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Transcriptional regulation of the open reading frame 35 encoded by Kaposi's Sarcoma-associated herpesvirus

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Abstract

Kaposi's sarcoma-associated herpesvirus (KSHV) is a member of the *Gammaherpesvirinae* and is causally associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. The KSHV genome encodes over 85 genes; the function of some is entirely unknown. We have characterized the transcriptional regulation of a conserved and uncharacterized *Gammaherpesvirinae* open reading frame, *orf35*, which lies in a cluster of several overlapping genes, *orf34* to *orf38*. We identified the transcription start site and analyzed upstream sequences. We found that expression of the KSHV lytic replication and transcription activator (RTA) strongly increased the *orf35* promoter activity through a 46-nucleotide region which includes a conserved AP-1 binding site. Electrophoretic mobility shift assay demonstrated direct binding of cJUN and cFOS to the predicted AP-1 binding site. Finally, using a mutated promoter lacking the AP-1 site and dominant-negative cFOS, we established that the RTA-mediated *orf35* transactivation is AP-1-dependent.

Keywords: KSHV, Kaposi's sarcoma-associated herpesvirus; HHV-8, human herpesvirus 8; ORF35, open reading frame 35; RTA, replication and transcription activator; AP-1, activator protein 1

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV), also referred to as human herpesvirus-8 (HHV-8), is a gamma-2 herpesvirus which is implicated in the etiology of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL; also known as body cavity-based lymphoma (BCBL)), and a subset of multicentric Castleman's disease (Cohen et al., 2005; Schulz, 2006; Hengge et al., 2002; Dourmishev et al., 2003). KSHV is closely related to herpesvirus saimiri (HVS), an oncogenic virus that naturally infects the squirrel monkey, and to a greater degree to non-human primate gammaherpesviruses (Russo et al., 1996; Whitby et al., 2003). Among human viruses, KSHV displays the highest resemblance to the Epstein-Barr virus (EBV), a tumorigenic lymphotropic gamma-1 herpesvirus known to be associated with lymphomas and nasopharyngeal carcinoma. The KSHV genome is encoded on a linear double-stranded DNA, of approximately 165-kbp, which contains over 85 open reading frames (ORFs) (Russo et al., 1996) and an array of 12 microRNAs (Pfeffer et al., 2005; Cai et al., 2005; Samols et al., 2005; Pearce et al., 2005; Grundhoff et al., 2006). The genomic nomenclature of KSHV genes, which has been adapted for most gammaherpesviruses, is based on the homology to the HVS ORFs that are consecutively designated according to their location on the genome from left to right. Divergent KSHV genes are designated with the prefix K according to their position within the viral genome. Like all herpesviruses, KSHV encodes both enzymes involved in nucleic acid metabolism and DNA synthesis, and structural proteins encompassing the viral particle. A large number of KSHV-encoded proteins share sequence and functional similarities with host proteins, and were presumably captured from the host by molecular piracy. To date, the functions of several KSHV genes remain unknown, even among homologues of KSHV.

In common with all other herpesviruses, KSHV exhibits two divergent phases of infection: lytic (productive) and latent (non-productive), characterized by distinct gene expression programs

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(Sarid et al., 1998). The lytic viral program can be triggered experimentally by a number of physiologic, biological, or chemical stimuli, among which, the phorbol ester, 12-*O*-tetradecanoyl 13-acetate (TPA), is the most broad, efficient, and useful inducer (Moore et al., 1996; Renne et al., 1996; Miller et al., 1997; Davis et al., 2001). All lytic stimuli induce expression of the replication and transcription activator (RTA) encoded by *orf50* (Lukac et al., 1998; West and Wood, 2003; Deng et al., 2007). In turn, RTA activates a cascade of viral lytic program, resulting in the ordered expression of viral proteins, and ultimately leading to the release of progeny viruses and host cell death. Initiation of the lytic viral cascade by RTA is mediated through the transcriptional activation of selected viral promoters, including *orfK1*, *orf6* (single-stranded-DNA-binding protein), *orf9* (DNA polymerase), *orfK2* (viral interleukin-6 homolog), *orfK5* (modulator of immune regulation, MIR2), *orfK6* (viral MIP-1), polyadenylated nuclear (PAN) RNA (nut-1), *orf50* (RTA), *orfK8* (replication-associated protein, RAP), *orf57* (mRNA transport and accumulation protein, MTA), *orfK9* (viral IRF), *orf59* (DNA replication protein), *orf21* (thymidine kinase), *orfK12* (Kaposin), *orfK14* (viral OX-2), and *orf74* (viral G protein-coupled receptor) (Bowser et al., 2006; Chen et al., 2000; Deng et al., 2002; Haque et al., 2000, 2006; Jeong et al., 2001; Lukac et al., 1998; Song et al., 2001, 2003; Wang et al., 2003b). This wide range of promoter transactivation occurs through the direct binding of RTA to selective DNA motifs, termed RTA-responsive elements (RREs) (Liao et al., 2003; Bowser et al., 2006; Chang et al., 2002), or through the binding to other cellular transcription factors that tether RTA to the DNA, such as cAMP response element binding protein (CREB)-binding protein (CBP), Oct-1, CAAT/enhancer-binding protein alpha (C/EBP-α), RBP-Jκ (CSL), Sp1, MGC2663, and TATA-binding protein (TBP) (Bowser et al., 2006; Chang et al., 2005a; Gwack et al., 2001; Liang and Ganem, 2003; Liang et al., 2002; Sakakibara et al., 2001; Wang et al., 2001, 2003a, 2003b; Ye et al., 2005; Zhang et al., 1998). In addition, RTA may manipulate viral and cellular gene transcription through the modulation of key signaling proteins, such as cJUN and STAT3 (Yu et al., 2005; Gwack et al., 2002).

In an effort to increase our understanding of KSHV pathogenesis, we focused our attention on *orf35*, a previously uncharacterized open reading frame located in a lytic cluster of genes. Since *orf35* is included within a limited set of genes that are transiently expressed prior to the establishment of KSHV latency and early after infection of endothelial and fibroblastic cell cultures (Krishnan et al., 2004), we assume that it may mediate crucial functions during infection. Consistent with this hypothesis, a high abundance of *orf35* transcripts is detected early after infection with the rhesus monkey rhadinovirus (RRV) (Dittmer et al., 2005). An additional hint for the importance of ORF35 comes from a recent study, which employed signature-tagged transposon mutagenesis to generate murine gammaherpesvirus-68 (MHV-68) mutants, and demonstrated that ORF35 is required for in vitro infection (Song et al., 2005).

Understanding the mechanisms by which ORF35 expression is regulated may shed light on its role in the life cycle of KSHV; we therefore examined its transcriptional kinetics and control. In the present study, we identified the 5′ and 3′ ends of the major ORF35 transcript, and characterized its transcription kinetics in PEL cells upon virus reactivation by TPA. We also analyzed sequences upstream of the transcription start site for their ability to activate a luciferase reporter construct. We show that the KSHV immediate-early protein RTA transactivates the *orf35* promoter element and that this transactivation is AP-1-dependent.

Results

Sequence analysis of ORF35 encoded by KSHV

Like its homologs from other gammaherpesviruses, the KSHV *orf35* coding sequence partially overlaps its adjacent genes, namely *orf34* and *orf36*. It is the second-stream of a cluster of overlapping *orf34* to *orf38* lytic genes, all oriented in the same direction (Russo et al., 1996). The KSHV *orf35* encodes a protein of 150 amino acids with a calculated molecular mass of 16.8 kDa and a p*I* of 5.97. ORF35 protein is present in all complete genomes of the *Gammaherpesvirinae*, and shares high degree of sequence similarity at the amino acid level. Fig. 1 depicts the alignment of the amino acid sequences encoded by the ORF35 proteins of KSHV, RRV, HVS, AthV-3, BHV-4, mHV-68, and EBV, which was performed using the ClustalW program. Like other KSHV-encoded proteins, KSHV ORF35 is most similar to the ORF35 encoded by rhesus rhadinovirus 17577 (RRV17577), with which it shares 60% similarity and 35% identity. Lower similarity and identity scores were found between KSHV ORF35 and corresponding homologs encoded by other *Gammaherpesvirinae*. The relatively high sequence conservation of ORF35 in these related viruses suggests that it may exhibit functional conservation during the course of viral infection.

A recent PSI-BLAST search revealed similarity between *Gammaherpesvirinae* ORF35 and the ORF48 protein of the alphaherpesvirus, equine herpesvirus 4, and further PSI-BLAST iterations identified similar homology in all members of this subfamily (Mills et al., 2003). The ORF48 protein belongs to the UL14 family of viral proteins, which are present as a minor component of the virion tegument and possess multiple functions, including molecular chaperone activity (Cunningham et al., 2000; Yamauchi et al., 2001, 2003). Nevertheless, we found that unlike UL14, the ORF35 protein encoded by KSHV, lacks homology with the substrate-binding domain included in the heat-shock protein (HSP) 70 family of proteins.

No mammalian homologues of ORF35 proteins have been identified. The KSHV ORF35 protein sequence does not include a signal peptide to target the protein to a specific subcellular compartment, nor a nuclear localization signal. Comparison of the KSHV ORF35 protein sequence with the Eukaryotic Linear Motif (ELM) database identified several potential phosphorylation sites. Three putative sumoylation sites (ψKXE, where ψ is a hydrophobic amino acid) were found using a Sumoylation site prediction tool. Of note, several gammaherpesviruses contain at

A

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KSHV            -MDSTNSK-REFIKSALEANINRRAAVSLFDRFGGSSAVFEKQFQDAQHAVRAHGALKRE 58
RRV             -MASAAAK-KMLIKSELESEINKKLSISVFDRFGADSAVFNAQYKGTRESLRSYNSLKKK 58
HVS             -MNSNKK---EFLYSAFETEINKKASVSLFDRFGGKSCIFLHQLDHTKKSLIKHENLKRQ 56
AthV-3          -MNSNRK---KFLCSAFEAEINKKTSVSLFDRFGESNCLFLHQLDTTKKSLIKHENLKKQ 56
BHV-4           -MESRNKKIAELFKINLLSEVNKKTSVSLFDRFGEHSDIFLQQFEVTQKNLQDCNQLRQS 59
mHV68           -MDTKLLA-KKLIGSSLRADIEKRAAVSLTDRFGKSHSLTQFQYTKAKRAERTASSAREH 58
EBV             LMKMSSSK-RDLVAQQLRASVEKRAAVSARDRFGRDHALFETQFTSARGALESLRHARET 59
                 *         :.   : :.:::: ::*  ****    :   *   ::         :.

