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REVIEW

KSHV episomes: rugged individualists on the factory floor

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We have recently developed tools to study Kaposi's sarcoma-associated virus (KSHV) reactivation at the single-episome level. Using immunofluorescent labeling of latent nuclear antigen (LANA) protein to localize viral episomes, combined with fluorescence *in situ* RNA hybridization (RNA-FISH) of an intron region of immediate early transcripts, we have visualized active transcription of viral genomes in infected cells. At this level, we observed that not all episomes within a single cell were uniformly transcribed following reactivation stimuli. However, those episomes that were transcribed, formed large aggregates containing a significant fraction of cellular RNA polymerase II (RNAPII), foci consistent with previously described viral transcriptional factories. This focal assembly of RNAPII on viral episomes was accompanied by an overall decrease in the pool of cellular RNAPII. Additionally, the viral transcriptional factories localized with replicating viral genomic DNAs. This co-localization suggests that KSHV may assemble an "all-in-one" workroom for both gene transcription and DNA replication. While previous studies have reported on the variable response of individual KSHV infected cells or episomes derived from a population during reactivation, our results expose this variation further by demonstrating heterogeneity in the response of individual KSHV episomes within a single reactivating cell.

Keywords: KSHV; transcription; reactivation; RNA polymerase II; transcription factory

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KSHV is the eighth member of the human herpesvirus family identified in 1994 [1] and is etiologically linked to the development of Kaposi's sarcoma (KS), an angioproliferative and inflammatory lesion of the endothelium. KSHV is also strongly associated with two human lymphoproliferative diseases, primary effusion lymphoma (PEL) and multicentric Castleman's disease [2, 3]. Similar to all herpesviruses, the KSHV life cycle consists of two phases, known as latency and lytic replication. In latency, the viral genome persists in the host as nuclear episomes, and its expression is largely silenced except for a few genes [4, 5]. The KSHV lytic replication phase is initiated by the expression of a single viral protein, K-Rta. K-Rta is both necessary and sufficient to induce lytic reactivation of the latent KSHV genome [6-9]. K-Rta is classified as an immediate early gene and its coding sequence is separated into two exons [10, 11]. Various K-Rta responsive promoters have been identified *in vitro* and *in vivo* [10, 12, 13] thus expression of K-Rta triggers a cascade of viral gene expression. Lytic reactivation can also be induced experimentally by histone deacetylase inhibitors or phorbol esters, such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [14] and this pathway is accompanied by changes in viral chromatin structure [15, 16].

Previous single cell analysis of cells undergoing reactivation have noted that only 20% of K-Rta positive cells also expressed the late gene K8.1 suggesting the existence of additional incentive factors required for K-Rta positive cells to advance through complete reactivation [17]. Although K-Rta expression drives lytic reactivation, it has been considered an inefficient reactivating lever, subject to positive and negative regulation by viral and cellular factors [18].

The heterogeneous response of latent KSHV infected cells to reactivation stimuli has also been documented at the individual episome level by Darst *et al*. 2013 [19]. Methylation accessibility probing for individual template (MAPit) single molecule footprinting approaches were used to characterize locus-specific chromatin architectural diversity. By assessing the accessibility of a given locus to a DNA methyltransferase enzyme in combination with bisulfite sequencing, changes in viral chromatin structure in response to TPA or Rta transgene expression were evaluated. Their results estimated that only a subset of viral episomes (~10% of the total pool) can transcribe Rta following a TPA reactivation stimulus. The results also indicated that an open chromatin structure is a prerequisite for an episomal response to the inducer of lytic transcription. However, this study did not distinguish as to whether all episomes in 10% of cell population possessed an open chromatin structure versus whether 10% of the episomes within a cell can be accessed by RNAPII.

Against this background of reactivation heterogeneity, we wished to visualize the response of individual episomes during reactivation within a single cell. We utilized an RNA-FISH approach to visualize viral transcripts in combination with LANA immune staining to mark the location of the viral episomes in infected cells. Our studies utilized a PEL cell line (BCBL-1) or a derivative containing a doxycycline-inducible K-Rta expression cassette [8]. Using these models we have previously reported that LANA rapidly dissociates from the unique region of the KSHV genome during reactivation, however, in the same report we also showed that LANA remains tightly bound to the KSHV terminal repeat (TR) region during this time [20]; this LANA-TR interaction formed the basis to mark viral episomes. The use of RNA-FISH in this regard was first described in a study of cellular gene regulation, in which target transcripts were marked near the transcribing genomic locus *in situ* by generating probes specific to intronic regions [21, 22]. Using this same approach, we designed a panel of RNA-FISH probes for the K-Rta intron region allowing us to specifically tag pre-mRNAs immediately after transcription from viral DNA. KSHV reactivation was induced for 24h by incubation with TPA and the cells were fixed, permeabilized and subjected to immunostaining (LANA, localizes episomes) followed by RNA-FISH (localizes K-Rta intron, viral transcription sites). Using this combination, we successfully obtained the first images of reactivating KSHV episomes *in situ*. The images revealed that not all of the episomes in a single cell react uniformly to TPA stimulation. Three-dimensional (3D) imaging showed that some KSHV episomes were adjacent to transcripts, suggesting that these episomes were the likely origin of the RNAs, whereas other episomes were devoid of a transcript signal. The intra-nuclear variation in episomal participation in reactivation was striking. Reactivating cells could be found with high levels of Rta transcription involving multiple episomes, whereas Rta message production in other cells involved one or a few viral genomes, however in all cases, there were always episomes present in reactivating nuclei without adjacent transcription detected, suggesting these episomes remained latent.

