Functional co-operation between the Kaposi’s sarcoma-associated herpesvirus ORF57 and ORF50 regulatory proteins

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Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV; also known as human herpesvirus 8, H8) and ORF50 (also known as RTA) act post-transcriptionally and transcriptionally to regulate viral lytic gene expression and synergistically activate certain early and late KSHV promoters. When ORF57 and ORF50 were co-expressed, they co-operatively stimulated expression from the promoter of the immediate-early ORF50 gene itself. Co-immunoprecipitations with extracts of KSHV-infected cells showed that ORF57 and ORF50 proteins were present in the same complex. Using the pull-down assay with extracts of KSHV-infected cells, ORF50 protein was shown to interact with a glutathione S-transferase–ORF57 fusion protein. A chromatin immunoprecipitation assay showed that ORF50 promoter sequences were preferentially associated with immunoprecipitated chromatin using both anti-ORF50 and anti-ORF57 antibodies consistent with both an in vivo physical association between ORF57 and ORF50 and a potential role for ORF57 at the transcriptional level. This is the first demonstration of an interaction between these two lytic regulatory proteins in a gammaherpesvirus. Expression of ORF50 protein is sufficient to induce lytic replication in latently infected cells and may determine viral host range, spread and KS pathogenesis in vivo. A new insight into the co-ordinated activities of these two key regulatory proteins is provided in which upregulation of the ORF50 promoter with augmentation of ORF50 activity by ORF57 protein, and vice versa, would facilitate the cascade of lytic viral gene expression, thereby breaking latency. A functional and physical interaction between these two gammaherpesvirus regulatory protein counterparts could be a general feature of the herpesviruses.

INTRODUCTION

Kaposi’s sarcoma (KS)-associated herpesvirus (KSHV; also known as human herpesvirus 8) is classified as a gamma-herpesvirus and in the same subfamily as Epstein–Barr virus (EBV) and herpesvirus saimiri (HVS), is the most recently identified human herpesvirus (Chang et al., 1994). KSHV causes KS and perhaps a rare form of primary effusion lymphoma (PEL), especially in individuals infected with human immunodeficiency virus (reviewed by Schulz, 1998; Boshoff & Chang, 2001; Moore & Chang, 2001). Overwhelming molecular epidemiological evidence supports the association of KSHV with KS (reviewed by Sarid et al., 1999; Schulz, 1999; Boshoff & Weiss, 2001; Dourmishev et al., 2003). KSHV can establish latent and lytic replication cycles, and examination of KS biopsy material shows virus localized to the tumorous endothelial cells (Boshoff et al., 1995), the majority of which are latently infected (Staskus et al., 1997).

Currently there is no efficient cell culture system for KSHV; the only viable experimental system uses naturally infected B cell lines, such as BCBL-1 (Renne et al., 1996), generated by culture of PELs that contain the genome of KSHV. The majority of PEL cells are latently infected with KSHV, with a low level of spontaneous virus reactivation. Addition of the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) to BCBL-1 cells efficiently induces the lytic cycle, producing virions (Renne et al., 1996). ORF50 (also known as RTA), a replication and transcription activator, and ORF57 (also known as MTA), immediate-early proteins, the earliest KSHV regulatory genes to be induced and expressed (Lukac et al., 1999; Paulose-Murphy et al., 2001), are both essential for lytic virus replication (Sun et al., 1999; Zhu et al., 1999).

ORF57 protein exhibits nucleocytoplasmic shuttling (Bello et al., 1999) and, like its EBV MTA (BMLF-1) and HVS...
ORF57 homologues (Lieberman et al., 1986; Nicholas et al., 1988; Kenney et al., 1989; Whitehouse et al., 1998), has a post-transcriptional mode of action (Gupta et al., 2000; Kirshner et al., 2000). An ORF57 counterpart is present in every herpesvirus sequenced, reflecting the importance of this signature viral protein. ORF50 protein is analogous to gammaherpesvirus transactivators HVS ORF50 and EBV RTA (BRLF-1) (Hardwick et al., 1988; Whitehouse et al., 1997; Lukac et al., 1999). Expression of ORF50 alone is necessary and sufficient for the switch from latent to lytic KSHV replication (Lukac et al., 1998; Sun et al., 1998; Gradoville et al., 2000) in cultured cells (Liang & Ganem, 2003) and may determine viral host range and spread in vivo (Bechtel et al., 2003). ORF50 protein exhibits sequence-specific DNA binding (Lukac et al., 2001; Song et al., 2001) and positively regulates its own promoter (Seaman et al., 1999; Deng et al., 2000) via an octamer element and Oct-1 protein (Sakakibara et al., 2001). Overexpression of ORF57 and ORF50 proteins together, driven by a human cytomegalovirus (HCMV) promoter, synergistically enhanced expression from several viral lytic gene promoters; however, at that time no physical association was demonstrated between the two proteins (Kirshner et al., 2000).

