible pMEP4 system also allows us to circumvent the potential cell growth inhibition that has been described for several E2 proteins (13). By using this system, ~85% of mitotic cells have BPV1 E2 protein associated with the condensed chromosomes (6).

**Codon Optimization Increases Expression of Select E2 Proteins.** Most E2 proteins showed good nuclear expression and were of the expected molecular weight. However, HPV4, HPV11, HPV16, and HPV31 proteins were poorly expressed, even when induced with higher concentrations of CdSO4. To increase the levels of these proteins, the 5' ends of HPV4, HPV11, and HPV16 genes and the entire HPV31 gene were resynthesized to change the codons to those most frequent in mammalian cells. In theory, this strategy increases translation because of the abundance of tRNAs available to the most common codons. However, protein expression can also increase because of the removal of negative RNA regulatory elements. A substantial increase in E2 protein levels was observed by immunoblotting and immunofluorescence for each of the recoded E2 proteins. Fig. 9, which is published as supporting information on the PNAS web site, shows the increase in E2 expression, as observed by immunoblotting and immunofluorescence. The resulting expression levels of all 13 papillomavirus E2 proteins in CV-1 cells are shown in the immunoblot in Fig. 2. The molecular weight differences observed result from variable lengths of the E2 hinge regions.

**All 13 E2 Proteins Activate Transcription from an E2-Dependent Promoter.** To ensure that the E2 proteins were functional and that the FLAG epitope did not interfere with the properties of the transactivation domain, the proteins were tested for their ability to activate transcription from the reporter plasmid, pBS1073, which contains four E2 binding sites upstream from the thymidine kinase promoter (16). As shown in Fig. 3, all E2 proteins showed substantial transactivation activity, demonstrating that the FLAG epitope does not interfere with E2 function and that all of the E2 proteins are efficient transcriptional transactivators.

**Most Papillomavirus E2 Proteins Bind Mitotic Chromosomes in a Pattern Similar to That of BPV1 E2.** To further ensure that the FLAG epitope did not interfere with the ability of the E2 proteins to associate with mitotic chromosomes, the localization of BPV1 E2 and BPV1 FLAG-E2 was compared. As shown in Fig. 4, the BPV1 E2 protein, with or without the FLAG epitope tag, was localized in punctate speckles on mitotic chromosomes. Therefore, the presence of the FLAG tag on the N terminus of the BPV1 E2 protein does not interfere with its nuclear localization and association with mitotic chromatin. To further ensure that
the tag did not interfere with the localization of specific classes of papillomavirus E2 proteins, the FLAG tag was also inserted in the hinge region of the HPV31 E2 protein (data not shown). No differences were observed in expression or localization patterns of HPV31 E2 proteins with N-terminal or hinge epitope tags (see below).

To determine the chromosomal association of each E2 protein, their localization was assessed by immunofluorescence. Representative images of each E2 protein are shown in Fig. 5.

The mitotic levels of the proteins ranged from low levels of COPV and HPV4 E2 to high, and often aggregated, DPV E2 protein. Nevertheless, mitotic chromosomal association could be observed for the majority of the papillomavirus E2 proteins. E2 proteins from EEPV, DPV, ROPV, CRPV, COPV, HPV1a, HPV4, and HPV8 were found to be localized as punctate dots on the mitotic chromosomes. The percentage of mitotic cells with E2 associated with chromosomes varied among the different E2 proteins, but this result is most likely to be due to difficulties in expressing the proteins in an optimal manner rather than inherent differences in the ability of these proteins to bind mitotic chromatin. These values are summarized in Table 1, which is published as supporting information on the PNAS website.

Certain HPV E2 Proteins Bind to the Pericentromeric Region of Mitotic Chromosomes. The pattern of staining of E2 on mitotic chromosomes appeared somewhat different for HPV8 and CRPV E2 proteins. For each of these E2 proteins, there were fewer, but larger, speckles of E2 staining that were often located in pairs. The level of HPV8 E2 protein was better for detailed analysis, and so only HPV8 was investigated further. Analysis of cells at different stages of mitosis suggested that these speckles were located close to centromeres. To further analyze the location of HPV8 E2, cells were stained for $α$-tubulin to visualize the position of the E2 protein with respect to the mitotic spindle. The HPV8 protein could be seen to be located on mitotic chromosomes, very close to the point of attachment of the mitotic spindle (see Fig. 6A). To verify this observation, cells were costained with antiserum against centromeres. As shown in Fig. 6B, this experiment demonstrated that each region of HPV8 E2 staining was located immediately adjacent to, but not directly overlapping, the centromere. In contrast, staining of the BPV1 E2 protein with respect to centromeres was random. Thus, certain E2 proteins have evolved a similar, yet distinct, mechanism to attach to mitotic chromosomes.

