On the control of late gene expression in Kaposi’s sarcoma-associated herpesvirus (human herpesvirus-8)

Jean Chang and Don Ganem

Howard Hughes Medical Institute and Departments of Microbiology and Medicine, University of California, San Francisco, CA 94143-0414, USA

Herpesvirus late genes require viral DNA replication for maximal expression. Although late gene expression appears to require DNA replication in cis in alphaherpesviruses, studies in Epstein–Barr virus (EBV) suggest that this cis-requirement might not pertain to the gammaherpesviruses. Based on these findings, a system was created to investigate the elements required for the regulation of Kaposi’s sarcoma-associated herpesvirus (KSHV; human herpesvirus-8) late gene expression. The transcript of a classic late gene encoding the viral assembly protein was characterized and reporter genes driven by the assembly protein promoter region were constructed. Unlike the EBV case, expression of a reporter gene under the control of the assembly protein promoter did not display authentic regulation when removed from the context of the viral genome. Although reporter expression rose in cells displaying lytic replication, this expression was not diminished by specific inhibitors of viral DNA synthesis. Minimal core promoters were similarly unable to reproduce late gene regulation. These results suggest that proper KSHV late gene expression is likely to be dependent upon virus lytic replication in cis and indicate that the regulation of KSHV late genes more closely resembles that observed in herpes simplex virus than that described for EBV.

Introduction

Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV; human herpesvirus-8) is the proposed aetiological agent of KS (Beral et al., 1990). Epidemiological evidence shows that infection is not ubiquitous, is strongly correlated with increased risk of disease and precedes disease development (Gao et al., 1996a, b; Kedes et al., 1996; Martin et al., 1998; Whitby et al., 1995). Viral DNA is present in KS lesions of all epidemiological types: classical, HIV-associated, African endemic and transplant-associated (Boshoff & Weiss, 1998; Moore & Chang, 1995). KSHV is also implicated in at least two lymphoproliferative disorders, primary effusion lymphoma (PEL) (Cesarman et al., 1995a) and multicentric Castleman’s disease (Soulier et al., 1995), consistent with its classification as a lymphotropic gammaherpesvirus (Moore et al., 1996a; Russo et al., 1996). Viral sequences can also be detected in CD19+ peripheral B cells of infected individuals (Ambroziak et al., 1995; Mesri et al., 1996; Moore et al., 1996b; Whitby et al., 1995), again bespeaking its lymph tropism. The majority of cells in B cell lines derived from PEL are latently infected with KSHV (Cesarman et al., 1995b; Renne et al., 1996). These latently infected cells can be induced to lytic reactivation by chemical inducers such as phorbol ester, sodium butyrate or calcium ionophores (Miller et al., 1997; Moore et al., 1996a; Renne et al., 1996).

In herpesviruses, activation (or reactivation) of the virus lytic cycle initiates a temporally regulated cascade of gene expression, ultimately resulting in the release of infectious virions (Honess & Roizman, 1974). Lytic cycle genes can be categorized roughly into three classes: immediate-early (IE), delayed early (DE) and late genes. IE gene products direct the expression of DE genes, including components of the virus replication machinery. After replication of the viral genome, late genes, generally structural proteins, are expressed and virions are packaged and released. KSHV gene expression is no exception to this paradigm (Sun et al., 1999). In KSHV, the ORF50 protein (a homologue of the EBV IE R protein) has been shown to trigger reactivation from latency in B cell lines derived from PEL and direct expression of KSHV DE genes (Lukac et al., 1998, 1999; Sun et al., 1998). KSHV assembly protein (ORF17.5) and small viral capsid antigen (ORF5) are triggered by the homologues of which in herpes simplex virus (HSV) and Epstein–Barr virus (EBV) are classified as late genes, have been shown to behave with late kinetics in that they are induced by
activators such as TPA but their induction is blocked by inhibitors of the viral polymerase (Lin et al., 1997; Unal et al., 1997). Thus, viral DNA replication seems to constitute an important temporal boundary between early and late gene expression in KSHV.

