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Keeping it quiet: chromatin control of gammaherpesvirus latency

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Summary

The human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) establish long-term latent infections associated with diverse human cancers. Viral oncogenesis depends on the ability of the latent viral genome to persist in host nuclei as episomes that express a restricted, yet dynamic pattern of viral genes. Multiple epigenetic events control viral episome generation and maintenance. This Review highlights some of the recent findings on the role of chromatin assembly, histone and DNA modifications, and higher-order chromosome structures that enable gammaherpesvirus to establish stable latent infections that mediate viral pathogenesis.

Introduction

Epstein-Barr Virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are distinguished among the herpesvirus family for their causal association with several human cancers^{1, 2}. The viral genomes and gene products can be detected in tumour cells of various tissue origins, including lymphomas, carcinomas and sarcomas. In general, gammaherpesvirus oncogenesis correlates with the capacity of viral genomes to persist in dividing cells and to express a limited set of viral genes that drive host cell proliferation and survival^{3, 4}.

Like other herpesviruses, EBV and KSHV can establish latent, non-productive infections (that is, infections during which viral genomes exist in host cells without the production of infectious viral particles). In contrast to other herpesviruses, gammaherpesviruses are particularly adept at establishing stable latent infections in proliferating host cells. During gammaherpesvirus latency, the viral genomes are maintained in the host nuclear compartment as multicopy, non-integrated circular genomes with chromatin structure similar to that of the host chromosome. These latent genomes, referred to as episomes or minichromosomes, have features similar to the host cell chromosome, that allow them to be transcribed and replicated by the host cell machinery. In addition, the viral genome is epigenetically modified, which allows the virus to fine tune its gene expression patterns in response to changes in the host cell environment. Moreover, epigenetic features provide the

Note: We apologize to our many colleagues for not citing numerous related studies due to manuscript space limitations.

heritable memory required to maintain a consistent gene expression pattern during multiple divisions of proliferating host cells^{5, 6}.

This Review focuses on recent work highlighting the importance of chromatin assembly, epigenetic modifications and chromatin-organizing factors that control the establishment of gammaherpesvirus latency. The Review highlights some of the key steps of primary viral infection, and considers how each stage of infection may contribute to the building of the latent viral episome. The major events include the assembly of viral chromatin, the patterning of histone post-translational modifications and DNA methylation, and the formation of higher-order chromosome conformations that coordinate gene expression programmes and maintain epigenetic memory during cell division.

Early epigenome establishment

Gammaherpesviruses enter the host cell nucleus as naked linear DNA genomes protected by a viral encoded protein capsid that is delivered to the nuclear compartment (Fig. 1). These early events, including receptor engagement and capsid transport, are likely to set the stage for viral gene expression in the nucleus (Box 1). How the naked, unmodified viral DNA is assembled in the nucleus into a functional circular minichromosome that is competent for programmed gene expression and DNA replication remains poorly understood. The processes of genome circularization and chromatinization are thought to be key regulatory events that are crucial for the establishment of latent infection.

Viral genome circularization—Genome circularization is likely to be important for protecting viral DNA ends and establishing a genome structure capable of completing the gammaherpesvirus life cycle⁷. For EBV, circularization is required to generate the templates for the terminal repeat transcripts encoding LMP2a and LMP2b, which promote B cell proliferation and suppress viral lytic cycle reactivation⁸. For KSHV, circularization is necessary to generate an intact episome maintenance element consisting of multiple tandem copies of the viral terminal repeats (TRs)⁹. Circularization is thought to be necessary for rolling-circle DNA replication, which is thought to be the conserved mechanism of all herpesvirus lytic cycle DNA replication¹⁰. Lytic cycle DNA replication may also be required for the amplification of the viral genome prior to the establishment of latency¹¹. This is consistent with observations that viral lytic gene products are produced transiently during the early stage of primary infection¹¹.

Circularization is an inefficient process and can be detected for only a subset of genomes at 24 hours post infection¹². Circularization of the gammaherpesvirus genomes is thought to involve some form of DNA end-processing and homologous recombination followed by ligation. In support of a requirement for DNA end processing is the finding that linear EBV virion DNA has asymmetric end structures - one blunt and one with a single base overhang¹³ - suggesting that additional end-processing is required for ligation. A role for homologous recombination is based on the finding that terminal repeat copy number changes upon circularization during primary EBV infection¹⁴. Genomes that fail to circularize can integrate into the host chromosome, but these integrated genomes frequently lose essential genetic information and viral function^{12, 15}.

Genome circularization might also be necessary for proper chromatinization (see below). Linear genomes are known to activate the host DNA damage response (DDR), which can function as a potent antiviral defense¹⁶. The host DNA damage response is tightly coordinated with chromatinization, as it is well established that double-strand breaks are subject to histone modifications, especially phosphorylation of histone H2AX mediated by ataxia-telangiectasia (ATM) kinase to generate γ H2AX¹⁷. The ATM pathway has been implicated as a control point for gammaherpesvirus primary infection¹⁸. Primary EBV infection activates ATM, and inhibitors of ATM increase the number of cells that become immortalized by EBV latent infection. Upon detection of damage, ATM phosphorylates histone H2AX to generate γ H2AX and initiate the DNA damage response¹⁹. For the mouse gammaherpesvirus MHV68, ATM kinase activity and γ H2AX formation support chronic infection^{20, 21}. For KSHV, γ H2AX is enriched at the terminal repeats and contributes to episome maintenance^{20, 22–23}. Together, these findings suggest that genome circularization is coordinated with host DDR-dependent histone modifications and chromatin assembly to promote the establishment of latency.

