Hitchhiking of Viral Genomes on Cellular Chromosomes

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Abstract
Persistent viral infections require a host cell reservoir that maintains functional copies of the viral genome. To this end, several DNA viruses maintain their genomes as extrachromosomal DNA minichromosomes in actively dividing cells. These viruses typically encode a viral protein that binds specifically to viral DNA genomes and tethers them to host mitotic chromosomes, thus enabling the viral genomes to hitchhike or piggyback into daughter cells. Viruses that use this tethering mechanism include papillomaviruses and the gammaherpesviruses Epstein-Barr virus and Kaposi’s sarcoma-associated herpesvirus. This review describes the advantages and consequences of persistent extrachromosomal viral genome replication.
1. STRATEGIES OF PERSISTENT INFECTION OF VIRUSES WITH EXTRACHROMOSOMAL GENOMES

1.1. Introduction

Papillomaviruses (PVs) and gammaherpesviruses are ancient viruses that have coevolved with their human hosts (1, 2). For the most part, the healthy host immune system controls these infections so that they are asymptomatic or harmless. However, the longevity of the viral-host association, as well as the manipulation of host pathways to ensure that virally infected cells survive, can sometimes result in disease. The Achilles’ heel of these infections is their reliance on a single viral protein to tether the extrachromosomal viral genomes to host chromosomes in latent or persistently infected cells. Disruption of this tether should lead to viral genome loss and cure of infection.

1.2. Papillomaviridae

PVs are a ubiquitous group of viruses that infect and replicate in the epidermis of vertebrates. The persistent replication cycle is tightly linked to the differentiation process of this stratified epithelium; the basal layers of the epithelium must frequently divide and differentiate to replenish the outermost tissue layer that protects against injury, infection, and dehydration. PVs infect the basal layer of the epithelium through a microabrasion and establish a persistent infection in these dividing cells. The viral genome replicates at low levels within these cells and has very limited gene expression. This low viral activity, combined with immune evasion functions of viral proteins, results in long-term infection that escapes immune detection (3).

Basal epidermal cells divide either symmetrically (self-renewal) or asymmetrically. In the latter case, one daughter cell leaves the basement membrane and begins the process of differentiation, moving up through the epithelium until it is sloughed from the surface. PVs exploit this process as productive viral DNA replication, late gene expression, and viral particle assembly occur sequentially as cells progress toward terminal differentiation and viral-laden squames are shed from the tissue surface. This resourceful strategy results in long-term infection (without immune clearance) and continual dissemination of viral particles.

To date, almost 500 different animal and human papillomavirus (HPV) types have been identified (4), most of which give rise to benign lesions (warts or papillomas) or asymptomatic infection in their hosts. Infections are usually self-limiting, as the host immune system eventually clears the virus, but can be debilitating in immunocompromised hosts (e.g., organ transplant recipients, human immunodeficiency virus infection, or genetic disease). In addition, a handful of PVs are oncogenic, and persistent infection with these types sometimes leads to cancer. In fact, oncogenic HPV are responsible for 5% of human cancers worldwide (e.g., cervical, anogenital, and oropharyngeal) (5).

Despite their complex infectious cycle, the small PV circular, dsDNA genomes (~7–8 kb) contain just four well-conserved genes; these encode for capsid proteins (L1 and L2) and nonstructural replication proteins (E1 and E2). Additional proteins (E4, E5, E6, and E7) encoded by some PVs influence cellular environments to optimize viral replication, virion release, and evasion of the host immune response and may be evolutionary adaptations to different niches in the host epithelium (1).

1.3. Gammaherpesvirinae: Human Herpesviruses-4 and -8

The gammaherpesviruses human herpesvirus 4 (HHV-4) (Epstein-Barr virus or EBV) and HHV-8 (Kaposi’s sarcoma-associated herpesvirus or KSHV) are also ancient viruses that coevolved with their hosts and give rise to lifelong infections (2). EBV infects 95% of the human population,
but KSHV is more geographically restricted (2). In most individuals, EBV or KSHV infection occurs in early childhood, resulting in subclinical infection. Both viruses are transmitted in saliva and transferred from the oral cavity to circulating B cells, where they establish persistent, latent infection (2, 6). This is best understood for EBV, which initially infects oral epithelial cells and is transferred to B cells in the oropharynx (6). EBV is relatively dormant in infected B cells but can be triggered to reactivate into the lytic phase to produce virions. Persistent EBV infection is rarely associated with disease in healthy individuals, but in rare cases, immunosuppression, cellular stress, or dysregulated viral gene expression gives rise to EBV-associated lymphomas (Hodgkin disease, immunoblastic lymphoma, and Burkitt lymphoma). Similarly, KSHV infection can cause the B cell malignancies primary effusion lymphoma and multicentric Castleman disease and the endothelial cell–associated cancer Kaposi’s sarcoma (2, 6). In contrast to PVs, the gammaherpesviruses use two or more cell types to switch from latency to transmission modes. The EBV and KSHV genomes are also large (EBV is \( \sim 172 \text{ kbp} \), and KSHV is \( \sim 165–170 \text{ kbp} \)). Both genomes are linear dsDNA in the virion but are circularized by ligation of their terminal ends shortly after infection and thereafter maintained as circular dsDNA (7). EBV and KSHV encode at least 85 gene products important for infection, immune evasion, B cell reprogramming, latency, viral DNA replication, and virion structure. Only a subset of genes is expressed in latency, but there is consistent expression of EBNA1 (Epstein-Barr virus nuclear antigen 1) and KSHV LANA (latency-associated nuclear antigen). A key function of these proteins is to maintain the viral genomes as extrachromosomal plasmids.

