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Alterations in Acetylation of Histone H4 Lysine 8 and Trimethylation of Lysine 20 Associated with Lytic Gene Promoters during Kaposi's Sarcoma-Associated Herpesvirus Reactivation

Sora Lim[1], Seho Cha[1], Jun Hyeong Jang[1], Dahye Yang[1], Joonho Choe[2], and Taegun Seo[1]*

[1]*Department of Life Science, Dongguk University-Seoul, Goyang 10326, Republic of Korea*
[2]*Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon 34141, Republic of Korea*

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**Corresponding author*
Phone: +82-31-961-5135;
Fax: +82-31-961-5108;
E-mail: tseo@dongguk.edu

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Kaposi's sarcoma-associated herpesvirus (KSHV) is associated with formation of Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Replication and transcription activator (RTA) genes are expressed upon reactivation of KSHV, which displays a biphasic life cycle consisting of latent and lytic replication phases. RTA protein expression results in KSHV genome amplification and successive viral lytic gene expression. Transcriptional activity of viral lytic genes is regulated through epigenetic modifications. In Raji cells latently infected with Epstein-Barr virus, various modifications, such as acetylation and methylation, have been identified at specific lysine residues in histone H4 during viral reactivation, supporting the theory that expression of specific lytic genes is controlled by histone modification processes. Data obtained from chromatin immunoprecipitation and quantitative real-time PCR analyses revealed alterations in the H4K8ac and H4K20me3 levels at lytic gene promoters during reactivation. Our results indicate that H4K20me3 is associated with the maintenance of latency, while H4K8ac contributes to KSHV reactivation in infected TREx BCBL-1 RTA cells.

Keywords: Kaposi's sarcoma-associated herpesvirus, histone modification, histone H4 lysine 8 acetylation, histone H4 lysine 20 trimethylation, viral reactivation

Introduction

Eight human herpesviruses have been identified to date, including herpes simplex virus 1, herpes simplex virus 2, varicella–zoster virus, Epstein–Barr virus, human cytomegalovirus, human herpesvirus 6, human herpesvirus 7, and Kaposi's sarcoma-associated herpesvirus (KSHV). Herpesviruses are classified into α-, β-, and γ-subfamilies, based on their biological features, organization, and genome sequence [1]. γ-Herpesviruses share a greater number of genes and genome organization than the α- and β-subfamilies. Similar to other herpesvirus families, γ-herpesviruses have established long-term latency in host cells, with occasional lytic replication. Epstein-Barr virus (EBV) and KSHV are two human γ-herpesvirinae that induce lymphoproliferative and neoplastic disorders [1, 2]. For example, KSHV, also designated as human herpesvirus 8 (HHV-8), is a known etiologic factor in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma [3–5].

KSHV has a biphasic life-cycle distinguished by distinct gene expression profiles within the host cell [6]. In infected cells, the KSHV genome is maintained as a circular episome, and few genes are expressed during the latency period [7]. Host immune responses tightly regulate latent infection of KSHV. The latent phase is reversible, and KSHV reactivation leads to entry into the lytic phase. Viral reactivation is followed by virion production and lytic DNA replication. During the lytic phase, three classes of lytic viral genes are activated: immediate early (IE), early (E), and late (L). Initial activation of IE lytic genes is followed by regulation of E and L genes encoding proteins

important for structure and genome replication [8–10]. Viral gene expression and DNA replication are achieved using cellular machinery, leading to capsid assembly and exit of mature virion particles capable of host cell infection. KSHV reactivation and lytic DNA replication are important for both viral propagation and tumorigenesis. During lytic gene expression, the replication and transcription activator (RTA) encoded by ORF50 is activated. RTA is an IE protein and viral transcription factor that plays a crucial role in lytic cycle gene expression by stimulating the activities of various lytic promoters and viral DNA replication [11]. Interestingly, RTA alone is reported to be sufficient to induce lytic gene expression [12]. RTA expression is repressed during latency but can be activated by pharmaceutical agents or hypoxia [13–15].