The prevailing model of herpesvirus late gene regulation is based on work done in HSV. Initial experiments, where late promoters were removed from the virus context to either plasmid vectors or the host genome, showed that such constructs were expressed with early kinetics (Dennis & Smiley, 1984; Homa et al., 1986; Silver & Roizman, 1985). Further experiments showed that unreplicated viral genomes cannot serve as templates for late gene expression, despite the probable presence of all trans-acting factors via a separate viral genome replicated in trans (Mavromara-Nazos & Roizman, 1987), highlighting the necessity for DNA replication in cis. Once a lytic origin was provided in cis to a late promoter on a plasmid vector, proper late gene regulation could be observed in infected cells (Johnson & Everett, 1986a). Several groups subsequently demonstrated that minimal late promoter regions (TATA box + cap site) were sufficient to reproduce late gene regulation in infected cells when a lytic origin was present in cis (Flanagan et al., 1991; Homa et al., 1986, 1988; Johnson & Everett, 1986b). However, such a model is doubtless an oversimplification, as further studies have shown that structural features other than the TATA box are able to influence late gene expression (Kibler et al., 1991; Mavromara-Nazos & Roizman, 1989; Steffy & Weir, 1991).

The nature of the dependence upon viral DNA replication for late gene expression may vary between herpesviruses. Recent studies of EBV, a gammaherpesvirus more closely related to KSHV, showed that EBV late promoter regions driving a CAT reporter gene exhibited late kinetics in infected cells even when the plasmid lacked an oriLyt in cis (Serio et al., 1997). It was subsequently shown that the region responsible for conferring late gene regulation in this context was the minimal core promoter, bearing a unique TATA box variant (TATTAAA) (Serio et al., 1998). These results suggested that DNA replication in cis might not be a strict requirement for late gene expression in all herpesviruses.

Sparked by these results, we set out to define the sequence requirements for properly regulated expression of KSHV late genes. In this work, we show that KSHV late promoters, unlike their EBV counterparts, do not reproduce correct late gene regulation when removed from the context of the viral genome. KSHV late gene expression therefore seems likely to require replication of the viral genome in cis.

**Methods**

**Plasmids.** pAP and pAP rev were constructed by cloning the 696 nt SacI–EcoRV fragment from genomic positions 31663 to 32359 in the forward or reverse orientations, respectively, into pGL3-basic that had been digested with SmaI. The SacI site was blunt-ended with Klenow polymerase.

pPr/AP and pPr/rev were constructed by cloning the 1313 nt HindIII–EcoRV fragment from genomic positions 31663 to 32976 in the forward or reverse orientations, respectively, into pGL3-basic that had been digested with SmaI. The HindIII site was blunt-ended with Klenow polymerase.

pAP TTG was constructed by changing the initiating ATG of the AP ORF (nt 31687) to TTG by PCR mutagenesis.

pGL3-gB has been described previously (Lučak et al., 1998). pGL3-MCP was constructed by cloning the 1935 nt Xnasl–NcoI fragment from genomic positions 32362 to 31666 into pGL3-basic that had been digested with the same enzymes.

pGL3—30 AP was constructed by PCR amplification of the pGL3 AP plasmid with primers AP—30 (5’ GAGCCACCGGTATTTAAA-

GGCCAGC) and pGL3rev (5’ CTTCATAGCCATATGCGTGCCTC). The PCR product was digested with MmlI and Nael and cloned into the corresponding sites in pGL3-basic.

**Cell lines and transfections.** BCBL-1 cells were propagated and maintained in RPMI 1640 medium as described by Renne et al. (1996). Cells were diluted to a density of 2.5 × 10^6 cells/ml 18–24 h prior to transfection. For Superfect transfection, cells were washed once with PBS and 1 × 10^5 cells were resuspended in 6 ml growth medium for each transfection. DNA was diluted in 150 µl unsupplemented RPMI 1640 medium and 20 µl Superfect (Qiagen) was added. Complexes were allowed to form for 10 min at room temperature and then 1 ml of growth medium was mixed with the DNA–Superfect complexes which were then added to the cells. Twenty-four h post-transfection, cells were split for chemical treatment. Cells were harvested 48 h post-induction.

