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Epstein-Barr virus and host cell methylation: Regulation of latency, replication and virus reactivation

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Abstract

Epigenetic mechanisms govern the different life phases of Epstein-Barr virus (EBV). In the first *prelatent phase* the viral DNA acquires nucleosomes, histone marks are established, and 5 methyl cytosine residues become detectable. In the *latent phase* repressive histone marks and extensive DNA methylation silence the majority of viral promoters sparing a few latent genes. DNA methylation is a prerequisite for the induction of EBV's *lytic phase* in order to escape from latency and give rise to viral progeny. All three phases rely on the different epigenetic states of viral DNA and the availability of viral and cellular factors. EBV exploits cellular mechanisms of epigenetic regulation for its different life phases and serves as a marvelous example of an intimate host-pathogen relationship.

Introduction

Epstein Barr Virus (EBV) is a human tumor virus and a model for viral latency. It is a very successful human herpesvirus: More than 95% of the adult population is infected with EBV and carries the virus lifelong. The key to EBV's success lies in its ingenious bipartite life cycle. Upon infection of B cells, EBV establishes a strictly latent infection in B cells. *In vitro*, it activates the quiescent B lymphocytes and induces their proliferation. During latency, no virus is synthesized and the viral genome is maintained in the infected cells as nuclear plasmid copies. Only a small subset of viral genes is expressed that supports the proliferation of the infected cells and ensures the maintenance and replication of its viral genome in synchrony with the infected host cell. The full set of viral genes is expressed in the lytic phase, only, during which the infected cell synthesizes viral progeny [1,2]. Epigenetic principles govern the diverse expression patterns in the different phases of EBV's life cycle. The virus has evolved to take advantage of the host cell's epigenetic machinery to establish a stable latent infection and to use a smart principle to escape from it.

This review intends to highlight the recently uncovered surprising concepts of epigenetic regulation of EBV. We believe that the new findings support a revised concept of EBV's life cycle.

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EBV acquires an epigenetic signature during the prelatent phase

Upon infection of primary B cells, the virus establishes a strictly latent infection but initially a transient, so-called prelatent phase ensues that is characterized by the expression of a small set of viral genes that has previously been classified as lytic genes [3–6] together with the set of ‘classical’ latent genes. The prelatent phase lasts about a week in *in vitro* infected B cells [4] but it now appears that the lytic gene products fulfill important functions in order to take the necessary steps towards stable latent infection. For example, EBV encoded miRNAs, viral members of the anti-apoptotic BCL-2 family, and the viral transcription factor BZLF1 protect the cell from apoptosis, activate the quiescent cells and induce cell cycle progression [4,7]. Importantly, viral genes, which are essential for lytic amplification of viral DNA and genes encoding EBV’s structural proteins are not detectably expressed [4]. Thus, viral progeny cannot be produced during the prelatent phase of infection.

What is the molecular basis for the early expression of these genes and the eventual shutdown once a stable latency is established? Incoming viral DNA is unmethylated and free of any epigenetic modification. It appears likely that it constitutes a template, which is freely accessible to the cellular transcription machinery. Viral transcripts include latent viral genes and an insufficiently defined subset of viral lytic genes. We hypothesize that throughout the course of the prelatent phase EBV DNA acquires an epigenetic pattern driven by the host cell epigenetic machinery [4,8]. The viral DNA gains nucleosomes and repressive chromatin marks leading to the eventual repression of the subset of lytic genes (and certain promoters of latent genes), which are expressed in the prelatent phase [4,9].

Promoter selection during latency is controlled by DNA methylation

EBV can use three different promoters for the expression of latent genes of members of the so-called EBNA family: the *W* promoter (*Wp*), the *C* promoter (*Cp*) and/or the *Q* promoter (*Qp*) (Fig. 1). It is well understood that epigenetic mechanisms control their activities. In the prelatent phase, B cells support the early expression of *EBNA* genes via *Wp* [10]. They code for EBV’s transforming genes, including *EBNA1*, *EBNA-LP*, *EBNA2*, and *EBNA3A-C* [1]. Later, EBNA1 protein induces their expression from the alternate EBNA promoter *Cp*, either directly or indirectly by recruiting cellular proteins [11]. The promoter switch is essential for the persistence of EBV in B cells, because *Wp* becomes inactive shortly after infection. How *Wp* is repressed is not completely understood and discussed controversially. DNA methylation was proposed to be responsible for the promoter shutdown [9], but studies with low-passage lymphoblastoid cell lines showed a variable methylation of *Wp*, only, whereas methylation increased with higher passage number [12]. Therefore, it was proposed that alternate repressive epigenetic mechanisms dominate and DNA methylation of *Wp* is a secondary event [13].