Epigenetics, including processes such as DNA methylation and histone modification, is defined as heritable alterations in genome function that occur with no induction of changes in DNA sequences [16]. Recent studies have focused on understanding epigenetic mechanisms, which involve histone modifications [17]. Histone modifications, including acetylation, phosphorylation, methylation, ubiquitination, and ADP ribosylation, play critical roles in the regulation of chromatin structure by influencing contacts not only between various histones but also between histones and DNA [18–22].

Lysine residues at the N-terminal of histone tails are acetylated by histone acetyltransferases or deacetylated by histone deacetylases for development of chromatin domains, including euchromatin and heterochromatin domains. Acetylation of lysine residues decreases the positive charge and, consequently, the affinity between histone and DNA. As a result, the chromatin structure is less compacted and transcription machineries are subsequently more accessible to the promoter region [20, 23–25]. To date, several epigenetic markers have been identified throughout the genome. Acetylation occurs at various lysines located within histone tails, including H3K9, H3K14, H3K18, H4K5, H4K8, and H4K12 [25, 26]. Histone methylation contributes to both transcriptional activation and inactivation. For instance, H3K4me, H3K36me, and H3K79me have been identified as activation markers at activated gene regions and H3K9me, H4K20me, and H4K27 as repressive markers at inactivated gene regions [27–29].

In EBV displaying high similarity to KSHV, the IF protein BZLF1 plays a crucial role in viral reactivation. H3K4me3 and H4K8ac have been identified as activation markers at the promoter region of BZLF1 during EBV reactivation, and, conversely, H3K27me and H4K20me3 are reported as negative markers during latency resulting in gene silencing [28, 30]. Previous studies on histone modifications in KSHV have mainly been performed on H3, and not H4. In the current study, we focused on H4, and based on earlier findings with EBV, investigated whether or not alterations in H4K8ac and H4K20me3 levels at lytic gene promoters during reactivation affect the regulation of lytic gene transactivation.

Materials and Methods

Cell Lines and Culture

TREx BCBL-1 pcDNA5 FRT/TO and TREx BCBL-1 RTA are KSHV-infected cell lines. A tetracycline-inducible RTA gene is present within the TREx BCBL-1 RTA cell line. Cells were cultured in RPMI 1640 (WelGENE, Korea) containing 10% fetal bovine serum (WelGENE) and 1% streptomycin-penicillin (WelGENE) at 37°C with 5% CO_2 and additionally maintained in the presence of 1 μg/ml doxycycline (Dox) for the indicated times.

Antibodies

Antibodies used for the western blot experiments were obtained from rabbit serum, including anti-K8 and anti-LANA. Anti-β-actin antibody was purchased from Santa Cruz (Cat. No. sc-47778). Rabbit anti-histone H4 (Millipore Cat. No. 04-858), rabbit anti-acetyl histone H4 (Lys8) (Millipore Cat. No. 07-328), and rabbit anti-trimethyl-histone H4 (Lys20) (Millipore Cat. No. 07-463) were used for chromatin immunoprecipitation.