Viral genome chromatinization—Cellular DNA is packaged with proteins known as histones to form chromatin. This condensed configuration, which is mediated by several host factors, ensures that the genome is protected from DNA damage and offers tight regulation of gene expression²⁴. Chromatin assembly after primary infection is likely to be a crucial determinant of gammaherpesvirus fate. Failure to establish the appropriate nucleosome positions and epigenetic modifications may account for variability in viral genome expression. Most infecting gammaherpesvirus linear genomes fail to establish stable episomal infections, and it is possible that improper circularization or chromatinization may account for this high failure rate^{25, 26}.

For alphaherpesviruses, specific histone chaperones (for example, ASF1) and histone variants (for example, H3.3) have been implicated as cofactors in lytic infection^{8, 27, 28}. The role of histone loading factors in gammaherpesvirus infection has not been addressed directly, but may be inferred from the interactions with some viral tegument proteins. All gammaherpesviruses encode a class of tegument protein that targets cellular factors involved in chromatinization. These include the EBV BNRF1, KSHV ORF75, murine herpesvirus 68 (MHV68) ORF75c, and herpesvirus saimiri (HVS) ORF3. Each of these proteins targets components of PML nuclear bodies (PML-NBs, also known as ND10) (Fig. 1), which consist of cellular proteins (PML, SP100, DAXX and ATR) that regulate chromatin assembly and have a central role in the intrinsic resistance to viral nuclear infection^{29–31}. For example, DAXX and ATRX form a protein complex that loads histone variant H3.3 to suppress transcription at repetitive DNA elements^{32–34}. DAXX alone can mediate transcriptional repression through its interaction with histone deacetylases^{35, 36}. Other components of PML NBs, including SP100, have also been implicated in transcriptional regulation of viral genomes^{37, 38}.

Interestingly, the gammaherpesvirus tegument proteins target different components of the PML-NBs through non-conserved regions (despite substantial evolutionary homology elsewhere in the proteins). EBV BNRF1 interacts with DAXX and prevents ATRX localization to PML-NBs³⁹. MHV68 ORF75c and HVS ORF3 interacts with and degrade

PML and SP100, respectively⁴⁰. Destruction of the PML-NBs is thought to be necessary for the completion of herpesvirus lytic cycle gene expression and DNA replication⁴¹. However, BNRF1 does not degrade any component of the PML-NBs, but merely prevents ATRX localization and binding. It is therefore tempting to speculate that BNRF1 remodels, but does not destroy PML-NBs to permit latent cycle viral gene expression, but still restrict lytic gene expression.

Control of latent cycle transcription

Human gammaherpesvirus primary infection typically results in a slow, abortive lytic cycle that competes with a more robust latent cycle gene expression programme. Chromatin and other epigenetic features have important roles in regulating the balance between different gene expression programmes. Below I discuss the gene expression programme of EBV and KSHV and consider how chromatin organization is a common regulatory mechanism for each virus.

EBV transcription regulation during latency—EBV can establish at least four distinct latency-associated transcription patterns, referred to as latency types^{42, 43}. The most restrictive transcription programme is latency type 0 (observed in non-cycling, resting memory B cells), during which no viral gene products are synthesized. Type I latency (observed in proliferating memory B cells and Burkitt lymphoma cells) involves the expression of EBNA1 and several non-coding RNAs. Type II latency is defined by the expression of one or several latency membrane proteins (LMP1, LMP2a or LMP2b), in addition to the type I gene products, and this type of latency is observed in Hodgkin's lymphomas and epithelial cell carcinomas. Type III latency (observed in highly proliferating B cells and immortalized cell lines) is the most permissive for transcription, during which all of the viral genes associated with latency are detected, including EBNA1, EBNA-LP, EBNA2, EBNA3a, EBNA3b, EBNA3c, LMP1, LMP2a and LMP2b.

During the early phase of EBV infection of naive B cells, the first latent viral gene detected is an *EBNA2* mRNA, transcription of which initiates at the W repeat promoter (Wp) (Fig 2). EBNA2 is essential for the transcriptional activation of host and viral genes required for B cell proliferation. This is achieved through interactions with the host transcription factors RBP-jK (also known as CBF1 and CSL) and PU.1⁴⁴. As EBNA2 accumulates, it associates with RBP-jK and PU.1 sites on viral promoters. This causes transcription to shift from the Wp to an upstream promoter known as Cp. Transcription that initiates from Cp gives rise to a much larger (~100kB) polycistronic and alternatively spliced transcript that generates EBNA-LP, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNA1. EBNA2 is also required for transcriptional activation of the LMP1 and the LMP2 promoters, through interactions with RBP-jK or Pu.1^{45, 46}. Additional host factors involved in this process include PAX5, which is required for early activation of Wp^{47, 48}, and OCT2, which has been implicated in the activation of Cp⁴⁹.

EBV early gene expression is more complicated since genetic studies indicate that EBNA1 is also required for the transcriptional activation of Cp during primary infection of B cells⁵⁰. This suggests that EBNA1 must be expressed prior to the switch to Cp-initiated

transcription⁵⁰. *EBNA1* mRNA is generated from Cp at later times in type III latency, but it is not clear how EBNA1 is generated during the earliest stages of B-cell infection. In type I latency, EBNA1 is expressed from a constitutive promoter, termed Qp. It not known, but tempting to speculate that EBNA1 transcripts during early phase infection may originate from Qp, rather than Wp^{51–53}. Qp has two high-affinity EBNA1 binding sites that result in auto-inhibition by EBNA1 protein, so it is possible that this inhibition of EBNA1 correlates with and may facilitate the switch to Cp-initiated transcription.

The temporal order of transcription events during primary viral infection is therefore not completely clear, and it is possible that Qp-driven EBNA1 and Wp-driven EBNA2 are generated simultaneously and cooperate to reinforce the type III latency programme⁵². How type III latency evolves into the more restrictive type I or type II latency is also not entirely clear. It is known that in type I latency, Cp-driven transcription is repressed, and only EBNA1 is transcribed by Qp. This promoter switch correlates with epigenetic changes at the Cp and LMP1 promoter, including DNA methylation of critical transcription factor binding sites^{54, 55}. Factors that inhibit EBNA2-mediated transcriptional activation might also have a role in the switch from type III to type I latency.