We have shown that simultaneous expression of ORF57 and ORF50 proteins stimulates reporter gene expression from the immediate-early ORF50 gene promoter itself by a further 13-fold above the level obtained with ORF50 protein alone. We have also shown that ORF57 and ORF50 proteins are present together in the same complex in extracts of KSHV-infected BCBL-1 cells, and a physical interaction was also demonstrated using a pull-down assay with a glutathione S-transferase (GST)–ORF57 fusion protein. A chromatin immunoprecipitation (ChIP) assay showed that ORF50 promoter sequences were preferentially associated with immunoprecipitated chromatin using both anti-ORF50 and anti-ORF57 antibodies (Abs). Thus, the coordinated activities of these two KSHV regulatory proteins will promote the cascade of lytic viral gene expression, overcoming the inefficiency of spontaneous lytic virus reactivation.

**METHODS**

**Plasmids.** Plasmid pGST-ORF57, expressing an N-terminal GST fused with the full-length (FL) ORF57 protein (aa 1–455) driven by a tac promoter, was constructed by cloning ORF57 cDNA into the EcoRI sites of pGEX-5X-3 vector (Pharmacia). ORF57 sequences were excised from pKS4 (a kind gift of L. Bello), which contained
ORF57 full-length cDNA cloned into the EcoRI site of pEGFP-C1 (Clontech). Plasmid pGBK7-ORF57 (FL) containing full-length ORF57 (aa 1–455), driven by a T7 promoter was used for in vitro translation of ORF57 protein. ORF57 cDNA sequences were excised as XhoI and PstI fragments from pKS4 and cloned into the SalI and PstI sites of pGBK7 (Clontech), resulting in loss of both XhoI and SalI sites from pGBK7-ORF57 (FL). Plasmids pcDNA4-gORF57 (FL) and pcDNA4-CORF57 (FL) contained full-length genomic and cDNA sequences, respectively, driven by an HCMV promoter encoding ORF57 protein (aa 1–455). ORF57 genomic and cDNA sequences were PCR amplified with primers containing BamHI and XhoI sites (shown in bold) (PM1 F, 5’-GGGCGGCTCCATT-GTTACAAGGCAATGGACATG; PM2 R, 5’-GGGCCGTGCAAG- TTAGAAGTGGATAAAGAAGAA) using pKS3 (Bello et al., 1999) and pKS4 as templates, respectively, and cloned into pcDNA4 / HisMax B (Invitrogen). Deletion mutants of full-length ORF57 (see Fig. 1b) were generated by PCR using primers containing BamHI and XhoI sites (ORF57N1 F, 5’-TCACAGGATGCTTGAAGTCTGAC- GTCC; ORF57N181 F, 5’-CTCGCTCGGATGATGAGTACGGTGAG; ORF57N329 F, 5’-CCAGATTTGGATGATGAGTACGGTGAG; ORF57N387 F, 5’-ACTATCGGATGATGAGTACGGTGAG- GC; ORF57N215 R, 5’-GTCGCTCGGATGATGAGTACGGTGAG; ORF57N328 R, 5’-AAGCACTCGGATGATGAGTACGGTGAG; ORF57N455 R, 5’-GGCCTTTGCGGATGATGAGTACGGTGAG) with pKS4 as the template, and amplified fragments were cloned into BamHI / XhoI sites of pcDNA4 / HisMax C. The encoded ORF57 aa 17–455, 1–215, 181–328, 329–455 and 387–455. The reporter plasmid containing the ORF50 promoter is referred to as pORF50/500-luc since it contains 500 bp of 5’-untranslated region (UTR) sequence directly upstream from the ORF50 translation initiation codon (Lukac et al., 1998; Sun et al., 1998; Seaman et al., 1998) of KSHV (BCBL-1), driving a firefly luciferase reporter gene in the pGL3-Basic vector (Promega). ORF50 promoter sequences were amplified by PCR with primers containing HindIII / XhoI sites (ORF50-500 F, 5’-GGGCGGCTCGGATGATGAGTACGGTGAG; ORF50-500 R, 5’-GGGCCGTGCAAGTGGATAAAGAAGAA) to create pORF50/500-luc. The identities of all the recombinant constructs were confirmed by DNA sequencing. Plasmid pKS3 (GFP-gORF57) contained ORF57 genomic DNA cloned into pEGFP-C1 (Clontech) and expressed an N-terminal enhanced green fluorescent protein (GFP)–ORF57 fusion (Bello et al., 1999). Plasmid pcDNA3-gORF57, encoding FL ORF57 genomic DNA driven by the HCMV promoter, kindly provided by D. Ganes, was as described previously (Lukac et al., 1998, 1999). Plasmid pSEW-R21 expressing a GST fusion with ORF50 FL (aa 1–691) has been described previously (Wang et al., 2001). Synthetic peptides representing the KSHV ORF57 segments between aa 40 and 54, 182 and 195 and 411 and 445 were used to raise anti-ORF57 Abs in rabbits (termed 718, GH and 721, respectively). The anti-ORF50 Ab raised against a synthetic peptide corresponding to KSHV ORF50 aa 527–539 has been described previously (Wang et al., 2003a).

