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EPIGENETIC REGULATION OF KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS REPLICATION

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is the causative agent of Kaposi's sarcoma and B-lymphocyte disorders, primary effusion lymphoma (PEL) and Multicentric Castleman's disease (MCD). KSHV usually exists in a latent form in which the viral genome is circularized into an extrachromosomal episome. However, induction of lytic replication by environmental stimuli or chemical agents is important for the spread of KSHV. The switch between latency and lytic replication is regulated by epigenetic factors. Hypomethylation of the promoter of replication and transcription activator (RTA), which is essential for the lytic switch, leads to KSHV reactivation. Histone acetylation induces KSHV replication by influencing protein-protein-associations and transcription factor binding. Histone modifications also determine chromatin structure and nucleosome positioning, which are important for KSHV DNA replication during latency. The association of KSHV proteins with chromatin remodeling complexes promotes the open chromatin structure needed for transcription factor binding and DNA replication. Additionally, post-translational modification of KSHV proteins is important for the regulation of RTA activity and KSHV replication. KSHV may also cause epigenetic modification of the host genome, contributing to promoter hypermethylation of tumor suppressor genes in KSHV-associated neoplasias.

Keywords

Kaposi's sarcoma-associated herpesvirus; epigenetics; histone acetylation; chromatin structure; DNA methylation

1. Epigenetic Regulation of Gene Expression

In eukaryotic cells, DNA methylation at CpG dinucleotides plays an important role in the regulation of gene expression and pathogen recognition. In the human genome CpG dinucleotides are relatively rare, occurring in frequencies approximately 25% as would be expected, because over time methylated cytosine residues undergo an irreversible deamination

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to become thymine (reviewed by [1]). However, some regions of the human genome contain stretches of DNA greater than 0.5kb with a C+G content of 55% or greater (reviewed by [2]). These regions, called CpG islands, make up ~1% of the human genome and are often associated with the promoters of genes. Methylation at CpG dinucleotides near or within promoters effectively diminishes gene transcription by blocking the access of transcriptional machinery to promoter regions by steric hindrance (reviewed [1]). This transcriptional repression is important for preventing the expression of foreign DNA, such as retrotransposons and proviral sequences (reviewed by [3]). Most CpG dinucleotides in the human genome are methylated. In contrast, unmethylated CpG dinucleotides are a signature of bacteria and other pathogens, and serve as potent stimulators of the vertebrate immune system.

Eukaryotic genomic material is packaged as chromatin, which is comprised of DNA wrapped around core histone proteins (reviewed by [2]). The tails of core histones which aid in chromatin packaging are often covalently modified after transcription (reviewed by [4]). These histone tail modifications play an important role in determining chromatin structure and condensation, both of which are important in regulating transcriptional activity. For example, acetylation of lysines on core histone tails, by histone acetyltransferases (HATs), leads to unraveling of chromatin and transcriptional activation. On the other hand, counteracting deacetylation by histone deacetylases (HDACs) leads to chromatin condensation and transcriptional silencing. Modified histones also recruit numerous other proteins, including transcription factors, and chromatin remodeling complexes, which use energy liberated from cleaved ATP molecules to move nucleosome along the length of DNA, resulting in assembly or disassembly of nucleosome cores and serve as an effective method of controlling transcription (reviewed by [5]).

The process of malignant transformation includes both genetic and epigenetic changes. In human cancers, methylation and histone modification patterns are often altered; this greatly influences chromatin structure and subsequent gene expression. Since a considerable amount of human cancers are attributed to viral infections, it is important to consider epigenetic modifications in the context of how it affects the function of tumor viruses. Here we will discuss the role of Kaposi's sarcoma-associated herpesvirus (KSHV) in neoplasia formation, as well as the mechanism which DNA methylation, histone acetylation, and chromatin structure affect viral gene expression. In brief, epigenetic alterations in KSHV associated neoplasias will also be discussed.

1. KSHV and neoplasia

KSHV, also known as Human Herpesvirus 8, is a member of the $\gamma 2$ herpesvirus family, which includes Epstein-Barr virus (EBV) and the simian Herpesvirus saimiri. Members of this family are characteristically able to induce the formation of distinct neoplasias in natural or experimental hosts (reviewed by [6]). KSHV was first discovered by its association with Kaposi's sarcoma, a common AIDS-associated neoplasia of endothelial origin [7]. Additionally, KSHV also causes two B-lymphocyte disorders, primary effusion lymphoma (PEL) and Multicentric Castlemans Disease (MCD), which are characterized by proliferation of B-cells in the body cavities and the lymph nodes, respectively [8].