Western Blot Analysis

TREx BCBL-1 cells (~4 × 10^6) were treated with Dox for the indicated times. Next, cells were washed in PBS and lysed with RIPA (50 mM Tris-HCl; pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride (PMSF)). SDS sample buffer (5×) was added to whole cell lysates and the samples were boiled at 100°C for 5 min. The samples were electrophoresed on 10–15% gradient sodium dodecyl sulfate-polyacrylamide gels at 120 V for 1–2 h, and transferred to nitrocellulose membranes (Whatman, GE Healthcare) at 300 mA for 1 h. The AccuRuler RGB PLUS Prestained Protein Ladder (Maestrogen) was used to estimate the sizes of proteins resolved with SDS-PAGE. Membranes were blocked with 3–5% skimmed milk in TBST (120 mM NaCl, 20 mM Tris-HCl (pH 7.5), 0.1% (v/v) Tween 20) for 1 h at room temperature and immunostained with specific antibodies (1:1,000–5,000 dilution) overnight at 4°C. Subsequently, membranes were washed 5 times for 5 min with TBST and incubated with the anti-rabbit IgG-HRP-linked secondary antibody (1:20,000 dilution) (Cell Signaling Cat. No. 7074S) or anti-mouse IgG-HRP-linked secondary antibody (1:10,000) (Santa Cruz Cat. No. H0415) in blocking buffer at room temperature for 1 h. Finally, the membranes were washed 5 times with TBST for 5 min each and developed using the chemiluminescence kit

(Western Lightning ECL; PerkinElmer).

Chromatin Immunoprecipitation (ChIP) Assay

Part of the ChIP assay was carried out as described previously [31]. A total of 2×10^7 cells treated with 1 μg/ml Dox for 18 h or left untreated were cross-linked with 1.42% formaldehyde for 15 min at room temperature (RT). Cross-linking was terminated with 125 mM glycine for 5 min at RT. Cells were washed twice with ice-cold phosphate-buffered saline and lysed in 1 ml of IP buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.5% (v/v) NP-40, 1.0% (v/v) Triton X-100 including protease inhibitors (100 mM/μl PMSF, 1 μg/ml leupeptin). After centrifugation at 3,000 ×*g* for 3 min, isolated nuclear pellets from cells were resuspended in 1 ml of IP buffer, followed by sonication (Sonics TM Vibra cell VCX750, USA). DNA (average length of 0.5–1 kb) was obtained under the following conditions: 10 cycles of 2 sec pulses and 18 sec rest per round at a power output setting of 30%. Supernatant fractions were collected after centrifugation at 4°C, and 10% was set aside for use as the input control. The remaining supernatant fraction was incubated with the corresponding antibodies (1–2 μg) at 4°C overnight with rotation, followed by centrifugation at 12,000 ×*g* for 10 min. Protein G Resin (GenScript) was washed 3 times in IP buffer to eliminate ethanol, and diluted 1:1 with IP buffer. The cleared immune complex was transferred to a tube with the resin slurry. The samples were rotated at 4°C for 45 min (30 rotations per minute), followed by centrifugation at 2,000 ×*g* for a few seconds. The supernatant was discarded and the precipitated resin was washed 5 times with 1 ml of cold IP buffer without protease inhibitors. To reverse the cross-linking, immunoprecipitated samples were eluted from the resin twice with elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 15 min at RT. Then, 5 M NaCl was supplemented with the elutes and inputs at 65°C overnight. DNA fragments were purified using the phenol:chloroform: isoamyl alcohol extraction method and precipitated with ethanol. Precipitated and input DNAs were dissolved in triple-distilled water for quantitative real-time PCR (qRT-PCR) analysis.

Quantitative Real-Time PCR

Input DNA was diluted and amplified for efficient quantification of RT-PCR. The PCRs included 2 μl of DNA template, 2 μl of 0.5 pM primer pairs, and 10 μl of KAPA SYBR FAST qPCR Master Mix (KAPA Biosystems, Cat. No. KK4602). Each promoter was amplified using specific primers (Table 1), with a view to evaluating the level of protein occupancy in various genomic regions. Quantitative RT-PCR was performed on a Rotor Gene Q machine (Qiagen) using Rotor-Gene Q Series software and the following amplification conditions: 3 min predenaturation at 95°C, 40 cycles of 3 sec denaturation at 95°C, 20 sec annealing and extension at 60°C.

Results

Effects of the Dox-Induced RTA System and KSHV Reactivation on Whole Cellular Histone Modification in TREx BCBL-1 Cells

To determine the global changes in cellular levels of

Table 1. Primers used for quantitative real-time PCR.