KSHV            AELGTLVRKAGQRFEALKRERSILRQPRDLPRVADIDALVDAVADLKEEVAVRLDALEE- 117
RRV             DDLATVVGTLETSLREKQSELGLLKG-FNRKKIEEFDAVADAVRDLKDELYGELEILGT- 116
HVS             KSIEGMLQAVDTSIQEKRKELSLLKN-FNRHKLTAAEDLQDKILELKEDIHFEIESLNN- 114
AthV-3          KSIEGMLQEVNLSIQEKKKELSLLKT-FDRHKLSDTEDLQDKISELTEDLQFEIEALNH- 114
BHV-4           TKVDNIISFVESTIRSQEKQLETLLK-FDKKKLERAEALTNRVSDLSEDIQAELSFLTSE 118
mHV68           CHRIENMVSTKRELSDCVSELTHLKEICQNFSVEDAERLIEETTVLKEELEDTVNTVSA- 117
EBV             FESKQLISTYQRVVTATKTQFPKINY----KQLERVEELREQELEARDELRQALEPFEE- 114
                      :      .     :   :         :   : : :      :::   :. .

KSHV            ---NGEETPTHSSSEIKDTIVRWRLDDLPPVCPETP--------- 150
RRV             ---LDNESVPVEEESPKDDIIRWKLERLPRVCPKSP--------- 149
HVS             GQPSSQEEENSSETSVPDTIMQWRIEALPRVPSAPF--------- 150
AthV-3          GQSSSQEEESSSENTVTGTIMRWRIEALPRVPSTTLQ-------- 151
BHV-4           GGDGTNISHGSEDDTTRDTIMHWRLGTIPDVPASPPSDP------ 157
mHV68           -ALQREESLSADSEQEESDITCWRLDGLPTVTARIG--------- 152
EBV             -----HGCEYGCGVEPDELLQQWRVECLPRTPSRDPGLFGEGDCH 154
                     .             :  *::  :* . .
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B

	RRV	HVS	AthV-3	BHV-4	MHV-68	EBV	
KSHV	60	58	61	52	44	44	% Amino acid similarity
	35	30	29	30	28	25	% Amino acid identity

Fig. 1. Alignment of the amino acid sequence of KSHV ORF35. (A) ClustalW alignment of the amino acid sequences of seven ORF35 homologs encoded by KSHV, RRV (Rhesus rhadinovirus), HVS (Herpesvirus saimiri), mHV-68 (Murine herpesvirus 68), BHV-4 (Bovine herpesvirus 4), AthV-3 (Ateline herpesvirus 3), and EBV (Epstein-Barr virus). Identical amino acids are indicated by stars, conserved substitutions are indicated by semicolons, and semi-conserved substitutions are indicated by single dots. Putative sumoylation sites are indicated by gray shading. (B) Percent amino acid similarity and identity of KSHV ORF35 to corresponding gammaherpesvirus ORF35 proteins, calculated using BLOSUM62 amino acid substitution matrix.

least one conserved sumoylation site in their ORF35 coding region, yet their functional significance remains to be elucidated (Fig. 1A).

Transcription of orf35 in PEL cells

To determine the kinetics of the synthesis of the KSHV-encoded *orf35* mRNA in PEL cells, we induced a lytic transcription program in the KSHV-infected BCP-1 cells with TPA, and employed RT-PCR to detect transcripts at 24, 48, and 72 h post induction. As shown in Fig. 2A, low expression of *orf35* transcript was observed in BCP-1 cells growing under standard growth conditions. Increased transcription of *orf35* was evident after 24 h of TPA treatment, peaking at 48 h and decreasing at 72 h. A minor inhibition of *orf*35 transcription was evident in cells treated with a combination of TPA and the viral DNA polymerase inhibitor PAA for 48 h, suggesting that the *orf35* transcript is not a late gene. Northern blot hybridization of total cellular RNA to detect the *orf35* transcript confirmed these results and allowed determination of the transcript size. Induction of a predominant 3.4 kb transcript was observed after TPA stimulation, peaking at 48 h after stimulation (Fig. 2B). A similar transcript size was previously described in lytic BC-3, JSC-1, and BCBL-1 cell lines (Haque et al., 2006; Cannon et al., 1999).

Identification of the intact orf35 cDNA

To further characterize and map the full-length *orf35* transcript, we screened a BC-1 cDNA library with a radiolabeled *orf35* PCR probe. The longest cDNA clone (ϕ5) of 3327 bp, included *orf35* to *orf38*, was assumed to represent an authentic full-length cDNA. Nucleotide sequence analysis of this cDNA clone gave no indication of splicing. A highly conserved hexanucleotide signal, AAUAAA, at positions 58,853 to 58,858 within the coding region of *orf38*, was identified; this sequence could potentially serve as a polyA signal for cleavage to generate the 3′ transcript end for polyadenylation. Using computer analysis of *orf35* and downstream sequences, we identified two additional potential polyA signals within *orf37* (positions 58,004 AAUAAA and 58,341 AUUAAA) and one within *orf38* (position 58,834 AUUAAA),

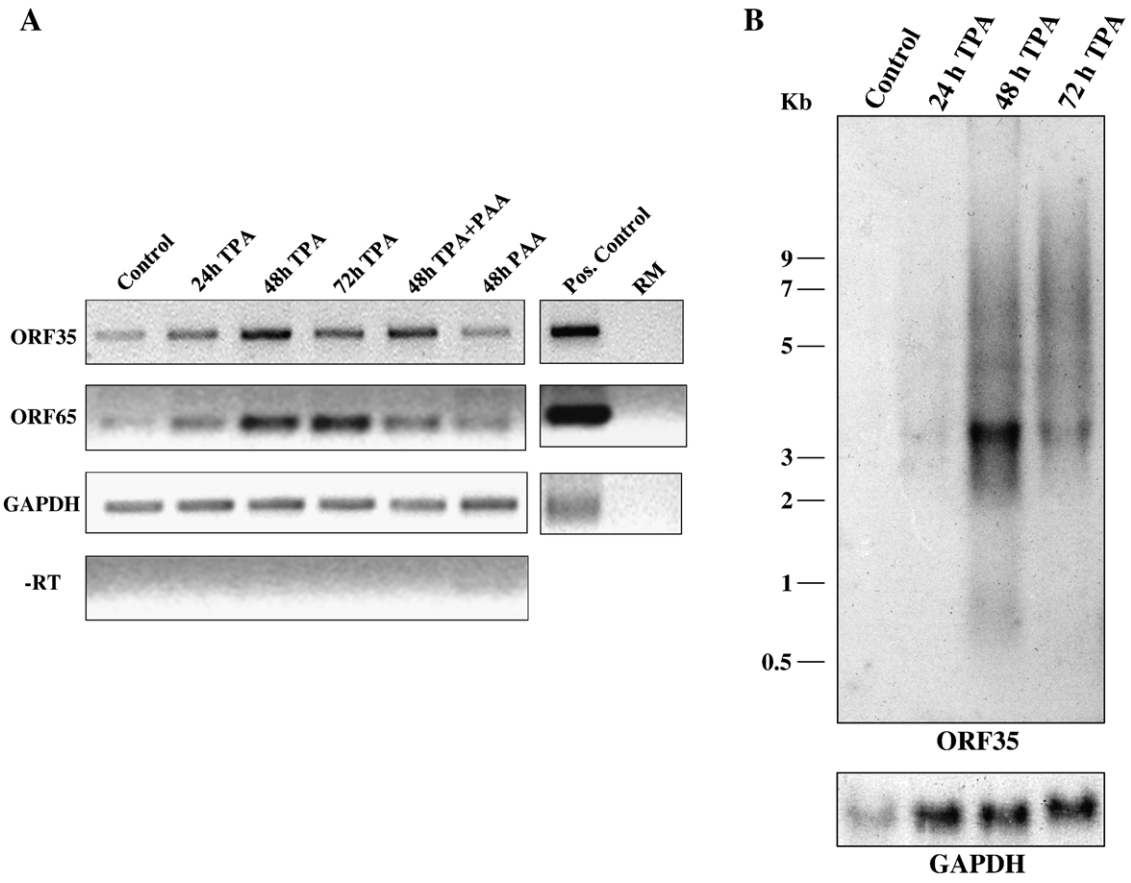

Fig. 2. Semi-quantitative RT-PCR and Northern blot hybridization demonstrating *orf35* transcription in PEL cells. (A) Total RNA was prepared from BCP-1 cells prior to and following induction with TPA for the indicated time periods, and from cells that were treated with TPA and PAA for 48 h. RNA was reverse transcribed using random primers and subjected to PCR using *orf35*, and GAPDH primers. PCR with primers that target the *orf65* late gene was used to control for the inhibitory activity of PAA. −RT reactions were used to control for DNA contamination. High molecular weight DNA from BCP-1 cells was used as a positive control for PCR, and reaction mixture (RM) with no template controlled for DNA contamination. (B) Northern blot of total cellular RNA from BCBL-1 cells prior to and following induction with TPA, probed with a ^{32}P-labeled DNA *orf35* probe. GAPDH was used as a control for RNA loading.

suggesting that more than one polyA signal may function in this genomic region. However, our findings together with previous reports (Haque et al., 2006) suggest that the major polyA signal used in PEL cells upon TPA stimulation is located at position 58853.

To accurately determine the transcription start site, we designed a primer (PE) that would anneal to a site located 51 bases 3′ to the *orf35* ATG and performed primer extension. Total RNA was isolated from non-induced and TPA-induced BC-1, BCBL-1 and BCP-1 cells 48 h after induction of lytic replication. PE synthesized four oligonucleotide products that were predominantly observed after TPA treatment (Fig. 3A); three extended several nucleotides upstream from the start site of the largest cDNA clone. Taken together, as depicted in Fig. 3B, our mapping of the intact KSHV *orf35* transcript revealed that it encompasses 3315–3367 nt and starts at positions 55,566–55,514, which are 73–125 nt upstream of *orf35* AUG and within the *orf34* coding region. The transcript is not spliced and contains the coding sequences of *orf35* to *orf38*. Similar findings have recently been reported by Haque et al. (2006); however, their transcript mapping indicates a single start site at position 55567. In addition, this group identified a second low abundance transcript that contains the *orf34* to *orf38* cluster of genes (Haque et al., 2006).