2. REPLICATION MECHANISMS OF PERSISTENT VIRUSES WITH EXTRACHROMOSOMAL GENOMES

2.1. Multiple Phases of Viral DNA Replication

The complex infectious cycles of the PVs and gammaherpesviruses require multiple phases and modes of viral genome replication (Figure 1). In the first phase (upon initial infection), the incoming viral genome undergoes limited DNA synthesis to amplify the genome to a low copy number. Next, these genomes are established in the host cell, a relatively rare event that requires evasion of antiviral restriction factors and epigenetic silencing and localization of the genomes to advantageous regions of the nucleus. Once established, viral genomes replicate at a constant copy number in dividing cells through synchronous replication with host DNA during S-phase, and these genomes are partitioned to daughter cells in association with host mitotic chromosomes. Infected cells can persist for many years (PVs) or for the life of the host (gammaherpesviruses), and a robust mechanism is required to retain the extrachromosomal viral genome in dividing cells. The final phase of viral DNA synthesis is productive DNA amplification that generates high numbers of genomes for packaging in progeny virions. This phase is usually induced by terminal differentiation of infected host lymphocytes or epithelial cells (2, 6, 8).

2.2. Initiation of Viral DNA Synthesis

In theory, initiation of viral DNA synthesis and partitioning of viral genomes are two independent, separable processes. DNA synthesis is initiated at a replication origin bound by an initiator protein such as cellular origin recognition complex (ORC) or PV E1, and a cascade of cellular proteins assemble to synthesize DNA in a semiconservative manner. Viral genomes are partitioned by a virally encoded DNA binding protein that binds viral DNA and tethers the genomes to host chromosomes. In practice, these processes are tightly linked, as viral tethering proteins also recruit factors required for DNA replication.
Figure 1
Phases of replication in gammaherpesvirus and papillomavirus infection. Upon infection, the virus undergoes limited DNA synthesis and becomes established as an extrachromosomal plasmid in the host nucleus. As the infected cells divide, viral genomes (purple circles) are maintained as stable copy number plasmids that are partitioned by binding to host chromosomes (green) in mitosis. Changes in the cell environment, such as differentiation, induce the productive or lytic phase, and large amounts of viral progeny genomes are synthesized.

2.3. Partitioning of Extrachromosomal DNA in Dividing Cells
There are numerous ways in which extrachromosomal DNA can be effectively partitioned to daughter cells (Figure 2). In bacteria, low copy plasmids use specialized partitioning mechanisms, but high copy plasmids are partitioned by random diffusion without tethering to host structures (Figure 2a). In eukaryotes, extrachromosomal DNA must additionally be retained in the nucleus after cell division, and so it is advantageous to directly associate with the spindle apparatus through centromere-like elements (Figure 2b) or piggyback on host chromosomes (Figure 2c,d). DNA synthesis of extrachromosomal DNA can take place in close association with host DNA, with replicated viral genomes remaining associated with chromatids until they are faithfully separated in mitosis (Figure 2d). Alternatively, extrachromosomal DNA can replicate in a separate nuclear location and subsequently randomly associate with host chromosomes (Figure 2e). The viruses reviewed here distribute their genomes using the schemes shown in Figure 2c,d. Almost 90% of EBV genomes are observed associated with host chromosomes as pairs until anaphase and so partition in a quasi-faithful manner (9) (Figure 2d). While KSHV genomes are synthesized while tethered to host chromatin, they partition nonequally in clusters (Figure 2e), resulting in a larger number of genomes in fewer cells (10). The small PV genome size makes analogous experiments extremely challenging.

2.4. Papillomavirus Replication
PV DNA synthesis requires two viral proteins and is dependent on cellular factors (11). E1 is a sequence-specific helicase that binds and unwinds the viral origin of replication. E2 is a multifunctional protein that regulates viral transcription, promotes initiation of DNA replication, and partitions the viral genomes by tethering them to mitotic chromosomes. The replication origin is in the upstream regulatory region (URR) of the genome and consists of overlapping E1 binding sites (E1BSs) flanked by E2 binding sites (E2BSs) (12) (Figure 3a). E1 binds to the
Figure 2

Different models of plasmid partitioning, showing viral genomes or extrachromosomal plasmids (purple circles) (a) Viral genomes or extrachromosomal plasmids (purple circles) randomly distributed throughout the mitotic cell (singly or in clusters), (b) attached to the microtubules of the spindle, (c) randomly attached to chromosomes, and (d) attached pairwise to sister chromatids. The pink bars represent topological or protein links between daughter molecules. Video 1 shows faithful partitioning of a viral genome.

origin cooperatively with E2 (13), E2 dissociates, and E1 converts into a double hexamer that unwinds the genome in a bidirectional manner (14). Cellular replication proteins are recruited to the replication fork to promote theta-mode replication of the viral DNA.