Promoter and coding regions		DNA sequence (5′ to 3′)	Coordinates on KSHV
RTA promoter (-0.25 kb to TSS)	Forward	ATCTCCAATACCCGGAATT	71445-71463
	Reverse	TTTTGTGGCTGCCTGGA	71678-71694
RTA coding region (+0.7 to +0.9 kb)	Forward	GAGAAACGCCGGCCAATT	73352-73369
	Reverse	GGGTTTGCTAATGCAAACT	73533-73552
Origin of lytic replication (Left end)	Forward	CATGGGGTTGGGATTTTT	23844-23861
	Reverse	AATGGGCGTAACCGTAG	24077-24093
PAN RNA promoter (-0.25 kb to TSS)	Forward	TAGTGATTCGGTAGATTTG	28569-28587
	Reverse	GCTAAACTGACTCAAGCT	28801-28818
ORF57 promoter (-0.25 kb to TSS)	Forward	AACAGTCCGTGTATTCCTT	81919-81937
	Reverse	GTCCTTTGGTTCTTATATTG	82149-82168
LANA promoter (-0.45 to -0.2 kb)	Forward	TTAGGTTCTAGGTTGTATTC	123607-123626
	Reverse	TCCAGGCTCTACAGGTAG	123839-123856
LANA promoter (-0.25 kb to TSS)	Forward	AGTTGCCATATAGACTGGC	123807-123825
	Reverse	AAGCCACACCTCTCCCC	124040-124056
LANA promoter (TSS to +0.2 kb)	Forward	ATGTCATTTCCTGTGGAGAGTCC	124059-124081
	Reverse	GCCCATAACTTATTGTGT	124241-124258

TSS : Transcriptional start site.

Primers were designed according to the sequence of KSHV (AF148805.2) in the GenBank database.

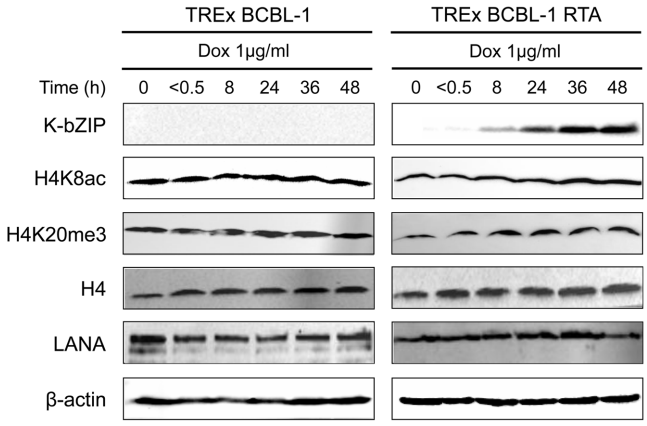

Fig. 1. Effects of the Dox-induced system and KSHV reactivation on cellular histone modification levels in TREx BCBL-1 cells.

TREx BCBL-1 and TREx BCBL-1 RTA cells were treated with Dox (1 μg/ml) and incubated for 0, 0.5, 8, 24, 32, and 48 h. Protein levels of K-bZIP, H4K8ac, H4K20me3, H4, and LANA were examined via western blot assay, with anti-β-actin as the control.

H4K8ac and H4K20me3 upon lytic reactivation, western blot assays were done in TREx BCBL-1 and TREx BCBL-1 RTA cells treated with Dox at the indicated times using specific antibodies (Fig. 1). We additionally examined whether the Dox-induced RTA system operates efficiently in these cells. K-bZIP protein expression was detected in TREx BCBL-1 RTA cells treated with Dox over 8 h. Our results showed protein expression of lytic genes induced through switching of the viral life cycle to the lytic phase in TREx BCBL-1 RTA cell treated with Dox. Expression patterns of H4 and H4K8ac proteins remained unchanged after Dox treatment at the indicated times, whereas H4K20me3 proteins increased slightly after Dox treatment. β-Actin, one of the housekeeping genes, and latent nuclear antigen (LANA), expressed in both latent and lytic cycles, were used as the controls. These findings collectively support efficient operation of the Dox-induced RTA system in TREx BCBL-1 RTA cells and confirm that lytic reactivation does not affect global levels of cellular H4K8ac and H4K20me3 in KSHV-infected cells.