3. KSHV Replication and Latency

KSHV genome structure and gene expression pattern varies depending on its replication state. Herpesviruses exist in two replication states: lytic or latent. After primary infection, KSHV actively replicates using viral machinery and new virus particles are produced and released by host cell lysis (reviewed by [9]). During this stage, the KSHV genome is linear and the entire viral genome is expressed. Lytic gene expression begins with the expression of immediate-early (IE) genes that regulate the expression of other viral genes [10]. Expression of IE genes

occurs independent of viral replication, and afterwards, early and late genes are expressed. These include genes important for genome replication as well as structural proteins.

The lytic switch of KSHV, RTA (replication and transcription activator), is encoded by ORF50 of the KSHV genome [11]. During latency, ORF50/RTA expression is repressed, however RTA may be activated by physiological conditions, such as hypoxia, or by pharmaceutical agents ([12], [13], and [14]). RTA activation triggers the start of the lytic replication cascade. RTA binds to numerous cellular and viral proteins and functions by directly transactivating various KSHV promoters. In a mechanism mediated by octamer binding protein-1, RTA also binds its own promoter and exhibits auto activation [15], possibly allowing KSHV to respond to cellular stimuli. Interestingly, expression of RTA alone is enough to disrupt KSHV latency and induce the expression of lytic genes [11] and the presence of dominant-negative RTA mutants eliminates viral reactivation [16].

After initial infection, KSHV may establish lifelong latency in B-cells ([8] and [17]). Throughout latency, viral gene expression is highly regulated and only a few viral genes are expressed. The latent KSHV genome is circularized by joining of GC rich terminal repeats (TRs) at the ends of the viral genome to form an extrachromosomal circular episome [18]. This episome is tethered to the host chromosome by the latency associated nuclear antigen (LANA), which also functions to regulate episome replication by host cell machinery [19]. LANA, a phosphoprotein expressed in latently infected cells (reviewed by [6]), is essential for the segregation of episomes to host daughter and persistent KSHV infection [19]. Additionally, LANA promotes the maintenance of latency by associating with the ORF50 promoter [20] or binding cellular factors which normally interact with ORF50. After extended periods of latency, KSHV infections may be reactivated and the lytic gene expression may restart, however, latency is the default pathway of KSHV.

A balance between latent and lytic gene expression is important for the pathogenesis of KSHV. Latent viral proteins, such as vFLIP and LANA serve to inactivate tumor suppressors and block apoptosis (reviewed by [9]). However, lytic replication is also important for transmission of the virus in the population and in the pathogenesis of KS. KSHV protein, vIL-6, which is more highly expressed during the lytic cycle, promotes cellular growth and angiogenesis, while protecting against apoptosis (reviewed by [6]). Additional evidence for the importance of lytic replication includes the fact that inhibition of active KSHV replication by gancyclovir reduces the incidence of KSHV in HIV-infected individuals (reviewed by [21]).

4. Regulation of KSHV latency by DNA methylation

In the majority of PEL cell lines, such as BCBL-1, BC3, and JSC1, HHV-8 is found in a latent state. Nevertheless, the lytic cycle may be induced *in vitro* by treatment with chemicals that induce epigenetic changes. More specifically, DNA methyltransferase inhibitor 5-Azacytidine (5-AzaC), HDAC inhibitor sodium butyrate (NaB) and HAT inducer, tetradecanoylphorbol acetate (TPA) are all stimulators of KSHV lytic replication ([14] and [22]). The ability to study latently infected cells in the context of reactivation by 5-AzaC, NaB, or TPA allows researchers to elucidate the mechanism by which different epigenetic modifications control the switch between KSHV latency and lytic cycle.

Deamination of methylated cytosines to form thymines occurs spontaneously, and results in a reduction in the frequency of CpGs in the human genome, or CpG suppression (reviewed by [21]). Low level of CpG suppression, which is expressed as a ratio of observed CpGs versus expected CpGs, suggests that a genome has been subject to extensive DNA methylation. As herpesviruses persist in their host for long periods of time, they are also subject to CpG methylation and may exhibit CpG suppression. Unlike other γ -herpesviruses, the KSHV genome does not exhibit global CpG suppression [14]. This suggests that KSHV is not subject

to extensive methylation. However, localized CpG suppression does occur at the promoters of specific genes, such as ORF50 and LANA.