![Fig. 4](image_url)  
**Fig. 4.** FLAG tag does not interfere with association of E2 with mitotic chromosomes. Shown is immunodetection of BPV1 E2 and BPV1 FLAG-E2 by using monoclonal antibodies B201 or anti-FLAG M2, respectively. E2 proteins are stained green, and cellular DNA is counterstained in red with propidium iodide.

![Fig. 5](image_url)  
**Fig. 5.** E2 proteins from several genera of papillomaviruses bind to mitotic chromosomes. Immunodetection of E2 proteins with anti-FLAG M2 (green) is shown. Cellular DNA is counterstained in red with propidium iodide.
HPV8 E2 Proteins Do Not Stably Interact with Mitotic Chromosomes in CV-1 Cells. Despite having quite different expression levels, the E2 proteins from HPV11, HPV16, HPV31, and HPV57, all members of the \(\text{HP9251}\)-papillomavirus genus, gave a distinct pattern of chromosomal association. Each of these proteins was found in close association with mitotic chromosomes in early mitosis (prophase) and was bound to chromosomes in late mitosis (telophase). In most metaphase and anaphase cells the E2 proteins could not be detected, despite good E2 interphase expression (Fig. 7 and Table 1). In the small percentage of cells in which E2 protein could be detected in mid-mitosis, it was present throughout the cell and had no specific association with the mitotic chromosomes. Van Tine et al. (12) reported that HPV11 E2 interacts with the mitotic spindle instead of the chromosomes. However, we observe no obvious association with the mitotic spindle in our cells. Therefore, the \(\text{HP9251}\)-E2 proteins are not stably attached to chromosomes throughout mitosis in cells in which the other E2 proteins are tightly associated.

Preextraction of Mitotic Cells Induces \(\alpha\)-Papillomavirus E2 Proteins to Bind Mitotic Chromosomes. Previous studies in our laboratory have shown that E2 localization can be influenced very significantly by fixation conditions (17) and that, in some cases, proteins that are temperature-sensitive and slightly misfolded can be induced to bind mitotic chromosomes with different fixation conditions. Therefore, we fixed cells using a technique that has been shown to stabilize and enhance staining of the mitotic spindle and involves preextraction in a buffer containing 0.1% Triton X-100 followed by standard paraformaldehyde fixation. As shown in Fig. 8, the \(\alpha\)-group E2s could be observed in prominent large speckles on several chromosomes after preextraction. It might be concluded that this staining was artifactual, because it was observed only after prefixation extraction. However, these speckles were very similar to those observed for HPV8 E2 and were closely associated with centromeric regions of chromosomes, as shown in Fig. 8c. Notably, the BPV1 E2 TR repressor protein, which is incapable of chromosome binding (3), did not associate with mitotic chromosomes upon the same prefixation treatment (data not shown), and the extraction had no effect on BPV1 or HPV8 E2 chromosomal binding patterns. Therefore, we postulate that the \(\alpha\)-E2 proteins do not associate with mitotic chromosomes \emph{in vivo} because of an incorrect or incompatible intracellular environment. However, the extraction technique promotes or permits associations that were not previously possible.

Discussion

Expression vectors were generated for 13 different papillomavirus E2 proteins. Each protein has an N-terminal FLAG tag, is expressed at moderate levels within cells, and functions as an efficient activator of transcription. This panel of proteins is extremely useful for analyzing and comparing papillomavirus E2 functions.

To date, the association of the E2 protein with mitotic chromosomes has been demonstrated only for BPV1. In this study we analyzed the mitotic chromosomal binding function of 13 E2 proteins. We observed that 9 of the 13 E2 proteins are observed as speckles on condensed chromosomes in mitotic cells. These nine proteins represent six different host species, and they
are derived from six different genera of the Papillomaviridae. Therefore, the association of the E2 proteins with mitotic chromosomes is a common mechanism among papillomaviruses. The mechanism of tethering genomes to mitotic chromosomes by a virally encoded protein is also observed in several episomal herpesviruses and so is a common mechanism of episomal viral replication.