Alterations in Histone H4K8 Acetylation at Viral Lytic Gene Promoters upon KSHV Reactivation

The chromatin immunoprecipitation assay was conducted to evaluate the changes in H4K8ac at a number of lytic gene promoter regions upon KSHV reactivation in recombinant TREx BCBL-1 cells treated with Dox. To obtain efficient ChIP data, cross-linked chromatin samples were sonicated for various times (data not shown). Sheared samples were de-crosslinked, treated with RNase, purified, and resolved on a 1% agarose gel stained with ethidium bromide. Sonication for 10 cycles of 2 sec pulses and 18 sec rests per round at a power output setting of 30% yielded DNA segments ranging from 200 to 1,000 bp in length, which is standard for ChIP experiments. Chromatin samples sonicated for over 30 cycles of 2 sec pulses and 18 sec rests per round at a power output setting of 30% did not show a significant decrease in DNA fragment sizes. Quantitative real-time PCR was further conducted to quantify the extent of histone modification changes at various lytic gene promoters. In previous histone modification studies by our group, primers specific for several lytic gene promoters were designed. Polyadenylated nuclear (PAN) RNA and ORF57 genes are located downstream of the RTA gene and linked to lytic gene replication [32, 33]. The Ori-Lyt gene is additionally reported to play a crucial role in lytic gene replication [34, 35]. The H4K8ac level was increased at a number of putative promoter and coding regions in KSHV reactivation-induced cells after Dox treatment compared with cells in which KSHV reactivation could not be induced (Fig. 2). Notably, the level of H4K8ac was altered at the RTA coding (+0.7 kb) and putative promoter (−0.25 kb) regions (Fig. 2A), as well as the putative PAN RNA and ORF57 promoter regions (Fig. 2B). In contrast, no differences were evident in the H4K8ac modification levels at the Ori-Lyt and LANA coding and putative promoter regions in reactivated cells (Fig. 2C). Our ChIP data indicate that alterations in H4K8ac affect transcriptional activities of lytic genes induced upon KSHV reactivation via RTA expression.

Alterations in Trimethylation of Histone H4K20 at Viral Lytic Gene Promoters upon KSHV Reactivation

The H4K20me3 level at various lytic gene promoter regions was determined in recombinant Dox-treated TREx BCBL-1 cells displaying KSHV reactivation, with the aid of the ChIP assay, followed by qRT-PCR. Similar to the trend previously observed with EBV, H4K20me3 was increased at a number of putative promoter and coding regions in KSHV reactivation-induced cells compared with cells in which KSHV reactivation could not be induced (Fig. 3). The H4K20me3 level was decreased at the RTA coding region (+0.7 kb), putative promoter region (−0.25 kb), and putative PAN RNA, ORF57 promoter regions (Figs. 3A and 3B). In contrast, the H4K8ac level remained constant at the Ori-Lyt promoter (Fig. 3B). Moreover, no changes in the H4K20me3 modification were observed at the LANA coding and putative regions in reactivated cells (Fig. 3C).