**Chemical induction of the KSHV lytic cycle and preparation of cell extracts.** BCBL-1 cells (0.2 × 10^6 cells ml^-1) were treated with TPA (20 ng ml^-1) (Renne et al., 1996) for 72 h or left untreated. Cell extracts prepared as described by Wadd et al. (1999) in 800 μl lysis buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0-1% NP-40, 0.5 mM PMSF) containing protease inhibitor cocktail (Roche) were passed five times through a 26-gauge needle. RNase treatment was with 10 U (ONE; Promega) at 37°C for 20 min.

**Recombinant protein expression, in vitro GST pull-down assays and Western immunoblotting.** GST–ORF57 (FL) fusion protein was prepared as described previously (Wang et al., 2003a). GST–ORF57 fusion protein was prepared as described for GST–HnRNP K (Wadd et al., 1999) with modifications: E. coli BL21 cells (500 ml) were treated with 1-0 mM IPTG for 16 h at 28°C and resuspended in 5 ml modified NETN lysis buffer (20 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0-1% NP-40, 10% w/v glycerol, 5 mM β-mercaptoethanol) with 100 μg lysozyme ml^-1 and sonicated on ice. Triton X-100 was added to 2% (v/v), extracts were kept at 4°C for 30 min and the GST–ORF57 fusion protein was purified on glutathione–Sepharose 4B beads. GST pull-down assays performed as described previously (Wadd et al., 1999; Koffa et al., 2001) used untreated or TPA-treated BCBL-1 extracts (200 μg protein) or 10 μl each of [35S]methionine-labelled ORF57 (FL), ORF57 deletion mutants and luciferase (Promega) proteins, synthesized in vitro using the TNT T7 Quick Coupled transcription/translation system (Promega) according to the manufacturer’s instructions. Bound proteins were resolved by SDS-PAGE and visualized either by Western blotting (Wadd et al., 1999) using anti-ORF57 (GH) Ab diluted 1:2500, with the ECL detection system (Amersham Pharmacia Biotech) or by autoradiography.

**In vitro co-immunoprecipitation assays.** For immunoprecipitations with [35S]methionine-labelled ORF57 and luciferase proteins synthesized in vitro, 10 μl [35S]methionine-labelled in vitro-translated protein was incubated with 6 μl rabbit anti-ORF57 (GH) Ab in 200 μl in vitro immunoprecipitation buffer (50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM EGTA, 0-1% NP-40, 1% BSA, 0-5 mM PMSF) and protease inhibitor cocktail (Roche) for 1 h at 4°C. Then, 75 μl of a 50% slurry in immunoprecipitation buffer of protein A–protein G (50:50 ratio) Sepharose beads (Amersham Pharmacia) was added to the mixture and incubated for 1 h at 4°C. The beads were washed four times with cold immunoprecipitation buffer at 15 min intervals, resuspended and boiled in 2× SDS gel loading buffer, and bound proteins were resolved by SDS-PAGE and visualized by autoradiography.

**In vivo co-immunoprecipitation assays.** BCBL-1 cell extracts (200 μg protein) were pre-cleared with 5 μl rabbit pre-immune serum and 100 μl of a 50% slurry of protein A–protein G (50:50) Sepharose beads for 1 h at 4°C. After pre-clearing, 3 μl each of 718, GH and 721 ORF57 Abs, 5 μl anti-ORF50 Ab or pre-immune rabbit serum was added to pre-cleared cell extracts in 300 μl immunoprecipitation buffer (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5% glycerol, 1 mM EDTA, 2 mM EGTA, 0-1% NP-40, 1% BSA, 0-5 mM PMSF) with protease inhibitor cocktail (Roche), and immunoprecipitations were performed as described previously (Koffa et al., 2001). Immunoprecipitated proteins were resolved by SDS-PAGE and visualized by Western blotting (Wadd et al., 1999) using anti-ORF57 (GH) Ab diluted 1:3000, with the ECL detection system (Amersham Pharmacia Biotech).

