Published in final edited form as:

Arch Virol. 2010 March ; 155(3): 361–366. doi:10.1007/s00705-009-0576-5.

Number of and distance between response elements in Kaposi's sarcoma-associated herpesvirus ORF57 promoter influence its activation by replication and transcription activator and its repression by interferon regulatory factor 7

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Abstract

Kaposi's sarcoma-associated herpesvirus ORF57 expression is highly responsive to replication and transcription activator (RTA) and interferon regulatory factor 7 (IRF-7). Three RTA response elements (RREs) have been identified in the ORF57 promoter. Here, we show evidence of another functional RRE located between nt 82003 and 82081, which can complement the loss of RTA activation resulting from RRE1 deletion. Repeats of a recombination signal-binding protein $J\kappa$ (RBP- $J\kappa$) site enhanced RTA activation, which could not be suppressed by IRF-7. Alteration of the distance between the RBP- $J\kappa$ site and RRE2 modulated responsiveness to RTA and IRF-7. These results will help to elucidate the precise regulation of viral gene expression.

Kaposi's sarcoma-associated herpesvirus (KSHV), also known as human herpesvirus 8 (HHV-8), is the etiologic agent of Kaposi's sarcoma (KS), the most common neoplasm in AIDS patients [5]. KSHV is also associated with two other lymphoproliferative disorders, primary effusion lymphoma and multicentric Castleman's disease [4,16]. Like other herpesviruses, KSHV goes through lytic and latent phases of infection, and the switch from latency to lytic replication is governed by the viral replication and transcription activator (RTA) [9,12,17]. RTA, encoded by ORF50, can activate promoters of a number of viral genes [7]. Using a ChIP-on-chip approach, 19 RTA binding sites have been identified in KSHV-infected BCBL-1 cells, and these are located in the regions of promoters, introns, or exons of KSHV genes, with the consensus binding motif TTCCAGGAT(N)₀₋₁₆TTCTGGGA [6]. Eight KSHV genes were shown to be the direct transcriptional targets of RTA in the absence of de novo protein synthesis. These include PAN, K2, K9, K14, ORF37, ORF52, ORF56, and ORF57, suggesting that the induction of other KSHV lytic genes requires additional protein expressions [2].

KSHV ORF57 encodes a viral early nuclear protein named mRNA transcript accumulation protein (MTA), which is essential for the expression of many KSHV genes and affects almost

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every step of viral gene expression [14,15]. The expression of ORF57 itself is highly responsive to RTA [12]. An RTA binding site was identified in the ORF57 promoter as a 52-bp DNA sequence located between nt -106 and -54, upstream of the first transcriptional start site (TSS1, genome position nt 82003). This region contains a 12-bp palindromic sequence that is critical for RTA binding and activation [13]. A 40-bp region encompassing nt 81904 to 81943 in the ORF57 promoter was simultaneously identified to be necessary for RTA transactivation [8]. Two non-conserved AT-rich RTA response elements (RREs) were found within this region: RRE1 shares homology with the promoter sequence of a delayed early gene K8; RRE2 binds not only RTA, but also interferon regulatory factor 7 (IRF-7), a key regulator of type-I interferon-dependent innate immune responses in host cells [10,13,18,19,23]. Moreover, the competition between RTA and IRF-7 for binding with RRE2 affects RTA transactivation and modulates viral activation [19]. Between RRE1 and RRE2 in the 40-bp region, a 7-bp recognition site for recombination signal-binding protein RBP-J κ was identified in the anti-sense orientation relative to the MTA ORF. The RTA is recruited to the RBP-J κ site through the interaction with RBP-J κ to activate the ORF57 promoter [11].

In addition, based on homology with the consensus sequence of the Epstein-Barr virus RRE, four potential conserved RREs have been predicted in the ORF57 promoter, which are GC-rich, with the consensus sequence CCN₉GG [8,21]. Among them, the one in close proximity to RRE1 and RRE2 has been shown to bind to RTA in vitro and in vivo and has been named RRE3 [21]. Although binding of RTA to RRE3 is weaker than to RRE1 and RRE2, and RRE3 alone is not sufficient for efficient RTA transactivation, the presence of two adjacent RREs is necessary for optimal RTA-mediated transactivation of the ORF57 promoter [21].