Expression of ORF35 protein

We cloned PCR products of *orf35* in-frame with an N-terminal HA or GFP tags into plasmids that allow gene expression in mammalian cells. In addition, we obtained rabbit antibodies to an ORF35 peptide (amino acids 3–17: STNSKRE-FIKSALEA). Using these reagents, we determined the apparent molecular weight of ORF35 on SDS-PAGE as ~17 kDa. In line, a 43-kDa protein was detected on immunoblot prepared from GFP-ORF35 transfected cell extracts (Fig. 4A). This protein size is compatible with the calculated molecular weight of ORF35, and was also detected by our ORF35 polyclonal antibody (Fig. 4A). We next examined the intracellular localization of the ORF35 protein by GFP fluorescence on cells transfected with GFP-ORF35 fusion protein expression plasmid. As shown in Fig. 4B, unlike GFP, GFP-ORF35 is predominantly cytoplasmic. Similar results were obtained on cells transfected with HA-ORF35 expression plasmid and anti-HA antibody (data not shown). Taken

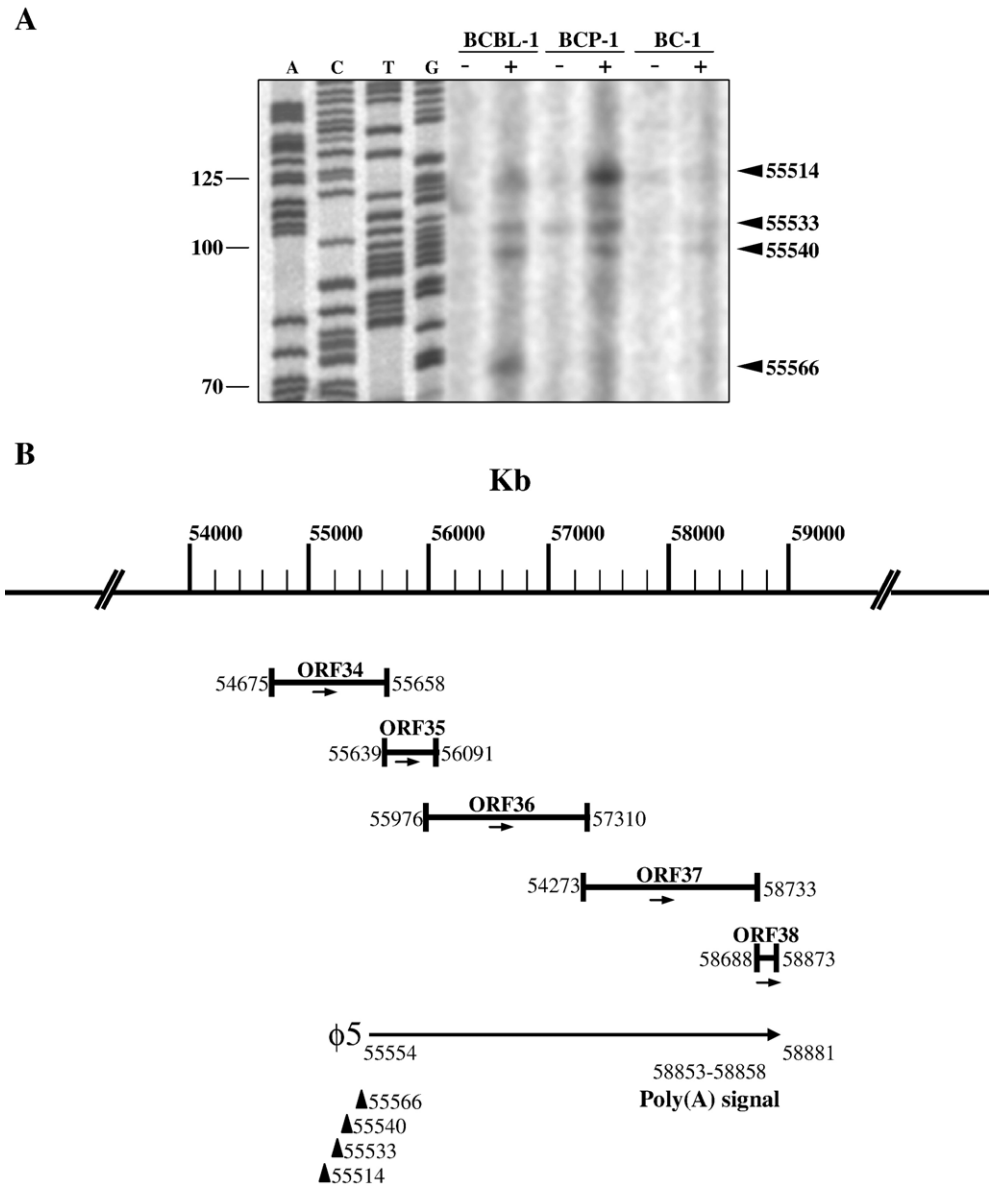

Fig. 3. Initiation site of the KSHV *orf35* transcript identified by primer extension and depiction of the transcript. (A) Total cellular RNA was prepared from BC-1, BCP-1 and BCBL-1 cells prior to (−) and 48 h after stimulation by TPA (+). Primer PE was end-labeled with [γ-^{32}P]ATP, hybridized to 10 μg of RNA at 60 °C, and extended at 42 °C. Reaction products were precipitated with ethanol and resolved on 6% polyacrylamide, 7 M urea gel in Tris–borate–EDTA. Sequencing ladder indicates the size of the primer extension product. The arrowheads in the right indicate the position of the cDNA products, and the numbers indicate their nucleotide position. (B) Map of genes in *orf35* region is shown with nucleotide positions as designated by Russo et al. (1996). The illustration is based on data obtained by screening of a cDNA library (ϕ5) and primer extension (vertical black arrows).

together, these findings show that the expression of ORF35 fits its predicted molecular weight with no apparent post-transcriptional modifications.

To determine if the cDNA phage clone (ϕ5), which was shown to contain the cluster of *orf35* to *orf38* genes, encodes ORF35 protein, we performed in vitro transcription/translation using a plasmid containing full-length HA-tagged ORF35 as the positive control. The results (Fig. 4C) showed two translation products of 19 and 17-kDa for pcDNA/HA-ORF35, suggesting that this plasmid can produce both HA-tagged ORF35 and ORF35 alone using the AUG translation initiation codons from the HA or from ORF35, respectively. A similar translation product of 17-kDa was evident from the phage clone, indicating its potential to produce ORF35. In addition, we observed a high abundance translation product of approximately 50-kDa. The expected molecular weights of ORF36, ORF37, and ORF38 proteins are 50.3-, 55.1-, and 6.8-kDa, respectively. Hence, the products of the coupled in vitro transcription and translation reaction suggest that the transcript encoded in phage clone ϕ5 can potentially serve as a template for the translation of ORF35 as well as proteins beyond ORF35.

Basal activity of orf35 promoter

The 533-nt upstream sequence from the *orf35* translation start site was scanned with Genomatix MatInspector program to

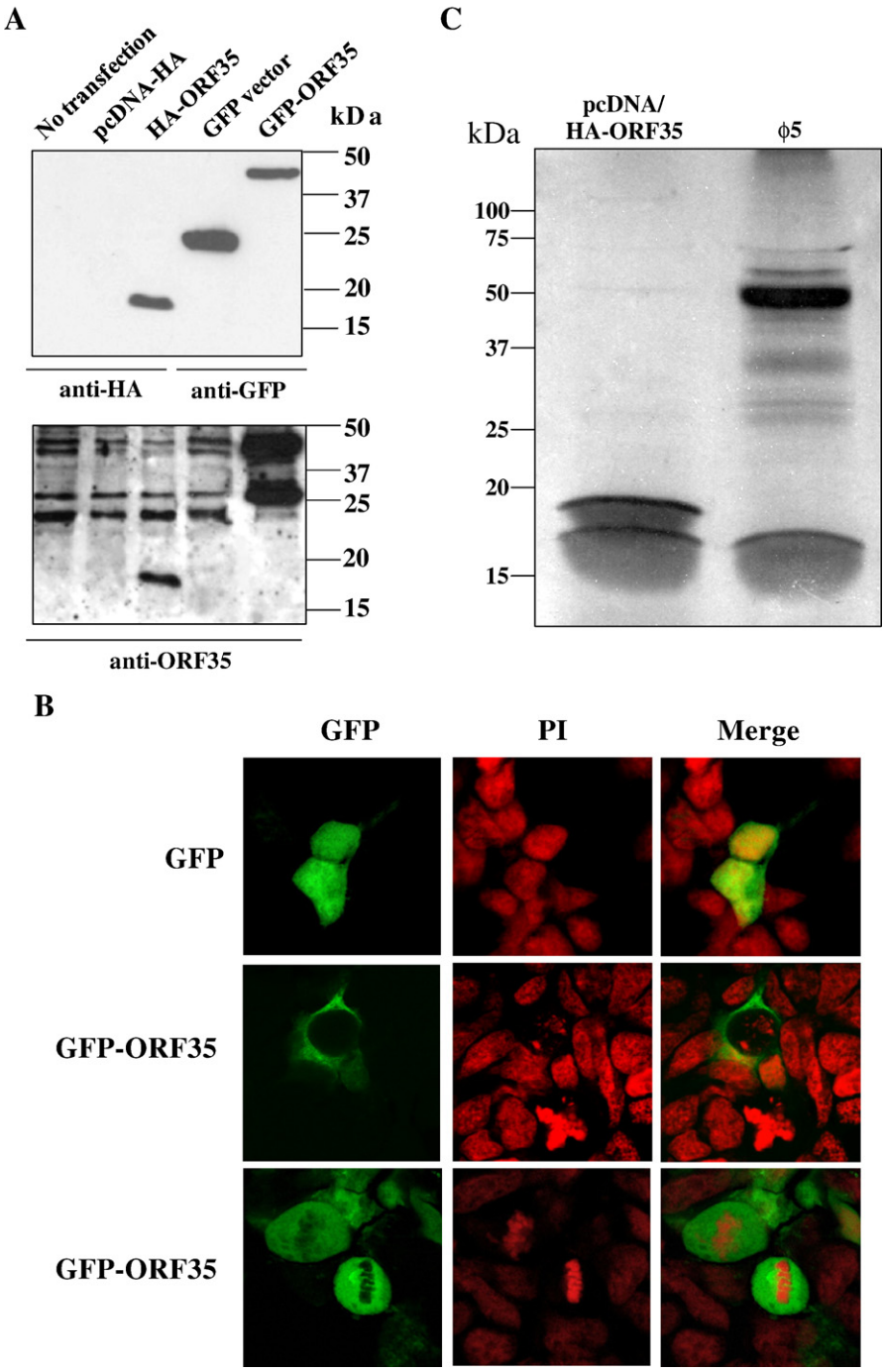

Fig. 4. Expression of ORF35 protein. HEK-293T cells were transfected with the indicated plasmids and cell extracts were analyzed by Western blot with anti-HA antibody and sequentially with anti-GFP antibody. Expression of 17-kDa and 43-kDa recombinant ORF35 proteins is clearly seen. The same bands were evident when a second Western blot was carried with antibodies to ORF35 peptide (A). Cells were transfected with pEGFP-C1 or pGFP-ORF35 and then fixed at 24 h post transfection. The fluorescence of GFP and GFP-ORF35 fusion protein was visualized in propidium iodide (PI) stained cells with a confocal laser microscope. As shown, ORF35 is predominantly cytoplasmic (B). The excised clone from phage screening was in vitro transcribed/translated, along with a plasmid containing HA-ORF35 as a positive control for ORF35. The resulting band of 17-kDa was identical in the T3/T7 polymerase translated ϕ5 clone and ORF35 control plasmid. Additional protein products were also produced (C).