BJAB cells were propagated and maintained in RPMI 1640 medium as described by Renne et al. (1996). For electroporation, cells were washed once with PBS and resuspended in unsupplemented medium. Cells (1 × 10^6) were aliquoted in 0.4 µl volumes to electroporation cuvettes (0.42 cm) containing 20 µg DNA. Cells were electroporated at 960 µF and 250 mV and subsequently transferred to 10 ml fresh medium. Superfect transfection was carried out as described above.

293 cells were maintained and propagated in Dulbecco’s modified Eagle’s medium H16 supplemented with 10% FCS, 2 mM glutamine and antibiotics. For transfections, cells were plated at 2 × 10^5 cells per 60 mm dish and grown overnight. On the day of transfection, cells were fed 3 h before DNA addition. DNA was precipitated by using the calcium phosphate procedure. Twenty-four h after DNA addition, cells were washed with PBS and fresh medium with or without TPA was added. Cells were harvested 48 h after addition of DNA.

**Preparation of RNA and CDNA cloning.** BCBL-1 cells were diluted to a density of 3 × 10^6 cells/ml and induced with 20 ng/ml TPA or mock treated. Twenty-four h post-induction, cells were harvested and RNA was isolated by using RNAzol B (Tel-Test) following the manufacturer’s directions.

mRNA from induced BCBL-1 cells was primed with poly(dT) and cloned into Lambda Zap (Stratagene) by R. Renne (UCSF, San Francisco, USA). The library was screened with a Clal–Sacl fragment from the assembly protein ORF by using a Random DNA Prime kit (Amersham).

**RNase protection assay.** The riboprobe plasmid was constructed by PCR amplification of a 256 nt region spanning genomic positions 31511–31766 with primers 5’ TGGGATTAACTGACAGAGGGTGGG and 5’ TCAGGCTACCGGGGAACATTCGACGCC. The PCR product was digested with KpnI and EcoRI and cloned into the corresponding sites in pBSII SK+. The riboprobe plasmid was linearized with EcoRI and transcribed with T7 RNA polymerase in the presence of [35S]UTP. Gel-purified riboprobes (4 × 10^6 or 8 × 10^6 c.p.m.) were hybridized to 10 µg total RNA overnight at 42 °C. RNase digestion was performed with a
KSHV late gene expression

Fig. 1. Mapping of the 5′ end of the AP transcript. (a) The nucleotide sequence of the riboprobe used for RPA is depicted. The asterisks show the locations of the 5′ ends of cloned cDNAs. The largest possible AP ORF is boxed. The candidate TATA box motif is underlined. The horizontal arrow represents the oligonucleotide used for primer extension analysis. The mapped transcription start sites are denoted by vertical arrows. (b) RPA of the start site of the AP transcript. Total RNA from TPA-treated and untreated BCBL-1 cells was isolated and hybridized to the riboprobe shown in (a). RNA hybrids were digested with RNase, denatured and electrophoresed. A sequencing ladder (right) was used for sizing. (c) Primer extension analysis of the start site of the AP transcript. Total BCBL-1 RNA from TPA-treated and untreated cells was isolated and primer extension was performed with the primer indicated in (a). Products were visualized as in (b). A sequencing ladder (right) provides a size reference.

1:100 dilution of the RNase A/RNase T1 solution from the RPA II kit (Ambion). Digested RNAs were precipitated and separated on an 8% denaturing acrylamide gel. The gel was exposed to Kodak BioMax film for 18 h.

**Primer extension.** Primer extension was performed as described by Zhong et al. (1996). Ten µg total RNA from BCBL-1 cells, induced with TPA for 48 h or uninduced, was hybridized with primer PE1 (5′ GGGATGGATATGATATCCTCTTGAC) or PE2 (5′ GGATGCCGA-CGGGAATTGGCTGGC). Samples were separated on an 8% denaturing acrylamide gel. The gel was exposed to Kodak BioMax film for 4 days.