Upon entry into the cell, the L1 capsid protein is dissociated from the virus particle, and the viral genome, in complex with the L2 minor capsid protein, accesses the nucleus encased in endocytic vesicles. After nuclear membrane breakdown in mitosis, the L2 genome vesicles bind to condensed host mitotic chromosomes (15, 16). After cell division, viral genomes associate with nuclear domain 10 bodies; paradoxically, these are important for antiviral defense, but many DNA viruses initiate the infectious cycle here by taking advantage of some components and disrupting or degrading others (17). E1 and E2 proteins then initiate low-level amplification of viral DNA. Successful establishment of viral genomes requires evasion of intrinsic immune defenses and association with regions of transcriptionally active host chromatin (18). In addition to its role in initiation of viral DNA synthesis, E2 binds and partitions the viral genomes in approximately equal numbers into daughter cells during the maintenance phase of replication by tethering them to mitotic chromosomes (19, 20).

Typically, both E1 and E2 are required at each stage of PV replication, but there is evidence that E1 might be dispensable during the maintenance phase (21, 22). E1 induces a DNA damage response (DDR) in host nuclei, and so its expression and nuclear localization are tightly regulated (23–25). Under certain circumstances, E1 is probably dispensable for maintenance replication, and viral DNA synthesis is initiated by cellular proteins. Cellular DNA is licensed so that it replicates once per cell cycle, and there is keen interest in whether viral genomes that replicate with a stable
Comparison of cis-elements required for stable genome maintenance. (a) The URRs from two PV types are shown. The consensus E2BSs are indicated by purple circles and the E1BS by a green rectangle. Minimal origins and MMEs are indicated. (b) EBV oriP contains a DS and an FR element. The DS element is required for initiation of replication and contains four EBNA1 BSs (green circles). The FR element contains multiple 30-bp tandem repeats (light purple box), each containing an EBNA1 BS, and is required for genome maintenance and partitioning. (c) Each repeat in the KSHV TR element (light purple box) contains three LANA binding sites (green circles), one of which (darker green) overlaps with an element required for initiation of DNA synthesis (42, 79). Abbreviations: BPV1, bovine papillomavirus; DS, dyad symmetry; E1BS, E1 binding site; E2BS, E2 binding site; EBNA1 BS, Epstein-Barr virus nuclear antigen 1 binding site; EBV, Epstein-Barr virus; FR, family of repeats; HPV16, human papillomavirus 16; KSHV TR, Kaposi's sarcoma-associated herpesvirus terminal repeat; LANA, latency-associated nuclear antigen; ME, minichromosome maintenance element; MME, minichromosome maintenance enhancer element; oriP, viral replication origin; PV, papillomavirus; URR, upstream regulatory region.

copy number are similarly restricted. Bovine papillomavirus (BPV1) replicates by a random choice mechanism whereby some molecules replicate more than once per cell cycle and others not at all (20, 26). For alpha-HPVs, the replication mode varies in different cell and HPV types, but overexpression of E1 results in unlicensed, random replication (27). Thus, we surmise that in the absence of E1, HPV replication is initiated by cellular replication proteins and is likely licensed, but in the presence of E1, random choice replication is more likely.

Vegetative (productive) replication of PV genomes occurs in differentiated keratinocytes. Activation of the late promoter generates high levels of E1 and E2 to rapidly amplify viral DNA. However, the dependence on cellular replication proteins is problematic in differentiated cells not in S-phase, and so PVs induce a cellular DDR (28). This response recruits cellular factors to replication foci to synthesize DNA and resolve replication intermediates, and replication switches from theta-mode to recombination-dependent replication (RDR) (29).

2.5. Epstein-Barr Virus Replication

The linear EBV genome is recircularized in the nucleus upon infection and amplified to a low copy number; successful genome establishment is relatively rare and requires association with beneficial regions of host chromatin (30). In the maintenance phase, viral genomes replicate once per cell cycle (licensed) in synchrony with the host DNA. Replication requires the viral replication origin (oriP) and EBNA1 protein (31). oriP contains two separable functional elements, the family of repeats (FRs) and the dyad symmetry (DS) (32), each of which contains multiple EBNA1 binding sites (Figure 3b). EBNA1 recruits cellular replication proteins to initiate DNA synthesis at the
DS element and binds to FR to retain and partition the viral genomes. EBNA1 does this by tethering the viral genomes to host chromosomes, so that the genomes are faithfully or quasi-faithfully partitioned to daughter cells by association with sister chromatids (9, 33). The final phase of EBV DNA replication is initiated upon reactivation of latent EBV genomes in resting memory B cells. EBV encodes many additional proteins required for productive viral DNA synthesis, and replication switches to a rolling circle (RC)/RDR mode (34). As in many other viruses, DDR proteins are recruited to replication foci (35).