**ORF50 promoter-driven luciferase assay.** Human 293 cells (6 × 10^4), a cell line useful for KSHV propagation from KS biopsy specimens (Foreman et al., 1997) and infectious virus production, following induction by overexpression of ORF50 from KSHV-infected cells (Bectel et al., 2003), were transfected using Polyfect (Qiagen) following the manufacturer’s protocol. Transfections used 230 ng pORF50/500-luc promoter DNA, 10 ng pRL–SV40 DNA (expressing Renilla luciferase), specified amounts of GFP–gORF57 and pcDNA4-gORF57 (or pcDNA-ORF57 deletion mutants) DNAs...
with or without pcDNA3-3-gORF50 DNA. The final amount of DNA in each transfection was kept constant with empty pcDNA3/pcDNA4 or pEGFP-C1 vector DNAs. Control experiments were performed with the heterologous promoter containing the interferon stimulatory response element driving a luciferase gene (pISRE-luc, 250 ng DNA), and recombinant IFN-α was added at 200 U ml⁻¹ at 5 h post-transfection. Transfected cells were harvested 48 h after transfection and frozen at −70 °C for 2 h. Luciferase activity of transfected cell extracts was measured using the Dual Luciferase Reporter assay kit (Promega). All firefly luciferase data were normalized with respect to Renilla luciferase activity expressed from a separate, constitutively active plasmid that was co-transfected with the firefly luciferase reporter plasmid. This normalization compensated for differences in transfection efficiencies between replicate cultures.

**Chromatin immunoprecipitation assay.** BCBL-1 cells were treated with TPA for 48 h to induce the KSHV lytic cycle before harvesting. Chromatin extracts, cross-linking, sonication, immunoprecipitation, agarose bead elution and protein removal were carried out with a ChIP assay kit (Upstate Biotechnology) based on the manufacturer’s protocol with slight modifications as described previously (Wang et al., 2004). DNA recovered from immunoprecipitates with anti-ORF50 or anti-ORF57 (GH) polyclonal Abs or a no-Ab control was used as a template for PCR amplifications. Primers LGH4354 (5'-GAACATCCGTGCTCCAAGCAGTGTCCTCACTTGACGCTGTCGTTCA-3') and LGH4355 (5'-GGACGTAAGCTTACAGTATTCTGGAGGACGATGAC-3'), specific for a 261 bp region in the KSHV ORF50 promoter from −241 to +20, were used to detect the ORF50 promoter region. Primers LGH5297 (5'-CGTGTTCGAACGCGCTGTCGTTCACTTGACGCTGTCGTTCA-3') and LGH5298 (5'-CTGGGAGTACACAGTATTCTGGAGGACGATGAC-3'), specific for KSHV ORF64 coding region (aa 1329–1425), were used as a negative control to detect a non-promoter region. The PCR products were analyzed by electrophoresis on a 2% agarose gel. Quantification of the PCR products was conducted with a Multilmage light cabinet (Alpha-Innotech Corp.) and the accompanying FluorChem (version 1.02) software.

**RESULTS**

**Expression of ORF57 protein increases activity from the ORF50 promoter**

The contribution of ORF57 protein alone to the transcriptional activity of the sequences upstream of the KSHV ORF50 gene, containing the putative promoter, was assessed in a functional assay. This analysis allowed examination of ORF57 effects on the ORF50 promoter independent of any possible effects on the ORF50 RNA sequences. We had a family of putative ORF50 promoter constructs encompassing various lengths upstream from the ORF50 translation initiation codon (Lukac et al., 1998; Sun et al., 1998; Seaman et al., 1999) up to 3000 bp. These putative ORF50 promoter elements were cloned into a luciferase reporter vector (pGL3-Basic; Promega). One of these (pORF50/250-luc) containing a 250 bp upstream region has been used to assay the effects of inflammatory cytokines on the transcriptional activity of the ORF50 promoter (Milligan et al., 2004). In the present study, a construct incorporating 500 bp of the ORF50 upstream sequence driving the firefly luciferase gene (pORF50/500-luc) was used, which had promoter activity. This pORF50/500-luc construct activated luciferase expression 4-fold in 293 cells transfected with pORF50/500-luc DNA compared with control vector lacking KSHV sequences (pGL3-Basic) and was responsive to TPA or sodium butyrate, which are inducers of KSHV lytic replication. The ORF57 expression plasmid (GFP-gORF57) was co-transfected into 293 cells with the ORF50 promoter reporter (pORF50/500-luc). Normalized luciferase activity from the ORF50 promoter increased with increasing amounts of ORF57 (Fig. 1a). At approximately 600 ng GFP-gORF57 DNA, luciferase activity from the ORF50 promoter reporter reached a plateau of 7.8-fold activation (Fig. 1a). Similar levels of luciferase induction were obtained by co-transfecting the pORF50/500-luc reporter with pcDNA4-ORF57 (FL) DNA (data not shown).