**Luciferase assay.** Cells were washed twice with 1 ml PBS. After the addition of 0.2 ml reporter lysis buffer (Promega), cells were harvested by scraping, vortexed and spun briefly to pellet cellular debris. Aliquots
Fig. 2. Characterization of AP promoter constructs. (a) Structure of reporter plasmids containing AP promoter regions. (b) The 1.3 kb (pPr/AP) and 700 bp (pAP) promoter regions of AP are both able to drive expression of a reporter gene. 293 cells were transfected with 4 µg of the indicated reporter plasmid and 2 µg β-galactosidase-expressing plasmid. Cells were harvested 48 h post-transfection and β-galactosidase (β-gal) and luciferase activities were measured. Luciferase activity, normalized to β-galactosidase activity for transfection efficiency, is plotted, with fold activation relative to pGL3-basic shown below. (c) AP promoter regions are not responsive to TPA in 293 cells. Cells were transfected as above and divided to two sets: treated with TPA (20 ng/ml; filled bars) or untreated (open bars). Cells were harvested and assayed as above. Luciferase activity is plotted (RLU, relative luciferase units), with fold activation calculated relative to untreated cells transfected with the same reporter plasmid. (d) AP promoter regions are mildly responsive to TPA in BCBL-1 cells. BCBL-1 were transfected by Superfect with 4 µg reporter plasmid and 1 µg β-galactosidase-expressing plasmid and treated as in (c). Cells were harvested and assayed with luciferase activity plotted and fold activation calculated relative to untreated cells transfected with the same reporter plasmid.
Results

Fine structure of the assembly protein (AP) mRNA

We chose a classic late gene, AP, for our initial study of late gene regulation in KSHV. Visual inspection of the region upstream of the AP ORF revealed a likely TATA box at nt 31747, 60 nt upstream of the first potential start codon (Fig. 1a). We used the KSHV-infected B cell line BCBL-1 as a source of AP mRNA. BCBL-1 is latently infected with KSHV; treatment of these cells with phorbol esters induces lytic reactivation, viral DNA replication and subsequent late gene expression. A cDNA library derived from poly(dT)-primed, induced BCBL-1 mRNA was screened with a 774 bp Clal–SacI fragment as probe, corresponding to a region within the AP ORF (genomic positions 30840–31614). Four positive clones were isolated, representing three independent clones, as determined by sequence analysis. All three cDNA clones shared the same poly(A) addition signal, AAUAAA, at nt 30761. Although two of three cloned cDNAs started 12 nt downstream of the first potential initiating ATG for AP ORF (Fig. 1b), this site was confirmed independently by primer extension. We used a labelled oligonucleotide (PE1) located 12 nt downstream of a possible initiation ATG in the AP promoter region as probe, corresponding to a region within the AP ORF (genomic positions 30840–31614). Four positive clones were isolated, representing three independent clones, as determined by sequence analysis. All three cDNA clones shared the same poly(A) addition signal, AAUAAA, at nt 30761. Although two of three cloned cDNAs started 12 nt upstream of the first AUG, the presence of a candidate TATA box suggested that the transcriptional start site might lie further upstream. To identify the start site of the AP transcript, we used RNase protection analysis (RPA) and primer extension analysis. For RPA, a 266 nt riboprobes was hybridized to BCBL-1 RNA and subjected to T2 ribonuclease digestion of unannealed, single-stranded regions. Two protected products were seen, of 166 and 171 bp, corresponding to genomic positions 31705 and 31710 (Fig. 1c).

The start site was confirmed independently by primer extension. We used a labelled oligonucleotide (PE1) located 12 nt downstream of the first potential initiating ATG for AP (Fig. 1a) and annealed it to induced and uninduced BCBL-1 RNA. Extension from the primer produced two products, of 66 and 71 bp, confirming our RPA results (Fig. 1c). Significantly, the major start site occurs 28 nt downstream of a possible TATA box.

KSHV late promoters taken out of the virus context are not dependent upon virus replication

In order to study the transcriptional regulation of KSHV late genes, we set out to identify regions of the AP promoter that could reproduce late gene regulation in a heterologous context. A 700 bp SacI–EcoRV fragment and a 1.3 kb HindIII–EcoRV fragment were cloned into a luciferase reporter plasmid in both orientations (Fig. 2a) and tested for their ability to drive luciferase expression. When tested in 293 cells, both pPr/AP and pAP were able to direct the expression of luciferase up to 292- and 89.9-fold over the promoterless, parent reporter vector, pGL3-basic (Fig. 2b). This activity was dependent upon orientation, as the same fragments cloned in the reverse direction, pPr/AP rev and pAP rev, were much less active (1.8- and 15.4-fold) in the same assay.