2.6. Kaposi’s Sarcoma-Associated Herpes Virus Replication

Similar to those of EBV, linear KSHV genomes are recircularized upon infection by ligation of the terminal repeats (TRs); however, unlike in EBV, these repeats also function as the latent replication origin and partitioning element (36) (Figure 3c). After initial genome amplification and establishment, viral genomes are maintained at a constant copy number through licensed DNA replication and partitioning by the viral LANA protein (37). LANA binds to the LANA binding sites (LBSs) in the TRs and tethers the genomes to host chromosomes (36, 38). Although KSHV genomes are partitioned in approximately equivalent numbers per cellular division, live cell imaging shows that viral genomes cluster at tethering sites and partition somewhat randomly, resulting in loss of KSHV genomes in some daughter cells (10). Finally, as in EBV, lytic reactivation leads to induction of virally encoded replication proteins, a switch from theta-mode DNA synthesis to RC/RDR replication (34) and recruitment of DDR factors to assist in DNA synthesis and resolution of replication intermediates (35).

3. VIRAL AND CELLULAR FACTORS INVOLVED IN TETHERING AND PARTITIONING VIRAL GENOMES

3.1. Viral Tethering Proteins

Tethering to host nuclear structures is critical for partitioning and nuclear retention of viral genomes in dividing cells. To this end, multifunctional viral proteins act as a physical tether between these nuclear structures and viral genomes. This section describes the viral tethering proteins.

3.1.1. Papillomavirus: E2 protein. The full-length PV E2 protein (E2-TA or E2) plays roles in replication and transcription but also tethers viral and host genomes together during maintenance replication. E2 contains two conserved domains linked by a flexible hinge (Figure 4). The N-terminal transcriptional activation domain forms a cashew-shaped structure important for transcriptional regulation and interaction with E1. This domain interacts with multiple cellular proteins that influence replication, transcription, and tethering of E2 to host chromatin (39). The C-terminal domain of E2 binds specifically to 12-bp E2BS motifs in the viral genome. This DNA binding domain (DBD) dimerizes to form a stable, antiparallel beta-barrel core with recognition alpha helices on the surface that insert into the major groove of DNA and specifically interact with nucleotides of the conserved E2BS sequence (40). This structure is well conserved in E2 proteins from all PV genera and has a strong similarity to the dimeric DBDs of LANA and EBNA1, despite little sequence similarity among the three proteins (40–42). The conserved E2 domains are connected by a flexible and unstructured hinge region that is not required for transcription or replication functions. The hinge has low sequence complexity and is not well conserved, in sequence or length, among the E2 proteins of different PV genera. However, the hinges contain sites of post-translational modification that are conserved within each genus and regulate E2 half-life, nuclear location, protein-protein interactions, and tethering to host chromatin (39).
3.1.2. Epstein-Barr virus: EBNA1. The N-terminal half of EBNA1 contains two regions important for replication, transcriptional regulation, and tethering (domains A and B, or linking regions 1 and 2) separated by a long glycine-alanine repeat region (Figure 4). The dimeric, sequence-specific DBD is located at the C terminus. EBNA1 chromatin association is mediated by two glycine-arginine-rich regions within domains A and B (43, 44). These regions function as AT hooks that bind AT-rich regions of DNA (45) and also to cellular proteins important for tethering to host chromatin (43, 46). The two domains are relatively equivalent in function, and two copies of either can mediate binding to host chromatin (44, 47, 48). EBNA1 binds to conserved motifs in the FR and DS region of oriP, the latent origin of replication. This is mediated by the C-terminal DBD that, akin to E2, forms a dimeric eight-stranded beta-barrel structure (41, 49). Site-specific DNA recognition is mediated by a surface alpha helix and an extended chain that extends into the DNA minor groove (41, 49, 50). During latent infection, EBNA1 is required to replicate and maintain the extrachromosomal viral genomes, and this provides a potential target for antigen recognition by circulating immune cells. The glycine-alanine repeat region is encoded by purine-rich sequences that inhibit antigen presentation. This is due to the formation of G-quadruplex structures that inhibit translation and reduce the formation of EBNA1-derived T cell epitopes (51).

3.1.3. Kaposi’s sarcoma-associated herpesvirus: LANA. LANA has three distinct regions: a small N-terminal domain, a large central region containing multiple interspersed repeat and unique sequences, and a C-terminal DNA binding/dimerization domain (Figure 4). The 23 residue N-terminal domain contains a bipartite nuclear localization signal and a short chromatin binding domain (CBD) (52). The N-terminal domain is important for LANA-mediated transactivation, and residues 5–13 are necessary for tethering to chromatin (53–55). As in E2 and EBNA, the C-terminal domain of LANA is the specific DNA binding/dimerization domain that forms a beta-barrel core with surface recognition alpha helices that contact residues in the LBS motif (42, 56, 57). On the face of the domain opposite from the recognition helix is a basic patch that interacts with cellular factors and impacts KSHV plasmid maintenance (58, 59). The N-terminal CBD is primarily responsible for host chromosome binding, but sequences in the C-terminal DBD can also mediate binding to pericentromeric and telomeric regions of host chromosomes.
The N- and C-terminal domains of LANA are separated by long stretches of repetitive sequences that, like EBNA1, inhibit antigen presentation (63), and computational analysis indicates that they may also form G-quadruplex structures that inhibit translation (63).

