Auto-activation of the rta gene of human herpesvirus-8/Kaposi’s sarcoma-associated herpesvirus

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Rta, mainly encoded by open reading frame 50 (ORF50), is the product of an immediate-early gene of human herpesvirus-8 (HHV-8)/Kaposi’s sarcoma-associated herpesvirus. Rta is a transcriptional activator that is both necessary and sufficient to disrupt viral latency and activate the expression of downstream viral lytic genes. We report that ectopically expressed Rta protein could also activate the rta promoter on a reporter plasmid up to 144-fold, both in latently infected B cells and in uninfected epithelial cells, and that this activation was dose-dependent. Furthermore, by analysing the 5’ untranslated region using ribonuclease protection assays, we demonstrated that transfection of an Rta expression plasmid into latently infected cells activated the expression of rta transcripts from endogenous viral genomes. We propose that auto-activation of the immediate-early gene, rta, is an important strategy for HHV-8 to effectively respond to environmental stimuli and maximally activate the virus lytic cycle.

Human herpesvirus 8 (HHV-8), also known as Kaposi’s sarcoma-associated herpesvirus, has been implicated in the pathogenesis of Kaposi’s sarcoma, primary effusion lymphoma (PEL) and multicentric Castleman’s disease (Cesarman et al., 1995a; Chang et al., 1994; Soulier et al., 1995) (reviewed in Neipel & Fleckenstein, 1999; Schulz & Moore, 1999). Like other herpesviruses, HHV-8 has two distinct phases in its life-cycle, latency and lytic replication. Latency is characterized by persistence of the viral genome with expression of a limited set of viral genes (Sarid et al., 1998; Zhong et al., 1996). Once the virus is reactivated from latency and enters the lytic cycle, most viral genes are expressed in an ordered fashion (immediate-early, early and late) (Sun et al., 1999), leading to production of infectious virions (Renne et al., 1996; Vieira et al., 1997).

A number of cell lines derived from PEL carry HHV-8 predominantly in a latent state (Arvanitakis et al., 1996; Boshoff et al., 1998; Cesaman et al., 1995b; Renne et al., 1996; Said et al., 1996). However, treatment of these cells with chemicals such as 12-O-tetradecanoylphorbol 13-acetate (TPA) or sodium butyrate induces HHV-8 to initiate lytic replication in a subset of the cell population (Miller et al., 1997; Nicholas et al., 1997; Renne et al., 1996; Sarid et al., 1998). Using these cell lines, several immediate-early genes have been identified (Lukac et al., 1999; Sun et al., 1998; Zhu et al., 1999). However, a biological function has only been demonstrated for the product of one immediate-early gene, rta. Expression of Rta alone in latently infected cells disrupted latency and activated the expression of viral lytic genes; induction of a viral late gene, ORF65, by Rta indicated that Rta drives the lytic cycle to completion (Lukac et al., 1998; Sun et al., 1998). In addition, introduction of a dominant-negative mutant of Rta into latently infected cells abolished viral reactivation (Lukac et al., 1999). Therefore, HHV-8 Rta is both necessary and sufficient to mediate the switch from latency to lytic replication.