In order to test these plasmids for their ability to be regulated as late genes, we planned to introduce them into BCBL-1 cells and then induce the lytic cycle with TPA and examine reporter (luciferase) expression. As a control, we first tested whether these promoters could be upregulated directly by TPA in the absence of virus replication. 293 cells, human embryonic kidney cells lacking the KSHV genome but semi-permissive for virus replication (Foreman et al., 1997; Renne et al., 1998), were transfected with the two plasmids in the absence or presence of TPA. As shown in Fig. 2(c), expression of either plasmid was induced substantially by TPA, as judged by luciferase production (induction ratio ≤ 2). In contrast, when the same constructs were transfected into BCBL-1 cells, a context wherein viral activators and other cofactors necessary for lytic reactivation and replication are present, these reporters were modestly responsive to TPA (Fig. 2d). Given the precedents from EBV (Serio et al., 1997, 1998), the relatively low level of TPA-induced activity in BCBL-1 cells was surprising. It concerned us that the initiating ATG from the AP ORF present in both pAP and pPr/AP might be causing initiation of translation upstream and out-of-frame of the luciferase ORF. To test this possibility, we used PCR mutagenesis to change the ATG to TTG in pAP, creating pAP TTG. When tested in BCBL-1 cells, the ablation of the upstream ATG resulted in a 12-fold induction of luciferase expression by TPA (Fig. 2e). All subsequent experiments were carried out with pAP TTG, as this construct provided us with greater dynamic range to detect inhibition of activation of this late promoter.

To determine whether this 12-fold TPA induction reflected faithful reproduction of late gene regulation, we transfected pAP TTG into BCBL-1 cells under four different conditions: chemical inducer alone (ionomycin or TPA), viral polymerase inhibitor ganciclovir (GCV) alone, inducer and inhibitor combined (ionomycin/GCV or TPA/GCV) or no treatment. Luciferase activity of untreated cells was set to 1. Fig. 3(b) shows that pAP TTG activation was not inhibited by GCV. Moreover, activation of this reporter construct was not entirely dependent upon viral cofactors, as pAP TTG was also activated modestly by TPA and ionomycin in BJAB cells, an uninfected B cell line (Fig. 3c), and behaved identically with regard to GCV in this background.

In order to confirm this result, we also tested the promoter regions of two other classic late genes: glycoprotein B (gB) and
Fig. 3. Late gene promoter constructs in BJAB and BCBL-1 cells. (a) Structure of reporter plasmids containing gB and MCP promoter regions. (b) pAP TTG is responsive to both TPA and ionomycin but insensitive to GCV. BCBL-1 cells were transfected by Superfect with 4 µg reporter plasmid pAP TTG and 1 µg β-galactosidase-expressing plasmid and then treated with the indicated chemical inducers, TPA (20 ng/ml) or ionomycin (Iono; 0–5 µM), and GCV (50 µM) as indicated. Fold activation is plotted, with all values calculated relative to untreated cells. (c) pAP TTG is responsive to both TPA and ionomycin in BJAB cells. Cells were transfected by electroporation with 8 µg reporter plasmid pAP TTG and 2 µg β-galactosidase-expressing plasmid. Cells were treated and analysed as in (b). (d) gB and MCP promoter regions are unresponsive to GCV in BCBL-1. BCBL-1 cells were transfected by Superfect with 5 µg reporter plasmid pGL3 gB or pGL3 MCP as shown and 1 µg β-galactosidase-expressing plasmid and then treated with the indicated chemical inducers, TPA (20 ng/ml) or ionomycin (0–5 µM), and viral inhibitors, GCV (50 µM) or PFA (0–5 mM), as indicated. Fold activation is plotted, with all values calculated relative to untreated cells. (e) gB and MCP promoter regions are also inducible in BJAB cells. BJAB cells were transfected by Superfect and treated and analysed as in (d).
major capsid protein (MCP) (Fig. 3a). Fig. 3(d) shows that these reporters were likewise insensitive to the viral polymerase inhibitors GCV and phosphonoformic acid (PFA) in BCBL-1 cells. pPr/AP, which contains a larger region upstream of AP than pAP TTG, was similarly unaffected by both GCV and cidofovir (data not shown). The gB and MCP promoters could be activated by both TPA and ionomycin in BJAB cells, again confirming that the activation of gene expression produced by these inducers in BCBL-1 cells was not dependent upon products of the KSHV genome. These results suggest that late gene regulation in KSHV cannot be faithfully reproduced when late promoters are taken out of the virus context.

