The herpesvirus saimiri Rta gene autostimulates via binding to a non-consensus response element

Matthew S. Walters,† Kersten T. Hall and Adrian Whitehouse

Herpesvirus saimiri ORF 50a protein expression is sufficient to reactivate the entire lytic-replication cycle. ORF 50a functions as a sequence-specific transactivator that is capable of activating delayed-early gene expression via direct binding to an ORF 50 response element (RE) within the respective promoter. Here, it is shown that ORF 50a is capable of transactivating its own promoter. Deletion analysis of the ORF 50a promoter showed that the ORF 50-responsive element is contained within an 80 bp fragment, situated 293–373 bp from the transcription initiation site. Gel-retardation analysis further mapped the RE to a 34 bp fragment that was able to confer ORF 50 responsiveness to an enhancerless SV40 minimal promoter. Sequence analysis showed that this RE has no direct similarity to previously identified ORF 50 REs. Therefore, it is concluded that ORF 50a is capable of stimulating its own promoter via a novel RE.

Gene expression in HVS is modulated by the two major transcriptional regulating genes, encoded by open reading frames (ORFs) 50 and 57 (Nicholas et al., 1988, 1991; Whitehouse et al., 1997a, 1998a, b). The ORF 57 gene product encodes a multifunctional protein that is capable of both transactivation and repression of viral gene expression at a post-transcriptional level (Whitehouse et al., 1998a, b; Goodwin et al., 1999). The ORF 50 gene produces two transcripts, termed ORF 50a and b. The larger, spliced ORF 50a gene product is the major transactivating protein. It activates transcription following direct binding to promoters containing a specific sequence motif, CCN_{3}GG, termed the ORF 50 response element (RE) (Nicholas et al., 1991; Whitehouse et al., 1997a, b, 1998a). This RE has significant similarity to the EBV Rta RE consensus sequence, GNCCN_{3}GGNG (Gruffat et al., 1990, 1992; Gruffat & Sergeant, 1994). Recently, we have identified a DNA-binding domain within ORF 50a that has a high degree of similarity to the DNA-binding domain encoded by mammalian high-mobility group A (HMGA) chromosomal proteins, termed an AT hook (Reeves & Nissen, 1990). This domain is required for ORF 50 transactivation and binding to the ORF 50 RE (Walters et al., 2004). Once bound, HVS ORF 50a recruits and interacts with the TATA binding protein (TBP) (Hall et al., 1999), suggesting that it recruits components of the TFIIID complex and allowing transcription initiation by RNA polymerase II.

Studies in EBV, KSHV, MHV-68 and HVS have shown that ORF 50 proteins are key regulators in the switch from the latent state to the lytic-replication cycle (Zalani et al., 1996; Lukac et al., 1998, 1999; Ragoczy et al., 1998; Sun et al., 1998; Ragoczy & Miller, 1999; Wu et al., 2000; Goodwin et al., 2001). Studies have shown that the Rta promoters of EBV (Zalani et al., 1992, 1995, 1996, 1997; Glaser et al., 1998; Chang & Liu, 2000; Ragoczy & Miller, 2001) and KSHV (Deng et al., 2000; Chen et al., 2001; Sakakibara et al., 2001; Brown et al., 2003; Haque et al., 2003; Liang & Ganem, 2003; Wang et al., 2003; Damania et al., 2004) are activated by many cellular factors and, more significantly, by the Rta proteins themselves. Therefore, in this report, we investigated whether HVS ORF 50a was capable of regulating its own promoter.

To determine whether ORF 50a is capable of stimulating its own promoter, the putative ORF 50a promoter situated between positions 68320 and 69878 of the HVS genome was PCR-amplified by using the primers DelFor1
(5′-ACATGCATGCGAACGACATAACGCGTAGA-3′) and DelRev (5′-ACATGCATGCGCTTGAGCTTACAGCTCAG-3′). These primers incorporated SpII and SalI sites, respectively, and the resulting products were cloned into pCAT-Basic (Promega), previously digested with Sall and SpII, deriving pCAT-DEL1. 293T cells were transfected by using Lipofectamine (Invitrogen) with 1 μg of either pEGFP, p50GFP or p50GFPAmAT-hook (which contains a deletion of the ORF 50 DNA-binding domain) (Walters et al., 2004) in the presence of 1 μg pCAT-DEL1. Cells were harvested 30 h post-transfection and the protein concentration of each sample was calculated by using a DC Protein Assay kit (Bio-Rad). Cell extracts corresponding to 10 μg protein were assayed for chloramphenicol acetyltransferase (CAT) activity by standard methods (Gorman et al., 1982). Levels of chloramphenicol converted to acetylated products were calculated by using a FUJIX BAS1000 Bio-Imaging analyser (Fuji Photo Film Co.). Raw data produced from the Image Reader BAS-1000 software were subsequently quantified by using Advanced Image Data Analyser (AIDA) version 2.31 software and represented as fold increase in CAT activity compared to vector control (pEGFP).