EBV can also show partial lytic cycle gene activation during de novo infection of primary B cells; this has been referred to as the pre-latency phase⁵⁶. In the process of establishing a latent infection, EBV transiently expresses some early phase lytic cycle gene products, including the immediate early protein Zta (also known as BZLF1; a DNA-binding protein that activates the transcription of most lytic cycle genes), but virion production is minimal¹¹. Similarly, early, but not late lytic cycle gene transcripts are detected in transcriptome analyses of latently infected lymphoblastoid cell lines (LCLs), suggesting partial lytic cycle gene activity occurs in these cell population⁵⁷. These findings suggest that partial lytic cycle gene expression is permitted during the establishment phase of latency and in some latently infected cells. Importantly, completion of the lytic cycle can be restricted at multiple levels, including inhibition of viral gene expression⁵⁸ and protein function^{59–61}(see below).

KSHV transcriptional regulation: KSHV seems to show less variation than EBV with regard to latency transcription, although heterogenous gene expression has been reported in some latency models and tumour-derived cells⁶². Like EBV, KSHV primary infection typically results in an abortive lytic infection, with infected cells producing low levels of viral particles and primarily expressing the latent transcriptional programme. The major latency-associated transcript consists of a multicistronic message encoding LANA, vCyclin and vFLIP. LANA is the KSHV orthologue of EBNA1, and can bind directly to the terminal repeat DNA to promote DNA replication and episome maintenance during latency^{63, 64}; vCyclin and vFLIP are required for host cell cycle proliferation and survival⁶⁵. A strong initiator element has been identified at the core promoter for these latency transcripts^{66, 67}. Alternative downstream promoters can initiate a transcript for vFLIP, K12 and a cluster of viral miRNAs^{68, 69}. In addition, the latency proteins vIL6 and vIRF3 are also detected in most models of KSHV latency and in KSHV-infected tumour cells^{70, 71}.

However, some viral genes associated with the lytic cycle can be detected in the context of tumours. For example, in tumour isolates from primary effusion lymphoma, the lytic cycle

gene vGPCR (also known as ORF74) can be detected along with latency transcripts⁷², and sustained vGPCR expression is thought to be crucial for B cell tumorigenesis^{73–76}. As vGPCR is typically considered a lytic viral gene product, its expression in tumour cells may reflect an aberrant control of latent infection⁷⁵.

More recent studies suggest that KSHV can adopt different transcription patterns depending on the host cell type⁶². In human lymphatic endothelial cells (LECs), the KSHV lytic cycle immediate early genes ORF45 and ORF50 are transcribed along with canonical latency genes (LANA, vCyclin and vFLIP), but other lytic genes are not detected. One phenotypic consequence of this different gene expression programme is that LECs are more sensitive to treatment with rapamycin (an immunosuppressant that activates the growth control protein mammalian target of rapamycin (mTOR), as the KSHV ORF45 protein induces chronic activation of mTOR⁶². These findings suggest that KSHV may have different latency types similar to that of EBV, and that both viruses may express some lytic genes without full commitment to lytic cycle DNA replication and viral production.

Ensuring expression of the viral epigenome

As mentioned above, EBV and KSHV can establish stable and distinct transcription programmes during latent infection, which in the case of EBV reflect the different latency types^{77–80}. In many cases, these different latency types have been shown to have correspondingly different epigenetic modification patterns, referred to as ‘epigenotypes’^{55, 80}.

Epigenetic stabilization of latency programmes—DNA methylation patterns have been shown to have a key role in regulating both KSHV⁸¹ and EBV latency types^{54, 56, 78}. DNA methylation, which typically represses gene expression, occurs gradually after primary infection. For EBV, the slow rate of DNA methylation restricts lytic cycle gene activation, as DNA methylation is required for transcription activation of some viral genes by Zta^{56, 82–89}. Zta is unusual in that it can bind selectively to DNA with methylated cytosine⁸⁶; in fact, methylation of some viral promoters is necessary for Zta-dependent binding and transcription activation and lytic gene expression⁸⁹. Thus, the lack of DNA methylation provides a paradoxical restriction to EBV lytic cycle gene expression^{56, 82}. In the case of KSHV, DNA methylation does not occur at constitutively active latency promoters, like the LANA promoter, but instead at several transcriptionally inactive regions. Similarly, in EBV DNA methylation is spared at transcriptionally active latency promoters, as well as other protected sites such as OriP and Qp, which constitutively bind the episome maintenance protein EBNA1⁹⁰. However, DNA methylation has been shown to repress Cp in type I latency, resulting in EBNA2 and EBNA3 silencing⁵⁴. The mechanisms that determine DNA methylation patterns are not yet understood, although it is possible that some sites are methylated owing to a lack of transcriptional activity (‘methylation by neglect’), whereas others are spared DNA methylation owing to the protective effects of some DNA binding proteins, like EBNA1.

Histone modifications also have a central role in regulating EBV and KSHV latency. Many studies have shown that gammaherpesvirus latency could be disrupted with histone

deacetylase inhibitors⁹¹. Transcriptional activation of both latent and lytic genes correlate with changes in histone tail modifications at active promoter regions^{92, 93}. These modifications include the well-established histone marks associated with eukaryotic gene activation, namely hyperacetylation of histone H3 and H4 N-terminal tails, and trimethylation of H3 at lysine 4 (H3K4me3)^{92, 93}. More recent genome-wide studies have indicated that EBV and KSHV have complex histone modification patterns during latent infection^{57, 77, 81, 94–97}. The epigenetic landscape of KSHV latent genomes has been examined in several cell types⁸¹ and compared with reactivating genomes⁹⁶. These studies revealed that the promoter region upstream of lytic immediate early gene ORF50 (encoding the lytic activator Rta) is enriched with both activating (H3K4me3) and repressing (H3K27me3) histone modifications^{81, 96}. This ‘bivalent’ control of gene expression is also found at promoters of cellular genes that remain poised for activation during developmental switches⁹⁸. The small molecule inhibitor of the H3K27me3 methylase EZH2, DZNep, was shown to stimulate KSHV lytic cycle gene activation⁹⁶, suggesting a role for H3K27me3 in promoting latency. The transcriptional repressive effects of H3K27me3 are known to be mediated by the chromatin modulator Polycomb⁹⁹, suggesting that these proteins have a central role in restricting the lytic cycle gene programme and chromatin structure of KSHV during latency.