By combining K-Rta intron RNA-FISH with differing antibodies, the relationship between K-Rta transcription and additional protein targets of interest could be interrogated. For example, we reasoned that K-Rta RNA, which has immediately been transcribed from the viral genome, should be in close proximity with RNAPII. Accordingly, we stained for RNAPII and probed for K-Rta RNA intron-containing transcripts by RNA-FISH. The results showed co-localization of K-Rta RNA with cellular RNAPII with many cells showing an aggregated/focal pattern of RNAPII expression. The results also showed a significantly lower overall RNAPII signal intensity in reactivating cells compared to non-reactivating cells. The decrease in RNAPII detected by immune-FISH was confirmed by western blot analysis probing lysates collected at 0-72 hour post-reactivation, while viral protein expression increased during this time frame. The RNAPII protein decrease could be partially rescued by MG132 or bortezomib treatment of reactivating cells implying that RNAPII degradation was at least in part mediated by the proteasome. The RNAPII data suggested that viral induced aggregation in the context of a decreased overall RNAPII intensity was a reflection of a biased shift of RNAPII to viral factories to transcribe viral genes at the expense of cellular gene transcription. When we probed several cellular and viral genes for expression levels we found that this was the case. Decreased expression of cellular GAPDH (glyceraldehyde 3-phosphate dehydrogenase), ACTB (beta-actin), and RBP1 (RNAPII subunit) was observed, while expression of viral genes such as K-Rta, PAN, K8.1, K-bZIP and LANA increased. The RT-qPCR results were consistent with RNAPII occupancy at these gene promoters, determined by RNAPII chromatin immunoprecipitation (ChIP), with occupancy at viral and cellular promoters increased and decreased, respectively.