identify potential transcription factor binding sites (Cartharius et al., 2005). Several conserved transcription factor recognition sites, including C/EBP, GATA-1, Sp-1, AP-1, and RBP-Jκ, were identified, whereas no conserved RTA-responsive elements (RREs) was found (Fig. 5). Potential hypoxia regulated elements have been recently identified upstream to this region and confirmed to mediate hypoxia response (Haque et al., 2006).

To identify the *cis*-acting elements necessary to activate *orf35* transcription, a series of 5′ truncated deletion constructs was synthesized. Fragments containing sequences starting adjacent to the *orf35* transcription initiation site (genomic

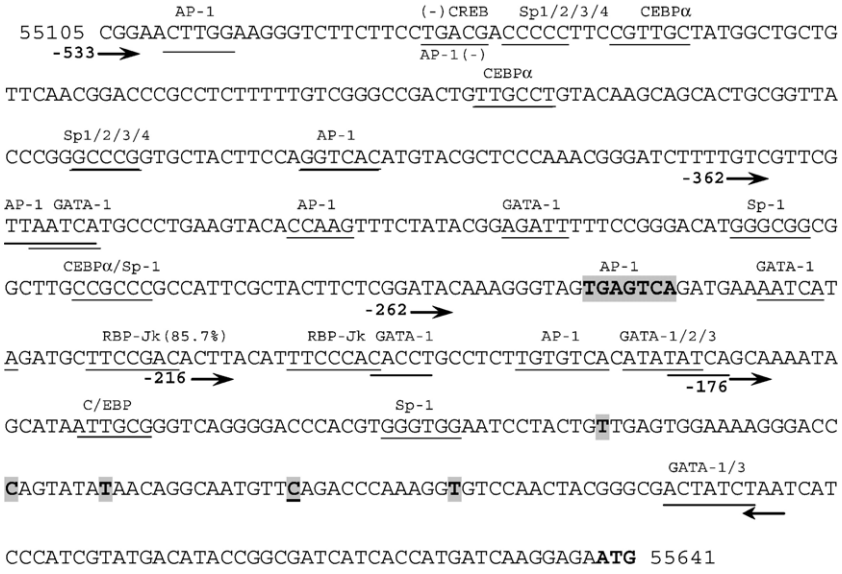

Fig. 5. Nucleotide sequence of the KSHV *orf35* promoter region. The sequence was taken from GeneBank KSU75698, nt 55105 to 55641, and corresponds to the region between −533 and +1 (ATG) relative to *orf35*. Potential transcription start sites that were identified by primer extension are boldface and indicated by gray shading while the 5′-end of ϕ5 is also underlined. Consensus motifs representing putative transcription factor binding sites that were identified with MatInspector program are depicted. Locations of truncated *orf35* promoter fragments used in luciferase reporter assays are indicated by arrows according to their position and designation. The AP-1 binding site which is included in the −262 promoter fragment (−262ORF35p) is marked by gray shading.

position 55,590 to 55,105, 55,277, 55,376, 55,422, and 55,462) were generated by PCR and cloned upstream to a luciferase reporter gene in the pGL3-Basic vector. The nomenclature for each plasmid (−533ORF35p, −362ORF35p, −262ORF35p, −216ORF35p, and −176ORF35p) was defined according to the distance from the ATG, and their positions are indicated in Fig. 5. These constructs were transiently transfected into HEK-293T cells which were previously shown to support KSHV infection in vitro (Krishnan et al., 2005). Transfections were performed in triplicate, repeated three times, and normalized against β-galactosidase reporter activity, which was used to control for transfection efficiency. Promoter activity, which was measured by luciferase units for each of the constructs, is presented as fold activation over pGL3-Basic control vector. With the exception of the −176ORF35p and −216ORF35p promoter constructs, the three other promoter constructs (−262ORF35p, −362ORF35p, and −533ORF35p) produced a significant transcriptional activity over the pGL3-Basic vector (Fig. 6A), indicating that sequences as close as 262 nucleotides upstream of the *orf35* transcription start site contain promoter activity. This finding also indicates that the *orf35* promoter may be active in HEK-293T cells in the complete absence of any KSHV gene product, though repression of this element in KSHV-infected cells is not excluded.

RTA activates the orf35 promoter in a reporter system

The early transcription pattern of *orf35* suggested that it might be activated by the KSHV-encoded protein RTA. To verify that the *orf35* promoter is a target for RTA, we examined the effect of RTA on the transcriptional activity of *orf35* promoter fragments. RTA was expressed from a pcDNA3-ORF50 vector, and the fold activation over pGL3-Basic for each promoter fragment was compared to that of a pcDNA3 control vector (Fig. 6B). All five reporters investigated were significantly stimulated by RTA. RTA could activate the −533ORF35p promoter fragment about ∼170-fold as compared to an empty vector. Likewise, RTA increased the activation of the −262ORF35p promoter construct ∼200-fold over cotransfection with pcDNA3 vector. However, for the −216ORF35p promoter construct, transactivation by RTA was only 18-fold over cotransfection with pcDNA3, suggesting that the 46-bp region included in the −262ORF35p construct, and absent in the −216ORF35p reporter construct, contains DNA elements that mediate a potent RTA response. Collectively, these reporter assays demonstrated that RTA can greatly activate the *orf35* promoter in HEK-293T cells, and suggest that a predominant RTA-responsive region resides in the 46-nt region which is included in the −262ORF35p reporter plasmid.

RBP-Jκ is dispensable for the activation of orf35 promoter by RTA

As indicated above and shown in Fig. 5, we identified two RBP-Jκ binding sites in the sequence upstream to the transcription initiation site of *orf35*. Both are included in −262ORF35p reporter construct whereas the more conserved one (TTCCCAC) is included also in the −216ORF35p reporter construct. Previous studies have shown that RTA can activate several KSHV lytic promoters through regulation of the activities involving the RBP-Jκ binding sites (Liang and Ganem, 2003; Liang et al., 2002; Chang et al., 2005b, Lan et al., 2005; Carroll et al., 2006). We therefore wanted to determine if RTA regulates *orf35* promoter through RBP-Jκ. Notably, as shown in Fig. 6B, the −216ORF35p reporter construct which contains the fully conserved RBP-Jκ binding

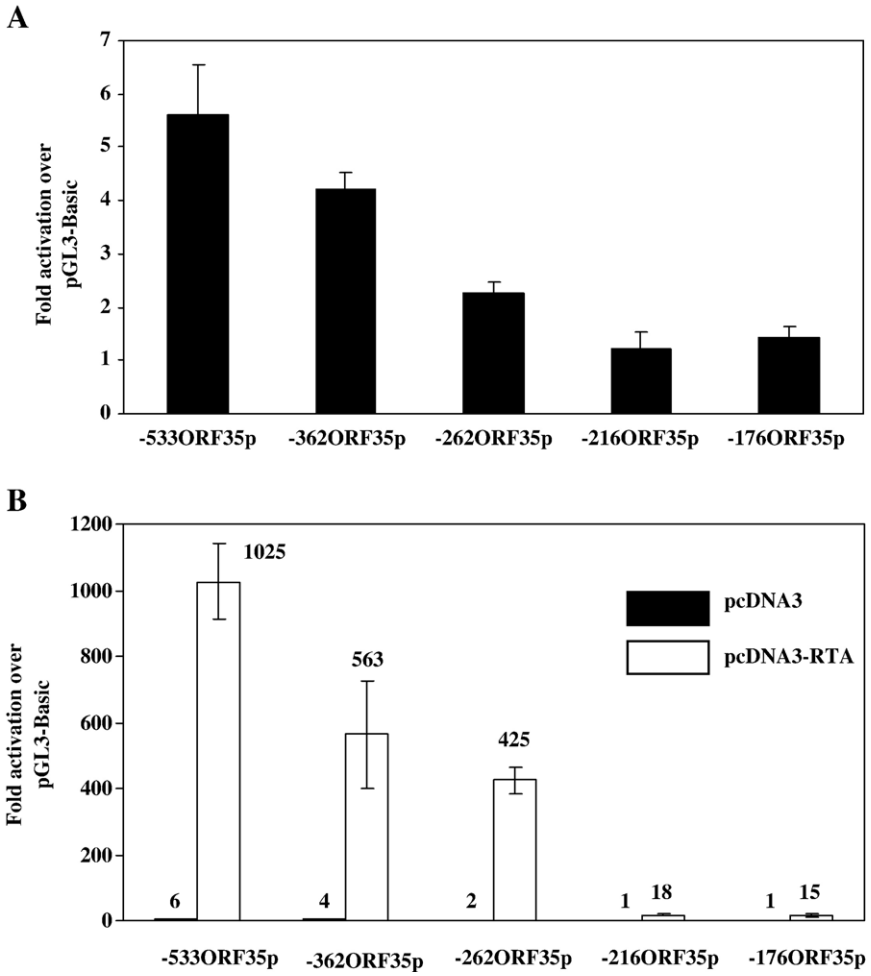

Fig. 6. ORF35 promoter activity in HEK-293T cells. (A) Basal activity of the *orf35* promoter deletion constructs in HEK-293T cells that were transfected with 0.5 μg of each of the *orf35* promoter constructs. (B) For RTA activation assay, 0.5 μg of each of the *orf35* promoter constructs and 0.25 μg of a β-galactosidase expression plasmid were transfected with either 1 μg of pcDNA3-RTA expression plasmid or pcDNA3 vector into the HEK-293T cell line. Cells were harvested 48 h post transfection and assayed for relative luciferase activity (RLU). Relative activation was calculated as normalized luciferase activity (Luciferase activity/β-galactosidase activity) divided by the normalized activity in cells transfected with the pGL3-Basic reporter construct alone. Error bars represent the standard deviation of triplicate samples. The results shown are representative of those from three similar experiments.