Bisulfite sequencing of latently infected BCBL-1 cell lines reveal that during latency the ORF50 promoter is highly methylated [14]. Additionally, this promoter methylation was seen in biopsy specimens from patients with MCD, KS, and PEL. In all cases, treatment with TPA resulted in demethylation of the ORF50 promoter. Furthermore, demethylation induced by 5-AzaC caused lytic reactivation and IE (ORF50), early (vIRF), and late (K8.1) gene expression. This demonstrates that control of ORF50 promoter methylation is important for the induction of lytic replication.

5. Histone Modification and Chromatin Remodeling affect KSHV Replication

Histone acetyltransferase inducer TPA functions to promote KSHV lytic cycle by activating transcription factors and enhancing their DNA-binding activity [23]. RTA responsive promoters, including the RTA promoter itself, often contain C/EBP α (CCAAT/enhancer-binding protein alpha) binding sites; the binding of the C/EBP α transcription factor to the ORF50 promoter is a key step in the TPA-mediated induction of KSHV lytic replication [22]. TPA stimulates the expression of the C/EBP α protein which is stabilized by KSHV RTA and RAP (replication-associated protein) and enhances its autoregulation. However, without RTA or RAP, TPA cannot induce C/EBP α transactivation [23]. Wang *et al.* found that the activity of the AP-1 transcription factor is also important in early activation of the RTA promoters during KSHV lytic cycle, in that AP1 DNA-binding activity was increased as early as one hour after TPA treatment [23]. This increased AP-1 activity may be a result of increased level of phosphorylated cJUN, a subunit of the AP1-1 transcriptional activating complex, after TPA treatment. Recent work by Yu *et al.* also implicates the Ets transcription factors in TPA mediated RTA activation [25]. The possibility exists that the induction of HATs by TPA affects the accessibility of transcription factors important in regulating the KSHV replication.

Transcriptional coactivators p300 and CBP (CREB-binding protein), which possess intrinsic HAT activity, are targeted by numerous KSHV proteins. Initially, it was reported that the viral homologue to interferon regulatory factor (vIRF) encoded by ORFK9 binds to p300/CBP [26]. Subsequent to this report, studies also implicated K-bZip, LANA, and RTA as p300/CBP interacting proteins [[24], [25], and [26]]. Binding of all four KSHV proteins occurs at the C/H3 domain of p300/CBP [[27], [24] and [25]] which is important for protein-protein interaction. In most cases, binding of the C/H3 domain by KSHV proteins inhibits of CBP transactivation of cellular genes, possibly by competing with cellular factors for the C/H3 binding site. Additionally, CBP binding by vIRF, K-bZip, and LANA leads to a reduction in the proteins HAT activity. Histone acetylation is important in regulating chromatin structure. In fact, Li *et al.* demonstrated that reduction of HAT activity induced by vIRF led to histone acetylation as well as alteration in chromosome structure, seen by immunofluorescence tests [28]. In the case of RTA, CBP binds at its carboxy-terminal transactivation domain and positively regulates its transcriptional activity, while association with HDAC1 represses its activity [26]. Additionally, CBP interacting protein c-Jun further increased this activity. The ability of KSHV proteins to interact with p300/CBP allows modulation of HAT activity and chromatin structure as well as modulation of the activity of its own viral ORF.

Sodium butyrate activates KSHV lytic cycle by a mechanism distinct from that of TPA or 5-AzaC. The NaB response element of the ORF50 promoter, about 115nt upstream of the ORF50 transcription start site, is a binding site for the Sp1 transcription factor [29]. Sp1 binding is required for ORF50 promoter activation by NaB; removal of the Sp1 binding site by insertion mutations abolishes the ability of NaB to activate the RTA promoter. Additionally, there is an increased recruitment of Sp1 and its coactivator CBP to the ORF50 promoter after NaB

treatment. CBP, which has intrinsic HAT activity, physically interacts with RTA via its LXXLL motif [26]; this interaction is necessary for RTA transactivation [9]. On the contrary, in its repressed state RTA interacts with HDAC-1, a repressor of its activity.