3.2. Viral Cis-Elements Required for Viral Plasmid Maintenance

Viral tethering proteins regulate replication, transcription, and plasmid maintenance by binding to distinct cis-elements located within the regulatory regions of PV, KSHV, and EBV genomes. The cis-elements required for initiation of viral DNA synthesis and plasmid maintenance are often separable.

3.2.1. Papillomavirus E2 binding sites and enhancer elements. E2 proteins bind consensus sites (ACCN6GGT) located within the URR (64) (Figure 3a). Two or three E2BSs flank an E1BS to form the replication origin (12, 13), and these E2BSs also regulate expression of the adjacent early promoter. There are additional E2BSs upstream in the URR, but the number varies greatly among different genera of PV (39). The deltapapillomavirus BPV1 contains 12 E2BSs in the URR. A minichromosome maintenance element (MME), containing a transcriptional enhancer and six of the E2BSs, and the minimal replication origin are required for maintenance replication (20, 65). However, multimerized E2BSs can substitute for the MME. In contrast, most alphapapillomaviruses contain four E2BSs, and only the two to three adjacent to the origin are required for genome maintenance (66, 67). In addition to the minimal replication origin, the URR transcriptional enhancer is necessary for maintenance of an alphapapillomavirus HPV18 replicon (66). This minichromosome maintenance enhancer element (MMEE) contains binding sites for several cellular factors, which may impact the maintenance of the HPV18 genome (68).

3.2.2. Epstein-Barr virus: oriP (dyad symmetry and family of repeats). EBV oriP, the latent origin of replication, is the only cis-element required to maintain the EBV genome; it contains multiple 30-bp repeats, each of which contains an EBNA1 binding site (69). oriP contains two separate elements, FR and DS (32, 70, 71). DS contains four 30-bp repeats and is necessary for initiation of DNA synthesis but unnecessary for genome maintenance (72, 73). FR contains 20 tandem repeats and is required for genome maintenance and partitioning (69, 70, 74, 75). At least seven EBNA1 BSs are required for EBV plasmid maintenance (76).

3.2.3. Kaposi's sarcoma-associated herpesvirus: terminal repeat. In contrast to that of EBV, the KSHV latent replication origin is formed by recircularization of the 801-bp TR repeats. On average, there are 35–40 repeats, each of which contains three 18–20-bp LANA BS motifs (one overlaps the site of replication initiation) (42). As in EBV oriP, the LBSs are spaced optimally apart on the same DNA helix face, thus facilitating cooperative binding of adjacent LANA dimers (42, 77). The KSHV TR cis-element is both necessary and sufficient for initiation of DNA synthesis and genome maintenance (77–79). However, little is known about how many of the individual TR repeats initiate DNA synthesis on a single viral genome.

3.3. Cellular Partners of the Viral Tethering Proteins

E2, EBNA1, and LANA interact with many cellular proteins to facilitate viral transcriptional regulation, DNA synthesis, and partitioning. Some of these are proposed to directly mediate, or assist, in tethering viral genomes to host chromosomes, but it is difficult to separate their tethering role from functions related to viral transcription and replication. The following section lists cellular targets that may be involved in chromatin tethering.
3.3.1. Papillomavirus E2 protein. Several cellular proteins have been postulated to be the anchor of the E2 tethering protein, but the strength and nature of these interactions vary among viral types and often occur only during discrete stages of mitosis. It is probable that multiple cellular factors participate in the tethering complex.

3.3.1.1. Transcriptional and epigenetic regulators: Brd4. Brd4 (bromodomain and extraterminal domain protein 4) is the best-studied candidate for PV chromosomal attachment. It binds acetylated histones in cellular enhancers and superenhancers, recruits transcription factors, and promotes transcriptional elongation (80, 81). Brd4 both activates and represses PV transcription and binds and colocalizes with many different E2 proteins on mitotic chromosomes (82–85). Two conserved residues in the E2 transactivation domain interact with the C terminus of Brd4 (82, 83, 86). The Brd4-E2 protein association modulates viral transcription in all PVs studied, but the importance of Brd4 in tethering and partitioning viral genomes is more complex. E2 proteins that bind Brd4 with high affinity colocalize in punctate foci with Brd4 on mitotic chromosomes (84, 85). However, Brd4 binding to E2 proteins from the alpha genus (HPV11, -16, -18, -31) is weak and difficult to detect on mitotic chromosomes except in late telophase and under specific fixation conditions (85, 87).

Figure 5 shows examples of different PV E2 proteins associated with host mitotic chromosomes. Although Brd4 is the most-studied E2 chromosomal partner, differences in this association among different PV types indicate that additional cellular factors are required.