Rta is highly conserved among gammaherpesviruses (Nicholas et al., 1991; Sun et al., 1998; Telford et al., 1995; van Santen, 1993; Wu et al., 2000). Recently, it was shown that Rta of murine gammaherpesvirus 68 is also capable of disrupting latency and driving the lytic cycle to completion (Wu et al., 2000). In Epstein–Barr virus, Rta (also called BRLF1 or R) and ZEBRA (also termed BZLF1, Zta, Z or EB1) are the earliest viral gene products synthesized during virus reactivation. They both exhibit auto-activation and cross-activation in certain cell lines. These two proteins in turn act in a cooperative manner to synergistically activate a cascade of lytic gene expression (Chevallier-Greco et al., 1986; Cox et al., 1990; Flemington & Speck, 1990; Hardwick et al., 1988; Holley-Guthrie et al., 1990; Manet et al., 1989; Ragocey et al., 1998; Zalani et al., 1996). However, unlike Rta, a homologue of the ZEBRA protein has only been identified in HHV-8 among gammaherpesviruses (Gruffat et al., 1999; Lin et al., 1999; Sun et al., 1998). Ectopic expression of this protein, K-bZip, did not disrupt latency (Sun et al., 1998). Moreover, K-bZip was shown...
Fig. 1. Rta activates its own promoter in a reporter system. (A) Rta activates the rta promoter plasmid in different cell lines. The reporter plasmid pPrluc contains a 3 kb sequence upstream of the translational initiation site of the rta gene that drives the expression of firefly luciferase (pPrluc). pPrluc was co-transfected into KS-1, BCBL-1 or 293T cells with either an Rta expression plasmid (pcDNA3/Rta) or pcDNA3, and a control vector, pRL-CMV. The pRL-CMV vector constitutively expresses Renilla luciferase driven by the CMV immediate-early enhancer/promoter. Forty-eight hours post-transfection, cells were harvested and dual-luciferase assays were performed. Firefly luciferase activities from pPrluc were normalized to the corresponding Renilla luciferase activities. For each cell line, fold activation of pPrluc by Rta was calculated by comparing the normalized firefly luciferase activity stimulated by Rta to that by pcDNA3. The values represent averages of three experiments, with the standard deviations shown. (B) Dose-dependence of Rta activation. A fixed amount of the reporter plasmid, pPrluc, was transfected into 293T cells with increasing amounts (from 0 to 16 ng) of pcDNA3/Rta, along with pRL-CMV. Decreasing amounts of pcDNA3 (from 16 to 0 ng) were included in each sample so that the total amount of pcDNA3 vector backbone remained the same. Normalized rta promoter activity was calculated as described for (A). The normalized rta promoter activity from cells transfected with 0 ng of pcDNA3/Rta and 16 ng of pcDNA3 was set at 1. Fold activation by different amounts of Rta was calculated by comparing the normalized rta promoter activities to that stimulated by 0 ng of pcDNA3/Rta and 16 ng of pcDNA3. The values represent averages of three experiments, with the standard deviations shown.

Fig. 2. Transfection of the Rta expression plasmid activates transcription of the rta gene from endogenous viral genomes. (A) Schematic representation of the Rta-related sequence in the HHV-8 genome and plasmids. Numbers indicate nucleotide positions in the viral genome (Russo et al., 1996). The methionine initiation codon for the Rta protein is located at position 71596, upstream of ORF49. The Rta expression plasmid, pcDNA3/Rta, contains the genomic sequence encoding the Rta protein (Sun et al., 1998). The 3′ portion of the riboprobe used for the ribonuclease protection assay is complementary to the sequence upstream of the methionine initiation codon (nucleotides 71429–71594). The fragment was amplified using primers RtaP1 (5′-acgcatggacggtcaccagc-3′) and BglRI, and inserted into pcDNA3, and used as a template to transcribe labelled antisense riboprobes, using a MAXScript kit (Ambion); therefore, the 5′ portion is derived from pcDNA3/KS(−) (fig. 2). The riboprobe hybridizes specifically to the rta transcripts from the endogenous viral genomes, but not to the transcripts from pcDNA3/Rta. (B) Analysis of the rta transcripts by ribonuclease protection assays. Two cell lines were used, KS-1 (lanes 1–4) and BCBL-1 (lanes 5–8), pcDNA3/Rta (lanes 1 and 5) or pcDNA3 (lanes 2 and 6) was transfected into cells, and total cellular RNA was harvested at 20 h post-transfection. As positive controls, cells were also induced by chemicals, and the RNA harvested at 12 h post-induction (lane 3, 20 ng/ml TPA; lane 6, 3 mM sodium butyrate). RNAs from untreated cells (lanes 4 and 8) were also prepared. Samples in lanes 9 and 10 contained only yeast RNA. RNAs were hybridized to radiolabelled, gel-purified riboprobes (see panel A), and a ribonuclease protection assay was performed. DNA marker (M, 100 bp ladder) and sequencing ladders (G, A, T, C) were run in parallel to determine the sizes of the protected fragments. An RNase A–RNase T1 mixture was added to samples 1–9, but not to sample 10. Only 2.5% of the undigested probes was loaded in lane 10. The arrowhead indicates the protected fragments representing rta transcripts initiated at nucleotides 71489 and 71490. Similar results were observed in three independent experiments. (C) Integrity of the RNA samples is shown in the ethidium bromide-stained agarose gel. The positions of the 28S and 18S rRNAs are indicated.