To identify the regions of ORF57 protein involved in stimulation of luciferase activity from the ORF50 promoter, pcDNA4-ORF57 DNA expressing full-length ORF57 or various deletion mutant proteins (Fig. 1b) were co-transfected with the pORF50/500-luc reporter construct (Fig. 1c). Compared with the level obtained with full-length ORF57 protein, the ORF50 N-terminal (aa 1–215) and middle region (aa 181–328) mutants showed low levels of luciferase activity (Fig. 1c). By contrast, a luciferase value of 108% was obtained with a mutant expressing ORF57 aa 329–455 (Fig. 1c). The activation was reduced to 65% with ORF57 aa 387–455 (Fig. 1c). Thus, residues at the ORF57 protein C terminus are important for the activation of luciferase expression.

**Simultaneous expression of ORF57 and ORF50 proteins co-operatively stimulates ORF50-mediated promoter activity**

During lytic KHSV replication, ORF57 is not expressed in isolation; rather it is produced with other viral regulatory proteins and is always accompanied by the KSHV transactivator protein ORF50. We therefore examined the effect of co-expressing ORF57 and ORF50 proteins on the ORF50 promoter. Human 293 cells were transfected with the pORF50/500-luc reporter plasmid and vectors expressing the following proteins: ORF57 alone (GFP-gORF57; 600 ng) or ORF50 alone (pcDNA3-gORF50; 500 ng) or ORF50 and ORF57 together. The functionality of the 5'-UTR of pORF50/500-luc was verified in co-transfection studies when, in several independent experiments, ORF50 protein expressed alone (pcDNA3-gORF50; 500 ng) activated basal promoter activity of pORF50/500-luc by a maximum of 4-fold (Fig. 2a). These data were consistent with levels seen by others for a comparable ORF50 5'-UTR reporter plasmid in KSHV-uninfected cells (Seaman et al., 1999; Sakakibara et al., 2001; Wang et al., 2003b). Expression of ORF57 alone activated luciferase expression by a maximum of 8-fold (Fig. 2a; also shown in Fig. 1a). Simultaneously, when ORF50 DNA (pcDNA3-gORF50; 500 ng), which resulted in maximum activity from the pORF50/500-luc reporter vector, was co-transfected with increasing amounts of ORF57 DNA (GFP-gORF57; 0–900 ng), normalized luciferase activity rose in a dose-dependent manner (Fig. 2b). ORF50 promoter activity was upregulated further to a
maximum of 13-fold with the addition of 600 ng ORF57 DNA (Fig. 2b), above the 4-fold level obtained with ORF50 DNA alone (as shown in Fig. 2a). Similar results were obtained using a pORF50/3000-luc reporter plasmid containing 3000 bp of DNA upstream of the ORF50 translation initiation codon (data not shown). These data demonstrated functional co-operation between ORF57 and ORF50 proteins on gene expression from the ORF50 promoter.

Neither ORF57 nor ORF50 alone (Fig. 2c, lanes 2 and 3) or in combination (Fig. 2c, lane 4) stimulated luciferase expression from heterologous promoters such as the interferon-stimulated response element (ISRE) promoter (pISRE-luc) (Fig. 2c, lane 1). However, the promoter was functional in the luciferase assays, since it responded to the addition of recombinant IFN-α (Fig. 2c, lane 3). In addition, ORF57 and ORF50 proteins together did not stimulate luciferase expression from the SV40 promoter (in the pGL3-Control plasmid) or Renilla luciferase activity (from the pRL-SV40 plasmid) used in luciferase assays (data not shown).

Kinetics of ORF57 and ORF50 expression in TPA-treated BCBL-1 cells

Given the functional co-operation between the ORF57 and ORF50 proteins on expression from the pORF50/500-luc reporter plasmid, we tested for simultaneous expression of the ORF57 and ORF50 proteins following TPA treatment of BCBL-1 cells. Western blotting with anti-ORF57 (GH) Ab