site had low responsiveness to RTA activation, suggesting that this site is dispensable for the activation by RTA. Yet, we hypothesized that it might be functional within the context of a larger promoter fragment, and therefore set to investigate the activity of −216ORF35p and −262ORF35p reporter constructs in mouse fibroblast cells that lack RBP-Jκ (OT11), a defect that can be reversed by the expression of RBP-Jκ. We selected this experimental system because it has been widely used to demonstrate the role of RBP-Jκ in promoter activation by RTA (Liang and Ganem, 2003, 2004; Liang et al., 2002; Chang et al., 2005b; Lan et al., 2005). Furthermore, PAN1 promoter, previously shown to be regulated by RBP-Jκ was used as a positive control in our experiments. As shown in Fig. 7, activation of PAN1 promoter was evident in OT11 cells upon RTA expression, and this activation was further augmented upon expression of RBP-Jκ. This is in line with previous findings (Liang et al., 2002). Similarly, the *orf35* promoter by RTA activity increased upon RTA expression, but the expression of RBP-Jκ did not enhance its activity. On the contrary, the expression of RBP-Jκ abolished the activation of *orf35* promoter by RTA. This finding, together with the relatively small transcriptional activation of −216ORF35p reporter construct in HEK-293T cells led us to conclude that the foremost activation of *orf35* promoter by RTA is not through RBP-Jκ.

The RTA-mediated orf35 transactivation is AP-1-dependent

To identify the predominant RTA response element in *orf35* promoter, we then focused on positions 55,376 to 55,422 that are included in the highly RTA responsive −262ORF35p reporter construct but absent in the shorter poorly RTA responsive −216ORF35p reporter construct. We hypothesized that RTA could activate the −262ORF35p reporter by direct or indirect modes. Direct activation of −262ORF35p reporter can take place through direct binding of RTA to response elements within this fragment; however, known RRE motifs could not be identified in this sequence.

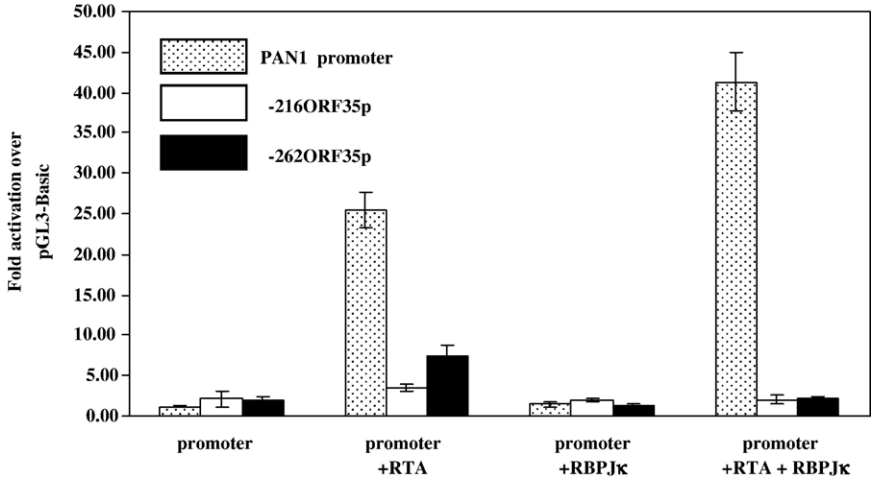

Fig. 7. Role of RBP-Jκ in the activation of *orf35* promoter by RTA. RBP-Jκ−/− OT11 mouse fibroblast cells were cotransfected with 0.5 μg PAN1, −216ORF35p, or −262ORF35p reporter construct together with 1.5 μg pcDNA-RTA, pSG5/Flag-RBP-Jκ, or with both pcDNA-RTA and pSG5/Flag-RBP-Jκ. The total amount of effector plasmid DNA used in each transfection was normalized to 3 μg by adding empty pcDNA3 vector DNA. Determination of the luciferase activity and calculation of the relative activation were performed as described in Fig. 6. The results shown are representative of those from three similar experiments.

To determine whether RTA activates the −262ORF35p reporter through direct contact, we performed an electrophoresis mobility shift assay (EMSA) with a ^{32}P-labeled oligonucleotide probe (ORF35-PWT) that included the nucleotides between positions −216 and −262 relative to the *orf35* start site. Direct binding experiments of RTA to this fragment using nuclear extracts from cells overexpressing RTA or in vitro translated reticulocyte lysates failed to demonstrate binding of RTA to this region (data not shown). However, using the MatInspector computer program, we identified a conserved AP-1 binding site (5′-TGAGTCA-3′) located between −262ORF35p and −216ORF35p (Fig. 5). AP-1 complexes are ubiquitous heterodimeric transcription factors composed of cJUN and cFOS subfamilies. The consensus AP-1 binding element is present in the promoter regions of several KSHV lytic genes, including the *orf50* (RTA), *orfK8* (RAP), and *orf57* (MTA) (Byun et al., 2002; Wang et al., 2004; Gonzalez et al., 2006). Furthermore, it has been shown that RTA increases cJUN expression and that these proteins interact to increase RTA transcriptional activation (Wang et al., 2004; Gwack et al., 2001). Therefore, we hypothesized that AP-1 family members may participate in transactivation of the *orf35* promoter by RTA protein. To test the ability of the AP-1 site to bind JUN-FOS complexes, we performed EMSA with in vitro-translated HA-tagged cFOS and untagged cJUN proteins and a ^{32}P-labeled oligonucleotide ORF35-PWT probe. An AP-1 shift band, which was successfully competed by excess unlabeled probe and further supershifted by the addition anti-HA antibody, was evident in the presence of cFOS and cJUN (Fig. 8). No additional shifted bands were noticed when in vitro translated RTA was added, suggesting that the AP-1 complex which is formed on the DNA does not tether RTA to the *orf35* promoter at this site.

We further evaluated the effect of AP-1 on the *orf35* promoter by cotransfecting cJUN and cFOS expression plasmids with the −262ORF35p reporter segment. The combined increase of transactivation by cotransfected cJUN and cFOS proteins on the −262ORF35p segment reached almost 5-fold at the higher doses of cJUN and cFOS (Fig. 9A), indicating that the AP-1 transcription factor complex affects the activity of the *orf35* promoter. The AP-1 complex is thought to be one of the most important targets through which TPA stimulates gene expression (Angel et al., 1987; Hess et al., 2004). When transfected into HEK-293T cells, the −262ORF35p reporter segment was responsive to TPA treatment after 24 h, showing 4-fold increased luciferase activity as compared to non-treated cells (Fig. 9A). When this promoter segment was cotransfected with cJUN plus cFOS plasmids and the transfected cells treated with TPA, promoter activity was stimulated 13-fold at the higher doses of cJUN and cFOS, indicating that TPA synergistically stimulates transactivation by AP-1 on the *orf35* promoter (Fig. 9A). Importantly, overexpression of cJUN and cFOS failed to further augment the activation of *orf35* promoter by RTA (Fig. 9B). Thus, these cotransfection results are consistent with the EMSA data and demonstrate that cJUN along with cFOS transactivate the *orf35* gene promoter by a mechanism that involves a direct binding to the AP-1 site at the position −249 to −243 relative to the *orf35* ATG; however, direct AP-1 DNA binding without RTA appears not to be the dominant determinant of transcriptional activation.

To further determine the effect of the AP-1 transcription complex on RTA-mediated transactivation of the *orf35* promoter, we constructed a reporter plasmid with mutations in the putative core AP-1 binding sequence within the −262ORF35p reporter segment (Fig. 10A). Reporter assays were carried in HEK-293T cells transfected with the wild-type −262ORF35p reporter segment or with a mutated −262ORF35mp reporter. As shown in Fig. 10B, the mutant *orf35* reporter segment showed a 4-fold decrease in the responsiveness to RTA in an RTA dose-dependent manner. This suggests that direct DNA binding of AP-1 is a dominant determinant of RTA-induction.

To further confirm the essential role of AP-1 complex in RTA-mediated *orf35* transactivation, we cotransfected HEK-

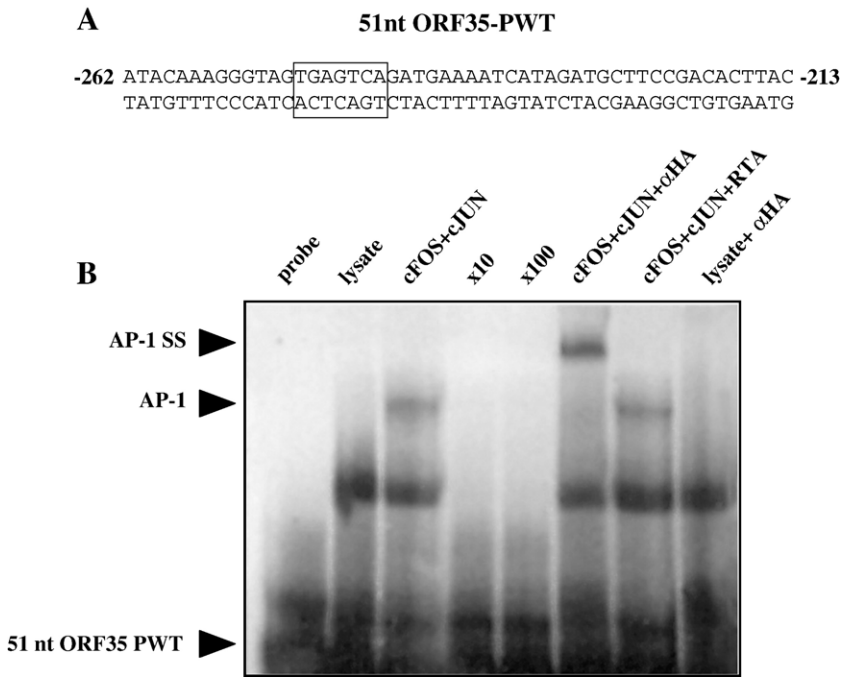

Fig. 8. Identification of an AP-1 binding site in the KSHV *orf35* promoter by EMSA assay. (A) Sequence of the *orf35* promoter wild-type (PWT) probe used in EMSA experiments. The putative AP-1 site is boxed. (B) Results of an EMSA experiment showing the AP-1-binding ability of the AP-1 motif in the *orf35* promoter. Lane 1, only ORF35-PWT; lane 2, unprogrammed reticulocyte lysate; lane 3, in vitro-cotranslated HA-tagged cFOS and cJUN; lanes 4 and 5, excess unlabeled probe (×10, ×100); lane 6, mouse anti-HA antibody added to generate a specific supershifted band; lane 7, in vitro-cotranslated HA-tagged cFOS and cJUN mixed with in vitro translated RTA; lane 8, antibody control. Protein samples were incubated with the ^{32}P-labeled ORF35-PWT probe. AP-1 specific shifted bands are marked; SS, antibody supershift.