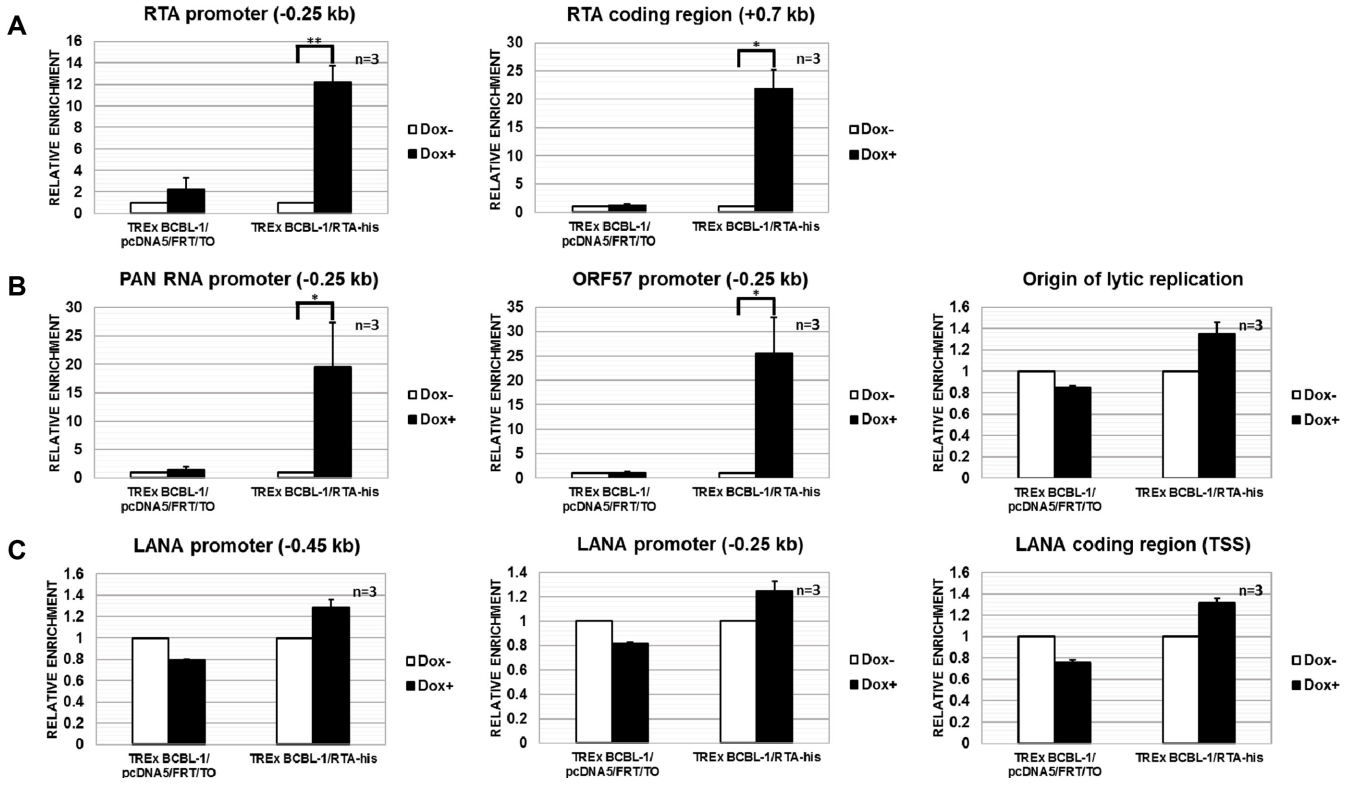

Fig. 2. Alterations in acetylated histone H4K8 levels at specific viral lytic gene promoters upon KSHV reactivation.
TREx BCBL-1 and TREx BCBL-1 RTA cells were treated with Dox (1 μg/ml) for 18 h or left untreated. The ChIP assay was conducted, as described in Materials and Methods, using anti-histone H4 or anti-H4K8ac antibody. Quantitative real-time PCR was performed to quantify the extent of histone modification changes at various lytic gene promoters using the primers specified in Table 1 (**A**–**C**). Average levels of at least three replicates are shown. *, $p < 0.05$; **, $p < 0.01$.

The ChIP data support the theory that changes in H4K20me3 affect the transcriptional activities of lytic genes induced upon KSHV reactivation via RTA expression.