Co-expression of alpha-PV E1 and E2 results in nuclear foci that recruit Brd4, and Brd4 localizes on the surface of HPV31 late replication foci (88). This localization could represent transcriptionally active regions of viral DNA or the interface between viral and host chromatin. Brd4 is required for foci formation but is not absolutely required for E1-E2-dependent replication or HPV31 genome maintenance (88–91).

Figure 5

Variations in the pattern of E2 proteins binding to mitotic chromosomes. Mitotic cells were briefly extracted before fixation to stabilize HPV31 E2 binding. Abbreviations: BPV1, bovine papillomavirus; HPV, human papillomavirus. Figure reproduced from Reference 140 and reused with permission under a CC-BY license.
3.3.1.2. Chromosomal architectural proteins. Replication, repair, condensation, and faithful partitioning of cellular chromosomes require the concerted efforts of the structural maintenance of chromosome (SMC) architectural proteins condensin (SMC2/4), cohesin (SMC1/3), and SMC5/6. Although these proteins have not yet been demonstrated to be directly involved in the PV genome partitioning mechanism, they do influence maintenance replication. SMC1/CTCF complexes bind the late region of HPV genomes and promote maintenance and vegetative replication (92). SMC5/6 bind to PV E2 proteins, localize to replication foci, and are required for maintenance replication (93). Another E2 interacting protein, the DNA helicase ChlR1, links replication to chromatid cohesion (94). ChlR1 promotes the establishment of E2 and viral genomes with chromatin in the early stages of mitosis, but this interaction does not persist through mitosis and thus ChlR1 does not directly partition viral DNA (95). Similarly, topoisomerase II binding protein 1 localizes with E2 on mitotic chromosomes only in telophase (87, 90).

3.3.1.3. Mitotic checkpoint proteins, mitotic spindles, and Mklp2. When overexpressed, HPV11, -16, and -18 E2 proteins colocalize with mitotic spindle fibers (96). Similarly, some E2s interact with spindle assembly checkpoint proteins BubR1, Mad2, and Cdc20 (97) and mitotic kinesin-like protein 2 (Mklp2) (98). These interactions have not been shown to mediate partitioning of viral DNA and may reflect the promiscuity of E2 binding.

3.3.2. EBNA1. EBNA1 interacts with both cellular proteins and nucleic acids. As described for PV E2, it is probable that multiple cellular factors participate in the EBV tethering complex.

3.3.2.1. Interaction with AT-rich host DNA by EBNA1 AT hooks. The GR-rich domains A/B of EBNA1 have AT hook DNA binding properties and bind to AT-rich regions of host chromatin to maintain EBV plasmids (45). Inhibiting AT hook binding blocks this association and reduces EBV plasmid maintenance (99). Furthermore, the AT hooks from high motility group 1a, when fused to the EBNA1 DBD, support EBV oriP-dependent plasmid maintenance (100, 101).

3.3.2.2. EBP2. EBNA1 binding protein 2 (EBP2) is a nucleolar protein involved in rRNA processing that binds the GR-rich CBDs of EBNA1 (43). This interaction was originally thought to mediate EBNA1 mitotic chromosome association and EBV plasmid maintenance (43). However, EBNA1 binds mitotic chromosomes throughout mitosis while EBP2 colocalizes with EBNA1 from late prophase onward (48). Therefore, EBP2 might stabilize EBNA1 chromosomal association but does not directly mediate attachment.

3.3.2.3. Transcriptional and epigenetic regulators: Brd4. EBNA1 interacts with Brd4 through a region close to CB1 (residues 61–83) and localizes to the FR element of oriP (102). This interaction is important for EBNA1-mediated transcriptional activation.

3.3.2.4. G-quadruplex RNA. EBNA1 binds to G-quadruplex RNA through RGG motifs in the chromatin binding regions; this is required for ORC recruitment for viral DNA synthesis (103). Small molecules that disrupt G-quadruplexes inhibit the ORC-EBNA1 association, viral DNA replication, and EBNA1 binding to mitotic chromosomes (104). It remains to be determined whether this interaction is a primary, additive, or alternative tethering mechanism.

3.3.2.5. Chromatin binding protein: RCC1. Regulator of chromosome condensation 1 (RCC1) is an interaction partner of EBNA1 (105). RCC1 interacts directly with cellular histones,
and EBNA1 binding to RCC1 is mediated through its CBDs; both proteins colocalize during interphase and mitosis, and inhibition of this interaction disrupts EBNA1 chromatin association (105). Further studies are required to determine the precise role of this essential protein in EBV genome partitioning.

3.3.3. Kaposi’s sarcoma-associated herpesvirus LANA. Similar to E2 and EBNA1, KSHV LANA associates with numerous cellular factors, and many have been proposed as relevant for viral genome maintenance.

3.3.3.1. Nucleosomes: histone H2A/H2B interface. The N-terminal LANA CBD residues form a hairpin structure that associates with the H2A/H2B interface of nucleosomes and mediates LANA’s association with mitotic chromosomes (52). This interaction is the dominant chromosome attachment but could be modulated by the factors described below.