Several E2 proteins gave a distinctive pattern of mitotic chromosomal staining. Further analysis indicated that HPV8 E2 bound primarily to the pericentromeric region of the mitotic chromosomes. This region is adjacent to the kinetochore, which constitutes the region of attachment of the chromosome to the mitotic spindle. The region of E2 staining is adjacent to but does not overlap staining with human ant centromere antibody, which recognizes the inner region of the kinetochore (18). Therefore, the mechanism of attachment of this subset of E2 proteins is distinctive from that of BPV1.

The four α-papillomavirus E2 proteins, from HPV11, HPV16, HPV31, and HPV57, were poorly expressed in mitosis. They could only be found associated with mitotic chromosomes at early (prophase and prometaphase) and late (telophase and cytokinesis) stages of mitosis. In metaphase and early anaphase these E2 proteins were usually undetectable, and, in the small percentage of cells that contained detectable E2, it was distributed throughout the cytosol. A similar expression pattern can be found with other papillomavirus E2 proteins in a small percentage of mitotic cells, and we have concluded that this result is due to overexpression and mislocalization of the proteins. Such cells are observed when BPV1 E2 is expressed from different expression vectors, but not when it is synthesized from the viral genome, indicating that they do not represent the natural localization of the protein.

These four E2 proteins belong to the α-genus of Papillomaviridae, which suggests that the mitotic instability of these proteins in our system is an inherent property of this group of viruses. We have shown that a short sequence adjacent to the DNA binding domain of the BPV1 E2 protein undergoes a conformational change upon phosphorylation that targets the protein for ubiquitination (19) and degradation (15). On the other hand, the transactivation domain is important for the regulation of the stability of the α-papillomavirus HPV18 E2 protein by the proteasome pathway (20). Therefore, the stability of this class of E2 proteins is regulated differently from other E2 proteins; presumably this inherent mitotic instability can be regulated by cellular and/or viral factors.

Nevertheless, when mitotic cells were preextracted before fixation, the α-group E2 proteins could be observed to be associated with mitotic chromosomes in a pattern similar to that of HPV5 E2. It is well established that nuclear protein localization can change dramatically upon biochemical manipulation, and we believe that this is a particular problem in mitosis, when many nuclear substrates are dissociated to prepare for chromosomal separation and division of nuclear components. Therefore, we conclude that the pre fixation treatment either induces or permits E2 to bind to the pericentromeric region and/or unmasks the E2 FLAG epitope in this location. However, we believe that this localization has biological significance because of its similarity with that of HPV8 E2. Furthermore, although they report that HPV11 E2 protein associates with the centrosome and the mitotic spindle, Van Tine et al. (12) show that, in the presence of HPV11 E2, plasmids containing E2 binding sites and visualized by a Gal4-GFP fusion protein are localized in punctate dots in a pattern very similar to that which we observe for the HPV8 E2 protein and the HPV11 E2 protein after preextraction. Therefore, we reason that attachment of the α-E2 proteins to this region of mitotic chromosomes after extraction likely reflects the bona fide binding region of these proteins.

We also predict that, under the correct biological conditions, the α-E2 proteins will be found stably associated with mitotic chromosomes in vivo like the other papillomaviruses and episomal herpesviruses. Most likely, the inability to detect α-papillomavirus E2 proteins stably associated with mitotic chromosomes after direct fixation is due to a missing viral or cellular factor. This factor could be the cellular environment of a specific cell type or a viral or cellular protein that either stabilizes a complex containing E2 on mitotic chromosomes or prevents mitotic degradation of the E2 protein. Notably, the genomes of this group of papillomaviruses are maintained only extrachromosomally in keratinocyte-derived cells and under specialized culture conditions. Furthermore, it has been reported that E6 and E7 gene functions are required for episomal genome maintenance of this group of viruses (21, 22). It is also possible that the presence of the viral genome containing E2 binding sites, and other potential cis elements, could stabilize mitotic chromosomal association. The ability of the different E2 proteins to associate with mitotic chromosomes is very similar in cervical carcinoma-derived C33 cells to that observed in CV-1 cells (see Fig. 10, which is published as supporting information on the PNAS web site). However, experiments designed to supply such factors in other keratinocyte cell types have been hampered by poor mitotic expression of E2 and growth inhibition of cells containing viral genomes (data not shown).