Discussion

A limited number of genes are expressed during latency to avoid the cellular immune system, while almost all the viral genes are expressed during the lytic phase. DNA methylation or histone modifications, such as methylation and acetylation, are known to play important roles in chromatin structure alterations and condensation. Thus, changes in chromatin structure and control of transcriptional activity via epigenetic modifications may underlie the distinct regulatory patterns of latent and lytic gene levels during viral reactivation. KSHV reactivation was previously shown to be caused by the increase of viral gene expression such as RTA, a viral transactivator, but the mechanism of viral gene expression is not well understood. Thus, more research is required in the genomic and chromatin levels. In our study, we provided the upregulation of H4K8ac and downregulation of H4K20me3 as key factors for the activation of RTA and other lytic genes. Our data may contribute to identifying the molecular mechanism of the KSHV reactivation.

KSHV reactivation induced alterations in histone modification levels at specific lytic gene promoters, as determined with ChIP (Figs. 2 and 3). Data showed that histone modification levels changed only at specific lytic promoter regions, not at whole-cell levels of histone modification.

qRT-PCR experiments were performed using primers specific for a number of lytic gene promoters. The RTA gene not only auto-activates its own promoter but also transactivates expression of lytic genes at multiple downstream regions, including K1, K2, K3, K5, K8, K8.1A, K9, K12, ORF6, ORF21, ORF37, ORF57, ORF59, ORF65, ORF74, vIL-6, PAN RNA, and vIRF1 [36]. RTA activates PAN RNA, the most abundant KSHV lytic transcript, via directly binding its DNA [33]. Moreover, RTA interacts