293T cells with a dominant-negative construct of c-FOS (A-FOS), RTA, and either the wild-type −262ORF35p reporter segment or the mutated −262ORF35mp reporter segment (Fig. 10C). Comparable levels of RTA expression were confirmed by Western blot analysis (Fig. 10D). The addition of equivalent amounts of A-FOS and RTA reduced the RTA-mediated −262ORF35p transactivation from 129-fold to 72-fold which is almost half of the fold activation observed, while addition of 2-equivalent amounts of A-FOS reduced this reporter transactivation to 33-fold, which is close to the RTA-mediated transactivation activity of the mutant reporter. Interestingly, the addition of A-FOS reduced the RTA-mediated −262ORF35mp transactivation from 36-fold to 20-fold, indicating that the AP-1 transcription complex contributes to *orf35* transactivation through additional sites besides the AP-1 site we investigated. Thus, these data confirm that the major RTA-mediated *orf35* transactivation is AP-1-dependent; nevertheless, there are likely to be additional weaker target sites.

Discussion

In this study, we identified and characterized the predominant KSHV *orf35* transcript in PEL cells. To identify the intact full-length *orf35* transcript, we performed RT-PCR, Northern blot hybridization, screening of a cDNA library, and primer extension assays. We found that *orf35* is transcribed as part of a polycistronic transcript that also contains *orf36* to *orf38* genes. This transcript is produced with early kinetics upon virus reactivation. The *orf35* transcript is 3315–3367 bases long, including a 73–125-nucleotide 5′-UTR which is within *orf34* coding sequences, and a 7-nucleotide 3′-UTR with a polyA signal within *orf38* coding sequences. Similar results were recently reported (Haque et al., 2006); nonetheless, we identified an extended 5′-UTR and demonstrated that *orf38* is also present in the *orf35* transcript. An additional transcript containing *orf35*, which also includes *orf34*, has been previously reported (Haque et al., 2006). However, it appears that in PEL cells the predominant transcript from this region contains the *orf35-orf38* gene cluster. Of note, upon virus infection the transcription pattern of *orf35* differs from the other genes in the cluster (Krishnan et al., 2004). Because the closest potential polyA signal downstream to *orf35* is localized to the *orf37* coding sequences, other alternatively spliced *orf35* transcripts probably exist during this phase.

The functions of the *orf36* and *orf37* gene products have been elucidated. *orf36* encodes a serine protein kinase, which is localized to the nucleus of infected cells, and is capable of phosphorylating ganciclovir and activating the cJUN N-terminal kinase signaling pathway (Cannon et al., 1999; Hamza et al., 2004; Park et al., 2000). ORF37, termed *s*hut*o*ff and e*x*onuclease (SOX) protein, possesses DNase and host shutoff activities (Glaunsinger and Ganem, 2004). *orf34*, *orf35*, and *orf38* encode proteins with unknown functions. The necessity of these gene products during various phases of infection is also unknown. *orf34* to *orf38* genes partially overlap each other and their translation route is currently unclear, though, it probably involves alternatives to the ordinary scanning mechanism. Furthermore, translation products from

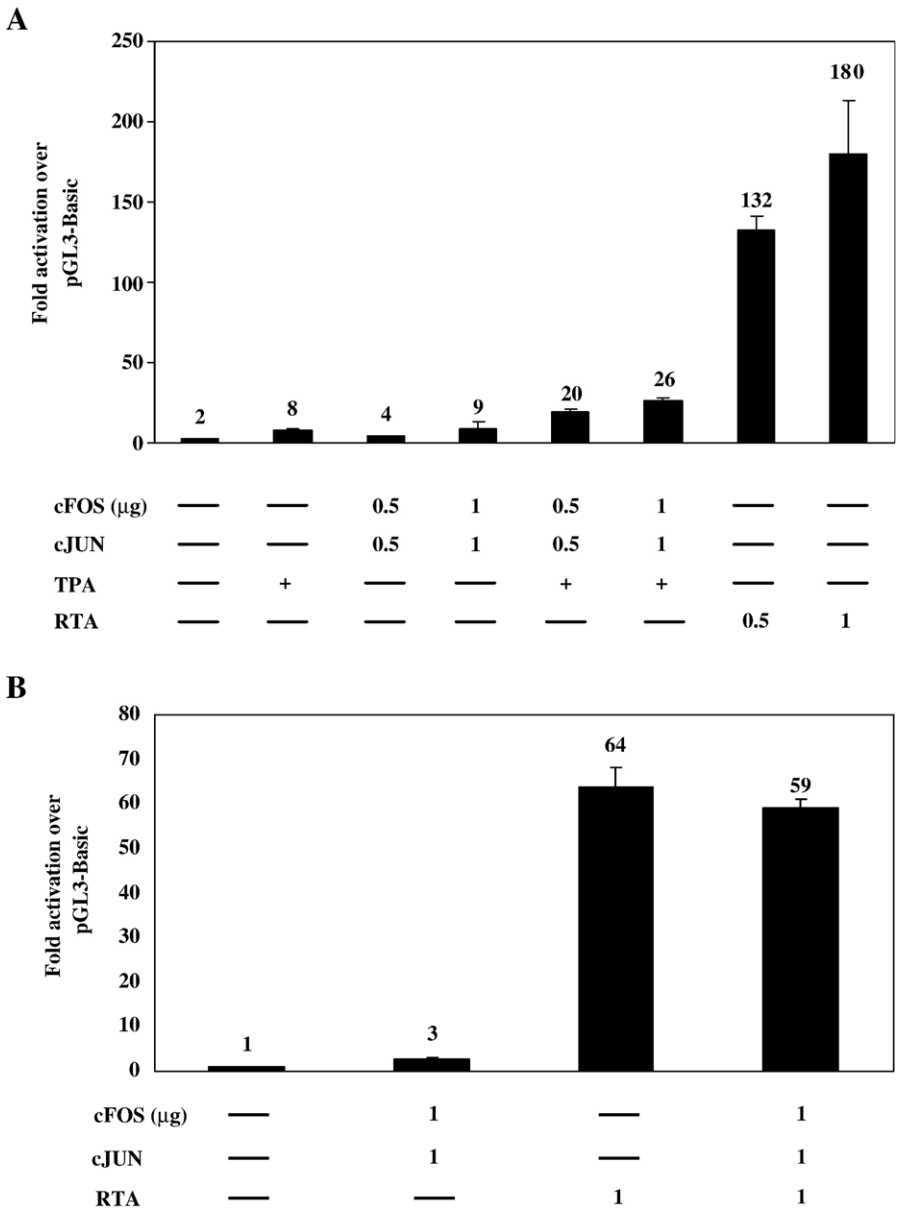

Fig. 9. KSHV *orf35* promoter is transcriptionally activated by AP-1. (A) cJUN and cFOS activate the −262ORF35p segment in luciferase reporter assays. HEK-293T cells were transfected with 0.25 μg of −262ORF35p reporter segment together with the indicated amounts of effector plasmid DNAs encoding cJUN, cFOS, and RTA. The total amount of effector plasmid DNA used in each transfection was normalized to 2.25 μg by adding empty pcDNA3 vector DNA. TPA was added into the medium, and the cells were incubated for 24 h before harvesting. (B) Overexpression of cJUN and cFOS fail to further augment RTA activation of *orf35* promoter. Error bars represent the standard deviation of triplicate samples. The results shown are representative of those from three similar experiments.

orf35 to *orf38* transcript may vary during different infection phases, according to the translation machinery involved in their production. Nevertheless, we show that a coupled in vitro transcription and translation reaction of a cDNA clone containing the *orf35* to *orf38* genes produced a predominant band of approximately 50-kDa, which probably corresponds to either the *orf36* or *orf37* gene products. A 17-kDa protein band, which likely matches the *orf35* gene product was also evident supporting the potential of this transcript to produce ORF35 protein.

ORF35 protein is highly conserved among all complete genomes of the *Gammaherpesvirinae* and shares a limited sequence similarity with the alphaherpesvirus UL14 gene product. Unlike UL14, ORF35 has not been detected in KSHV nor in mHV-68 or in EBV virion preparations that were analyzed by mass spectrometric analysis (Bechtel et al., 2005; Bortz et al., 2003; Johannsen et al., 2004; Zhu et al., 2005). However, ORF35 has been detected in purified RRV particles (O'Connor and Kedes, 2006). UL14 protein has been suggested to possess multiple functions, including a molecular chaperone activity. Its expression is mostly cytoplasmic, but it also accumulates in the nucleus upon induction of cellular stress conditions (Yamauchi et al., 2002). Likewise, we observed a predominant cytoplasmic localization of KSHV ORF35. UL14-deficient mutant exhibits an extended growth cycle and appears to be compromised in the efficient transit of virus particles (Cunningham et al., 2000). Significant suppression of apoptosis