In vivo in the immunocompetent host, the expression of all viral latent genes would lead to a strong antiviral immune response. DNA methylation and epigenetic silencing of the *C* promoter and a switch in the latency program ensures that EBV-infected cells can evade the antiviral cytotoxic T cell response. Transcription from *Qp* results in the expression of *EBNA1*, only. [14,15]. With the exception of the EBER locus *Qp* is the only promoter that is known to maintain an active chromatin configuration *in vivo*, which is probably supported by the binding of the chromatin insulator CTCF that prevents the spread of repressive epigenetic modifications into the *Q* promoter [16].

DNA methylation is a prerequisite for the escape from viral latency

A fascinating aspect of EBV’s life style is the epigenetic mechanism that controls the switch from latency to the productive lytic cycle. The lytic phase is initiated by the expression of

the viral *BZLF1* gene encoding the transcription factor BZLF1 (also called EB1, ZEBRA, Z, or Zta). High-throughput sequencing analysis of BZLF1-bound DNA confirmed that BZLF1 binds sequence-specifically to a previously known class of DNA motifs, termed ZREs, but prefers a second class that contains methylated 5'-cytosine residues (5mC). This class of binding motifs was termed meZREs (Fig. 2) [4,17–19]. meZREs prevail in early viral promoters like the *BBLF4* promoter, which is a prototypic target of BZLF1. The promoter is very rich in CpG dinucleotides, which are heavily methylated in the Burkitt lymphoma cell line Raji but also in memory B cells of healthy individuals, which are the natural reservoir of latent EBV [20]. Paradoxically, CpG methylation of meZREs is instrumental for the expression of essential lytic genes like *BBLF4* and thus indispensable for virus synthesis, changing the conventional view of DNA methylation solely as a repressive epigenetic mark. This peculiar feature explains why the virus fails to induce synthesis of progeny virus during the initial, prelatent phase of infection during which *BZLF1* is expressed: key lytic promoters that contain meZRE sites lack DNA methylation in the prelatent phase [4,19] hindering BZLF1 to bind its target sequences and activate the spectrum of all lytic genes. Thus, EBV invented a marvelous system to regulate its biphasic life cycle with the help of cell-mediated DNA methylation.

Induction of early lytic promoters results from an epigenetic switch

During latency the promoters of lytic genes are strongly repressed and silenced by epigenetic mechanisms. Lytic promoters are heavily DNA methylated, densely covered with nucleosomes, organized into compacted chromatin, and under control of Polycomb group proteins (PcGs) (Fig. 3), [20]. PcGs are defined as regulators that repress gene expression by keeping a transcriptionally inactive state that is mediated by trimethylation of histone H3 at lysine 27 (H3K27me3). There is so far no indication for the existence of bivalent, permissive chromatin marks in EBV promoters, which are prevalent in EBV's close relative, KSHV [20–22].

However, upon *BZLF1* expression, the epigenetic state of BZLF1 target genes changes dramatically (Fig. 3). Nucleosomes are removed locally at meZRE sites to open the chromatin. Epigenetic repression by Polycomb proteins is erased; instead, activation marks can be detected and transcription of the target genes is increased massively. Importantly, transactivation of lytic genes occurs on a fully methylated template without the need of active DNA demethylation at the BZLF1 responsive promoters [20]. These findings document that DNA methylation *per se* does not prevent the binding or block the activity of RNA-Polymerase II and provides a new paradigm for gene regulation.