Much of the data collected for the EBV epigenome has been derived from metadata analyses of the ENCODE ChIP-Seq data collection on LCLs containing the EBV B95.8 genome⁵⁷. The study indicated that type III latency EBV in LCLs has a complex organization of histone modifications, with high enrichment of H3K4me3 at the active promoters for Cp, LMP2A, LMP2 and at the RPMS1/BART promoter regions. In contrast to KSHV, these studies did not show a high level of repressive histone marks at lytic promoters, suggesting that EBV latency is regulated by other mechanisms⁹⁶.

Chromatin-organizing factors: CTCF and cohesins—Organization of histone modifications and nucleosome positioning is a key regulatory feature of eukaryotic chromosomes^{100, 101}. How this process occurs *de novo* on newly infecting viral genomes, and how these patterns are maintained during multiple cell divisions is of great relevance to understanding the epigenetic control of gammaherpesvirus latency. At least some of the nucleosome positions and histone tail modifications (see above) are directed by sequence-specific transcription factors and their cofactors. In addition, specialized factors such as CCCTC-binding factor (CTCF) are known to function as chromatin-organizing factors^{102–104}.

CTCF can prevent the spread of repressive or active chromatin from one regulatory domain into another, and can prevent enhancer communication with a specific promoter (acting as an insulator). CTCF can also function in DNA-loop formation, and it is possible that these structural loops serve as the molecular basis for other functions in chromatin boundary and enhancer insulator function^{105–107}. DNA loop formation by CTCF commonly involves another cellular protein complex, termed cohesin. Cohesin is a multiprotein complex that was originally characterized for its role in sister-chromatid cohesion, which keeps newly synthesized chromosomes in close contact in early mitosis and is necessary for faithful chromosome segregation during cell division^{102, 108, 109}. Cohesin has been found to

colocalize with CTCF at many cellular chromosomal positions and supports DNA-loop structures, which are important for gene regulation^{102, 108}. Co-occupancy of CTCF and cohesin has also been observed on the gammaherpesvirus episomes during latent infections^{57, 97, 110}.

In the KSHV episome, a cluster of three CTCF binding sites within the first intron of the major latency transcript show strong colocalization with cohesin¹¹⁰. The function of CTCF and cohesin binding within this position seems to be multifactorial. Genetic disruption of the CTCF sites in KSHV bacmids destabilizes episomal maintenance in some cell lines and reduces the efficiency of de novo infection¹¹⁰. CTCF-site mutant genomes show defects in latency transcript regulation, with a shift towards the unspliced form of the multicistronic transcript¹¹¹. Mutation of CTCF binding sites also disrupts cohesin and RNA polymerase II binding¹¹¹. The complexity of regulatory events surrounding CTCF binding sites suggests that they have a fundamental structural and organization role in the viral chromosome (Fig. 3).

One potential mechanism by which CTCF organizes chromatin may be by disrupting the normal positioning of nucleosomes. At the KSHV latency promoter, the cluster of three CTCF sites prevented the positioning of a second nucleosome downstream of the latency transcript initiation site¹¹¹. As the first nucleosome downstream of the transcription start site is commonly enriched in modifications associated with RNA polymerase II function (for example, H3K4me3 and H3K9ac)¹¹², it is possible that CTCF-binding sites prevents the processive spreading of these modifications and thereby modulate transcription elongation and mRNA splicing of the complex latency transcripts.

A chromatin boundary function for CTCF has been observed at two regulatory regions in the EBV episome. The CTCF-binding site upstream of Qp protects this core promoter from DNA methylation and consequent transcriptional silencing. This site also blocks the spread of the repressive histone modification H3K9me3 that appears just upstream of Qp^{90, 113}. A CTCF-binding site located between the Cp and the EBNA1-binding sites at OriP can regulate EBNA1-mediated transcription activation of Cp^{114, 115}. Specifically, CTCF binding between OriP and Cp might prevent the unwarranted spread of euchromatic H3K4me3 (which is enriched around OriP in most latency types) into Cp, especially in more restrictive latency types I and II where Cp is repressed.

CTCF-binding sites may also provide chromatin boundary functions at the lytic control region of the KSHV episome, which, as mentioned above, has bivalent histone modifications^{77, 81}. As CTCF-binding sites have been identified at the boundaries of lytic promoter regions, it is possible that CTCF, together with cohesin, might protect this bivalent chromatin organization¹¹⁶. Chromatin-immunoprecipitation (ChIP) assays also suggest that CTCF also functions to retain RNA polymerase II at the lytic promoter in a conformation poised for rapid response to reactivation signals¹¹⁶. Phosphorylated RNA polymerase II (associated with transcription initiation, but not yet competent for elongation) is enriched at the KSHV lytic control region¹¹⁶, so the presence of CTCF might provide a boundary for trapping poised, but not elongating RNA pol II, at this location.