To functionally confirm the response elements within the 40-bp region in the ORF57 promoter that mediate both RTA transactivation and IRF-7 repression, ORF57 promoter fragments were amplified using forward primers 1–5 and reverse primer 6 (Table 1) and then inserted into the pGL3-basic vector (Promega, Madison, WI). Reporters S1–S5 (Fig. 1a) contain promoter regions spanning from nt 81556, 81904, 81918, 81930, 81944, respectively, to the TSS1 (nt 82003). 293 T-cells were co-transfected with each of the S1–S5 ORF57 promoter-derived luciferase reporters (50 ng each), with or without 20 ng pCMV-Tag50 and 1 μ g pCMV-Tag7A [19,20], and luciferase activity was measured [8]. As expected, RTA activated p57pluc1 by 52.2-fold, and the activation was suppressed to 23.6-fold when IRF-7 expression plasmid was added (Fig. 1b). The other two reporters, S1 and S2, containing the full-length 40-bp region, were also responsive to RTA activation and IRF-7 suppression. The degree of activation was 38.7-fold and 16.3-fold, and the degree of suppression was 9-fold and 5.1-fold, respectively. However, when RRE1 was deleted in reporter S3, RTA could barely activate this reporter, and the activation was only 3.9-fold. Further deletions of the RBP-J κ site (reporter S4) or the entire 40-bp region (reporter S5) almost completely abolished transactivation by RTA, with only 2.5-fold and 3.4-fold activation, respectively. These results demonstrated that the 14-bp AT-rich RRE1 located in the 5' end of the 40-bp region was critical for the responsiveness to RTA transactivation. When IRF-7 expression plasmids were co-transfected with reporters S3, S4, and S5 activation was reduced to 1.4-fold, 1.2-fold, and 1.5-fold, respectively. Western blot analysis revealed that the variation in activation by RTA was not due to an IRF-7-mediated alteration in RTA expression, since similar levels of RTA were expressed (lower panel of Fig. 1b).

Using forward primers 1–5 and reverse primer 7 (Table 1), reporters L1–L5 were generated (Fig. 1a), which contain the ORF57 promoter regions extending to the second transcription start site (TSS2), nt 82081. Transfection experiments showed that reporters L1 and L2, which contained the full-length 40-bp region, were responsive to both RTA activation and IRF-7 repression (Fig. 1c). The degree of activation was 123.2-fold and 49.9-fold, and the degree of suppression was 18.6-fold and 21.3-fold, respectively. The degree of activation for the L1 and

L2 reporters was much higher than for S1 and S2 reporters, indicating that the 78-bp region between nt 82003 and 82081 might mediate RTA transactivation. Surprisingly, when RRE1 was deleted, the L3 reporter retained responsiveness to RTA (16.3-fold), suggesting the existence of a novel functional RRE in the 78-bp region that could complement the loss of RTA activation resulting from the deletion of RRE1. The sequence and the activation mechanism of this RRE need to be investigated further. When 293 T-cells were co-transfected with the IRF-7 expression plasmid, RTA-mediated transactivation for reporter L3 was suppressed to 5.1-fold, demonstrating that IRF-7 could effectively repress RTA transactivation mediated by RREs downstream of the RRE2. Therefore, the repression by IRF-7 of the adjacent RRE was orientation-independent. Reporters L4 and L5, from which the RBP-J κ site or the entire 40-bp region was further deleted, lost their responsiveness to RTA and IRF-7 (Fig. 1c), indicating that the novel RRE together with RRE2 and RRE3 was insufficient for efficient RTA transactivation. The expression levels of RTA were equivalent for different reporters (data not shown).

Reporters C1–C8 were constructed to investigate the effect of the number of and the distance between RREs on RTA transactivation and IRF-7 suppression. Schematic representations and partial sequences of reporters C1–C8 are shown in Fig. 2a. Slightly separating the RRE1 from the RBP-J κ site by a 6-bp spacer did not affect RTA transactivation in reporter C1 (Fig. 2b). The degree of activation was 14.3-fold, which was similar to that for reporter S2 (16.3-fold). However, the RTA activation was weakened if the RRE1 was put too far away from the RBP-J κ site: in reporter C4, where the spacer between RRE1 and the RBP-J κ site was elongated to 40-bp, RTA activation was reduced to 5.9-fold (Fig. 2b).

Addition of an extra RRE1 in reporter C2 did not notably affect the RTA transactivation, with the degree of activation similar to that seen for reporter C1 (14.3-fold for reporter C1 vs. 15-fold for reporter C2). Considered together with the transfection results for reporter S3, it would appear that the existence of RRE1 was necessary but not sufficient for RTA activation. However, addition of two more RBP-J κ sites enhanced the RTA transactivation to 30.9-fold (reporter C3), demonstrating that the RBP-J κ site was critical for RTA transactivation. Accordingly, the deletion of the RBP-J κ site in reporter C5 abolished the RTA transactivation (2.3-fold, Fig. 2b). These results are in agreement with the report of Liang et al. [11] showing that mutation of the RBP-J κ site in the ORF57 promoter strongly impaired RTA responsiveness.

It is likely that the distance between RRE1 and the RBP-J κ site did not affect IRF-7 repression. When co-transfected was done with the IRF-7 expression plasmid, RTA transactivation was repressed for both reporter C1 (1.2-fold) and C4 (0.6-fold) regardless of whether a 6-bp or 40-bp spacer was inserted. However, the degree of IRF-7 repression was weakened for reporter C2 with an additional copy of RRE1, which was 9.1-fold compared with 1.2-fold for reporter C1. Notably, IRF-7 could not suppress RTA activation for reporter C3 in which two extra copies of the RBP-J κ sites were added. The degree of activation was 33.2-fold compared with 30.9-fold when only the RTA expression plasmid was used for transfection (Fig. 2b), indicating that the repression of IRF-7 for RTA activation via competition for binding with RRE2 could be overcome by repeats of the RRE1 or RBP-J κ site.