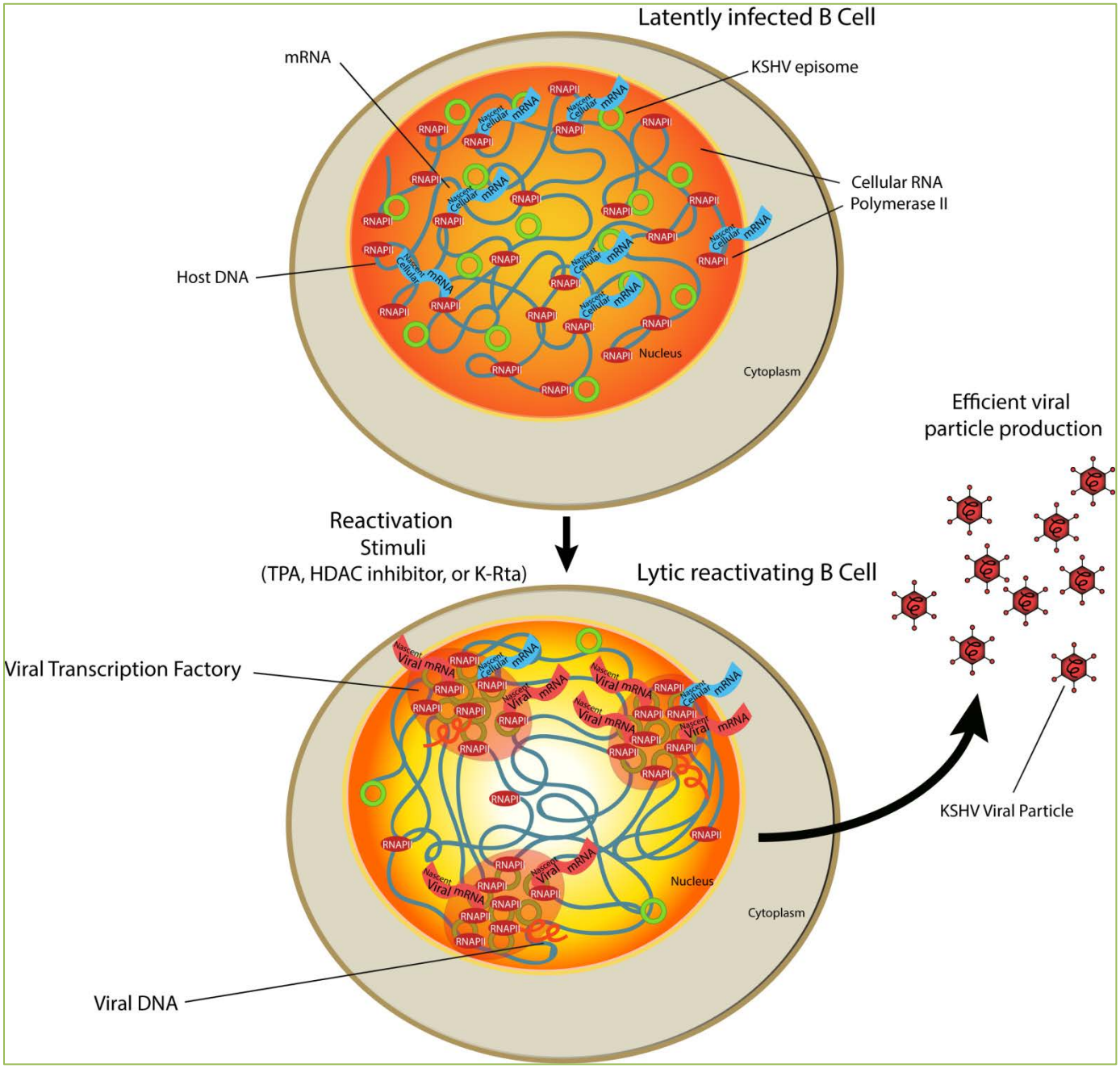

Figure 1. Individualized episomal response to reactivation signals. A latent B cell containing multiple KSHV episomes (green circles) and normal levels of RNAPII (red ovals) is shown in the top panel. Cellular mRNAs are transcribed by RNAPII and nascent RNAs are depicted. A lytic reactivating B cell is shown in the bottom panel. Reactivation is induced by TPA treatment and K-Rta expression (arrow). Focal aggregation of RNAPII (red ovals) at viral transcriptional factories and expression of viral RNAs are shown. Newly replicated viral DNA is depicted as red lines co-localized at the viral transcription factory. An overall decrease in cellular RNAPII is reflected by the gradient of orange shading (darker orange, more RNAPII) between the latent infected cell (top panel) and the lytic reactivating cell (bottom panel). Released viral particles are shown with viral DNA (black line).

Additional multiplex immune-FISH images localized RNAPII, LANA, and K-Rta transcription to overlapping sites within the nucleus. Taken together, the immune-FISH, gene expression, RNAPII ChIP and KSHV chromosome capture (Capture-C) data (Campbell *et al*., unpublished) suggest that during reactivation, (a) KSHV forms an active chromatin hub(s) (ACHs; [23]) to regulate viral gene expression, (b) containing a large fraction of the cellular RNAPII pool to (c) bias transcription in favor of viral genes at (d) the expense of cellular gene expression. Finally, TR fluorescence *in situ* DNA hybridization in conjunction with RNAPII staining detected an overlap between these molecules. RNAPII recruitment to sites of viral DNA replication suggests that KSHV lytic replication induces the formation of integrated

structures capable of both viral gene expression and lytic DNA replication (Figure 1).

One interesting question to arise from our study is how such a significant fraction of RNAPII is mobilized to viral episomes. One possibility is that this event is associated with the extremely high expression of the viral nuclear noncoding RNA, PAN. Although not directly assessed in our study, we speculate that one function of PAN may be to trap cellular RNAPII on the KSHV genome through DNA-RNA or RNA-protein interactions. PAN has been reported to interact with several viral and cellular proteins [24-27], and its communication network is probably incompletely defined. In addition, it is also possible that the process of transcription itself may contribute to PAN function through an enhancer-RNA-like activity to facilitate reactivation [28, 29]. However, previous nuclear PAN knock-down studies indicate that high levels of PAN RNA is the critical moiety for wild-type levels of virus production [27, 30], suggesting that partner interactions are an important basis of PAN function.

In summary, our recent publication [31] documents for the first time reactivating KSHV episomes at the single-episome level. Our findings demonstrate the individualistic pattern of behavior among episomes within a single nucleus containing up to 80 identical copies of the KSHV genome [32]. During the course of reactivation within the same nuclear space, some episomes reactivate, others remain quiescent. However, the individual responding episomes do succeed in the creation of an environment for maximal viral replication by assembly of all-inclusive factories to optimize viral transcription, decrease cellular transcription, and to proximally partition the events of viral transcription and viral DNA replication (Figure 1).

The reasons for this fascinating conduct are unknown but likely involve viral chromatin compaction, epigenetic modifications [15, 16, 19] and interactions with host factors [33-37]. Consistent with our results, previous studies have vividly demonstrated that only a limited number of herpesviral genomes give rise to progeny virus in studies using a Brainbow cassette to trace the lineage of incoming viral herpesviral genomes during *de novo* lytic infections [38, 39]. Although the mechanisms involved in these studies are likely different than we reported here, the theme of individual lytic responses among a population of herpesviral genomes is evident.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Author contributions

MC, CPC, and YI wrote the manuscript, revised and approved it.

Abbreviations

KSHV: Kaposi's sarcoma-associated virus; LANA: latent nuclear antigen; Rta: replication and transcription activator; RNA-FISH: fluorescence *in situ* RNA hybridization; RNAPII: RNA polymerase II; KS, Kaposi's sarcoma; PEL: primary effusion lymphoma; TPA: 12-O-tetradecanoylphorbol-13-acetate; BCBL-1: body-cavity-based lymphoma; TR: terminal repeat; 3D: three-dimensional; ChIP: chromatin immunoprecipitation; ACHs: active chromatin hubs.

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