Chromatin conformations regulating viral gene expression—Higher-order chromosome structures, such as promoter-enhancer DNA loop interactions, contribute to the coordinate control of eukaryotic gene expression¹⁰⁰. For gammaherpesviruses, it is not known how latent and lytic promoters located tens of kilobases away may coordinately regulate their transcription programmes. DNA loops between transcriptional regulatory elements have been identified in both EBV and KSHV latent genomes. In EBV type III latency, OriP functions as a transcriptional enhancer for both Cp and LMP1 promoters^{57, 117}. In both cases, the physical interaction involves the formation of DNA loops, which are dependent on CTCF-binding sites and cohesin⁵⁷. Cohesin colocalizes with both CTCF sites, and shRNA-mediated depletion of cohesin subunits disrupts loop formation and deregulates transcription from both promoters. Interestingly, in type I latency, when both Cp and LMP1 and LMP2 promoters are repressed, OriP forms a DNA loop with the active Qp¹¹⁷. This suggests that OriP functions as a transcriptional enhancer that selectively loops with the active promoters for each latency type (Fig. 2).

DNA loops involving OriP may be mediated, in part, by EBNA1, which is known to bind multiple sites within OriP and can form a short DNA loop between these sites within OriP^{118, 119}. The EBNA1 amino-terminal domain is known to have transcription enhancer function, as point mutations in the EBNA1 N-terminal unique region 1 (UR1) disrupt transcriptional activation of Cp⁵⁰. UR1 was shown to coordinate Zn through a pair of cysteine residues that are essential for EBNA1 homotypic interactions and transcriptional enhancer function¹²⁰. Furthermore, Zn binding and homotypic interactions were shown to be redox sensitive, suggesting that EBNA1 mediated loop formations are regulated by oxidative stress¹²⁰. These findings support a model whereby EBNA1 forms a Zn-hook like structure¹²¹ that may regulate long-distance interactions required for OriP loop formation and transcription enhancer function.

DNA regulatory loops have also been described for the KSHV episome¹²². Chromatin conformation capture (3C) studies revealed that the CTCF-cohesin site in the latency control region forms two loops: a short ~10kb loop between the CTCF cluster in the first intron of *LANA* and the region 3' of *K12*, encompassing the major latency transcripts; and a larger loop between the CTCF-cohesin sites and the control region for lytic transcripts of ORF50 and ORF45–46–47¹²². As noted above, the lytic control region is bracketed by CTCF-binding sites with a poised RNA polymerase II. shRNA-mediated depletion of cohesin subunits leads to a loss of DNA loop structures (as measured by 3C analysis) and a robust stimulation of lytic transcription¹²², which suggests that the CTCF-cohesin bound to the latency control region function as a repressor of lytic transcription. Thus, CTCF-cohesin mediated loops can function in both activation, as well as repression, of viral transcription.

DNA replication and episome maintenance

Gammaherpesviruses are unique in their ability to maintain a stable copy number of viral episomes in proliferating host cells. This is accomplished through the coordination of viral gene expression, DNA replication and genome segregation. Episome maintenance requires binding of the virus proteins EBNA1 (for EBV) or LANA (for KSHV) to repetitive

sequences in either OriP (for EBV) and the terminal repeats (TR; for KSHV)¹²³; thus, these proteins and DNA regions are referred to as episome maintenance elements.

Tethering to host chromosomes—A key feature of episome maintenance is the ability to tether the viral genome to the host metaphase chromosome (Fig. 4A), to retain viral genomes in the nuclear and chromosomal domains during host cell division. Thus, tethering allows gammaherpesviruses to ‘hitch a ride’ on the host chromosome and maintain a stable copy number after host cell division. Both EBNA1 and LANA have peptide motifs that confer metaphase chromosome tethering. EBNA1 tethering is mediated by RGG-like motifs in the EBNA1 N-terminal domain, which interacts with the host cell protein EBP2^{124–126}, with AT-rich DNA¹²⁷ and with G-quadruplex RNA¹²⁸. For KSHV, attachment to the metaphase chromosome is mediated by binding of the LANA N-terminal domain to core histones H2A and H2B¹²⁹, and of its C-terminal domain to host cell chromatin proteins, including the bromodomain proteins BRD2 and BRD4^{130, 131}, which recognize acetylated histones. Together, these observations suggest that in EBV and KSHV tethering is mediated through multiple interactions with host chromatin.

Promoting DNA replication—The gammaherpesvirus episome maintenance elements can also function as origins of DNA replication. Both EBNA1 and LANA can interact with the host replication factors, including the origin recognition complex (ORC) and replication protein A (RPA) to facilitate efficient replication origin formation. The precise mechanism of how EBNA1 and LANA stimulate origin formation remains poorly understood. EBNA1 and LANA can interact with ORC subunits, and the BAH domain of ORC1 has been implicated in EBNA1 binding¹³². The EBNA1 RGG-motifs are required for replication function, and this correlates with ORC recruitment. In addition, G-quadruplex RNA has been shown to stimulate the interaction between EBNA1 RGG-motifs and ORC¹³³.

Although viral episome maintenance elements such as EBV OriP and KSHV TR can act as efficient replication origins, their function depends on poorly understood epigenetic features¹³⁴. Histone acetylation and enrichment of H3K4 trimethylation are known to influence origin selection on host chromosomes^{135,136}. Similar modifications are observed at gammaherpesvirus episome maintenance elements in cells with established latent infections^{137, 138}, but this may not be sufficient for replication initiation, as single molecule studies indicate that DNA replication can initiate at sites outside of these episome maintenance elements^{139–143}. Thus, replication initiation may not be a primary essential function of episome maintenance elements.