The distance between the RBP-J κ site and RRE2 is crucial for RTA transactivation. In the wild-type ORF57 promoter, there are five base pairs between the RBP-J κ site and RRE2. If this 5-bp region was deleted (reporter C6), no responsiveness to RTA was observed (1.3-fold). Extension of the distance between the RBP-J κ site and RRE2 to 11 or 66-bp (reporters C7 and C8) also impaired the RTA responsiveness severely, with only 1.5- and 1.4-fold activity, respectively. Surprisingly, when the distance between the RBP-J κ site and the RRE2 was

altered, co-transfection with the IRF-7 expression plasmid increased the activation for reporters C6, C7, and C8 to 3.6-, 22.5- and 9.3-fold, respectively (Fig. 2b).

The KSHV ORF57 promoter has been studied extensively [8,13,21]. Our study provides insights into how viral gene expression is affected by response elements in the promoter region and is precisely regulated by viral and host protein factors. The existence of several response elements in tandem is a characteristic of the KSHV ORF57 promoter. Three RREs have been identified that bind to RTA and modulate RTA transactivation [11,13,21]. This study provides evidence of another functional RRE between TSS1 and TSS2 (Fig. 1c). The results also demonstrate that the 40-bp region can be divided, especially between RRE1 and the RBP-J κ site (Fig. 2b). However, the RBP-J κ site and RRE2 seem to be indivisible, since alteration of the distance between the RBP-J κ site and RRE2 affected the responsiveness to both RTA and IRF-7 (Fig. 2b). For sufficient transactivation and viral reactivation, RTA forms tetramers [1]. However, at this point, how RTA binds and transactivates the multiple RREs in vivo in the ORF57 promoter is not clear. The correlation between the polymerization of RTA and the linearity of response elements in the ORF57 promoter needs to be investigated further. In addition, several other viral and cellular factors are also involved in the regulation of ORF57 expression, including K-RBP [22], RBP-J κ [11], C/EBP α , c-Jun, Sp1, and Oct-1 [3]. Further studies on the contributions of these proteins in affecting RTA transactivation and IRF-7 repression of the ORF57 promoter will aid in the elucidation of the regulation of KSHV lytic gene expression.

Acknowledgments

This work was supported in part by NIH grant R01 TW007294, the Chinese National Program of Major Scientific Projects (2008ZX1001-1010), the National 973 Program (2005CB522903), Chinese NSF grants (30570083, 30870129 and 30970140) and Doctoral Fund of Chinese Ministry of Education (200800550021).

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Fig. 1.

Characterization of RREs in the ORF57 promoter and the responsiveness to RTA transactivation and IRF-7 suppression. **a** Schematic representation of deletion constructs used in transient transfection analysis. The locations of various putative promoter regulatory elements are indicated on the promoter segment, and the transcription start sites (TSS) 1 and 2 are indicated by *arrows*. The sequence of the 40-bp region is *boxed*, nucleotides of RRE1 and RRE2 are *shaded* and the RBP-J κ site *italicized*. *Solid lines* represent insertions of different deletion reporters, with the encompassed regions of the ORF57 promoter indicated by nt numbers. **b** Analysis of the responsiveness of reporters S1–S5 to RTA activation and IRF-7 repression. 293 T-cells were co-transfected with each of the reporter plasmids S1–S5, RTA and IRF-7 expression plasmids. Luciferase activities were measured 48 h post-transfection, and transfection efficiency was normalized by using the pCMV- β expression plasmid as an internal control. The activation fold was normalized to one in the absence of RTA and IRF-7 (*white columns*). The degree of activation when the reporters were co-transfected with RTA expression plasmid (*grey columns*) or with both RTA and IRF-7 expression plasmids (*black columns*) is shown. Results are averages of three independent experiments, and the standard deviations are shown. In parallel, the expression of RTA, IRF-7, and β -tubulin was detected by western blotting using anti-RTA, anti-Flag, and anti- β -tubulin antibodies. **c** Analysis of the responsiveness of reporters L1–L5 to RTA activation and IRF-7 repression

Fig. 2. Effect of the number of and the distance between response elements in mediating RTA transactivation and IRF-7 suppression. **a** Schematic representation of constructs used in transient transfection assays. Nucleotide sequences replacing the DNA fragment from nt 81904 to 81943 in reporter plasmid S2 are shown, with RRE1, RBP-Jκ site and RRE2 indicated. **b** Analysis of the responsiveness of reporters C1–C8 to RTA activation and IRF-7 repression

Table 1

Primers used in this study

Oligonucleotides	Sequences ^a
1	5'-CGGAGCTCAAGACCATTAGCTATC-3'
2	5'-GCGAGCTCGTGTAACAATAATGTTC-3'
3	5'-CAGAGCTCTTCCCACGGCCCATT-3'
4	5'-CAGAGCTCATTTTCGTTTGTGGTAC-3'
5	5'-CGGAGCTCGTACCAGTGGGACTG-3'
6	5'-TGCCATGGGCTATTTGGGAAC-3'
7	5'-TACCATGGCTTGTACCATGTCCTTTG-3'

^a SacI sites (GAGCTC) and NcoI sites (CCATGG) are underlined