3.3.3.2. Chromatin binding proteins: MeCP2 and DEK. The C-terminal domain of LANA localizes to the heterochromatin at pericentromeric regions of chromosomes, and this correlates with binding to methyl CpG binding protein 2 (MeCP2) (106). The abundant chromatin architectural protein DEK also interacts with the C-terminal LANA domain but does not display the same punctate patterning on mitotic chromosomes (60).

3.3.3.3. Mitotic checkpoint proteins, spindle, and kinetochore. The nuclear mitotic apparatus interacts with the LANA C-terminal domain during interphase, but this interaction is lost at the onset of mitosis (107). LANA also forms a complex with Bub1 and CENP-F, but only Bub1 and LANA remain colocalized throughout mitosis (108). Further study is needed to determine the role of Bub1 in KSHV genome maintenance.

3.3.3.4. Transcriptional and epigenetic regulators: Brd2/Brd4. The bromodomain and extraterminal domain proteins Brd2 and Brd4 interact with the electrostatic patch on the LANA DBD (38, 109). Brd4 and LANA colocalize with KSHV genomes on cellular chromosomes throughout mitosis, while Brd2 partially binds chromosomes and only in the presence of KSHV TR plasmids (109). Notably, Brd4 is one of the few cellular target interactions shared by all three viral tethering proteins.

4. CHROMOSOME REGIONS BOUND BY THE VIRAL TETHERING COMPLEX

4.1. Specific Chromosome Regions

Viral tethering proteins are detected on mitotic chromosomes either alone or in complex with their respective viral genome. Full-length LANA and EBNA1 show a diffuse binding pattern in the absence of viral DNA, likely reflecting the ubiquitous nature of their histone- or AT-rich DNA targets (55). In the presence of viral DNA, the protein-DNA complexes are observed as punctate dots across all chromosomes (33).

PV E2 proteins that bind with high affinity to Brd4 are observed colocalized with Brd4 in punctate speckles distributed randomly over mitotic chromosomes, and PV genomes are distributed in a similar pattern (85). As described below, this binding may be related to regions of chromosomes undergoing replication stress. Betapapillomavirus E2 (HPV8) proteins bind to the rDNA
loci located on acrocentric chromosomes; this is mediated by the E2 DBD and a chromatin binding peptide from the hinge region. When this peptide is mutated, HPV8 E2 binds to chromosomes with Brd4 as observed for other E2 proteins (110; A.A. McBride, unpublished data). The alphapapillomavirus E2 proteins bind Brd4 with low affinity and are not easily detected on mitotic chromosomes (111). However, when analyzed by immunofluorescence after a pre-extraction technique, these E2s are also observed bound to the short arms of acrocentric chromosomes (85). Notably, the LANA DBD also binds pericentromeric/telomeric regions of specific mitotic chromosomes through the secondary C-terminal CBD (60, 61).

4.2. Euchromatin and Transcriptionally Active Regions

All three viral tethering proteins bind to transcriptionally active host chromatin, although this might be associated with their role in viral transcriptional regulation. For example, E2 binds with Brd4 to transcriptionally active cellular promoters but does not alter gene expression, and binding does not persist through mitosis (18, 89). E2 binding to these promoters is Brd4 dependent and not due to direct DNA binding; it likely reflects the importance of associating with euchromatic, active chromatin that is unlikely to be silenced (18).

LANA also associates with sites of host active transcription and does not alter the transcriptional profile (112, 113). In contrast, EBNA1 binds to both active and repressed host chromatin, including long interspersed nuclear element-1 retrotransposons (114). EBNA1 also upregulates transcription of many associated cellular genes and relieves compaction at targeted sites through H1 displacement (115).

In situ Hi-C analysis of interaction between viral and host DNA revealed differences between the chromosomal interactions of the gammaherpesviruses and PVs. As predicted, HPV plasmids are tethered to gene-rich, euchromatic regions, but EBV interacted with gene-poor, heterochromatic regions (116). This could be because HPVs undergo low-level, persistent infection in the cell types analyzed while gammaherpesviruses establish a more silent, latent infection. Upon reactivation, the EBV genomes move toward active euchromatin, presumably to facilitate the productive phase of the viral replication cycle. Thus, the host chromatin tethering site may not be static through the viral replication cycle.

4.3. Common Fragile Sites

The punctate PV E2-Brd4 chromosomal target sites mapped by chromatin immunoprecipitation techniques have properties characteristic of common fragile sites (CFSs) (117). CFSs are regions of the host genome undergoing replication stress and could represent an advantageous location for a virus that uses the DDR for productive DNA replication. Late replication foci form more frequently adjacent to these sites (117). One consequence of viral genome tethering at CFSs is the risk of integration into the host genome, as observed in many HPV-associated cancers (118).