Lytic replication of EBV's DNA resets its genome to an epigenetically naïve state

The methylation-dependent target genes of BZLF1 encompass EBV's own replication machinery that supports the autonomous amplification of viral DNA in the lytic phase [18,23]. The newly synthesized DNA templates lack epigenetic modifications because EBV does not encode a viral cytosine methyltransferases and lytic DNA amplification is uncoupled from cellular methyltransferases that maintain the methylation pattern during cellular DNA replication. Late lytic genes are defined as genes that are expressed after the onset of lytic DNA replication because compounds that inhibit lytic replication also block gene expression of the late class of viral genes. This finding suggests that DNA replication *in cis* might be required for their transcription [24] similar to herpes simplex virus [25]. How late gene expression and lytic DNA amplification are coupled is enigmatic but late gene expression might depend on viral DNA free of methylated cytosine residues. This statement is highly speculative but epigenetically naïve templates, which originate from lytic DNA

amplification, could provide a spatial and temporal gait to control the timely synthesis of EBV's structural components perhaps in conjunction with unidentified viral factors.

Conclusion: Epigenetic principles govern the life cycle of EBV

Research on the epigenetic hallmarks of EBV's life cycle identified a crafty mechanism and might lead to a rather simple model in which the epigenetic states and the dependency on cofactors classify EBV promoters. These two general parameters are sufficient to describe the functions of viral promoters during the different phases of EBV's life cycle as "naïve", "conditional", or "meZRE" promoters (Fig. 4).

Naïve promoters need a DNA template that is epigenetically unmodified; they do not depend on viral cofactors but rely on cellular factors, only. In the prelatent phase, the virus encounters a cellular environment, in which the basal cellular transcription machinery binds and activates naïve promoter elements. Naïve promoters encompass the *W* promoter, for example, which initiates early expression of latent *EBNA* genes, and a number of "leaky" lytic promoters including the *BZLF1* promoter. The prelatent phase comes to an end when the viral genome acquires components of cellular chromatin. Reactivation of naïve promoters in the lytic phase probably depends on lytic DNA replication that erases all epigenetic signatures to yield an epigenetically unmodified template of viral DNA again accessible to cellular factors.

Conditional promoters are in a permissive epigenetic state but their activities depend on viral factors. The *C* and *Q* promoters, which are induced by *W_p*-initiated EBNA1, are examples of this class of promoters. Promoters of genes coding for EBV's structural proteins that are not expressed in the prelatent phase also fall into this class as they probably require a viral cofactor for their activation. This unknown cofactor (or cofactors), which likely belongs to the early class of viral genes, is not available in the prelatent but only in the lytic phase. Inducible promoters that are not actively held in an open configuration acquire repressive chromatin marks and are kept in an inactive state during latency. Promoter reactivation requires lytic DNA replication in *cis* resetting the viral genome to its epigenetically naïve state.

meZRE promoters encompass BZLF1-regulated, methylation-dependent promoters with meZRE sites. During the prelatent phase the essential cofactor BZLF1 is expressed, but the viral DNA is unmethylated and therefore not in the correct epigenetic state to allow binding of BZLF1. Later, *BZLF1*'s own promoter is repressed upon chromatinization and occupied by cellular repressors. As a consequence, the expression of this molecular switch gene is shut off during latency. Its reactivation requires an exogenous trigger such as antigen encounter that can overcome transcriptional repression and epigenetic silencing. Upon re-expression, the BZLF1 protein rescues meZRE promoters from their repressed state and activates the expression of the BZLF1-responsive genes.

Repressive epigenetic modifications introduced by the host cell intend to silence the invading pathogen. Presumably, EBV's marvelous epigenetic regulation has evolved as a viral counter strategy in order to overcome the fundamental measure, which is part of an antiviral host response. We believe that EBV's BZLF1 is not unique in its capacity to selectively bind sequence motifs with methylated cytosine residues. Rather, certain cues might require the immediate reactivation of cellular promoters from a methylated template similar to the situation in meZRE promoters upon induction of the lytic phase of EBV's life cycle. We can only speculate but BZLF1 might have evolved from a cellular predecessor that binds to and transactivates epigenetically repressed and methylated cellular genes in situations that need immediate action. Future work will be necessary to assess this

hypothesis, which might lead to the discovery of a novel paradigm for cellular gene regulation.