Results showed that p50GFP was able to stimulate the ORF 50a promoter to high levels (36-fold), suggesting that the ORF 50a protein can stimulate its own promoter. In contrast, stimulation was reduced dramatically when using p50GFPAmAT-hook (Fig. 1a), suggesting that ORF 50a stimulates its own promoter via direct binding of the promoter. To confirm that the transfection efficiency of these experiments was normalized, immunofluorescence was undertaken to ensure that a comparable number of cells were transfected. In addition, 100 μg cell extract was analysed by Western blot using a primary monoclonal green fluorescent protein (GFP) antibody (Clontech) to demonstrate comparable expression between assays (data not shown).

Analysis of the ORF 50a promoter failed to identify a consensus ORF 50 RE that was previously identified in HVS ORF 50-responsive promoters. Therefore, to map the alternative ORF 50a-responsive regions within the ORF 50a promoter, a series of further promoter deletions was generated (Fig. 1b). The promoter fragments were PCR-amplified by using the forward primers DelFor8–9 (DelFor8, 5′-ACATGCATGCGAACGACATATGCTTACAGCTCAGGAGCAACAC-3′; DelFor9, 5′-ACATGCATGCGCTTGAGCTTACAGCTCAGGAGCAACAC-3′) and the reverse primer DelRev and subsequently cloned into pCAT-Basic as described previously, deriving pCAT-DEL6.1 and pCAT-DEL6.2. 293T cells were then transfected with 1 μg p50GFP in the presence of 1 μg pCAT-DEL6 or pCAT-DEL7 and assayed for CAT activity as described previously (Fig. 1e).

The results demonstrated that p50GFP was capable of stimulating DEL6 and DEL6.1 to similar levels. However, deletion of the region between DEL6.1 and DEL6.2 resulted in ORF 50a stimulation returning to levels comparable to those obtained with DEL7. This, therefore, suggests that the element(s) responsible for ORF 50a stimulation lie between DEL6.1 and DEL6.2 of the ORF 50a promoter.

To fine-map the ORF 50a-responsive element, further deletions of this region were generated (Fig. 1d). The promoter fragments were PCR-amplified by using the forward primers DelFor8–9 (DelFor8, 5′-ACATGCATGCGAACGACATATGCTTACAGCTCAGGAGCAACAC-3′; DelFor9, 5′-ACATGCATGCGCTTGAGCTTACAGCTCAGGAGCAACAC-3′) and the reverse primer DelRev, and subsequently cloned into pCAT-Basic as described previously, deriving pCAT-DEL6.1 and pCAT-DEL6.2. 293T cells were then transfected with 1 μg p50GFP in the presence of 1 μg pCAT-DEL6 or pCAT-DEL7 and assayed for CAT activity as described previously (Fig. 1e). The results demonstrated that p50GFP was capable of stimulating DEL6 and DEL6.1 to similar levels. However, deletion of the region between DEL6.1 and DEL6.2 resulted in ORF 50a stimulation returning to levels comparable to those obtained with DEL7. This, therefore, suggests that the element(s) responsible for ORF 50a stimulation lie between DEL6.1 and DEL6.2 of the ORF 50a promoter.

To assess whether the ORF 50a protein binds directly to this region encompassing DEL6.1 to DEL6.2 of the ORF 50a promoter, gel-retardation experiments were performed by using three sets of overlapping oligonucleotides spanning the region, termed sets (I)–(III) (Fig. 2a). The oligonucleotides were annealed and labelled by using a DIG Gel Shift kit (Roche) according to the manufacturer’s protocol. The digoxigenin (DIG)-labelled oligonucleotides were then incubated with 2–5 μl nuclear extract from untransfected and p50GFP-transfected 293T cells, prepared by using a NucBuster Protein Extraction kit (Novagen). The protein–nucleic acid complexes were separated on a 4 % polyacrylamide gel and detected by immunoblotting using the DIG Gel Shift kit protocol (Fig. 2b). Results show the formation of a retarded complex with p50GFP-transfected cell extracts when incubated with oligonucleotide set (I). No other complexes were identified with oligonucleotide sets (II) and (III), suggesting that ORF 50a binds to the ORF 50a promoter directly between positions 69526 and 69560 of the published sequence (GenBank accession no. X64346). Moreover, to demonstrate that the retarded
Fig. 1. The region encompassing deletions 6.1 and 6.2 is required for ORF 50 autostimulation. (a) Effect of the ORF 50a wild-type and mutant proteins on stimulation of the ORF 50a promoter. 293T cells were cotransfected with pCAT-DEL1 in the presence of pEGFP, p50GFP or p50GFPΔAT-hook. Variations between three replicated assays, each performed in triplicate, are indicated. (b) Schematic representation of the ORF 50a promoter deletion series, cloned upstream of the CAT reporter gene. (c) Effect of the ORF 50a wild-type and mutant proteins on stimulation of the ORF 50a promoter deletion series. 293T cells were cotransfected with pCAT-DEL1–7 in the presence of pEGFP, p50GFP (filled bars) or p50GFPΔAT-hook (empty bars). Variations between three replicated assays, each performed in triplicate, are indicated. (d) Schematic representation of more refined ORF 50a promoter deletions, cloned upstream of the CAT reporter gene. (e) Effect of the ORF 50a wild-type protein on stimulation of the refined ORF 50a promoter deletions. 293T cells were cotransfected with pCAT-DEL6–7 in the presence of pEGFP or p50GFP. Variations between three replicated assays, each performed in triplicate, are indicated. All CAT assay data are represented as fold increase in activity compared to vector control.
complex detected is specific for ORF 50a, two further assays were performed. A supershift assay was undertaken by using a monoclonal GFP antibody (Clontech) and the set (I) oligonucleotides. GFP antibody (2 µg) was added to the binding reaction (described previously) and incubated for 15 min. A clear shift of the p50GFP-retarded complex was detected, demonstrating specificity for ORF 50a. Secondly, competition assays were performed by using increasing quantities of unlabelled oligonucleotides spanning the ORF 50 RE present within the ORF 6 promoter (Whitehouse et al., 1997b). This out-competed the binding reaction with labelled set (I) oligonucleotides, demonstrating that this retarded complex is specific for the ORF 50a protein (Fig. 2b).