to be an early protein whose expression was activated by Rta, placing K-bZip kinetically downstream of Rta (Sun et al., 1999). These studies highlight the conservation and critical role of Rta in controlling the balance between latency and lytic replication. Functional identification of the rta gene of HHV-8 has laid the foundation for us to dissect the mechanism of HHV-8 reactivation. Two groups of experiments have been undertaken, one to identify viral genes that are activated by Rta, and the other to study the regulation of rta gene expression. The convergence of these two groups of studies led to our discovery that Rta positively regulates its own expression. The major coding region for the Rta protein is located in ORF50 of the HHV-8 genome. A splicing event that removes ORF49 introduces a new methionine initiation codon at
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Fig. 2. For legend see opposite.
nucleotide 71596 [all positions cited are according to the published sequence (Russo et al., 1996), adding 6 amino acids in exon 1 plus an additional 54 amino acids in exon 2 to ORF50 (Lukac et al., 1999; Sun et al., 1998; Zhu et al., 1999)]. A 3 kb sequence upstream of the Rta translation initiation codon was amplified from total cellular DNA prepared from BC-1 cells, using primers NheRta (5′-acctgaggcgtcctcattggagca 3′) and BglRta2 (5′-acagagatttggtggctgctgg-ACAGTATTC 3′). The PCR fragment was cloned into the pGL2-basic vector (Promega), with Nhel and BglII as cloning sites, to derive pRPluc. pGL2-basic has only the coding sequence for firefly luciferase. pRPluc was responsive to TPA or sodium butyrate, which are inducers of HHV-8 lytic replication (H. Deng & R. Sun, unpublished results). Therefore, pRPluc can be used to study the transcriptional regulation of the rta promoter.

The reporter plasmid pRPluc (7.5 µg) was electroporated into 10^7 HHV-8 latently infected KS-1 or BCBL-1 cells, with either an Rta expression plasmid, pcDNA3/Rta (Sun et al., 1998), or the pcDNA3 vector (2.5 µg). Alternatively, 200 ng each of pRPluc and expression plasmid were co-transfected into an HHV-8-negative cell line, 293T, in 24-well plates, using LipofectAmine PLUS (Gibco). A control plasmid, pRL-CMV, which constitutively expresses Renilla luciferase, was included in each transfection. Cells were harvested at 48 h post-transfection, and Dual-Luciferase Reporter Assays (Promega) were performed. The rta promoter was activated 144 ± 4-fold by the Rta protein in BCBL-1 cells, and 38 ± 7-fold in KS-1 cells. In 293T, the rta promoter was activated 40 ± 3-fold (Fig. 1A). Therefore, the Rta protein activated the rta promoter independently of a B cell-specific or other virus-specific factor. Seaman et al. (1999) previously reported that in BCBL-1 cells an rta promoter construct, p50p-CAT, was activated 11 ± 6-fold by Rta. However, activation of p50p-CAT by pcDNA3 was 4 ± 27-fold. Thus, the -fold activation by Rta was 2 ± 7, in contrast to the 144 ± 4-fold activation of pRPluc by Rta that we demonstrated using the same cell line. One difference between these two studies is that p50p-CAT only contains a 655 bp fragment upstream of the Rta coding region.

To test the dose-dependence of Rta activation, a fixed amount of pRPluc (200 ng) was co-transfected with increasing amounts of pcDNA3/Rta into 293T cells. Decreasing amounts of pcDNA3 vector were also included in each sample so that the total amount of pcDNA3 vector backbone remained the same. As the amount of pcDNA3/Rta increased (from 0 to 16 ng), so did the normalized luciferase activity (Fig. 1B). Higher doses of pcDNA3/Rta (25, 50, 100 and 200 ng) resulted in progressively higher levels of rta promoter activity (200 ng of pcDNA3/Rta shown in Fig. 1A, 293T cells; other data not shown). Similar results were obtained in BCBL-1 cells (data not shown), demonstrating that Rta specifically activated the rta promoter. In addition, Rta did not activate promoters from other viruses, such as the SV40 promoter (in plasmids pGL3-control and pRL-SV40) or the HCMV immediate-early promoter (in plasmid pRL-CMV) (data not shown), indicating that activation of the rta promoter by Rta is specific.

Results from the reporter system demonstrated that Rta activated the rta promoter from a plasmid that lacks chromatin structure. We next examined whether Rta can activate the expression of the rta gene from endogenous viral genomes. To distinguish between the rta transcripts from the endogenous viral genomes and the transcripts from transfected pcDNA3/Rta (Fig. 2A), we cloned the 5′ untranslated region (nucleotides 71429–71594) of rta into pBluescript KS(−) (Stratagene), to generate riboprobes for ribonuclease protection assays (RPAs).