LANA regulates ORF50 activity by directly binding the ORF50 promoter and via various protein interactions; treatment of latently infected PEL cell lines with NaB greatly affects these associations [20]. During latency, LANA associates with ORF50, histone H2B, and Sp1. However, treatment of PEL cells with NaB results in LANA lysine acetylation and a subsequent disruption of LANA's association with the ORF50 promoter, a phenomenon not seen with TPA treatment. Moreover, treatment of BCBL-1 cells with NaB treatment also abolishes the interaction between LANA and Sp1, a critical regulator of NaB response. It is probable that the removal of LANA from ORF50 and Sp1 allows for a Sp1/ORF50 interaction and transcriptional activation.

Chromatin structure plays an important role in regulating ORF50 transactivating ability. In PEL cell lines, the chromatin around the immediate promoters are relatively devoid of acetylation, and chromatin condensation is maintained (reviewed by [9]). Additionally, in BCBL-1 and JSC1 cells, a nucleosome is strategically positioned near the ORF50 transcriptional start site and the Sp1 binding site contributes to transcriptional repression [30]. RTA interacts with the BRG component of the human SWI/SNF chromatin remodeling complexes, which physically alters chromatin structure and nucleosome positioning; this chromatin remodeling is an event essential for transcription initiation [31]. Coexpression of RTA and BRG1 *in vitro* enhances RTA activity, and RTA mutants which have a diminished interaction between RTA and SWI/SNF show drastically reduced RTA promoter activity. Additionally, there is reduced activity of RTA responsive promoters in the KSHV genome. NaB treatment of latently infected cells and subsequent lysine acetylation, also lead to the BRG1 recruitment and promoter activation [30]. ORFK8 of KSHV encodes K-bZip [32], a basic region leucine zipper protein that binds and represses ORF50 [33] and functions as a transactivator [34]. Transcriptional activation by K-bZip was found to be dependent on its association with hSNF5, a component of the SWI/SNF chromatin remodeling complex. These findings suggest that alteration of chromatin structure is an important event in controlling KSHV lytic genes as well as the transactivating ability of RTA and K-bZip.

Histone modification patterns and nucleosome positioning, in part, control DNA replication during latency. For eukaryotic DNA replication to occur, the origin recognition complex (ORC) and the MCM (mini-chromosome maintenance complex) must be present (reviewed by [35]). The ORC functions to recruit prereplication complex proteins to the origin of replication; one of the recruited proteins is MCM (mini-chromosome maintenance complex) that functions as a replicative helicase. The KSHV latent origin of replication is embedded within the GC rich terminal repeats of the viral genome [36]; chromatin structure at the TRs consists of four nucleosomes and two LANA binding sites [37]. During the G1/S phase of the cell cycle, chromatin structure at the TRs is altered and DNA becomes more accessible to replication machinery. Additionally, there is an alteration in histone modification patterns. The TR typically possesses high levels of acetylation at histones H3 and H4; this is in contrast to the internal region of the genome which is subjected to H3K4 methylation (MeH3K4). During G1/S, however, there is a significant reduction in MeH3K4, while histone hyperacetylation remains constant. Increase in MeH3K4 appears to be correlated with an increased recruitment of MCM3 to the latent origin of replication, which was elevated during the G1/S phase. LANA is also critical to the recruitment of MCM3, in addition to ORC and histone acetyltransferase, HBO1, to the TR and subsequent DNA replication. Elimination of either of these TR-associated proteins, by siRNA knockdown reduced the level of LANA-dependent DNA replication. This data presents a complex situation in which chromatin structure and histone modification

regulates DNA replication, by controlling the recruitment of replication proteins as well as the access of replicative machinery to the KSHV genome.