Partitioning the viral genome during cell division—The precise mechanism through which gammaherpesviruses partition their genomes to maintain a stable copy number is not yet known. Studies have shown that partitioning is non-random and coupled to DNA replication¹⁴⁴. One potential mechanism for active partitioning may involve the formation of DNA catenations between newly replicated DNA molecules^{145–148}. Two-dimensional agarose gel analyses reveal that DNA catenation-like structures form specifically at OriP (in EBV) and TR (in KSHV)^{145, 146, 146}. DNA catenations are thought to provide a mechanism to attach newly replicated sister chromosomes to each other, similar to sister chromatid cohesion for cellular chromosomes. This mechanism has been shown to be important for

plasmid partitioning in yeast and bacteria, and has been referred to as ‘chromosome kissing’¹⁵⁰. Both EBNA1 and LANA may induce DNA catenation formation at OriP and TR by perturbing DNA polymerase and replication fork structure^{145–148}. Evidence to support this model is provided by studies that show that host replication fork protection proteins, Timeless and Tipin, colocalize with OriP and provide essential functions in EBV and KSHV episome maintenance^{145, 146}. These studies suggest that gammaherpesvirus episome maintenance elements can form replication-dependent catenated structures that are required for faithful segregation of viral episomes during cell division. (Fig. 4B).

Reactivation and virus production

Reactivation from latency is required for the completion of the gammaherpesvirus life cycle and to produce new infectious viral particles. Reactivation can be stimulated by various stress responses, ranging from the unfolded protein response to hypoxia, as well as cell differentiation signals. These pathways typically converge by activating transcription of the immediate early genes, which can recruit numerous host cell regulators that modulate viral chromatin structure and function (for reviews see^{151–156}).

Disruption of repressive chromatin may be an essential pathway for gammaherpesvirus reactivation. The viral immediate early proteins Zta (for EBV) and Rta (for both EBV and KSHV) are required for transcription activation of lytic cycle genes¹⁵⁷. Zta and Rta can associate with histone acetyltransferases and chromatin remodeling proteins to stimulate transcription of chromatinized viral episomes^{158,159}. KSHV Rta can function as a E3 ubiquitin ligase to degrade transcriptional repressor complexes, like K-RBP and Hey1, that block KSHV lytic cycle transcription^{160, 161}. In addition to these viral immediate early proteins, recent studies have revealed that viral non-coding RNAs can also regulate the lytic switch. The KSHV polyadenylated nuclear non-coding RNA PAN can facilitate lytic cycle gene expression by disrupting polycomb-mediated mediated chromatin repression. PAN RNA was found to bind and recruit the histone H3K27 demethylases UTX and JMJD3 to reverse the polycomb-mediated repression of KSHV immediate early transcripts¹⁶². Interestingly, polycomb repression is also the target of the EBV-encoded EBNA3C, but in this case, EBNA3C promotes polycomb repression on host tumor suppressor genes^{163, 164}. It is not yet known whether EBNA3C recruits H3K27 methylases and polycomb to repress viral lytic genes, and thus stabilize latency.

One of the emerging themes in the regulation of gammaherpesvirus reactivation is that factors that promote latency can also repress lytic reactivation. For example, loss of EBNA1 or LANA increases viral lytic cycle gene expression, suggesting that these latency maintenance proteins also repress lytic gene expression^{165–167}. LANA functions as a transcriptional repressor that can interact with RBP-jK sites at lytic promoters, including the promoters for immediate early gene transcripts^{168, 169}. EBNA1 can also repress lytic gene transcription during latency, since its depletion leads to lytic cycle activation¹⁷⁰. However, the mechanism for EBNA1 transcriptional repression of EBV lytic gene transcription is not yet understood. (Fig. 5)

Many other factors contribute to the balance between latent and lytic gene expression. Since gammaherpesviruses encode numerous miRNAs, it is not surprising that one of the functions

of these non-coding RNAs is to stabilize latency by maintaining repressive epigenetic marks. For example, the KSHV miRNA K12–5 was shown to prevent lytic cycle gene expression by increasing global viral and cellular DNA methylation levels¹⁷¹. This was achieved through downregulation of the host protein RBL2, which represses the DNA methyltransferase DNMT3b¹⁷¹..

Heterogeneity of genomes and host cells is also an important consideration in gammaherpesvirus gene regulation. It is well known that gammaherpesvirus reactivation from latency is stochastic and multifactorial¹⁷², as only a subset of cells and genomes may respond to an activation signal. Global epigenetic regulators such as HDAC inhibitors and demethylating agents and histone methylase inhibitors can stimulate partial lytic reactivation, and the extent of reactivation varies among cell and latency types. The refractory nature of viral latency in some cell types has been difficult to explain, and remains a challenge for lytic therapies, during which some cells fail to respond to a reactivation signal. In one study the refractory cells showed high levels of STAT3 expression^{173, 174}, whereas in another study the block to reactivation correlated with increased levels of EBF1¹⁷⁵. Several additional host proteins, including the repressor ZEB^{58, 176, 177}, Jun dimerization protein¹⁷⁸ and OCT2⁶⁰, have been shown to block lytic reactivation. In addition to these, viral immediate early proteins can be inhibited by post-translational modifications⁵⁹ and by epigenetic modifications of the viral genome¹⁷⁹. Taken together, these studies suggest that combinatorial control and epigenetic variations of the viral genome may explain the sporadic and stochastic process of reactivation from latency.

Conclusions

Gammaherpesvirus latency is a complex and sophisticated form of genetic parasitism that involves the formation of a stable mini-chromosome that is highly responsive to the host cell environment and developmental status.

The establishment of latency involves a competition between lytic gene expression and a more dominating class of latency gene products. Stable latent infection depends on the acquisition of several epigenetic features, including circularization, chromatinization and post-translational modifications of histones and DNA. In addition, higher-order chromatin structures, such as DNA loops and catenations, may have a role in gene regulation and episome maintenance. These epigenetic features are necessary for stable gene expression programmes and faithful transmission of viral genomes to daughter host cells.

Despite the enormous wealth of information on gammaherpesvirus latency, there are considerable gaps in our knowledge of how latency is established and maintained. For instance, it is not yet known what host cell factors are primarily responsible for the restriction of gammaherpesvirus lytic gene expression during primary infection. We also do not know what epigenetic events are principle drivers of viral latency. Although we know that the formation of a stable viral episome involves nucleosome assembly and histone modifications, it remains unclear how nucleosome position and histone modification patterns are established on the newly infecting viral genomes, or how these patterns of chromatin organization are maintained over cell division cycles.