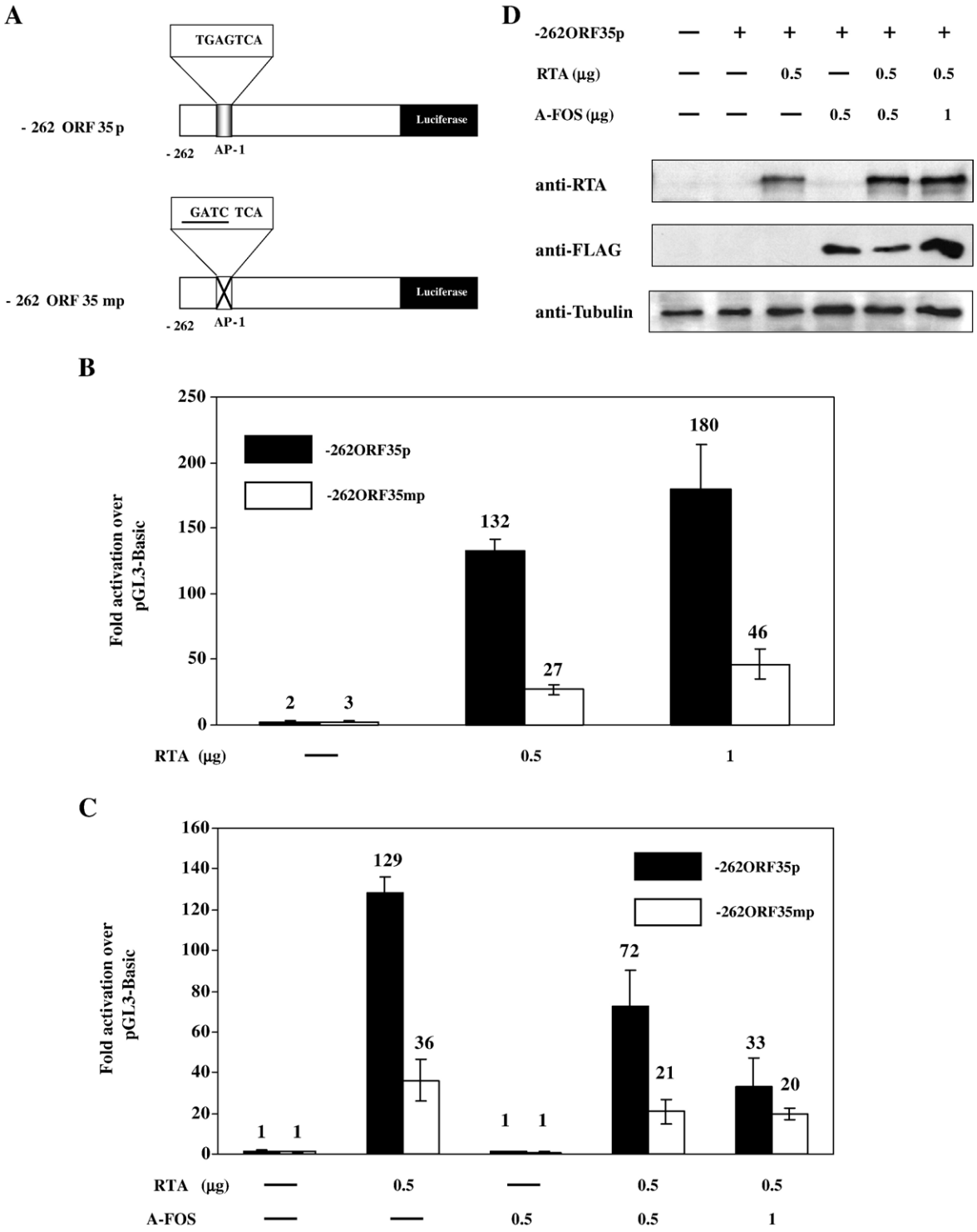

Fig. 10. RTA-mediated *orf35* transactivation is AP-1-dependent. (A) Schematic diagram of the wild-type and the mutated *orf35* promoter region used in transfection experiments. (B) HEK-293T cells were cotransfected with 0.25 μg target reporter plasmid DNA encoding either wild-type −262ORF35p (solid bars) or the point mutated version −262ORF35mp (open bars), and 0.5 or 1 μg of effector plasmid DNA encoding RTA. The total amount of effector plasmid DNA used in each transfection was normalized to 2.25 μg by adding empty pcDNA3 vector DNA. (C) Dominant negative c-FOS (A-FOS) inhibited induction of the RTA-mediated *orf35* transactivation. HEK-293T cells were cotransfected with 0.375 μg target reporter plasmid DNA encoding either wild-type −262ORF35p (solid bars) or the point mutant −262ORF35mp (open bars), 1 μg of RTA expression plasmid, and 1 or 2 μg of A-FOS expression plasmid. The total amount of effector plasmid DNA used in each transfection was normalized to 3.375 μg by adding empty pcDNA3 vector DNA. (D) Protein extracts were prepared from transfected cells, and equal amounts of protein (30 μg) were loaded per lane. Following SDS-PAGE and transfer of proteins to nitrocellulose, blots were probed for RTA, Flag (to detect A-FOS) and Tubulin indicating comparable amounts of RTA in each transfection condition.

and caspase activation is evident in cells expressing UL14 (Yamauchi et al., 2003), and nuclear translocation of two viral cytoplasmic encapsidation proteins (VP26 and UL33) is induced in UL14 expressing cells (Yamauchi et al., 2002). The functional similarity between UL14 and ORF35 proteins remains to be demonstrated; however, ORF35 may possess different functions because the domain included in the heat-shock proteins and UL14 is missing in KSHV ORF35.

In this study, we have documented a strong transcriptional activation of the *orf35* promoter by RTA. This promoter has been recently shown to contain a functional hypoxia-responsive element which plays a critical role in its hypoxic transcriptional activation (Haque et al., 2006). *orf35* promoter also contains conserved binding sites to RBP-Jκ; nonetheless, we have demonstrated that the activation by RTA is not through the interaction with this cellular transcription factor as shown for several other RTA-activated KSHV promoters (Liang and Ganem, 2003; Liang et al., 2002; Chang et al., 2005b, Lan et al., 2005; Carroll et al., 2006). We revealed a 46-nucleotide region which includes a conserved AP-1 binding site and possesses the predominant responsiveness to RTA. However, neither the RTA-responsive 46-nucleotide region nor the intact *orf35* promoter appears to share sequence similarity with the RTA-responsive elements (RREs) found in the PAN, Kaposin, *orfK8*, *orf57*, *orfK1*, and vIL-6 promoters (Chang et al., 2002, 2005a; Deng et al., 2002; Duan et al., 2001; Lukac et al., 1998, 1999; Song et al., 2002a, 2002b; Bowser et al., 2006). Moreover, RTA did not bind this 46-nucleotide fragment when tested by EMSA and added to the DNA probe alone or together with cJUN and cFOS. On the other hand, given that (i) RTA transcriptional activation of *orf35* promoter has not been further augmented by cJUN and cFOS overexpression, (ii) mutations in the putative AP-1 binding sequences markedly reduced the responsiveness of the *orf35* promoter to RTA, and (iii) expression of dominant-negative cFOS decreased RTA activation of *orf35* promoter, our data establish the requirement of AP-1 binding for RTA activation of *orf35* promoter.

AP-1 is a dimeric transcription factor that consists of members of the cFOS and cJUN gene subfamilies which bind consensus DNA sites in the promoter region of target genes (Shaulian and Karin, 2002; Karamouzis et al., 2007). The activity of AP-1 is regulated at multiple levels, including transcription of the genes encoding AP-1 subunits, control of the stability of their mRNAs, posttranslational modifications, turnover of the AP-1 components, and specific interactions with other transcription factors and cofactors.

Previous studies established the involvement of AP-1 in KSHV reactivation (Wang et al., 2004; Cohen et al., 2006), and two groups demonstrated the activation of AP-1 by virus entry events (Xie et al., 2005; Sharma-Walia et al., 2005). Furthermore, several KSHV-encoded proteins can potentially manipulate the activities of AP-1 in KSHV-infected cells. For example, endogenous unphosphorylated cJUN is induced upon expression of RTA which in turn is capable of engaging in a protein interaction with cJUN (Wang et al., 2004), while a dose-dependent stimulation of RTA activity by cJUN was reported by Gwack and colleagues (Gwack et al., 2001). Furthermore, a cooperative enhancement of the total cJUN transcriptional activation potential by RTA and ORF49 has been suggested (Gonzalez et al., 2006). Accordingly, RTA increases total cJUN protein and ORF49 induces phosphorylation and activation of cJUN, resulting in a synergistic activation of several KSHV lytic promoters containing AP-1 sites. Wang and colleagues confirmed a synergistic activation of RTA, MTA, and RAP promoters by cJUN plus cFOS with RTA (Wang et al., 2004). However, unlike the *orf35* promoter, these promoters are strongly activated by cJUN plus cFOS even in the absence of RTA, and a more than additive effect is evident when RTA is expressed with cJUN plus cFOS and vice versa. Furthermore, the dependence of RTA activity in the DNA binding of AP-1 has not been investigated in this study. To the best of our knowledge, our report is the first to describe a dependence of RTA transcriptional activation in the DNA binding of AP-1.

The mechanism by which RTA activates *orf35* promoter by means of AP-1 remains to be determined. Recruitment of RTA to *orf35* promoter by AP-1 transcription cofactors is possible. Mutual modulation of AP-1 and RTA transcriptional activities might also take place. Thus, binding of AP-1 and RTA to different sites in the *orf35* promoter may cooperatively yield high transcriptional activation. The binding of both factors may either generate an active ternary transcriptional complex or induce conformational changes that allow the recruitment and binding of additional cellular transcription factors. It is also possible that RTA modulates the expression and/or activity of the AP-1 transcription factor, which in turn regulate the *orf35* promoter. This possibility is supported by Wang et al. (2004) study, showing that RTA increases cJUN expression and is capable of forming protein interaction with cJUN.

During primary infection, KSHV entry events activate AP-1, and early stimulation of the RTA promoter is accomplished predominantly via this pathway (Xie et al., 2005; Pan et al., 2006). Unlike most other herpesviruses, KSHV quickly adopts a latent program of gene expression following de novo infection in vitro. However, the transient expression of a limited set of lytic viral genes takes place rapidly after infection, prior to the establishment of latency (Krishnan et al., 2004). Many of the transiently expressed KSHV lytic genes possess immune modulation and antiapoptotic functions, suggesting a role in providing conditions necessary for the coexistence of the virus with the host cell during the initial time of infection and/or during the establishment of latency. *orf35* has been included among this set of transiently expressed genes (Krishnan et al., 2004), and involvement of the AP-1 complex in its promoter activation further supports its primary role during the initial phases of infection. Further studies will reveal whether and in what manner the *orf35* gene product provides functional support for the early phases of KSHV infection and/or during the establishment of latency.

Materials and methods

Sequence analyses

Alignments of KSHV ORF35 protein sequences were compiled using ClustalW (Pearson, 1990). Additional sequence

analyses were done using BLASTP, ELM, and the Sumoylation site prediction algorithm (Xue et al., 2006). KSHV ORF35 promoter sequences were analyzed using the MatInspector program (Cartharius et al., 2005).