Minimal promoter constructs are also insufficient to direct gene expression with late gene kinetics

Serio et al. (1998) showed that, for EBV late gene regulation, the minimal core of a late promoter was able to confer late gene kinetics on a reporter construct. Their observation that upstream enhancers could override the temporal control provided by a core EBV late promoter led us to consider whether the KSHV promoter regions we tested may have been too large, encompassing enhancer regions that would mask the ability of a KSHV core late promoter to confer regulated late gene expression. Moreover, many of the EBV late promoters have an unusual TATA element (TATTAAA) (Serio et al., 1998). Inspection of the promoter regions of various KSHV late promoters showed that a subset of the late promoters, including AP, also had unusual TATA elements (TATTTAAA) reminiscent of the EBV late promoter TATA motif identified by the Miller group (Serio et al., 1998). To test this hypothesis, we created a deletion mutant of pAP TTG, truncating the upstream region to —30 from the start site. Fig. 4 shows that, unlike EBV, this minimal promoter region was unresponsive to viral polymerase inhibitors in BCBL-1 cells following lytic induction.

Discussion

These results show that late gene expression in KSHV cannot be faithfully reproduced when viral late promoters are taken out of their natural context. In transient reporter gene assays, cloned viral late promoters are unperturbed by viral polymerase inhibitors, despite authentic virus replication in trans. Thus, these viral promoter regions, ranging in size from 1.4 to 2 kb, may lack the complete repertoire of cis-acting signals required for late gene regulation. Moreover, unlike results in EBV, these promoter regions were modestly responsive to TPA and ionomycin even in the KSHV-negative B cell line BJAB, suggesting that activation of these regions does not require direct interaction with a virus-encoded product. In contrast, these regions were not responsive to TPA when transfected into 293 cells, implying that B cell-specific factors may play a role in the activation of KSHV late genes. Such cell-specific factors could contribute to the tissue specificity of lymphotropic viruses like KSHV. It would be interesting to see whether endothelial cell lines such as SLK could also support TPA or ionomycin responsiveness, as B lymphocytes and endothelial cells are the two major cell types infected by KSHV.

The presence of a candidate variant TATA motif, restricted to a subset of late promoters, suggested a possible explanation for our inability to reproduce late gene regulation; that our promoter regions incorporated upstream elements that do not normally play a role in the regulation of late genes but, in our assay, when the region is taken out of its native context, would override the genuine regulation that is directed by minimal late gene promoters during the natural virus lytic cycle. Our results, however, show that a core late gene promoter, while responsive to TPA, is not responsive to viral polymerase inhibitors, confirming our experience that late gene expression in KSHV differs from that in EBV.

Our data suggest that late gene expression in KSHV may follow more closely the HSV model of late gene regulation—namely a dependence upon virus replication in cis to facilitate control of late gene expression. Our results are reminiscent of early HSV experiments, which showed that HSV late promoters, when transferred to the host genome, behave as early promoters.

One possible model for the requirement of DNA synthesis in cis is that replication alters an inhibitory state such as methylation in promoter regions, restricted access of promoters packaged in nucleosomes or repression of transcription initiation by negative-regulatory proteins bound to the promoter. Replication of the viral genome would result in
promoter regions free of inhibitory elements due either to the
generation of DNA that has not been post-transcriptionally
modified (i.e. methylated) or to the displacement of inhibitors
(histones or negative-regulatory proteins) by the virus rep-
lication machinery. For any of these scenarios, the act of
replication is central to the ability to express late genes. If this
is so, further study of late gene regulation will be dependent
upon the identification of the KSHV lytic origin.

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