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Highlights

- EBV's life cycle consists of a prelatent, a latent, and a lytic phase.
- Each phase is characterized by a unique set of active and silenced viral genes.
- Cell-driven epigenetic modifications govern viral gene expression in all phases.
- EBV's transcription factor BZLF1 is the key to switch from latency to the lytic phase.
- BZLF1 binds to methylated viral promoters and reverts epigenetically repressed genes.

Fig. 1. Promoter usage and expression of EBNA genes depends on DNA methylation

The *C* promoter (*Cp*), the *W* promoter (*Wp*) and the *Q* promoter (*Qp*) are three latent promoters that promote the expression of EBV encoded nuclear antigens (EBNAs). All promoters are epigenetically naïve upon infection, but transcription starts exclusively at *Wp*. *Wp* activation leads to the so-called latency III expression profile, which includes the expression of *EBNA-LP*, *EBNA2*, *EBNA3a-c*, and *EBNA1* (upper panel). The presence of EBNA1 causes a promoter switch to *Cp*, and *Wp* becomes epigenetically silenced by DNA methylation and other repressive modifications (middle panel). This latency III profile is a hallmark of EBV-infected lymphoblastoid cell lines (LCLs) *in vitro*, but *in vivo* the latency I profile dominates, which supports the expression of only *EBNA1* from *Qp*. In this stage, *Cp* is kept in a repressed state because it acquires a repressive DNA methylation pattern (lower panel).

Fig. 2. BZLF1 binds to methylated target sequences in viral promoters

The viral transcription factor BZLF1 is able to bind to two different classes of recognition motifs in promoter elements of target genes to transactivate their expression. The canonical ZRE (left panel) does not contain a CpG site and resembles the AP-1 motif. BZLF1 binds a second target site, called meZRE site, in a methylation dependent manner, which contains a central CpG dinucleotide (right panel).

Fig. 3. An epigenetic switch controls the induction of the lytic phase

Lytic promoters are kept in an inactive state during latency. Inactivation is achieved by compaction of chromatin in dense nucleosomal arrays, Polycomb-mediated epigenetic silencing and DNA methylation. BZLF1 expression induces a chromatin remodeling at target promoters. Nucleosomes are removed locally, Polycomb repression is lost and activation marks are established. Importantly, DNA methylation is not erased but fully CpG methylated template DNA supports transcription by the cellular RNA-Polymerase II.

Fig. 4. EBV's life cycle is governed by the epigenetic state of its DNA genome and viral cofactors The two dimensional matrix shows the chromatin state of a promoter on the x-axis and the presence or absence of cofactors on the y-axis. Active promoters are indicated as red circles and are matched into the appropriate matrix area according to the requirements for their activation. The figure shows one matrix for each phase of EBV's life cycle.

Naïve promoters rely on permissive, epigenetically naïve chromatin for their activation. They are not dependent on a viral cofactor and are immediately active upon infection during the so-called prelatent phase. A typical example is the *W*promoter (*Wp*) (left panel).

Conditional promoters require open, permissive chromatin but they depend on at least one viral cofactor. During EBV's latent phase, the *C*promoter (*Cp*), the *Q*promoter (*Qp*) and the *LMPI,2*promoter (*LMPI,2p*) are active, because their cofactors (EBNA1 and 2) are provided in the prelatent phase. Certain naïve promoters like the *EBERp* remain active; others like the *Wp* become silenced by epigenetic mechanisms (middle panel).

meZRE promoters (right panel) require methylated DNA sequence motifs for their activation and the presence of the transcription factor BZLF1 as the viral cofactor. Upon EBV's switch to the lytic phase, BZLF1 protein activates transcription of members of the class of early genes, e.g. *BBLF4*. Early genes encode EBV's own replication machinery supporting autonomous lytic viral DNA replication. Lytic DNA replication resets the epigenetic modifications of the viral genome and yields an epigenetically naïve viral template, which supports the transcription mediated by naïve and conditional promoters. The expression of the complete set of viral genes including viral structural components fosters the packaging of viral DNA into capsids and the release of infectious viral progeny.