Five µg of pcDNA3/Rta or pcDNA3 was transfected into KS-1 or BCBL-1 cells. Total cellular RNA was harvested at 20 h post-transfection. As controls, RNAs were also prepared from untreated cells and from chemically induced cells at 12 h post-induction. The 8 h difference was chosen to compensate for possible delay of Rta action due to the transfection. Twenty µg of sample RNAs was hybridized to equal amounts of radiolabelled, gel-purified riboprobes, and an RPA was performed using a HybSpeed RPA kit (Ambion). Protected RNA fragments were resolved on a 6% denaturing polyacrylamide gel (Fig. 2B). Only 25 µg of the undigested probes were loaded in lane 10, showing the size of the undigested full-length rta probe. Unprotected probes were digested to near completion, as the residual full-length probes accounted for less than 0.02% of the input probes by quantitative phosphorimaging analysis. No rta transcripts from the viral genomes were detected in the RNA from untreated KS-1 cells (lane 4). When cells were induced with TPA, specific protected fragments were detected (lane 3), indicating that rta transcripts were expressed from the endogenous viral genomes. No rta transcripts were observed when cells were transfected with pcDNA3 vector alone (lane 2). However, introduction of pcDNA3/Rta into KS-1 cells yielded specific fragments that were protected from ribonuclease digestion (lane 1). The pattern of the bands on the polyacrylamide gel was the same as that seen in the chemically induced sample, suggesting that Rta and TPA activate expression of the rta gene from the endogenous viral genomes in a similar way. Activation of the endogenous rta gene was also observed in a different cell line, BCBL-1 (lanes 5–8).

Previous studies have identified the transcription initiation site of the rta gene at nucleotide 71513 in BC-1 cells using 5′ RACE (Sun et al., 1998; Zhu et al., 1999), or at nucleotide 71560 in BC-1 cells using 5′ RACE and primer extension (Lukac et al., 1999). In our RPAs, sequencing ladders were run in parallel to determine the sizes of the protected fragments. With correction for the slower mobility of RNA compared to DNA of the same length in a denaturing polyacrylamide gel, the shortest protected fragments (the doublet bands in Fig. 2B, arrowhead) represented transcripts initiated at nucleotides 71489 and 71490 in both KS-1 and BCBL-1 cells. This result was reproducible in three independent experiments. No shorter specific bands were observed. The discrepancy between previous results and our RPA result most likely stems from

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differences in the experimental methods. In 5’ RACE and primer extension experiments, reverse transcription reactions are usually performed at 37 or 42 °C, temperatures at which RNA secondary structures may not be completely disrupted. Moreover, consistent with our result, there is a putative TATA box (TAAAATA) at nucleotide 71452, 37–38 nucleotides upstream of our mapped transcription initiation sites.

The upper bands in RPAs represent protection of the complete viral sequence in the riboprobe (reproducible in three independent experiments). The appearance of the multiple bands was likely caused by end-breathing of the RNA–RNA hybrids and partial protection at the end of the RNA duplexes. Since gel-purified, full-length riboprobes were utilized, the hybrids and partial protection at the end of the RNA duplexes. bands was likely caused by end-breathing of the RNA–RNA in independent experiments). The appearance of the multiple complete viral sequence in the riboprobe (reproducible in three

rta box (TAAATA) at nucleotide 71452, 37–38 nucleotides upstream of our mapped transcription initiation sites. Further analysis of the multiple transcripts. Several open reading frames immediately upstream of nucleotide position 71489 (ORFs 45–48) are all transcribed in the opposite orientation to ORF50, and would not be protected by the antisense rta probe. Furthermore, all the protected transcripts from chemically induced cells were resistant to cycloheximide, a protein synthesis inhibitor (H. Deng & R. Sun, unpublished results), indicating that there are multiple transcription initiation sites for the rta gene. Our results are also consistent with previous reports of a complex transcription pattern across the rta locus of HHV-8 (Sun et al., 1998; Zhu et al., 1999). A precedent for this phenomenon is the herpesvirus saimiri rta homologue, which is transcribed from two distinct promoters (Whitehouse et al., 1997).

The rta gene was classified as an immediate-early gene, based on the resistance of its expression to cycloheximide (Sun et al., 1998; Zhu et al., 1999). However, it was noted that although treatment with cycloheximide did not abolish expression of the rta gene, it did cause a reduction in the level of rta gene expression in BC-1 cells. Rta auto-activation provides one explanation for this observation.

In summary, we have demonstrated Rta auto-activation of HHV-8, using two independent approaches. Thus, Rta establishes a positive feedback loop in the cascade of viral lytic gene expression. Auto-activation of rta, the master switch gene, may be an important strategy for the virus to amplify environmental stimuli, thereby allowing the virus to be efficiently reactivated from latency. Further analysis of the regulation of HHV-8 rta gene expression will help elucidate the mechanism controlling the dynamic balance between latency and lytic replication.

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References


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