4.4. rDNA

Betapapillomavirus E2s associate with the repetitive ribosomal DNA genes on the short arms of acrocentric chromosomes (110, 119). This interaction is mitosis specific, as rDNA resides in the nucleolus during interphase, and E2 binds only after nucleolar disassembly in mitosis (120). Under specific fixation conditions, alpha-PV E2s also bind this location (85, 120). The repetitive rDNA genes are prone to genetic instability and could serve as a DNA damage sensor (121), making them an attractive target for a virus that requires the DDR to facilitate viral DNA amplification.
5. REGULATION OF VIRAL GENOME PARTITIONING

5.1. Post-Translational Modification

Post-translational modifications of the viral tethering proteins impact stability, replication, and transactivation functions, nuclear localization, and chromatin interaction. Multiple phosphorylation sites have been identified within the hinges of the PV E2 proteins. In BPV1, CK2 phosphorylation induces proteasomal degradation and regulates BPV1 genome copy number during maintenance replication (122, 123). In contrast, phosphorylation of the HPV8 E2 hinge by PKA at a conserved RXXS motif stabilizes E2 and is essential for E2 host chromosome association (110, 120). In alphapapillomaviruses, phosphorylation at a nonconserved RXXS motif in the HPV16 E2 hinge increases protein stability and promotes interaction with Brd4 and host chromosomes (124).

EBNA1 is modified by serine/threonine phosphorylation and arginine methylation, particularly in the RXXS motif in GR-rich domain A. Phosphorylation regulates EBNA1 functions in plasmid maintenance and interaction with cellular proteins (i.e., EBP2) (125, 126). EBNA1 methylation is important for proper nuclear distribution of EBNA1; loss of EBNA1 methylation results in EBNA1 localization to the nucleolar periphery (125).

LANA has a multitude of post-translational modifications. RSK3 phosphorylates serine/threonine residues 13/14 within the CBD to stabilize the protein and promote H2B interaction (127). DNA-PK phosphorylates LANA between residues 31–52 and 91–340, and a kinase recruited by Brd2 phosphorylates the C-terminal domain. LANA is also arginine methylated, acetylated, sumoylated, and poly ADP-ribosylated (38). These modifications impact protein interactions and viral genome copy number.

Notably, all three tethering proteins contain RXXS motifs (often located in the chromosomal attachment domains) targeted by arginine methylation and/or serine phosphorylation. Post-translational modifications of these motifs impact protein stability and protein function.

5.2. Truncated Viral Repressor Proteins

All PVs encode a second E2 protein (E8^E2) that consists of a short basic peptide (E8) fused to the hinge and DBD of E2 (128). E8^E2 antagonizes the function of E2-TA in transcription and replication by forming heterodimers and competitively binding to E2BSs. The E8 moiety also recruits transcriptional repressor complexes to viral genomes (129). In the absence of E8^E2 expression, HPV genomes cannot stably maintain extrachromosomal viral genomes because of the propensity to enter unrestricted, productive DNA replication (130–132).

5.3. Higher-Order Multimerization

The formation of higher-order structures of the viral tethering proteins is central to genome tethering and partitioning. The DNA binding function of BPV1 E2 is not required for its association with Brd4 and host chromatin, but the dimerization function greatly enhances this activity (133). Accordingly, a dimeric E2 protein with a single transactivation domain cannot support maintenance replication (134). Dimerization of E2 through the C-terminal domain allows two transactivation domains to interact with chromosomal targets, promoting the formation of multimeric complexes consistent with the punctate E2 speckles on mitotic chromosomes. The CBDs of E2 and EBNA also self-associate to form DNA loops between individual DNA-bound dimers, although the significance of this is not known (47, 135, 136). The dimeric DBDs of LANA and EBNA1 form higher-order oligomeric structures (42, 57, 58, 137). This oligomerization is required for cooperative DNA binding and maintenance replication and is responsible for the
nuclear speckles formed by the proteins (47, 135, 136). LANA bound to KSHV DNA binds the histone H2A/B interface on cellular chromatin but can also interact with histones on viral chromatin, promoting the observed clusters of viral genomes associated with mitotic chromosomes (10).

5.4. Epigenetic Modifications of Host and Viral DNA

During persistent/latent infection, viral DNA is packaged in cellular nucleosomes that are subject to a wide range of epigenetic modifications (138). The viral tethering proteins directly influence these modifications to modulate the viral transcriptional program as well as the efficiency of genome maintenance and partitioning (139).

### SUMMARY POINTS

1. Several dsDNA oncogenic viruses cause persistent infection that can last the lifetime of the host.
2. These viruses often have viral genomes that replicate as stable copy number, extrachromosomal dsDNA circular plasmids in the nucleus of infected cells.
3. A robust mechanism is required to maintain extrachromosomal viral genomes in dividing cells; a viral tethering protein binds to viral DNA and links it to host chromosomes to partition the genomes to daughter cells.

### FUTURE ISSUES

1. Disruption of binding of the viral tethering complex to host chromosomes is an attractive therapeutic target that could cure viral infection.
2. Novel, more sensitive technologies will allow a more detailed understanding of the tethering and partitioning process in living cells and tissues.
3. Understanding how extrachromosomal viral DNA escapes detection has important implications for gene therapy.

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