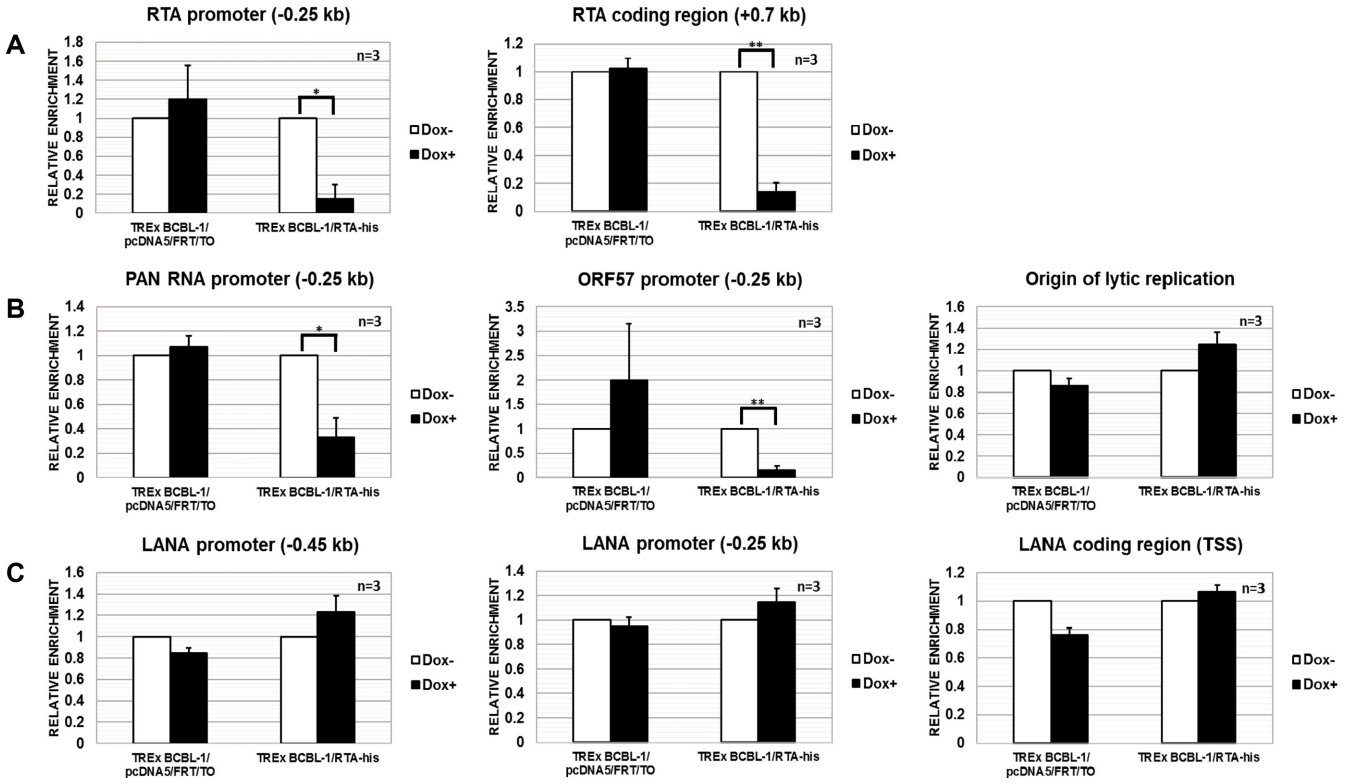

Fig. 3. Alterations in trimethylated histone H4K20 levels at specific viral lytic gene promoters upon KSHV reactivation.
TREx BCBL-1 and TREx BCBL-1 RTA cells were treated with Dox (1 μg/ml) for 18 h or left untreated. The ChIP assay was conducted, as described in Materials and Methods, using an anti-histone H4 or anti-H4K20me3 antibody. Quantitative real-time PCR was performed to quantify the extent of histone modification changes at various lytic gene promoters using the primers specified in Table 1 (**A**–**C**). Average levels of at least three replicates are shown. *, $p < 0.05$; **, $p < 0.01$.

with ORF57 to enhance the KSHV lytic gene expression cascade and increase the efficiency of viral reactivation [32]. The lytic origin of replication (Ori-Lyt) is composed of origin binding protein, which recruits core replication complexes to the replication site. Ori-Lyt incorporates several regions of transcription factor binding sites and the RTA-responsive element, which is crucial for RTA binding and Ori-Lyt-dependent DNA replication [34, 35].

Different histone modification patterns are related to expression of multiple viral gene classes during the latent phase. Data from the current study showed an increase in H4K8ac and decrease in H4K20me3 (high levels of which are maintained) at the RTA, PAN RNA, and ORF57 promoters during KSHV reactivation (Figs. 2 and 3). However, against our expectations, H4K8ac and H4K20me3 levels were not changed at the Ori-Lyt promoter region. Further research is warranted to establish whether histone modifications occur at other Ori-Lyt regions, which include RRE or K-bZIP binding sites [37].

H4K8ac and H4K20me3 modification levels also remained unchanged at the LANA promoter, consistent with the finding that expression levels of this gene seldom change during viral reactivation.

In EBV-infected cells, H4K8 acetylation occurs when TAF-1β, which is associated with acetylation activity, binds to the p300/CBP complex containing a histone acetyltransferase domain. Additional experiments are essential to establish whether TAF-1β influences H4K8ac and the effects of other coactivators, such as p300/CPB, in KSHV-infected cells [28, 38, 39]. Suppressor of variegation 420h (Suv420h) containing a SET domain has been identified as an H4K20me3 methyltransferase. In EBV, knockdown of Suv420h1 suppressed the BZLF1 promoter via methylation. The potential effect of SUV420h1 on the H4K20me3 level in KSHV is yet to be ascertained [30, 40].

In recent years, various studies on KSHV reactivation have been conducted with a view to developing effective treatments for KSHV-associated diseases. These earlier findings, in conjunction with data from the current study, support a novel means of developing therapeutic agents

for cancer-inducing viruses through modulation of specific factors associated with viral chromatin structure changes.

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