**Fig. 2.** ORF50 expression activates expression from the ORF50 promoter, and ORF57 when co-expressed with ORF50 co-operatively augments ORF50-mediated expression from this promoter. (a) ORF50 alone activates expression from the ORF50 promoter in the pORF50/500-luc reporter vector. Human 293 cells were co-transfected with the pORF50/500-luc reporter plasmid and plasmids expressing either ORF57 (GFP-gORF57; 600 ng DNA) or ORF50 (pcDNA3-gORF50; 500 ng DNA). In each sample, the total amount of vector backbone DNA was kept constant by adding empty vector (without insert) DNA. Final luciferase values were based on normalized luciferase activity (see Methods), and fold activation of firefly luciferase activity was calculated relative to the firefly luciferase activity measured when cells were transfected with the pORF50/500-luc reporter plus empty vector DNA in parallel and assigned an activity of 1. (b) The effect of ORF57 on the basal transcription activity of ORF50 promoter in pORF50/500-luc reporter in the presence of a constant amount of ORF50. In the same experiment as in (a), human 293 cells were simultaneously co-transfected with the pORF50/500-luc reporter plasmid and pcDNA3-gORF50 (500 ng DNA). The GFP-gORF57 expression vector was also added in increasing amounts of 0–900 ng DNA. Decreasing amounts of empty vector (without insert) were also included in each sample so that the total amount of vector backbone remained the same. Fold activation of firefly luciferase activity was calculated relative to the firefly luciferase activity of cells transfected with the pORF50/500-luc reporter plus pcDNA3-gORF50 DNA in parallel and assigned an activity of 1. (c) The effect of ORF57 and ORF50 alone and together on the transcription activity of an IFN-α-responsive promoter in plasmid pISRE-luc. Human 293 cells were co-transfected with a mixture of plasmids including the pISRE-luc reporter with either GFP-gORF57 or pcDNA3-gORF50 or both. Recombinant IFN-α was added at 5 h post-transfection, as appropriate. Fold activation of normalized firefly luciferase activity was calculated relative to the normalized firefly luciferase activity measured when cells were transfected with the pISRE-luc reporter plus empty vector DNA in parallel and assigned an activity of 1. Error bars in (a)–(c) show the standard deviations of the data derived from three separate transfections with luciferase assays each performed in duplicate.
on BCBL-1 cell extracts revealed that an increase in the amount of ORF57 protein was detected between 2 and 4 h post-TPA treatment (Fig. 3a, compare lanes 3 and 4). Up to 17 h after TPA treatment, anti-ORF57 Ab stained one major band of 50–52 kDa, and, between 17 and 24 h after TPA treatment, a faster-migrating protein band (45–48 kDa) was detected (Fig. 3a, compare lanes 6 and 7), suggestive of ORF57 protein processing. The ORF57 Ab showed some non-specific staining of a faint protein band present above ORF57 in both untreated and TPA-treated BCBL-1 cell extracts (Fig. 3a, lanes 1–8). The amount of this protein band did not increase with TPA treatment.

Parallel experiments with anti-ORF50 Ab showed that an increase in the level of ORF50 protein was detected at 2 h post-TPA treatment (Fig. 3b, lane 3). The anti-ORF50 Ab stained two bands in treated cell extracts (Fig. 3b, lanes 2–7): an upper band corresponding in size to ORF50 protein (~110–120 kDa) and a fainter lower band (~100 kDa) also present in untreated cells (Fig. 3b, lanes 1 and 8). The molecular mass of the ORF50 protein expressed in mammalian cells from transfected pcDNA3-gORF50 DNA is approximately 110 kDa, similar in size to the native ORF50 protein (~110 kDa) present in BCBL-1 cells and about 36 kDa larger than the predicted size of 73–7 kDa, indicating protein modification (Lukac et al., 1999). However, full-length ORF50 protein transcribed and translated in vitro in rabbit reticulocyte lysate from ORF50 cDNA has an apparent molecular mass of approximately 90 kDa, indicative of differences in its post-translational modification (Lukac et al., 1999). In BCBL-1 cells, some ORF50 protein was found in untreated cells (Fig. 3b, lanes 1 and 8), presumably reflecting spontaneous virus reactivation in the cells (Renne et al., 1996). ORF50 expression occurs in approximately 1% of untreated BCBL-1 cells (Wang et al., 2003b).

To search for a possible ORF57–ORF50 interaction, anti-ORF50 Ab was used in immunoprecipitations with untreated and TPA-treated BCBL-1 cell extracts, and ORF50 protein was detected by Western blotting. ORF50 protein was readily immunoprecipitated from TPA-treated cell extracts (Fig. 4a, lane 2) and some was also detected in untreated cell extracts (Fig. 4a, lane 1). Control pre-immune rabbit serum did not bring down ORF50 (Fig. 4a, lanes 3 and 4). ORF50 protein was present in input TPA-treated cell extracts and a small quantity was also in untreated extracts (Fig. 4a, lanes 5 and 6) due to spontaneous viral reactivation, as discussed above. The presence of ORF57 in immunoprecipitates obtained with anti-ORF50 Ab could not be shown by Western blotting with anti-ORF57 (GH) Ab as the IgG heavy chain of the available anti-ORF50 and ORF57 rabbit Abs is of similar size to ORF57 protein and gave a strong masking signal on Western blots (data not shown). The rabbit heavy chain IgG that masks the ORF57 protein band can be seen in the Western blot obtained with anti-ORF50 Ab (Fig. 4a, lanes 1–4).