6. Post-translational modifications of Viral Proteins

It is also important to note that post-translational modification of viral proteins, rather than histone proteins, is also important for the regulation of RTA activity and the control of KSHV latency. Post-translational modifications, such as sumoylation, phosphorylation and ADP-ribosylation, are important for transcriptional regulation. In 2003, Gwack *et al.* demonstrated that RTA interacts with poly (ADP-ribose) polymerase 1 (PARP-1) and Ste20-20 like kinase, hKFC via its serine/threonine rich region [38]. The two work synergistically to cause polyADP ribosylation and phosphorylation of RTA. These post-transcriptional modifications of RTA serve to repress RTA transactivation ability by reducing its recruitment to RTA responsive viral promoters. Furthermore, elimination of PARP-1 and hKFC interaction enhanced viral lytic replication. More recently, Izumiya *et al* also reported that the K-bZip protein is sumoylated [39]. K-bZip is an early lytic gene located downstream of RTA, which physically interacts with RTA and results in repression of RTA activation of specific KSHV promoters *in vitro* and *in vivo*, K-bZip binds Ubc9, an E2 SUMO conjugation enzyme, and is sumoylated at a specific lysine residue (K158) within its basic domain. Sumoylation plays an important role in K-bZip mediated RTA repression, in that reduction of sumoylation by SUMO-specific protease 1 and expression of a K-bZipK158R mutant both resulted in reduced transcriptional repression. K-bZip may also be phosphorylated by cellular cyclin dependent kinases or the KSHV viral protein kinase (vPK) ([40] and [41]). Interestingly, phosphorylation of K-bZip decreases its ability to act as a transcriptional repressor and decreases its ability to be sumoylated [41]. Together these results show that post-transcriptional modifications are another epigenetic mechanism by which KSHV lytic replication may be regulated.

7. KSHV Manipulation of Host Epigenetics

Promoter hypermethylation of tumor suppressor genes and resultant gene inactivation is frequently observed in human cancers (Reviewed by [42]). Interestingly, KSHV has mechanisms by which it is able to regulated host DNA methylation. In 2006, Shamay *et al* reported that LANA associates with DNA methyltransferases, DNMT1, DNMT3a, and DNMT3b, which establish and maintain CpG methylation patterns [43]. This interaction was abolished when the LANA chromatin binding motif was deleted. LANA, however, preferentially associates with DNMT3a, a *de novo* methyltransferase, and recruits it from the nuclear compartment to chromatin fractions of cells co-transfected with LANA and DNMTs. This association and relocalization of DNMT3a induced by LANA also resulted in *de novo* promoter methylation of the H-cadherin gene. More recently, it has also been reported that LANA associates with the TGF- β type II receptor (T β RII) promoter and induced its methylation [44]. Reduction of T β RII expression in PEL cells results in defective TGF- β signaling [44], which is important for preventing the development of tumors because it inhibits growth and promotes apoptosis [45]. Another tumor suppressor, p16INK4a, is also found to be inactivated by promoter hypermethylation [49]. The possibility exists that LANA also mediates this methylation, however, this issue has not yet been addressed in the literature.

8. Summary

Based on sequence homology, KSHV is closely related to EBV, another oncogenic herpesvirus. Intriguingly, the regulation of EBV latency and reactivation by epigenetic factors parallels that of KSHV (reviewed by [46]). The lytic switch and bZip proteins of the two are homologues, and the lytic cycle of both viruses may be induced by DNA methylation or HDAC inhibitors, such as 5-AzaC, NaB, and TPA. Studies performed to date using these chemical agents have given researchers great insight as to how both KSHV and EBV utilize host epigenetic

modifications to regulate their own replication. The similarity between the two, however, leaves a caveat which needs to be investigated. As PEL cell lines are often positive for both KSHV and EBV [8], the effect of epigenetic modifications should be studied in the presence of proteins from both viruses. Furthermore, the question still remains as to how HIV infection and/or immunosuppression lead to the alteration or deregulation of epigenetic modifications. Investigations as to how physiological and environmental stimuli, such as hypoxia and hypothermia, affect DNA methylation, histone modification, and chromatin structure still need to be performed, as both are believed to lead to herpesvirus reactivation. More importantly, the use of demethylating agents and HDAC inhibitors as treatment for cancers is currently being tested (reviewed by [47]). These agents appear promising due to the fact that neoplasias typically exhibit localized promoter hypermethylation as well as an elevation of HDAC activity; however, these treatments may not be applicable for KSHV- or EBV-associated cancers. Therapy may actually lead to virus reactivation and spread to nearby cells.

Abbreviations

5-AzaC, 5-Azacytidine
 DNMT, DNA methyltransferase
 EBV, Epstein-Barr virus
 HAT, histone acetyltransferase
 HDAC, histone deacetylase
 IE, immediate-early
 KSHV, Kaposi's sarcoma-associated herpesvirus
 LANA, latency associated nuclear antigen
 MCD, Multicentric Castleman's disease
 PEL, primary effusion lymphoma
 NaB, sodium butyrate
 TR, terminal repeat
 TPA, tetradecanoylphorbol acetate

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