Culture conditions

BCBL-1 (KSHV-positive cell line) (Renne et al., 1996), BC-1 (KSHV and EBV-positive cell line) (Cesarman et al., 1995), and BCP-1 (Boshoff et al., 1998) cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 50 IU/ml of penicillin, and 50 μg/ml of streptomycin. Human embryonic 293T kidney cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS and antibiotics. Mouse RBP-Jκ−/− (OT11) fibroblast cell line was kindly provided by T. Honjo (Kyoto University, Japan) (Oka et al., 1995), and were grown in high glucose DMEM supplemented with 10% FCS. Cells were maintained at 37 °C in humidified atmosphere with 5% CO_2. To induce lytic KSHV infection, cells were seeded at 4×10^5 cells/ml and 12-*O*-tetradecanoyl 13-acetate (TPA; Sigma) was added at 20 ng/ml. To inhibit virus DNA replication, phosphonoacetic acid (PAA) (Sigma) was added at a concentration of 0.5 mM.

Construction and screening of BC-1 cell line cDNA library

A cDNA phage library of BC-1 cells induced with TPA was kindly provided by Prof. Patrick Moore and Prof. Yuan Chang (Pittsburgh University, PA) (Sarid et al., 1999). Clones identified with a KSHV *orf35* PCR probe were plaque purified; positive phages were converted into phagemids by employing the ExAssist helper phage (Stratagene), and inserts were sequenced.

Reverse transcription (RT)-PCR and Northern blot analysis

Total cellular RNA was extracted with the use of EZ-RNA Total RNA Isolation kit (Biological Industries, Kibbutz Beit Haemek, Israel), according to the manufacturer's instructions. DNase I (Ambion)-digested RNA (1 μg) was reverse transcribed with random hexamers and RevertAid H Minus First Strand cDNA synthesis kit (Fermentas) according to the manufacturer's protocol. −RT reactions were used to control for DNA contamination. For PCR amplification, 10% of the reverse transcribed RNA was added to a reaction buffer containing a mixture of dNTPs (200 μM each), 1 μM of oligonucleotide primers for *orf35*, *orf65*, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 2 U Taq polymerase. Following 2 min denaturation at 94 °C, 35 amplification cycles were performed as follows: 94 °C for 30 sec, 58 °C for 1 min, 72 °C for 1 min, and 72 °C for 10 min. Samples were collected after 15, 20, 25, 30, and 35 amplification cycles and products were analyzed by gel electrophoresis using a 1.5% agarose gel stained with ethidium bromide.

For Northern blot hybridization, RNA was fractionated by electrophoresis on 1% agarose-1.6% formaldehyde in MOPS-EDTA buffer, and then transferred to nylon membrane (Schliecher and Schuell, Germany) over night in 10× SSC. PCR products from *orf35* and GAPDH were ^{32}P-labeled by random priming (Biological Industries, Kibbutz Beit Haemek, Israel) and hybridized to the blots at 42 °C in [5× SSC, 50% formamide, 5× Denhardt's solution, 2% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 100 μg denatured sheared salmon sperm DNA/ml]. Multiple post hybridization washes were carried out in 1× SSC, 0.1% SDS at 68 °C.

Primer extension

Total cellular RNA was prepared with EZ-RNA according to the manufacturer's protocol (Biological Industries, Kibbutz Beit Haemek, Israel). Primer PE 5′-GGCCTCCAGAGCCGACTTAATAAAC-3′ was end-labeled with [γ-^{32}P]ATP (10^5 cpm/pmol) and hybridized to 10 μg of RNA at 60 °C for 15 min. The sample was then kept on ice for 1 min. Next, 1 unit of reverse transcriptase (Expand RT, Roche) and 1 unit of RNase inhibitor (Promega) were added, and extension was performed at 42 °C for 90 min. Reaction products were precipitated with ethanol and resolved on a 6% polyacrylamide, 7 M urea gel in Tris–borate–EDTA. DNA sequencing reactions were performed with Sequenase 2.0 (United States Biochemical Corporation, Cleveland, Ohio).

Plasmids

The viral DNA from KSHV-infected BCBL-1 cells was used as a template for all PCR amplifications. The KSHV *orf35* gene fragment was amplified by PCR primers with *Bam*HI and *Eco*RI restriction sites at their 5′ and 3′ ends (5′-TTGGGATCCATGGACTCAACCAACTCT-3′ and 5′-TGGGAATTCTTAGGGAGTTTCAGGGCA-3′), respectively, and inserted into pcDNA/HA (Invitrogen) to obtain pcDNA/HA-ORF35, which allows gene expression in mammalian cells in-frame with an N-terminal HA tag. PCR products that were generated with primers containing *Xho*I and *Eco*RI restriction sites at their 5′ and 3′ ends (5′-TTGCTCGAGGAATGGACTCAACCAACT-3′ and 5′-TGGGAATTCTTAGGGAGTTTCAGGGCA-3′) were cloned into pEGFP-C1 (BD Biosciences) to obtain pEGFP-ORF35. The RTA expression plasmid (pcDNA-ORF50) (Lukac et al., 1998) and PAN1 promoter reporter construct (Kirshner et al., 2000) were kindly provided by Prof. Don Ganem (University of California, San Francisco, CA). Human HA-cFOS and cJUN expression pcDNA3 plasmids were kindly provided by Prof. Yosef Shaul (Weizmann Institute of Science, Israel). Flag-tagged cFOS dominant-negative (A-FOS) expression vector was obtained from Prof. Charles Vinson (National Institutes of Health, Bethesda, Maryland) (Olive et al., 1997). Human full-length RBP-Jκ expression plasmid was kindly provided by Prof. Delphine Ndiaye (Pasteur Institute, Paris).

To generate deletion mutant promoter constructs, the KSHV *orf35* promoter region was amplified with the anti-sense primer 5′ AGGCTCGAGTTAGATAGTCGCCCGTAGTT-3′ and sense primers 5′-ACCACGCGTGCAAAATAGCATAATTGCGG-3′, 5′-AAAACGCGTCTTACATTTCCCACACCTGC-3′,

5′-GGGACGCGTATACAAAGGGTAGTGAGTCA-3′, 5′-GGGACGCGTGTCGTTCGTTAATCATGCCCT-3′, and 5′-AAAACGCGTCGGAACTTGGAAGGGTCTTC-3′ to obtain fragments that are 176, 216, 262, 362, and 533 nt from the *orf35* ATG codon. PCR products were cloned into pGL3-basic (Promega) with *Xho*I and *Mlu*I cloning sites. The mutated reporter clone −262ORF35mp, with TGAG at positions −249 to −246 replaced with GATC to destroy the AP-1-binding site, was generated by using the −262 deletion mutant promoter plasmid as a template, a mutated AP-1 sense primer 5′-GGGACGCGTATACAAAGGGTAGGATCTCAGATGAAA-3′ and the antisense primer described above.

Western blot analysis

Cells were washed twice in cold phosphate-buffered saline (PBS), suspended in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonident P-40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μM leupeptin, 0.2 mM Na_3VO_4, 50 mM NaF) and incubated on ice for 30 min. Then cell debris was removed by centrifugation at 12,000×*g* for 15 min at 4 °C. Loading buffer (2×; 2% SDS, 20% glycerol, 125 mM Tris (pH 6.8), 0.02% bromophenol blue, and 10% β-mercaptoethanol) was added and the samples were boiled for 5 min. Protein lysates were resolved by SDS-polyacrylamide gel (PAGE) and transferred to nitrocellulose membranes (Schliecher and Schuell). The protein contents of different samples were verified to be similar by Ponceau staining. The nitrocellulose membranes were blocked with 5% dry milk in TBS and subsequently incubated with primary antibody (mouse anti-HA (HA.11) (Covance Research Products), anti-GFP (Covance Research Products), rabbit anti-ORF35, anti-RTA (kindly obtained from Dr. Keiji Ueda, Osaka University School of Medicine, Osaka, Japan), anti-Tubulin (Sigma), or anti-Flag (Sigma). Specific reactive bands were detected using goat anti-rabbit IgG or goat anti-mouse conjugated to horseradish peroxidase. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL) Western blotting detection kit (Amersham, Arlington Heights, IL).

In vitro transcription/translation

In vitro transcription and translation were carried out using a T7/T3 in vitro transcription and translation kit according to the manufacturer's protocol (Promega). Radiolabeled in vitro-synthesized proteins were obtained by adding [^{35}S]methionine. Proteins from each sample were assayed by Western blot and used for EMSA or electrophoresed in SDS-PAGE, dried, and exposed to film.

DNA transfection and reporter assays

DNA transfections into HEK-293T cells were carried out with the calcium phosphate method. DNA transfections into OT11 cells were performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's protocol. Cell lysates were prepared 48 h after transfection and luciferase activity was measured using the Promega luciferase kit in a Spectralfluor Plus luminometer (Tecan) according to the manufacturer's instructions. To control for transfection efficiency, we performed cotransfection with plasmid pcDNA-LacZ expressing β-galactosidase. In all cases, three or more separate transfections were performed, and results shown are representative of at least three experiments.

Electrophoretic mobility shift assay (EMSA)

RTA, cJUN, and cFOS protein samples used for EMSA were in vitro translated with the T7-TNT quick-coupled transcription-translation system (Promega) according to the manufacturer's procedures, and using the corresponding expression plasmids pcDNA-RTA, pcDNA3-HA-hcFOS, and pcDNA3-hcJUN as templates. Next, 10 pmol of annealed double-stranded oligonucleotides was end radiolabled with [γ-^{32}P]ATP using T4 polynucleotide kinase (PNK). For gel shift assays, 4 μl of in vitro-translated proteins was incubated for 10 min at R.T. in a binding buffer containing 50 mM Tris–HCl (pH 7.5), 250 mM NaCl, 5 mM $MgCl_2$, 20% glycerol, 2.5 mM EDTA, 2.5 mM dithiothreitol, and 0.25 μg/μl of poly(dI/dC). The ^{32}P-labled probe was then added and incubated for 20 min at R.T. Competition assays were carried out in the same manner, except that the above reaction mixture was preincubated with excess cold probe before the addition of the labeled probe. For supershift experiments, 3 μg of mouse anti-HA monoclonal antibody (MMS-101R, Covence) was added to the mixture and allowed to incubate for another 30 min before gel loading. Samples were separated by 5% nondenaturing PAGE. The gels were dried and subjected to autoradiography.

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