To further confirm the specificity of ORF 50a binding, the sequences between positions 69526 and 69560 of the published sequence were cloned into a heterologous promoter to determine whether they can confer ORF 50a responsiveness. Set (I) oligonucleotides were cloned into pGL3-Promoter (Promega), which contains the luciferase reporter gene under the control of a minimal SV40 promoter (Fig. 3a). To aid cloning into the polylinker of pGL3 (which is situated upstream of the minimal SV40 promoter), the oligonucleotides contained KpnI and SmaI restriction sites, deriving pGL3-50RE. Moreover, to compare the specificity of the ORF 50 promoter RE, a similar construct was produced containing the RE from the ORF 6 promoter (Whitehouse et al., 1997b). 293T cells were then transfected with 1 µg pGL3-Promoter, pGL3-50RE or pGL3-6RE in the presence of 1 µg pEGFP or p50GFP and assayed for luciferase activity by standard methods (Fig. 3b). Data are represented as fold increase in luciferase activity compared to vector control. Results demonstrated that p50GFP was unable to increase luciferase activity of approximately

![Fig. 2. ORF 50a is capable of binding a novel element encompassing nt 69526–69560 of the ORF 50a promoter. (a) Schematic representation of the oligonucleotide primers spanning the ORF 50a-responsive region within the ORF 50a promoter. (b) (i) Electrophoretic mobility-shift assays were performed by using DIG-labelled oligonucleotides spanning the ORF 50a promoter. Each labelled set of oligonucleotides was incubated with nuclear extracts prepared from untransfected or p50GFP-transfected 293T cells. (ii) To demonstrate the specificity of ORF 50a binding, a supershift assay was performed by using a primary monoclonal GFP antibody. (iii) To demonstrate the specificity of the ORF 50 interaction, increased quantities of unlabelled oligonucleotides spanning the ORF 50 RE present within the ORF 6 promoter were used to out-compete the binding reaction.](image-url)
threefold for pGL3-50RE, in comparison to an approximately 4.5-fold increase from the consensus ORF 6 promoter RE. This demonstrates that the sequences with the ORF 50 promoter RE are able to confer ORF 50a responsiveness. However, it may be less effective than the canonical ORF 6 promoter RE.

We have previously demonstrated that the ORF 50a protein is sufficient to reactivate the entire HVS lytic-replication cycle (Goodwin et al., 2001). Moreover, ORF 50a is capable of activating transcription following direct binding to promoters containing a specific sequence motif, CCN9GG, termed the ORF 50 RE (Nicholas et al., 1991; Whitehouse et al., 1997a, b, 1998a). Direct binding of the ORF 50 RE requires the ORF 50 DNA-binding domain, which has a high degree of similarity to the DNA-binding domain encoded by mammalian HMGA chromosomal proteins, termed an AT hook (Walters et al., 2004). In this study, we have demonstrated that ORF 50a is capable of stimulating its own promoter via direct binding of a novel 34 bp RE that is present within the promoter. Sequence analysis of the RE demonstrates that it has no direct similarity to a previously identified ORF 50 RE that was identified in other HVS promoters; however, both types of RE are recognized by the ORF 50 AT hook DNA-binding domain. Previous studies have shown that mammalian AT hook DNA-binding domains have a dynamic conformation, which allows them to produce optimal contact with the narrow, minor-groove regions of AT-rich DNA (Reeves & Nissen, 1999; Himes et al., 2000). Further analysis of the ORF 50 REs have shown that, in the absence of direct similarity, both contain AT-rich DNA sequences. Therefore, we suggest that the ORF 50 AT hook is capable of recognizing different REs via the presence of AT-rich regions. Interestingly, the alternative REs both confer ORF 50 responsiveness to an enhancerless promoter; however, stimulation levels vary. Therefore, analysis is now required to elucidate the binding affinity of ORF 50a to both types of RE and to determine whether they have a role in a further regulatory step of the lytic temporal cascade.

Acknowledgements

This work was supported in part from grants to A.W. from the Association of International Cancer Research and Yorkshire Cancer Research.

References