In summary, these data show that the mechanism of segregating viral genomes by mitotic chromosomal tethering is common among papillomaviruses as well as the persistent episomal herpesviruses. Furthermore, the E2 chromosomal binding target is different for different viruses, and some viruses may need a specialized cellular environment and/or other factors for stable mitotic chromosomal association.
The phylogenetic tree was drawn with the DrawTree component of PHYLIP (University of Washington, Seattle).

All E2 genes were generated by PCR. Plasmid templates, HPV1a, HPV4, HPV11, HPV16, HPV57, CRPV, EEPV, and DPV, were described in refs. 23–27. HPV57 DNA was originally described as HPV2 (23). The HPV8 (28), HPV31 (29, 30), COPV (31), and ROPV (32) genomes were obtained from originally described as HPV2 (23). The HPV8 (28), HPV31 (29, 30), COPV (31), and ROPV (32) genomes were obtained from T. Iftner (University of Tübingen, Tübingen, Germany), L. Laimins (Northwestern University, Chicago), R. Schlegel (Georgetown University, Washington, DC), and N. Christensen (Pennsylvania State University, Hershey, PA), respectively. Re-circularization of HPV1a, HPV4, HPV31, HPV57, and DPV genomes was necessary because they were cloned in the E2 gene. The E2 genes were amplified with an N-terminal FLAG tag [MVDYKDDDDK; a valine was inserted at the second position to potentially stabilize the protein (33)] and cloned into pMEP4. BPV1 pMEP4-E2 has been described previously (15). Each E2–FLAG gene and template was sequenced to confirm that they contained the correct sequence. The DPV E2 sequence differed substantially from that of the M11910 GenBank sequence, and this corrected sequence has been submitted to the GenBank database (accession no. AY803295).

Codon Optimization of E2 Genes. The HPV4, HPV11, HPV16, and HPV31 E2 genes were either partially or completely recoded. All codons from the 5′ end region of the HPV4 (first 131 bp), HPV11 (first 445 bp), HPV16 (first 171 bp), and HPV31 (entire E2) genes were replaced by the most common mammalian codons by gene synthesis.

Establishment of pMEP-E2 Cell Lines. CV-1-derived lines were generated by transfection of the pMEP-E2 plasmid by using FuGENE (Roche) and selection with 200 μg/ml hygromycin B (Roche). After 2 weeks, drug-resistant colonies were pooled and cultures were expanded.

Immunoblotting. E2 expression was induced with 1 μM CdSO4 for 3–4 h, and proteins were extracted in 50 mM Tris-Cl, pH 6.8/2% SDS/10% glycerol containing Complete (Roche). E2 proteins were detected with anti-M2 FLAG monoclonal antibody (Sigma) by using standard immunoblotting procedures.

Transactivation Assay. CV-1 cells were cotransfected with 50 ng of pMEP-E2 constructs and 2.0 μg of pBS1073, an E2-responsive luciferase reporter plasmid, and were assayed for luciferase activity as described in ref. 6.

Immunofluorescence. pMEP-E2 cell lines were plated on slides and synchronized with a thymidine block and release. E2 expression was induced with 1–2 μM CdSO4 for 3–4 h before fixation. Cells were fixed for 20 min in 4% paraformaldehyde/PBS and permeabilized with 0.1% Triton X-100 in PBS for 15 min. Where indicated, cells were permeabilized before fixation with 0.1% Triton X-100 in 80 mM Pipes, pH 6.8/5 mM EGTA/1 mM MgCl2. E2–FLAG proteins were detected with Sigma monoclonal or polyclonal anti-FLAG M2 (1:500) or polyclonal anti-FLAG M2 (1:500) antibodies. BPV1 E2 was detected by using mouse monoclonal antibody B201 (1:10). Centromeres were detected with human anti-centromere antiserum (Immunovision), and the mitotic spindle was detected with anti-α tubulin monoclonal antibody T5168 (Sigma). Immunofluorescence was performed as described in ref. 6. Digital images were captured with a Leica TCS-SP2 laser scanning confocal imaging system.

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