It will also be important to determine how higher-order chromosome conformations are established and how these structures facilitate interactions between enhancers, like OriP, and the appropriate promoter elements selected for transcription activation, like Cp or Qp.

How the viral episomes are replicated and segregated during each cell cycle may also be subject to important epigenetic control, including the formation of DNA catenations that promote sister chromatid cohesion after DNA replication. Whether these epigenetic factors allow the gammaherpesvirus genomes to survive as stable episomes and maintain a stable copy number in proliferating cells is an important unanswered question.

Finally, the mechanism of gammaherpesvirus persistence in cancer cells may be different from that in normal cells¹⁸⁰. Abberations in the prototypical epigenetic programmes may account for the rare incidence of virus-associated tumour formation. At present, we do not know whether specific epigenetic modifications correlate with cancer cells and whether these are inherently different than latency associated with normal, non-malignant cells. Understanding the detailed mechanisms of each of these processes discussed in this Review, and their potential aberrations in virus-associated cancers may provide insights into the oncogenic potential of gammaherpesvirus latency, and may provide novel strategies for therapeutic interventions that target latent infection and viral carcinogenesis.

Biography

Paul M. Lieberman is Professor in the Gene Expression and Regulation Program at the Wistar Institute. He is also the Director of the Center for Chemical Biology and Translational Medicine at the Wistar Institute. His recent work focuses on the chromosome biology of EBV and KSHV latency, and the development of small molecule regulators of latent virus infection.

Glossary

histone deacetylase	Histone deacetylases (HDAC) are a family of enzymes that remove an acetyl group from lysines on histone tails. HDACs typically promote “closed” or repressive chromatin, and reverse the action of histone acetylases that promote “open” chromatin. Small molecule inhibitors of HDACs, like sodium butyrate, trichostatin A, and valproic acid, are commonly used to reactivate latent gammaherpesviruses
Bacmids	Recombinant gammaherpesvirus genomes can be propagated as large bacterial plasmids referred to as Bacmids. Bacmids have antibiotic selection resistance and fluorescent protein markers that greatly facilitate the engineering of site-directed mutations in gammaherpesvirus genomes. Mutations are made when Bacmids are grown in recombinogenic strains of <i>E. coli</i> , and then passaged into mammalian host cells where infectious recombinant virus can be generated

Euchromatic	<p>Euchromatic refers to regions of “open” chromatin that are more accessible to DNA binding proteins and transcription initiation. Euchromatic chromatin (euchromatin) tends to be enriched in acetylated histones and di and tri-methylation of histone H3 lysine 4 (H3K4me2 and H3K4me3). In contrast, heterochromatin refers to “closed” chromatin that is less accessible to transcription factors. Heterochromatin tends to be enriched in trimethylation of histone H3K9me3 (constitutive heterochromatin) and H3K27me3 (facultative heterochromatin)</p>
Zn hook	<p>Some proteins, like EBNA1, can bind to Zn through an interaction with two amino acids, like cysteine and histidine. A Zn hook occurs when Zn mediates the interaction between two different molecules of the same protein through homotypic interactions, such that four amino acids (two from each protein monomer) form a cage to coordinate with a Zn atom at its center</p>
Chromatin conformation capture (3C)	<p>Chromatin conformation capture is a method developed by Job Dekker to measure the interaction between different DNA sites on the same or different chromosomes in vivo. The method involves cross-linking, restriction enzyme digestion, dilution, and relegation of cross-linked molecules. The method determines whether two different DNA regions are in close proximity to each other in vivo, and is used to show that promoters and enhancers form interactions through DNA looping</p>
G-quadruplex	<p>G-quadruplex (or G-quartets or G4-DNA) are DNA or RNA structures that can form in guanine rich stretches where four guanine molecules can form a planar structure that can be stacked to form higher-order stable structures. G-quadruplexes can form between one (intramolecular) or more (intermolecular) DNA or RNA molecules. Repetitive G-rich sequences like telomere DNA and RNA (TERRA) can form G-quadruplex structures. EBNA1 RGG-motif can bind selectively to G-quadruplex RNA</p>
DNA catenations	<p>DNA catenations can form when DNA strands are entangled, as occurs at when two replication forks collide to terminate DNA replication. Most DNA catenations can be decatenated by topoisomerases. Some catenations, including hemi-catenanes, form when the newly-replicated DNA strands are entangled. Catenated, and possibly hemicatenated, structures have been observed at the EBV and KSHV episome maintenance elements</p>
unfolded protein response	<p>The unfolded protein response (UPR) is a stress response that occurs in the endoplasmic reticulum (ER) when numerous proteins are misfolded or the ER is overwhelmed, as occurs during viral infection and during immunoglobulin production in plasma B cells. The XPB-1 protein is a</p>

	central regulator of the UPR and has been implicated in the reactivation of both EBV and KSHV from latency
Hypoxia	Hypoxia refers to a condition of reduced oxygen which can typically occur in tumor cells lacking sufficient oxygenation. Hypoxia is sensed by the hypoxia-inducible factor 1 (HIF1) that can activate transcription in response to low oxygen due to chemical modification of proline residues in HIF1. HIF1 has been shown to play important roles in both EBV and KSHV reactivation from latency
TOC blurb	EBV and KSHV establish latent infections, during which the viral genomes are maintained in the host cell as viral episomes. As discussed by Lieberman, latency depends on numerous factors, including viral genome chromatinization and epigenetic modification, as well as tight control of the latency transcription programme

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OnLine Summary

- Gammaherpesvirus latency is a programmed event with multiple epigenetic steps
- During latency, the viral genome establishes a meta-stable epigenetic pattern of histone modifications and DNA methylation.
- Epigenetic patterns and chromosome conformations are stabilized by host chromatin boundary factors CTCF and cohesin.
- Lytic reactivation is a stochastic process controlled by multiple epigenetic barriers.