Next, reciprocal immunoprecipitations were performed with anti-ORF57 Ab in KSHV-infected BCBL-1 cell extracts and probed by Western blotting with anti-ORF50 Ab. A 110–120 kDa band that reacted specifically with anti-ORF50 Ab was present in the immunoprecipitates from TPA-treated cell extracts (Fig. 4b, lane 4) and not from untreated BCBL-1 cell extracts (Fig. 4b, lane 3). The 110–120 kDa band was also absent from immunoprecipitates of TPA-treated and untreated cell extracts obtained using control pre-immune serum (Fig. 4b, lanes 6 and 7). As expected ORF50 protein was also detected in input TPA-treated cell extracts and to a lesser extent in untreated cell extracts (Fig. 4b, lanes 2 and 1). As some ORF57 homologues, such as ICP27, bind RNA (Mears & Rice, 1996), RNase treatment of the KSHV-infected TPA-treated cell extracts was performed before immunoprecipitations with anti-ORF57 Ab. Addition of RNase did not affect the interaction (Fig. 4b, compare lanes 5 and 4). Thus, ORF57 and ORF50 proteins are present in the same complex and it is unlikely that the co-immunoprecipitation of ORF50 with ORF57 is due to an interaction of these proteins that depends on RNA. The presence of ORF57 protein immunoprecipitated by anti-ORF57 Ab (GH) could not be shown by Western blotting as again the rabbit heavy chain IgG masked the position of ORF57 protein (data not shown).

To confirm the specificity of the anti-ORF57 Ab, an immunoprecipitation assay was performed using 35S-labelled ORF57 protein synthesized in vitro. Autoradiography showed that 35S-labelled ORF57 protein (Fig. 4c, input, lane 3) was immunoprecipitated with anti-ORF57 (GH) Ab (Fig. 4c, lane 1) but not with control pre-immune rabbit serum.
indicated by an arrow. A control unrelated protein, 35S-labelled luciferase, which is of similar size to ORF57, was not immunoprecipitated with anti-ORF57 (GH) Ab (Fig. 4c, lane 2). A control unrelated protein, 35S-labelled luciferase, was not pulled down with GST–ORF57 fusion protein (Fig. 5d, lane 2), and GST alone showed no interaction with ORF50 protein (Fig. 5d, lane 2). The existence of multiple forms of ORF50 protein in BCBL-1 cells (Fig. 5b, lanes 5 and 6) has been reported (Wang et al., 2003a) and is due in part to extensive post-translational modification (Lukac et al., 1999). Full-length ORF57 protein interacted with ORF50 protein in GST pull-down assays. To map the interacting ORF57 domain(s), ORF57 deletion mutants (see Fig. 1b) synthesized by in vitro translation and labelled with [35S]methionine were tested for their abilities to interact with GST–ORF50 (FL, 1–691): ORF57 (FL) synthesized in vitro from pcDNA4-ORF57 gave two truncation products. Bound proteins were separated by SDS-PAGE and analysed by autoradiography. A region of ORF57 between aa 17 and 215 was involved in the binding to ORF50 protein (Fig. 5c). ORF57 (FL) (aa 1–455) and ORF57 deletion mutants containing aa 17–455 and 1–215 interacted with GST–ORF50 (Fig. 5d, lanes 5 and 6). However, ORF57 aa 181–328, aa 329–455 and aa 387–455 failed to interact with ORF50 protein (Fig. 5d, lanes 8, 3 and 4). A control unrelated protein, 35S-labelled luciferase, was not pulled down with GST–ORF50 (Fig. 5d, lane 2), and GST alone showed no interaction with 35S-labelled ORF57 (FL) protein (Fig. 5c, lane 1).

**Association of ORF57 with the ORF50 promoter by ChIP assay**

The apparent physical binding of ORF57 with ORF50 in vitro and by co-immunoprecipitation suggested that the transcriptional events mediated by ORF50 and the post-transcriptional events mediated by ORF57 might be more closely associated processes than previously appreciated. Other studies have demonstrated that ORF50 (and replication-associated protein, RAP) can be found selectively associated with the chromatin of several KSHV early and immediate-early promoters after (but not before) TPA-induction of the lytic cycle in KSHV latently infected PEL cell lines (Wang et al., 2003a, b; Wu et al., 2003). Therefore, we asked whether endogenous ORF57 might also be found in association with the KSHV ORF50 promoter in sonicated nuclear extracts from induced BCBL-1

(Fig. 4c, lane 2). A control unrelated protein, 35S-labelled luciferase, which is of similar size to ORF57, was not immunoprecipitated with anti-ORF57 (GH) Ab (Fig. 4c, lane 4).
The ORF50 upstream region has intrinsic promoter activity (Seaman et al., 1999; Deng et al., 2000; Sakakibara et al., 2001). However, its precise boundaries have not yet been defined. Consistent with previous reports, we observed stimulation of the ORF50 promoter by ORF50 alone (Seaman et al., 1999; Deng et al., 2000; Sakakibara et al., 2001). The ORF50 promoter region (−500 bp) used in the present study contains the octamer binding sequence (−227/−190 bp) for the octamer-binding protein 1, involved in autoregulation of ORF50 (Sakakibara et al., 2001). Several KSHV promoters, including that of ORF57, contain ORF50-specific DNA-binding sites and are activated by ORF50 (Lukac et al., 2001; Song et al., 2001; Chang et al., 2002). However, the mechanism of ORF50-mediated activation of its responsive elements in KSHV promoters is not yet fully understood. ORF50 can activate certain viral promoters by interacting with the cellular DNA-binding protein RBP-Jk and replacing its repressing activity with activation (Liang et al., 2002).