Box 1 | Viral entry and recognition

The earliest event that can influence the outcome of gammaherpesvirus infection is the interaction of the viral particle with host cell surface receptors (Fig. 1). EBV binding to CD21¹⁸¹ and KSHV binding to ephrin A2^{182, 183} initiate cell signals, such as PI3K and AKT activation, that can modify nuclear factors which are important for viral gene expression. The viral genomes are then transported to the nuclear pore through a protective viral capsid such that viral DNA is never exposed to cytoplasmic DNA sensors¹⁸⁴. Interaction of the viral capsid with the nuclear pore results in the injection of a single naked viral genome into the nucleus¹⁸⁵. How these cytoplasmic events influence the outcome of viral DNA in the nucleus is not yet known, but they likely to be important for the establishment of successful latent infection

In the case of KSHV, one of the earliest nuclear events is the interaction of viral DNA with IFI16^{186,187}, an AIM2-like receptor (ALR) that recognizes double-stranded DNA through its HIN200 domain¹⁸⁸. Binding to viral DNA activates the inflammasome, **through a mechanism involving** the release of nuclear ASC and procaspase 1 into the cytoplasm¹⁸⁹; similar IFI16 recognition mechanisms have been described during the early stages of HSV infection^{190,191}. IFI16 has also been shown to associate with the EBV genome at all stages of latent infection, resulting in constitutive induction of the inflammasome¹⁹². It is not known how nuclear IFI16 interacts with the viral DNA nor how IFI16 activation of the inflammatory response alters the fate of viral infection. During early HSV infection, IFI16 is degraded by the viral tegument protein ICP0, resulting in a block to this innate immune pathway¹⁹³. One possibility is that the failure to inactivate IFI16 during KSHV and EBV infection may suggest that IFI16 is important for directing gammaherpesvirus to latency, rather than lytic cycle gene expression.

Figure 1. Early events that regulate the establishment of a latent gammaherpesvirus minichromosome

Depiction of the early events affecting the fate of gammaherpesvirus genomes. Receptor-mediated signaling sets the stage for viral gene expression programmes. Entry of the viral DNA into the nucleus activates IFI16, leading to inflammasome activation (in the case of KSHV) and may trigger the DNA damage response (DDR) following recognition by ATM kinase. The linear genome then undergoes circularization and chromatinization, which results in the formation of a stable episome. The viral tegument proteins ORF75 (for KSHV) and BNRF1 (for EBV) have a key role in promoting chromatinization, by disrupting proteins in PML-NBs, for example Daxx and ATRX.

Fig. 2. Establishment of the EBV epigenome

(A) EBV primary infection initiates with transcription from the latent W promoter (Wp) and the lytic Zta promoter. Lower levels of Qp and LMP1 transcripts are also detected. PAX5 and host immediate early factors have an important role in early transcription programming of viral gene expression. (B) Type III latency is established when EBNA2 expression is sufficient to stabilize Cp and LMP1 and LMP2 promoter initiation. EBNA2 binds RBP-jK at Cp and PU.1 at LMP1 promoter. Silencing of the Zta promoter is mediated by repressors, like ZEB. Loop formation is detected between OriP and Cp, and OriP and LMP1/LMP2 region. (C) Type I latency occurs when Cp is silenced by DNA methylation. OriP forms an exclusive loop with Qp, but not Cp

Fig. 3. Establishment of the KSHV epigenome

(A) Primary infection of KSHV results in combination of both lytic and latent transcription.

(B) Latency is stabilized by a CTCFI-cohesin loop between immediate early promoter region (ORF50–ORF45–46–47) and latency control region (LANA–vCyc–vFLIP). RBP-jK functions as a scaffold for activation by Rta (encoded by ORF50) during lytic and for repression by LANA during latency.

Fig. 4. Episome Maintenance and Viral Chromosome Structure

(A) Both EBNA1 and LANA tether the viral episomes to metaphase chromosomes. (Top) The tethering domains of EBNA1 are RGG-like and capable of binding to G-quadruplex RNA and ORC, EBP2, and AT-rich DNA. (Bottom) The N-terminal tethering domain of LANA interacts with histones H2A/H2B. LANA can also interact with other candidate chromatin mediators, including DEK, RBP-jK, and BRD2/4 proteins capable of binding acetylated lysines in the H3 or H4 N-terminal tails. (B) Both EBV and KSHV form sister chromatid catenations that depend on replication fork stalling or termination at the episome maintenance elements. EBV OriP and KSHV TR function as a replication fork blocks that recruit Timeless-Tipin replication fork protection complex and promote sister-chromatid linkages (catenations). Timeless and Tipin are essential for episome maintenance, and may be required for the establishment of stable epigenomes.

Fig. 5. Chromosome Control of Lytic Reactivation

(A) Factors that restrict lytic replication of KSHV include the histone H3K27 trimethylase EZH2 and polycomb repression complex that prevents the transcription of the ORF50/Rta immediate early gene. Additional controls include the CTCF-cohesin binding sites upstream and downstream of the ORF50 IE promoter control region, and the cohesin linkage between IE and lytic control regions. (B) EBV lytic control is provided by host-cell factors that repress the BZLF1/Zta IE control region, including ZEB, Jun dimer binding protein (Jun DBP), and CTCF. A cohesin loop between the Zta IE region and the LMP1/2 control region may repress lytic, similar to that observed in KSHV. Additional restrictions to lytic activation include the repressive interaction of Zta with Oct2, and the requirement for methylated DNA at Zta responsive promoters. Stat1/2 and EBF1 have also been implicated in restricting lytic cycle reactivation.