In the present study, the contribution of the ORF57 protein to the transcriptional activity from the ORF50 promoter in the absence of the downstream ORF50 RNA sequences was assessed independently of its possible effects on ORF50 promoter independently of cell-specific factors and other virus-specific factors. The ORF57 C-terminal region, comprising aa 329–455, stimulated expression from the ORF50 promoter as efficiently as full-length protein, whereas ORF57 aa 387–455 showed much reduced reporter gene activity. However, the N terminus of ORF57 and not the C terminus was shown to interact with ORF50, indicating that activation by the ORF57 C terminus may be via a cellular factor. Interestingly, KSHV ORF57, unlike its homologues in other herpesviruses, contains a putative leucine zipper motif located between aa 343 and 364 (our unpublished observations; Gupta et al., 2000) with a...
possible role in protein dimerization, and KSHV ORF57 is capable of interacting with itself via the C terminus (data not shown). Repressing and enhancing functions have both been shown to map to the C-terminal regions of HVS ORF57 and the herpes simplex virus 1 (HSV-1) ICP27 proteins (McMahan & Schaffer, 1990; Goodwin et al., 2000). These regions are required to modulate viral gene expression very early in HSV-1 infection (McMahan & Schaffer, 1990).

When ORF57 and ORF50 proteins were co-expressed, activity from the ORF50 promoter was further upregulated by 13-fold compared with the 4-fold level obtained with ORF50 alone. Thus, ORF57 augments the ORF50-inducible effect on its own promoter and these two proteins act cooperatively to promote expression from the ORF50 promoter. The synergistic effects of ORF57 and ORF50 protein co-expression, previously observed with viral early promoters, used ORF50 expressed under the control of the HCMV promoter (Kirshner et al., 2000), which is unaffected by ORF57 (Gupta et al., 2000; Kirshner et al., 2000). The synergy was attributed to a post-translational enhancement by ORF57 of ORF50 transcriptional activity (Kirshner et al., 2000).

We have shown by co-immunoprecipitation and pull-down assays that ORF57 and ORF50 proteins are present in the same complex and therefore interact physically. To ensure the biological relevance of this interaction, studies were performed with naturally infected BCBL-1 cells, in which these two proteins are expressed during the course of KSHV reactivation following TPA treatment. Kirshner et al. (2000) were unable to detect an interaction between the products of the ORF57 and ORF50 genes (Kirshner et al., 2000) but no experimental details were provided. A possible explanation could reflect the fact that our binding assays used recently generated Abs specific to viral ORF57 and ORF50 proteins, and that studies were performed in KSHV-infected TPA-treated BCBL-1 cells, where other viral proteins are also expressed. A ChIP assay using ORF50 promoter region primers revealed that ORF50 promoter sequences were indeed preferentially associated with immunoprecipitated chromatin using both anti-ORF50 and anti-ORF57 Abs. This result is fully consistent with both an in vivo physical association between ORF57 and ORF50 and a potential role for ORF57 at the transcriptional level, although a post-transcriptional action could also explain the presence of ORF57 in close association with active promoter-associated complexes. In the case of the ORF50 promoter, ORF50 itself may not bind directly to the promoter DNA sequences, but rather be present in a complex with enhanced levels of both the cellular C/EBPs and cJUN/cFOS proteins (Wang et al., 2003a, b, 2004), and ORF57 might also be a part of either these or other similar large complexes.

This is the first demonstration of an interaction between these two immediate-early regulatory proteins in the gammaherpesviruses. However, in alphaherpesviruses, the HSV-1 counterpart of ORF57, ICP27, complexes with the ICP4 protein, the HSV-1 counterpart of ORF50 (Panagiotidis et al., 1997). ICP27 affects intracellular localization (Zhu & Schaffer, 1995) and electrophoretic mobility of ICP4 (Rice & Knipe, 1988; Su & Knipe, 1989). ICP27 acts at transcriptional (Jean et al., 2001) and post-transcriptional levels, influencing pre-mRNA processing and promoting export of viral RNAs (Sandri-Goldin & Mendoza, 1992; Kofla et al., 2000).
Kaposi’s sarcoma-associated herpesvirus.

Epidemiology and pathogenesis of Kaposi’s sarcoma.

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